

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](https://doi.org/10.1016/j.scitotenv.2021.146016)

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**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

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

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16 **# The contribution of these two authors is equal.**

## 17 1. Introduction

18 Constructed wetlands (CWs) are well-established, nature-based, and robust wastewater treatment  
19 technology, which have been applied worldwide in the last decades (Carvalho et al., 2017; Ilyas and  
20 Masih, 2017). The physical, chemical, and biological processes in CWs can efficiently remove different  
21 kinds of pollutants, including organic matter, suspended solids, metals, and even emerging contaminants  
22 (Almeida et al., 2017; He et al., 2018; Józwiakowski et al., 2019). Among these pollutants, nitrogen (N)  
23 removal efficiency in previous studies are ambiguous (Ilyas and Masih, 2017; Vymazal, 2013). It has  
24 been reported that the total nitrogen (TN) removal in several investigated CWs in Poland, Brazil, and  
25 China ranged from lower than 10% to 80% (Józwiakowski et al., 2019; Li et al., 2018; Machado et al.,  
26 2017). These CWs may fail to comply with the increasingly stringent effluent TN standards. A  
27 combination of different types of CWs, namely hybrid CWs, is more efficient in N removal than a single  
28 CW (Vymazal, 2013). A previous study shows that a full-scale VBFW-HSFW (Vertical Baffled Flow  
29 Wetland – Horizontal Subsurface Flow Wetland) system can achieve 83% of  $\text{NH}_4^+$  removal and 77% of  
30 total nitrogen (TN) removal (Zhai et al., 2016). The high nitrogen removal efficiency in the hybrid CWs  
31 is probably attributed to the presence of novel nitrogen removal pathway like sulfur-based autotrophic  
32 denitrification, anaerobic ammonia oxidation and dissimilatory nitrate reduction to ammonium (Zhai  
33 et al., 2016).

34 In the wetlands, the most commonly known N transformation processes are nitrification and  
35 denitrification. However, recent studies reported other N transformation processes, including anaerobic  
36 ammonia oxidation (anammox), autotrophic denitrification, and dissimilatory nitrate reduction to  
37 ammonia (DNRA), are also found in the CWs (Ma et al., 2020; Nizzoli et al., 2010; Valipour and Ahn,  
38 2017; Zhai et al., 2016). The different C/N ratios affect C and N removal pathways in CWs. During



39 anammox,  $\text{NH}_4^+$  can be oxidized by  $\text{NO}_2^-$  into  $\text{N}_2$ . As reported previously, over 40% of  $\text{NH}_4^+$  could be  
40 removed attributing to anammox in a plant-bed/ditch system of a constructed wetland (Wang et al., 2018).  
41 Instead of  $\text{NO}_2^-$ ,  $\text{SO}_4^{2-}$  can also be used to oxidize  $\text{NH}_4^+$  in anammox, namely  $\text{SO}_4^{2-}$ -dependent anammox  
42 process (Rios-Del Toro et al., 2018). In this process,  $\text{NH}_4^+$  is oxidized to  $\text{N}_2$  coupled to the reduction of  
43  $\text{SO}_4^{2-}$  to  $\text{S}^0$ . Autotrophic denitrification is another important N transformation pathway. The autotrophic  
44 denitrification uses sulfur-compounds, such as elemental sulfur ( $\text{S}^0$ ),  $\text{S}_2\text{O}_3^-$ , and  $\text{S}^{2-}$  or FeS, as the electron  
45 donor to convert  $\text{NO}_3^-$  or  $\text{NO}_2^-$  into  $\text{N}_2$ . Other electron donors like hydrogen ( $\text{H}_2$ ) or reduced metal can  
46 also be used in the autotrophic denitrification (Guo et al., 2020; Tang et al., 2020). It was reported in an  
47 anaerobic digester that the contribution of autotrophic denitrification to TN removal can reach 90%  
48 without organic supplementation (Qiu et al., 2020). This result showed that autotrophic denitrification  
49 has great potential for TN removal in CWs when organic supply was limited. However, autotrophic  
50 denitrification may cause  $\text{NO}_2^-$  accumulation, limiting TN removal efficiency (Chen et al., 2018; Qiu et  
51 al., 2020). The dissimilatory nitrate reduction to ammonia (DNRA) is an alternative  $\text{NO}_3^-$  reduction  
52 pathway observed in the constructed wetland (Zhang et al., 2021). Previous studies showed that the  
53 DNRA could outcompete denitrification in natural system like subtropical pasture soils, freshwater  
54 sediment and marine sediment under certain conditions like high C/N ratios, high concentrations of  
55 sulfide, low concentrations of iron, or high temperature (Friedl et al., 2018; Holmes et al., 2019). The N  
56 removal in the CWs is coupled with C and S compound transformation. The sulfate reduction using  $\text{SO}_4^{2-}$   
57 to oxidize organic carbon shows the coupling transformation of C and S compounds in the CWs (Guo et  
58 al., 2020). Thus, the carbon, nitrogen and sulfur transformation in CWs is intertwined with one another  
59 (Baldwin, 2016; Zhou, 2007). Table 1 summarized the biochemical reactions involved in coupling carbon,  
60 nitrogen, and sulfur transformation commonly observed in the CWs. However, experiments on coupling



61 carbon, nitrogen, and sulfur transformation were primarily conducted in lab-scale CWs, and studies on  
 62 full-scale systems are limited. Investigation on carbon, nitrogen, and sulfur transformation in full-scale  
 63 systems, especially in long-term operated CWs, is required.

64 **Table 1 Potential biochemical reactions involved in coupling carbon, nitrogen, and sulfur**  
 65 **transformation in the CWs <sup>a</sup>**

Biochemical reactions	No.	Type <sup>b</sup>	E acceptor	E donor	Functional Microorganisms	Ref.
<b>Methanogenesis</b> $CH_3COO^- + H_2O \rightarrow CH_4 + HCO_3^-$	(1)	H	$CH_3COO^-$	$CH_3COO^-$	Methanogens	(Fenchel et al., 2012)
<b>Nitrification</b> $2NH_4^+ + 3O_2 \rightarrow 2NO_2^- + 4H^+ + 2H_2O$	(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)
<b>Anaerobic ammonia oxidation (Anammox)</b> $NH_4^+ + NO_2^- \rightarrow N_2 + 2H_2O$	(4)	A	$NO_2^-$	$NH_4^+$	Anammox bacteria	(Holmes et al., 2019)
<b>Sulfate-reducing anaerobic ammonia oxidation</b> $8NH_4^+ + 3SO_4^{2-} \rightarrow 4N_2 + 3HS^- + 12H_2O + 5H^+$	(5)	A	$SO_4^{2-}$	$NH_4^+$	Anammox bacteria	(Rikmann et al., 2012)
<b>Heterotrophic denitrification</b> $4NO_3^- + CH_3COO^- \rightarrow 4NO_2^- + 2HCO_3^- + H^+$	(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)
<b>Autotrophic denitrification</b> $HS^- + 4NO_3^- \rightarrow 4NO_2^- + SO_4^{2-} + H^+$	(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)
<b>Dissimilatory nitrate reduction to ammonium (DNRA)</b> $NO_3^- + CH_3COO^- + H^+ + H_2O \rightarrow NH_4^+ + 2HCO_3^-$	(12)	H	$NO_3^-$	$CH_3COO^-$	Sulfide-oxidizing bacteria	(Castro-Barros et al., 2017)
<b>Dissimilatory sulfate reduction</b> $SO_4^{2-} + CH_3COO^- \rightarrow HS^- + 2HCO_3^-$	(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

66

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

73 operated CWs.

## 74 **2. Materials and Methods**

### 75 **2.1 Study site**

76 The sediments used in this study were collected from a full-scale hybrid CW in Chongqing, China  
77 which is operating since January 2011. This hybrid CW consists of pre-treatment stages, a vertical-  
78 baffled flow wetland (VBFW, first stage), a horizontal subsurface flow wetland (HSFW, second stage)  
79 and a clean water pond for post-treatment (Zhai et al., 2016) (Figure S1). The influent of the CW was  
80 mainly composed of the municipal wastewater and partial agriculture wastewater at the flow rate of 500-  
81 600 m<sup>3</sup>/d. The studied HSFW contained 3-5 sections of HSFW bed (Figure S1) with a water depth of  
82 0.4-0.6m (Zhai et al., 2016). The HSFW was operated under a combination of anoxic and aerobic  
83 conditions (DO < 0.5mg/L, 100mV < ORP < 150 mV) with effective TN removal (~77%) (Zhai et al.,  
84 2016).

### 85 **2.2 Sediment sampling**

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

90 During each sampling period, composite sediment samples were collected from 6 locations by  
91 opening the HSFW bed at the depth of approximately 10cm below the sediment surface (50-70cm below  
92 the water surface). Therefore, the anaerobic sediment samples were taken. The collected sediment  
93 samples were immediately transferred into the containers which were flushed by pure N<sub>2</sub> and sealed  
94 before use. The collected sediment samples from 6 location will be mixed homogenously after pre-



95 treatment. The pre-treatment of the samples included two steps: (1) the sediment was sieved using a 100-  
96 mesh sieve to remove small stones, grass, and other waste; and (2) the sieved samples were further mixed  
97 and settled for solid-liquid separation. The liquid portion was discarded, and the solid portion was used  
98 as inoculum in the batch experiments and subsequent microbial investigations. All the sediment samples  
99 were stored in an icebox and kept at  $-4\text{ }^{\circ}\text{C}$  during the transport to the laboratory. Based on the temperature  
100 during the sampling campaign, the whole experiment was broadly divided into the high-temperature  
101 season (HT), from May to October with an average temperature of  $30\pm 2\text{ }^{\circ}\text{C}$ , and the low-temperature  
102 season (LT), from November to April next year with an average temperature of  $16\pm 2\text{ }^{\circ}\text{C}$ .

### 103 **2.3. Batch experiments**

104 The collected sediments were used as inoculum in the batch experiments to investigate coupling  
105 transformation of carbon, nitrogen, and sulfur compounds by using various initial concentrations.  
106 Approximately 100 mL pre-treated sediment samples were added into a 1000 mL bottle containing 500  
107 mL medium (the recipe is shown in Table S1). All the bottles were flushed with helium for 20 min to  
108 ensure anaerobic condition. The bottles were cultivated at the temperature which was same as the water  
109 temperature of the water layer above the sediment. The temperature was maintained by the air bath for  
110 48h.

111 During the experiments, sodium acetate ( $\text{CH}_3\text{COONa}$ ), ammonium chloride ( $\text{NH}_4\text{Cl}$ ), sodium nitrite  
112 ( $\text{NaNO}_2$ ), sodium nitrate ( $\text{NaNO}_3$ ), sodium sulfide ( $\text{Na}_2\text{S}$ ) and potassium sulfate ( $\text{K}_2\text{SO}_4$ ) were added at  
113 three different initial concentrations (Low, Medium, and High). The low concentration groups did not  
114 add target compounds while the medium concentration group added sufficient amount of target  
115 compounds. In the high concentration groups, the supplied amounts of target compounds was doubled.  
116 These concentrations were selected to reveal what biochemical process was present in the CW for the



117 transformation of carbon, nitrogen and sulfur, and also reveal the potential maximum capacity of  
 118 transformation. Using the same approach, the blank control with autoclaved sediments was carried out  
 119 in parallel with the other experiments. In total, 33 experiments in six trials were carried out to investigate  
 120 the key carbon, nitrogen, and sulfur transformation processes (Table 2).

121 **Table 2** Feed composition in the batch test trials

Test trial	Batch No.	CH <sub>3</sub> COONa	NH <sub>4</sub> Cl	NaNO <sub>2</sub>	NaNO <sub>3</sub>	Na <sub>2</sub> S	K <sub>2</sub> SO <sub>4</sub>
		(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 <sub>4</sub> <sup>+</sup> 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 <sub>2</sub> <sup>-</sup> 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 <sub>3</sub> <sup>-</sup> 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 <sup>2-</sup> 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 <sub>4</sub> <sup>2-</sup> 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

122  
 123 During the batch experiments, samples were collected at 0, 24 and 48 h. The bottles were mixed  
 124 gently and settled for at least 30 min. Then, 50 mL water samples were taken using a syringe, filtered  
 125 immediately on a 0.45µm pore size membrane filter, and analyzed for total organic carbon (TOC),  
 126 dissolved inorganic carbon (DIC), total carbon (TC), NH<sub>4</sub><sup>+</sup>, NO<sub>2</sub><sup>-</sup>, NO<sub>3</sub><sup>-</sup>, total dissolved inorganic nitrogen



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

#### 131 **2.4 Microbiological investigations**

132 The sediments were centrifuged and washed by sterilized Mili Q water for 2 times at 5000 rpm for  
133 15min. The liquid parts after centrifugation were collected and mixed, and the mixture was centrifuged  
134 at 8000 rpm for 5 min at 4 °C. Then, the solid part was used for DNA extraction in the microbial analysis.  
135 The DNA extraction was performed with an EZNA<sup>®</sup> Soil DNA Kit (Omega Bio-tek, Norcross, GA, USA)  
136 according to the manufacturer's instructions.

137 The extracted DNA was amplified with a pair of primers 338F (5'-AC TCC TAC GGG AGG CAG  
138 A-3') and 806R (5'-GG ACT ACH VGG GTW TCT AAT-3'). Reaction mixtures (20 $\mu$ L) for PCR  
139 contained: 10 ng of template DNA, 0.8  $\mu$ L of each primer (5  $\mu$ M), 4 $\mu$ L of 5-fold Fast*Pfu* Buffer, 2  $\mu$ L of  
140 dNTPs (2.5 mM), 0.4  $\mu$ L of Fast *Pfu* Polymerase and ultrapure H<sub>2</sub>O. The PCR procedure was: initial  
141 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  
142 s; final extension at 72 °C for 10 min. The PCR products were extracted, purified with an AxyPrep DNA  
143 Gel Extraction Kit (Axygen Biosciences, Union City, CA, USA), quantified with QuantiFluor<sup>™</sup> -ST  
144 (Promega, USA), and then sequenced on the Illumina MiSeq platform (Shanghai Majirbio Technology  
145 Co., Ltd., China). The operational taxonomic units (OTU) of the identified 16S rRNA gene sequences  
146 were analyzed by the Majorbio I-Sanger Cloud online platform ([www.i-sanger.com](http://www.i-sanger.com)), using RDP  
147 classifier Bayesian Algorithms (3% difference of the sequence as classification standards).

#### 148 **2.5 Analytical methods**

149 Analysis of gas samples for CH<sub>4</sub> and CO<sub>2</sub> was performed by a gas chromatograph (7820A, Agilent,  
150 USA) equipped with a thermal conductivity detector (TCD), a hydrogen flame ionization detector (HFID)  
151 and packed columns of MolSieve 5A, Heyesep Q. Pure helium was supplied as the carrier gas during the  
152 measurement. The Ideal-Gas Equation was used to convert the percentage of CH<sub>4</sub> and CO<sub>2</sub> into the  
153 concentration of the gaseous C (mg C/L).

154 Analysis of total organic carbon (TOC) and dissolved inorganic carbon (DIC) was performed with  
155 a TOC analyzer (TOC-L, SHIMADZU, Japan). The analysis of anions in the liquid phase including NO<sub>2</sub><sup>-</sup>,  
156 NO<sub>3</sub><sup>-</sup>, and SO<sub>4</sub><sup>2-</sup> were performed by an ion chromatograph equipped with anion self-regenerating  
157 suppressor (Dionex ASRS 600, 4 mm), an IonPac AS19 separation column (Dionex AS19, 4 × 250  
158 mm) and an IonPac AG19 anion guard column (Dionex AG19, 4 × 50 mm) according to the standard  
159 methods (Rice et al., 2012). The details can be found in supplementary materials (Text S1). The S<sup>2-</sup>  
160 analysis was performed by methylene blue colorimetric method based on the standard methods (Rice et  
161 al., 2012).

## 162 2.6. Data analysis

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



171 produced via Origin Pro 2018 (OriginLab co., USA).

## 172 **3. Results and Discussion**

### 173 **3.1 Effects of organic carbon on N and S transformation**

174 The effects of organic carbon (TOC) on N and S transformation (Batch 1) were shown in Figure 1.

175 In the low temperature (LT) season, increasing initial TOC dosages led to decrease  $\text{NO}_2^-$  generation and

176 obtained slight removal (34.05% of generation to 10.98% of removal,  $p=0.12$ ), and higher  $\text{NO}_3^-$  removal

177 (0.26% to 30.06%,  $p=0.41$ ). The  $\text{SO}_4^{2-}$  generation was declined from 18.85% to 10.81% ( $p=0.73$ ) at high

178 TOC dosage. However, increasing TOC dosages had no effects on the removal of  $\text{NH}_4^+$  (1.10% to 1.66%,

179  $p=0.72$ ) and  $\text{S}^{2-}$  (51.21% to 59.60%,  $p=0.95$ ). The results also showed that the differences of N and S

180 compound transformation at different TOC levels in LT seasons were insignificant ( $p > 0.05$ ).

181 Similar to the LT, in high temperature (HT) season, increasing initial TOC dosages had no effects

182 on  $\text{NH}_4^+$  removal (5.63% to 5.92%,  $p=0.66$ ) and  $\text{S}^{2-}$  removal (51.05% to 58.45%,  $p=0.52$ ). But increasing

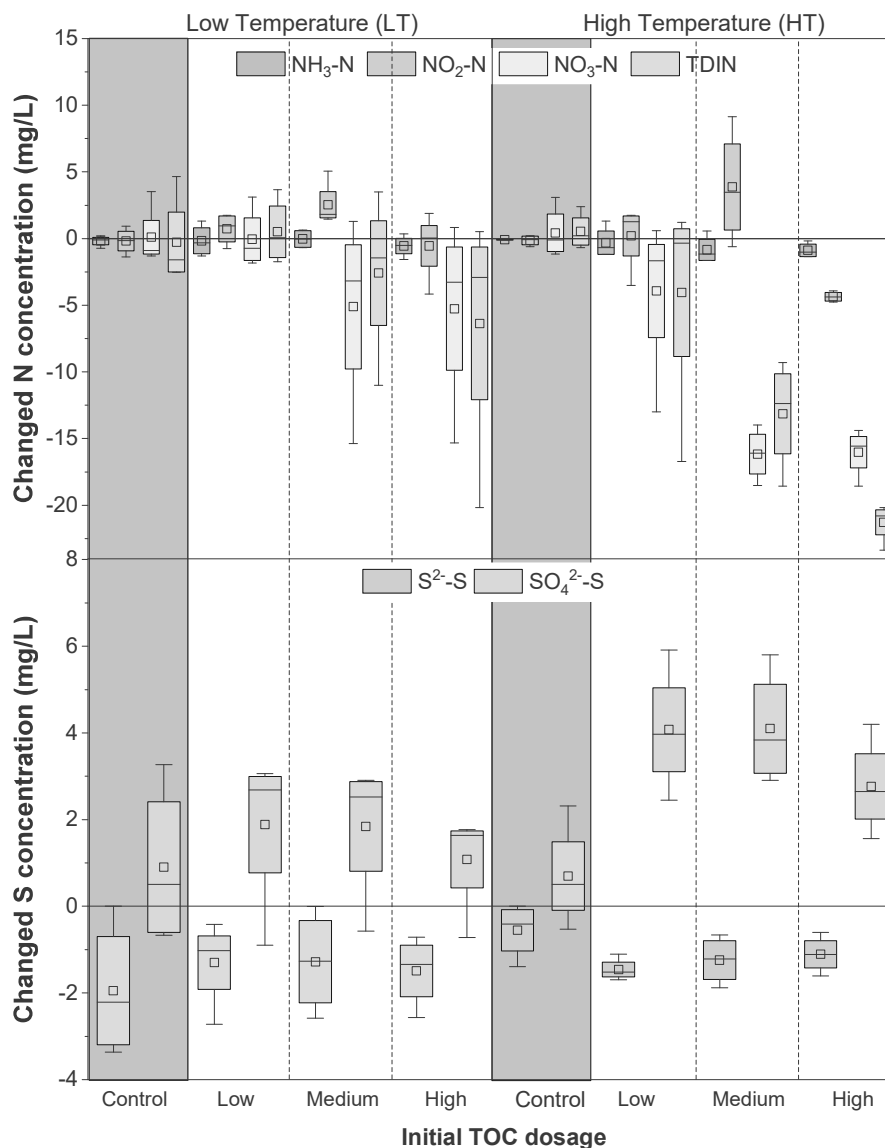
183 TOC dosages significantly promote both  $\text{NO}_2^-$  transformation from 77.33% of generation to 87.23% of

184 removal ( $p=0.009 < 0.01$ ), and  $\text{NO}_3^-$  removal increased from 31.06% to 92.36% ( $p=0.008 < 0.01$ ). Besides,

185 the generation of  $\text{SO}_4^{2-}$  was lowered at higher TOC dosages (40.97% to 27.64%), though the difference

186 was insignificant ( $p=0.29$ ).

187



188

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

193

### 194 3.2 Effects of N compounds on carbon, nitrogen, and sulfur transformation

#### 195 (1) $NH_4^+$

196 Effects of  $NH_4^+$  on N and S transformation with and without organic carbon (Batch 2) was shown

197 in Figure 2. Without organic carbon, increasing  $\text{NH}_4^+$  dosages had no significant effects on the  $\text{NH}_4^+$   
198 removal (LT:1.63% to 2.74%,  $p=0.96$ ; HT: 1.97% to 2.96%,  $p=0.96$ ),  $\text{NO}_3^-$  removal (LT: 3.99% to 7.07%,  
199  $p=0.94$ ; HT:15.79% to 22.43%,  $p=0.89$ ),  $\text{S}^{2-}$  removal (LT: 40.65% to 44.52%,  $p=0.97$ ; HT:43.59% to  
200 55.29%,  $p=0.38$ ), and  $\text{SO}_4^{2-}$  generation (LT: 2.27% to 8.39%,  $p=0.81$ ; HT:36.34% to 40.74%,  $p=0.92$ ) in  
201 both LT and HT season. The  $\text{NO}_2^-$  generation in LT season was stable (15.35% to 19.84%,  $p=0.51$ ) but  
202 insignificant decrease from 11.13% to 3.89% ( $p=0.92$ ) in the HT season.

203 When organic carbon added, increasing  $\text{NH}_4^+$  dosages led to stable removal of TOC (LT: 24.65% to  
204 31.64%,  $p=0.96$ ; HT: 76.62% to 84.76%,  $p=0.19$ ). Similarly, removal of  $\text{NH}_4^+$  (LT: 2.66% to 4.89%,  
205  $p=0.85$ ; HT:5.26% to 6.05%,  $p=0.44$ ),  $\text{NO}_3^-$  (LT:37.31% to 37.69%,  $p=0.99$ ; HT: 98.89% to 99.39%,  
206  $p=0.82$ ),  $\text{S}^{2-}$  (LT: 31.56% to 39.65%,  $p=0.91$ ; HT: 47.19% to 57.44%,  $p=0.80$ ), and generation of  $\text{NO}_2^-$   
207 (LT: 68.59% to 74.19%,  $p=0.14$ ; HT: 262.82% to 323.25%,  $p=0.23$ ) and  $\text{SO}_4^{2-}$  (LT: 12.94% to  
208 18.64%, $p=0.91$ ; HT:24.98% to 35.49%,  $p=0.51$ ) at different  $\text{NH}_4^+$  dosages had no significant differences.

## 209 (2) $\text{NO}_2^-$

210 Effects of  $\text{NO}_2^-$  on N and S transformation with and without organic carbon (Batch 3) was shown  
211 in Figure 2. Without organic carbon, increasing initial  $\text{NO}_2^-$  dosages did not affect  $\text{NH}_4^+$  removal (LT:  
212  $p=0.80$ ; HT:  $p=0.08$ ). Besides, the  $\text{NO}_2^-$  accumulation (LT:  $p=0.78$ ; HT:  $p=0.29$ ) and  $\text{NO}_3^-$  removal (LT:  
213  $p=0.83$ ; HT:  $p=0.70$ ) were stable, as well as the  $\text{S}^{2-}$  removal (LT:  $p=0.79$ ; HT:  $p=0.40$ ) and  $\text{SO}_4^{2-}$   
214 generation (LT:  $p=0.63$ ; HT:  $p=0.65$ ) were also stable at various initial  $\text{NO}_2^-$  dosages.

215 Adding organic carbon to  $\text{TOC}=25\text{mgC/L}$  led to higher  $\text{NO}_3^-$  removal and  $\text{NO}_2^-$  accumulation (Figure  
216 2, S3). However, increasing initial  $\text{NO}_2^-$  in presence of TOC still had no effects on TOC removal (LT:  
217 19.80% to 20.54%,  $p=0.86$ ; HT: 77.96% to 82.61%,  $p=0.75$ ),  $\text{NH}_4^+$  (LT:  $p=0.91$ ; HT:  $p=0.12$ ) and  $\text{NO}_3^-$   
218 removal (LT:  $p=0.96$ ; HT:  $p=0.91$ ) and  $\text{NO}_2^-$  generation (LT:  $p=0.53$ ; HT:  $p=0.89$ ) transformation, and



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

### 220 (3) $NO_3^-$

221 In Batch 4, different dosages of  $NO_3^-$  (0, 10, 20 mgN/L) were added. When no organic carbon was  
222 added, increasing initial  $NO_3^-$  dosages had no significant influence on the transformation of carbon,  
223 nitrogen, and sulfur compounds in both LT and HT seasons (Figure 2, S3,  $P > 0.10$ , Table S2). When  
224 organic carbon was added (TOC=25 mgC/L), increasing initial  $NO_3^-$  dosages will promote TOC removal  
225 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  
226 season. Besides, differences of  $NO_3^-$  removal (LT:  $p=0.19$ , HT:  $p=1.35 \times 10^{-6}$ ) and  $NO_2^-$  accumulation  
227 (LT:  $p=0.21$ , HT:  $p=0.008$ ) at different  $NO_3^-$  dosages was also significant, especially in the HT season.  
228 The  $S^{2-}$  removal and  $SO_4^{2-}$  generation was not significantly different at various  $NO_3^-$  dosages ( $p > 0.52$ ).  
229 Overall, current results indicate that the variation in initial N compounds had little or no significant  
230 influences on carbon, nitrogen, and sulfur transformation throughout the batch experimental periods.

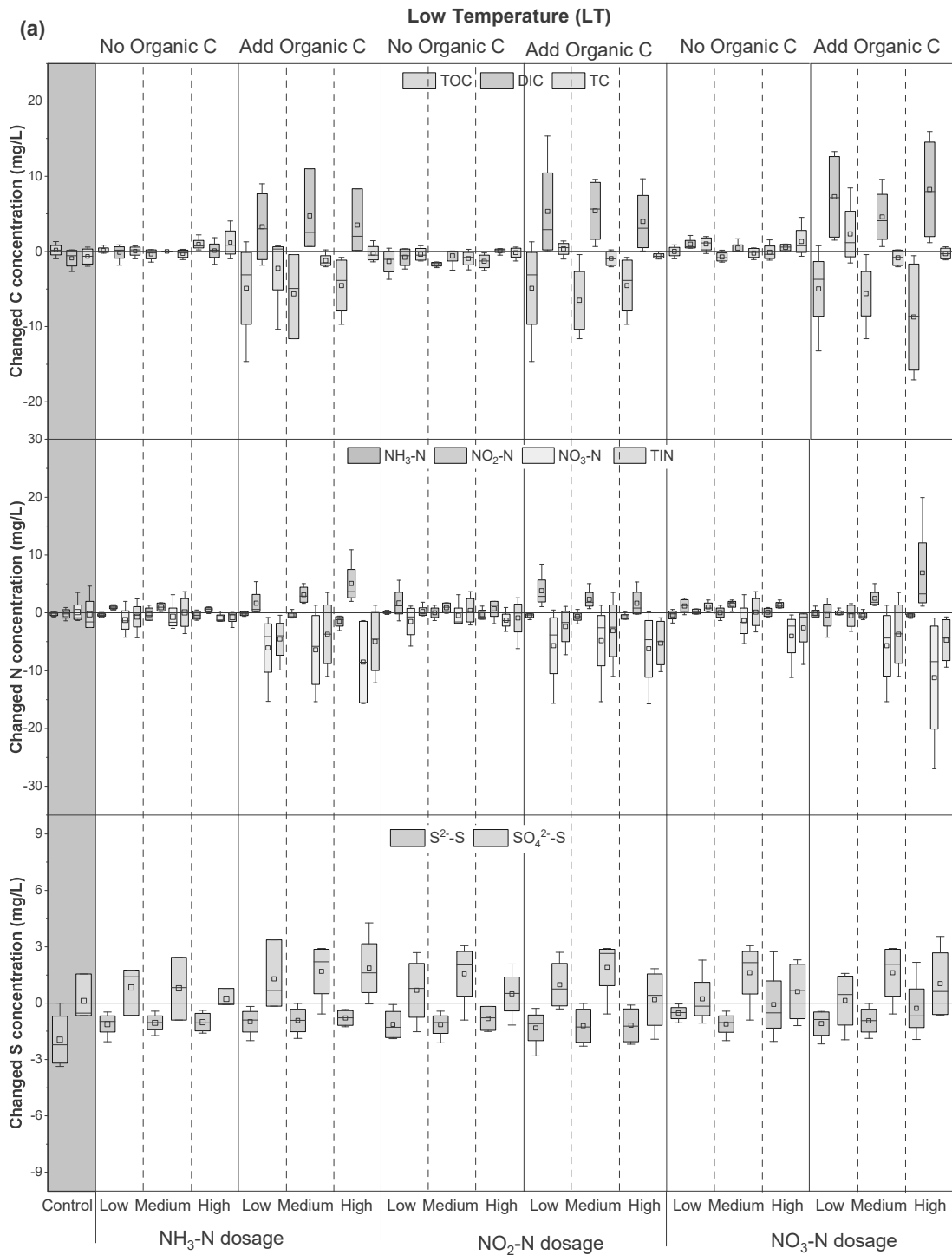
231 At neutral pH used in our experiment, the main form of ammonia nitrogen was  $NH_4^+$ . A high  
232 concentration of  $NH_4^+$  was regarded as an inhibitor in anaerobic digestion but the ammonium toxicity  
233 was very reversible (Yenigün and Demirel, 2013). The high  $NH_4^+$  concentration is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

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



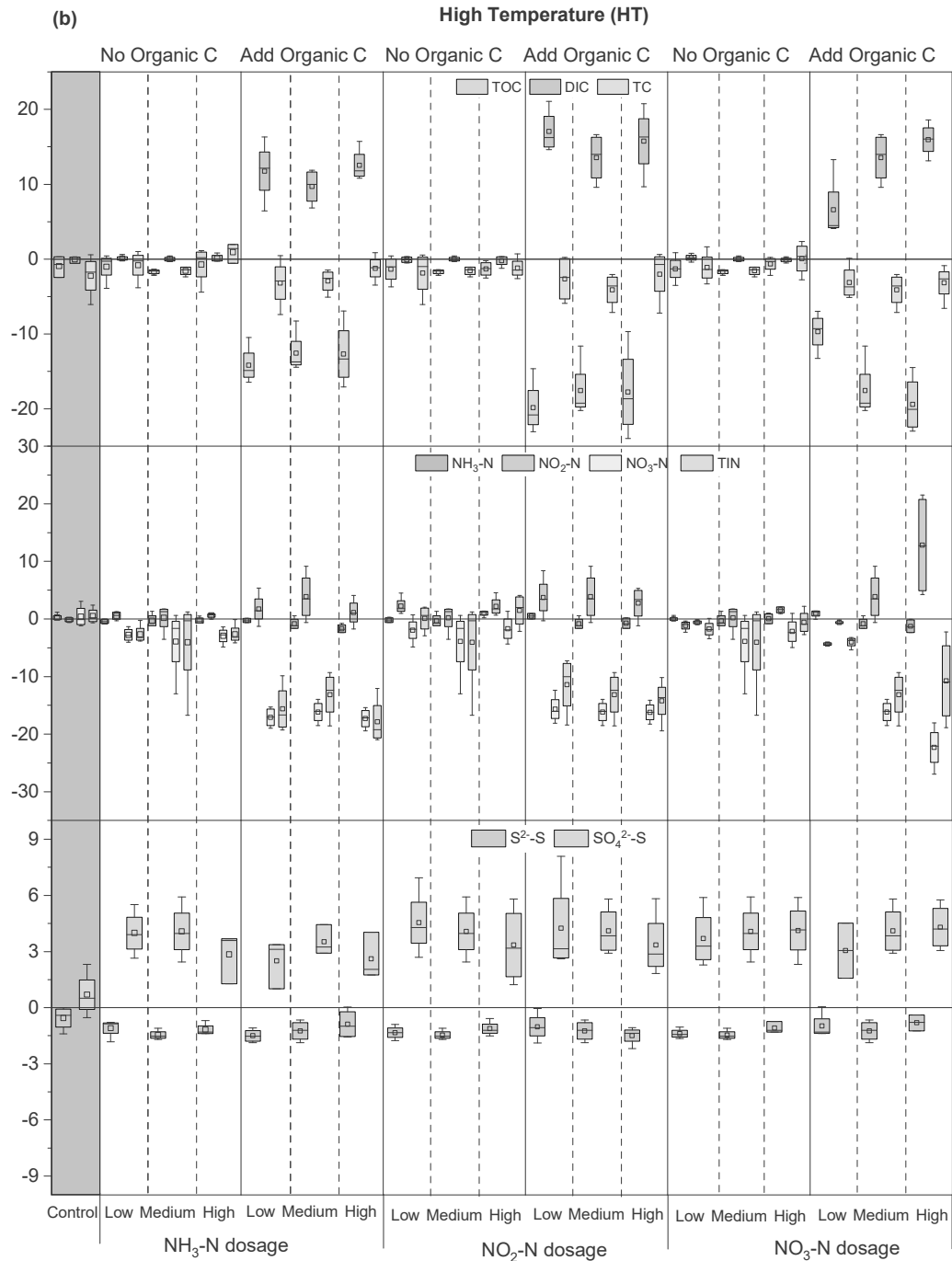
241 (Glass et al., 1997; Tang et al., 2020). In our experiment, increasing  $\text{NO}_2^-$  dosage showed no obvious

242 inhibiting effects on microbial carbon, nitrogen, and sulfur transformation.



243

244



245

246 **Figure 2. Transformation of Carbon (C), Nitrogen (N) and Sulfur (S) compounds at different**

247 **initial dosage of N compounds. (a) in the low temperature seasons; (b) in the high temperature**

248 **seasons. Control: Abiotic control group; Low: [NH<sub>4</sub><sup>+</sup>]<sub>0</sub>=0 mgN/L, [NO<sub>2</sub><sup>-</sup>]<sub>0</sub>=0 mgN/L, [NO<sub>3</sub><sup>-</sup>]<sub>0</sub>=0**

249 **mgN/L; Medium: [NH<sub>4</sub><sup>+</sup>]<sub>0</sub>=15 mgN/L, [NO<sub>2</sub><sup>-</sup>]<sub>0</sub>=5 mgN/L, [NO<sub>3</sub><sup>-</sup>]<sub>0</sub>=10 mgN/L; High: [NH<sub>4</sub><sup>+</sup>]<sub>0</sub>=30**





250 mgN/L,  $[\text{NO}_2^-]_0=10$  mgN/L,  $[\text{NO}_3^-]_0=20$  mgN/L. The positive values represent generation and the  
251 negative values represent removal. The small square represents for the average value.

252

### 253 3.3 Effects of S compounds on carbon, nitrogen, and sulfur transformation

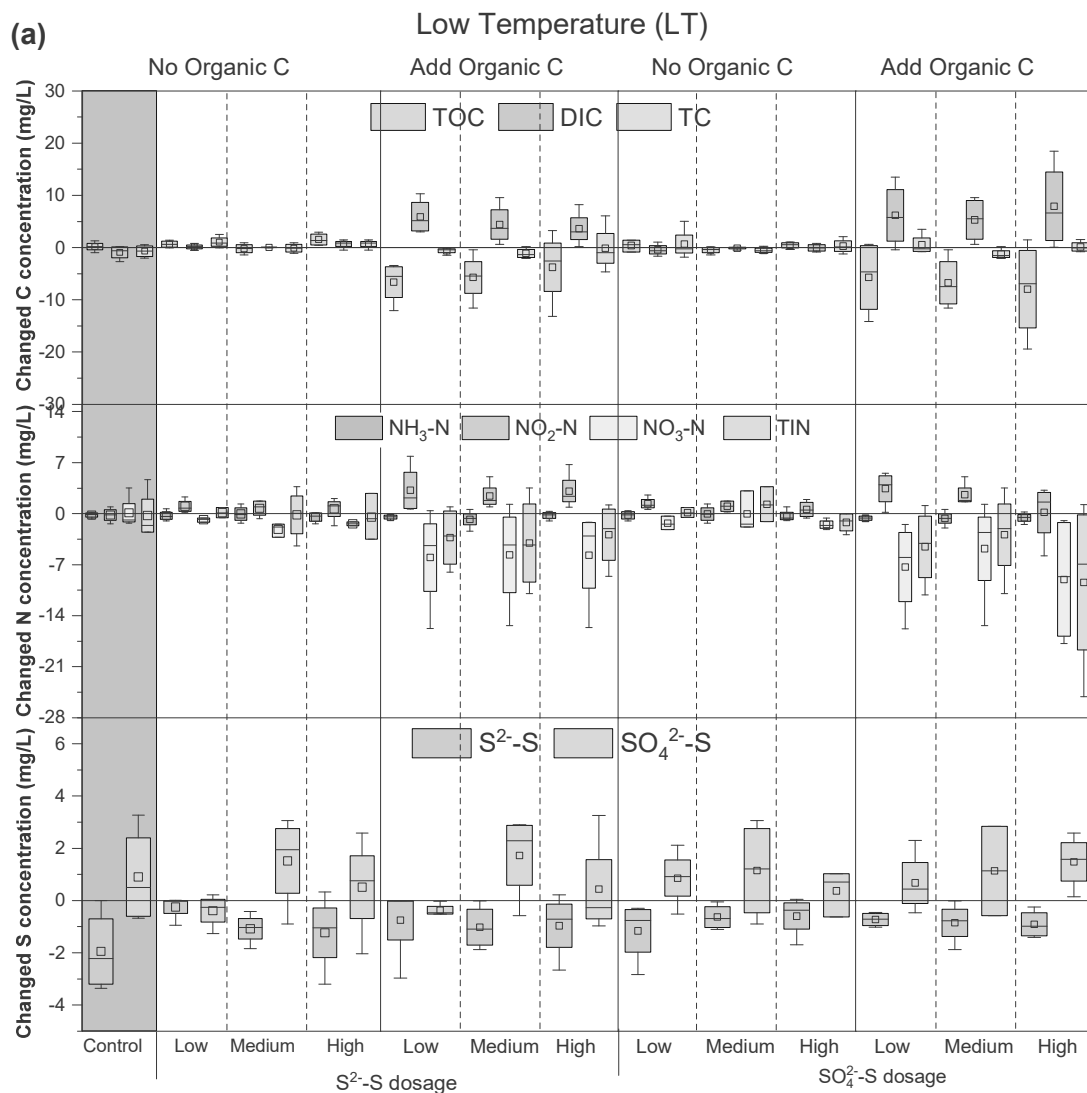
#### 254 (1) $\text{S}^{2-}$

255 In Batch 5, various initial  $\text{S}^{2-}$  dosages (0, 2.5, and 5 mgS/L) were added into the batches. When there  
256 was no organic carbon added, increasing initial  $\text{S}^{2-}$  dosages led to higher  $\text{NO}_3^-$  removal and  $\text{NO}_2^-$   
257 generation (Figure 3, S4) but the differences were not significant. The removal efficiency of  $\text{NO}_3^-$   
258 increased from 2.8% to 11.9% in LT season ( $p=0.98$ ) and from 7.1% to 25.2% in HT season ( $p=0.53$ ).  
259 Meanwhile, the  $\text{S}^{2-}$  removal (LT:  $p=0.19$ , HT:  $p=0.19$ ) increased but  $\text{SO}_4^{2-}$  generation decreased (LT:  
260  $p=0.14$ , HT:  $p=0.09$ ). Adding organic carbon improved  $\text{NO}_3^-$  removal and  $\text{NO}_2^-$  generation (Figure 3, S4),  
261 but increasing initial  $\text{S}^{2-}$  dosages resulted in no significant differences in  $\text{NO}_3^-$  removal (LT:  $p=0.99$ , HT:  
262  $p=0.95$ ) and  $\text{NO}_2^-$  generation (LT:  $p=0.90$ , HT:  $p=0.96$ ). As to the S compounds, higher initial  $\text{S}^{2-}$  dosages  
263 promoted  $\text{S}^{2-}$  removal (LT:  $p=0.81$ , HT:  $p=0.02$ ) but led to less  $\text{SO}_4^{2-}$  generation (LT:  $p=0.07$ , HT:  $p=0.27$ ).

#### 264 (2) $\text{SO}_4^{2-}$

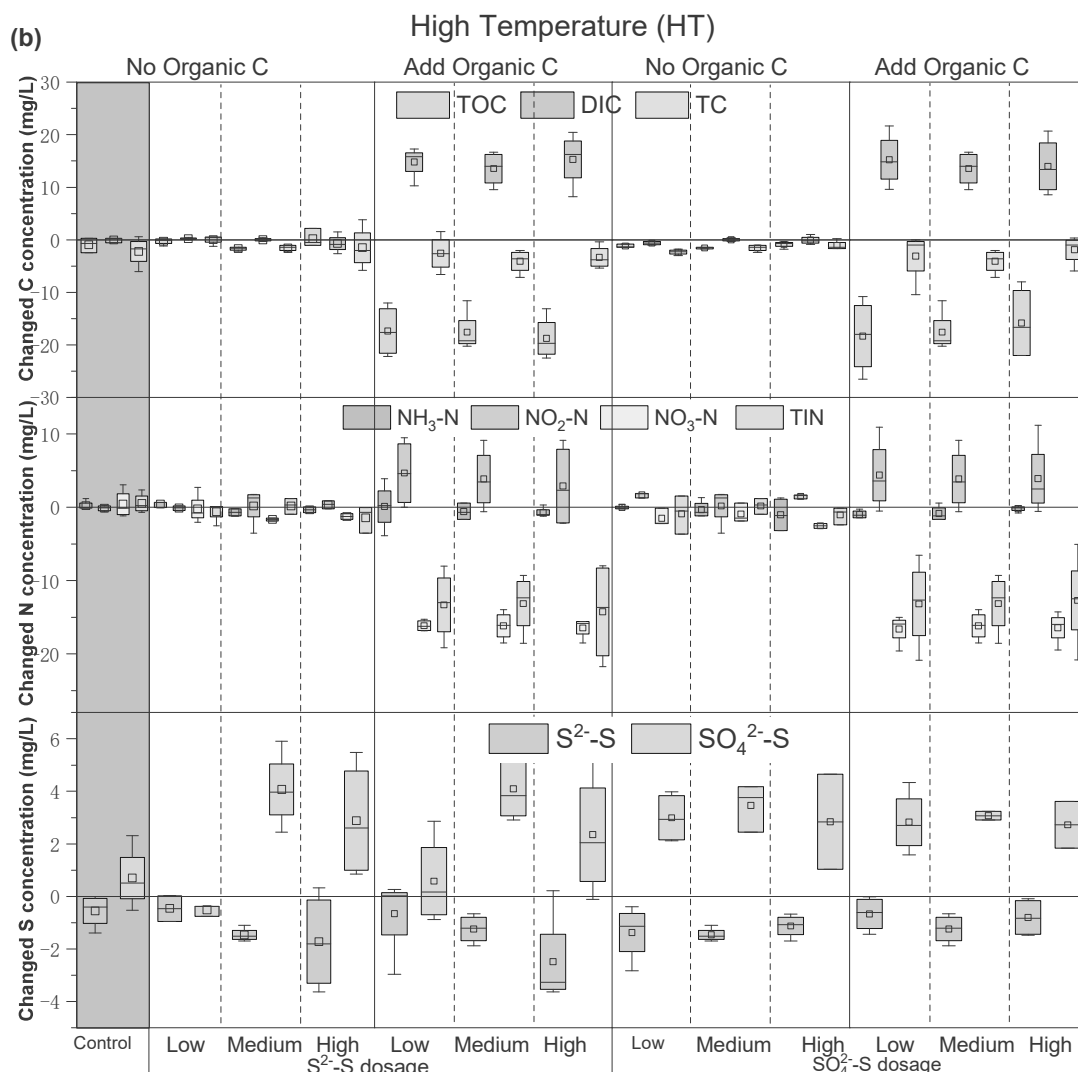
265 In the Batch 6, initial  $\text{SO}_4^{2-}$  dosages were varied in the experiment (0, 10, 20 mgS/L). When no  
266 organic carbon was added, increasing initial  $\text{SO}_4^{2-}$  dosages had no influence on carbon, nitrogen, and  
267 sulfur transformation ( $p>0.16$ , Figure 3, S4, Table S2). When organic carbon was added to 25 mgC/L  
268 (TOC), increasing initial  $\text{SO}_4^{2-}$  dosages had no influence on TOC removal (LT:  $p=0.90$ , HT:  $p=0.77$ ),  
269  $\text{NO}_3^-$  removal (LT:  $p=0.98$ , HT:  $p=0.95$ ) and  $\text{NO}_2^-$  generation (LT:  $p=0.75$ , HT:  $p=0.98$ ). Even though the  
270  $\text{S}^{2-}$  removal was stable (LT:  $p=0.89$ , HT:  $p=0.47$ ), higher  $\text{SO}_4^{2-}$  dosages seems lead to less  $\text{SO}_4^{2-}$  generation  
271 (LT:  $p=0.69$ , HT:  $p=0.95$ ).

272 It is notable that the amount of S forming  $\text{SO}_4^{2-}$  was higher than the detected  $\text{S}^{2-}$  converted. This is  
 273 because  $\text{S}^{2-}$  was easily form precipitations like FeS and retained in solid form, leading the total amount  
 274 of  $\text{S}^{2-}$  is higher than the amount in the liquid phase. In our experiments, the detected  $\text{S}^{2-}$  initial  
 275 concentration was  $1.31 \pm 0.25$  mg S/L, lower than the 2.5 mg S/L supplied by adding  $\text{Na}_2\text{S}$  (Table 2).  
 276 The solid  $\text{S}^{2-}$  was also feasible electron donors for sulfide-based autotrophic denitrification (Eq. 12) (Hu  
 277 et al., 2020; Wei et al., 2017) and converted into  $\text{SO}_4^{2-}$ . Generally, varying S compounds will affect the  
 278 transformation of N but the differences were insignificant.



279

280



281

282 **Figure 3. Transformation of Carbon (C), Nitrogen (N) and Sulfur (S) compounds at different initial**  
 283 **dosage of S compounds. (a) in the low temperature seasons; (b) in the high temperature seasons.**

284 Control: Abiotic control group; Low: [S<sup>2-</sup>]<sub>0</sub>=0 mgS/L, [SO<sub>4</sub><sup>2-</sup>]<sub>0</sub>=0 mgS/L; Medium: [S<sup>2-</sup>]<sub>0</sub>=2.5 mgS/L,  
 285 [SO<sub>4</sub><sup>2-</sup>]<sub>0</sub>=10 mgS/L; High: [S<sup>2-</sup>]<sub>0</sub>=5 mgS/L, [SO<sub>4</sub><sup>2-</sup>]<sub>0</sub>=20 mgS/L. The positive values represent generation  
 286 and the negative values represent removal. The small square represents for the average value.

287

### 288 3.4 Electron balance analysis and potential biochemical processes.

289 The electron balance analysis was performed to help identify the potential biochemical processes  
 290 that occurred in the batch experiments. When there was no organic carbon added, the electron donation

291 was mainly from  $S^{2-}$  oxidation to  $SO_4^{2-}$ . Even though  $NH_4^+$  was also an electron donor in processes like  
292 anammox, the electrons donated from  $NH_4^+$  oxidation could be neglectable because the transformation  
293 of  $NH_4^+$  was low ( $< 8\%$ ) and insignificant in all batches (Figure 1-3, S3, Table S2). The theoretical  
294 electron requirement for 1 mol  $NO_2^-$  and 1 mol  $NO_3^-$  converting into  $N_2$  was 3 mol and 5 mol, respectively.

295 In the experiment without adding organic carbon, the donated electron calculated based on the  $SO_4^{2-}$   
296 generation was close to the accepted electrons calculated by  $NO_3^-$  and  $NO_2^-$  reduction (Details in Text  
297 S1). The results showed that the sulfide-based autotrophic denitrification was present in the studied CW.  
298 In our experiment, both increasing  $NO_2^-$  dosages (Batch 3) and increasing  $NO_3^-$  dosages (Batch 4) had  
299 no effects on  $S^{2-}$  removal or  $SO_4^{2-}$  generation, but increasing initial  $S^{2-}$  dosages (Batch 5) led to higher  
300  $NO_3^-$  removal and  $NO_2^-$  generation. The result was different from previous studies, in which increasing  
301  $NO_3^-$  could promote sulfide-based autotrophic denitrification (Sun and Nemati, 2012). In our experiment,  
302 the limiting factor for sulfide-based denitrification was the concentration of  $S^{2-}$  instead of concentrations  
303 of electron acceptors. The  $NO_2^-$  accumulation was also observed (Figure 2,3) as reported previously in  
304 sulfur/sulfide-based autotrophic denitrification (Ge et al., 2012; Liu et al., 2017). That was because nitrate  
305 reductase (Nar) is more competitive to obtain electrons comparing with nitrite reductase (Nir) (Ge et al.,  
306 2012), leading to  $NO_3^-$  a preferable electron acceptor in sulfide-based autotrophic denitrification.

307 When organic carbon ( $CH_3COO^-$  in the experiment) was added, TOC was also an important electron  
308 donor. In theory, oxidizing 1 mol TOC into  $CO_2$  could donate 4 mol electron. Electron balance analysis  
309 showed that the heterotrophic denitrification occurred when organic carbon was added (Text S1). The  $S^{2-}$   
310 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  
311 organic carbon was almost the same in all batches (Table S2), indicating that the sulfide-based  
312 autotrophic denitrification was co-existed with heterotrophic denitrification. Simultaneous sulfur/sulfide-



313 based autotrophic and heterotrophic denitrification has been reported in CWs and other anaerobic  
314 bioreactors (Guerrero et al., 2016; Ma et al., 2020; Oh et al., 2001). Initial TOC dosage had no effects on  
315  $S^{2-}$  removal (LT:  $p=0.94$ ; HT:  $p=0.52$ ) and the slight decrease of  $SO_4^{2-}$  generation (LT:  $p=0.73$ ; HT:  
316  $p=0.29$ ), showing that promote heterotrophic denitrification will not inhibit sulfide-based autotrophic  
317 denitrification.

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

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

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

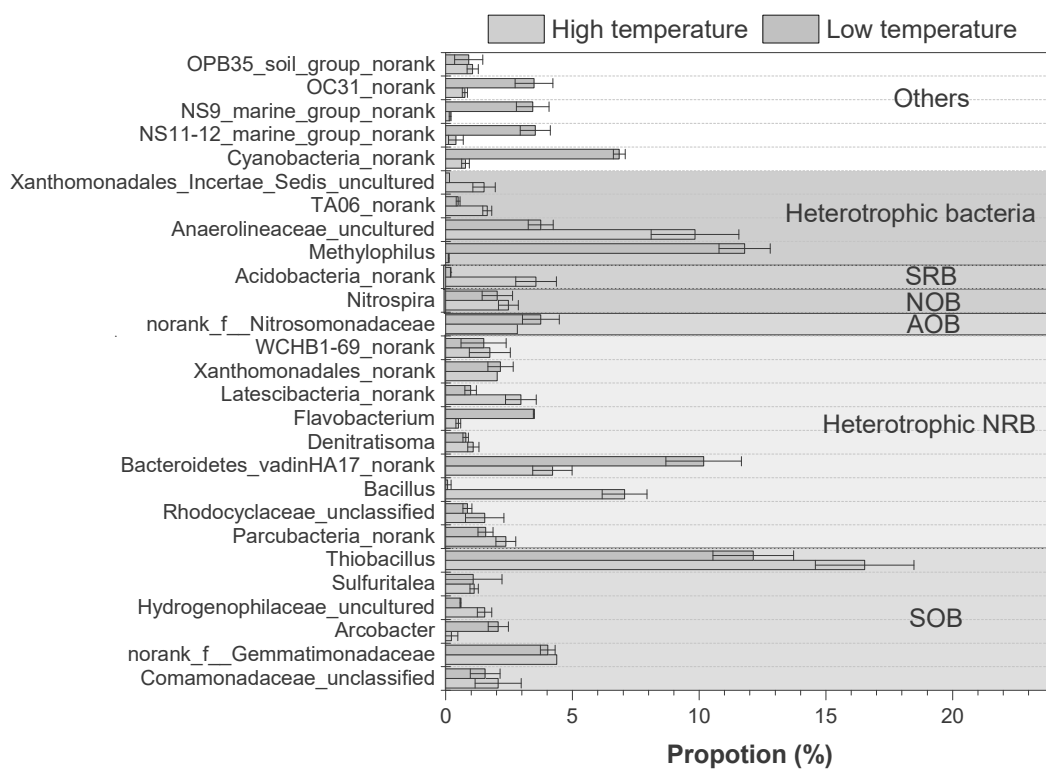
334 As shown in Figure 4, the dominant bacteria (proportion > 1% in the genus level) were categorized

335 based on their function in carbon, nitrogen, and sulfur transformation processes. The complete list of  
336 different functional bacteria was listed in Table S3. The dominant genus in the community was  
337 *Thiobacillus* in both HT season (16.5%) and LT season (12.1%). This genus was associated with sulfide  
338 oxidation and the *T. denitrificans* was a well-known bacterium mediated sulfide-based autotrophic  
339 denitrification (Lens, 2009; Ma et al., 2020). Previous studies showed that the *Thiobacillus* genus would  
340 be enriched in sulfide(FeS)-based autotrophic denitrification (Yang et al., 2017). In HT season,  
341 *Anaerolineaceae\_uncultured* (9.8 %) and *Bacillus* (7.1%) were both dominant genera. The  
342 *Anaerolineaceae\_uncultured* and *Thiobacillus* were both ubiquitous and abundant in both autotrophic  
343 and heterotrophic denitrification (Huang et al., 2021). This genus could consume organic carbon, creating  
344 C-limiting conditions for autotrophic denitrification, and it could convert the complex organic  
345 compounds into small molecular organic matters, promoting heterotrophic denitrification. The *Bacillus*  
346 contained strains could mediate denitrification (Lu et al., 2012) and sulfur oxidation (Ryu et al., 2009).  
347 In the LT season, *Methylophilus* (11.8%) was the dominant genus followed by  
348 *Bacteroidetes\_vadinHA17\_norank* (10.2%). The *Methylophilus* was a heterotrophic bacterium whose  
349 role was expected as *Anaerolineaceae\_uncultured* to create C-limiting conditions for autotrophic  
350 denitrification and to provide small organic compounds for heterotrophic denitrification. The  
351 *Bacteroidetes\_vadinHA17\_norank* was also heterotrophic bacteria commonly detected in anaerobic  
352 systems, which was also found in the denitrification system (Zhang et al., 2018). The presence of  
353 *norank\_f\_\_Nitrosomonadaceae* (AOB), *Nitrospira* (NOB), *Acidobacteria\_norank* (SRB) showed the  
354 potential presence of ammonia oxidation (Prosser et al., 2014), nitrification (Daims et al., 2015), and  
355 dissimilatory sulfate reduction (Anantharaman et al., 2018) in the experiment, even though they are not  
356 observed. Similarly, the genus mediating anammox, *Candidatus Brocadia*, was observed at 0.06% in the

357 microbial community. The genus *Desulfovibrio*, probably mediating DNRA, was also found in the  
 358 microbial community (Su et al., 2020).

359 The abundance of heterotrophic NRB (LT: 21.59%; HT: 23.50%) and SOB (LT: 21.45%; HT:  
 360 25.86%) were higher than other functional groups. Besides, all the SOB genus listed in Figure 4 could  
 361 mediate sulfide/sulfur-based autotrophic denitrification (Ma et al., 2020; Xing et al., 2017). This was in  
 362 accordance with results from the batch experiments in which heterotrophic denitrification and sulfide-  
 363 based autotrophic denitrification were observed, but other processes contributed little to carbon, nitrogen,  
 364 and sulfur transformation.

365



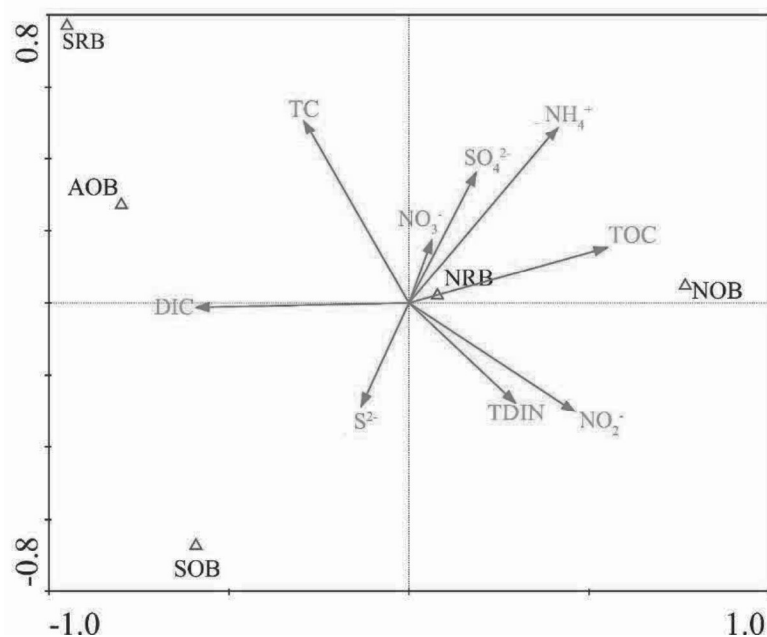
366

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

371 heterotrophic denitrification or dissimilatory  $\text{SO}_4^{2-}$  reduction. The bacteria in the square could mediate  
372 sulfide-based autotrophic denitrification.

373

374 The correlation between functional bacteria and the carbon, nitrogen, and sulfur transformation was  
375 further revealed by the Spearman's correlation coefficient ( $\rho$ , Table S4) and canonical correlation analysis  
376 (CCA, Figure 5). The results clearly showed that  $\text{NO}_3^-$  removal was closely related to TOC removal  
377 ( $\rho=0.786$ ,  $p=0.04$ ) and  $\text{SO}_4^{2-}$  generation ( $\rho=-0.643$ ,  $p=0.12$ ), and strongly correlated with NRB ( $\rho=0.571$ ,  
378  $p=0.18$ ). The correlation coefficient and CCA further confirmed that the presence of heterotrophic  
379 denitrification and sulfide-based autotrophic denitrification in the studied CW.



380

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



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

387

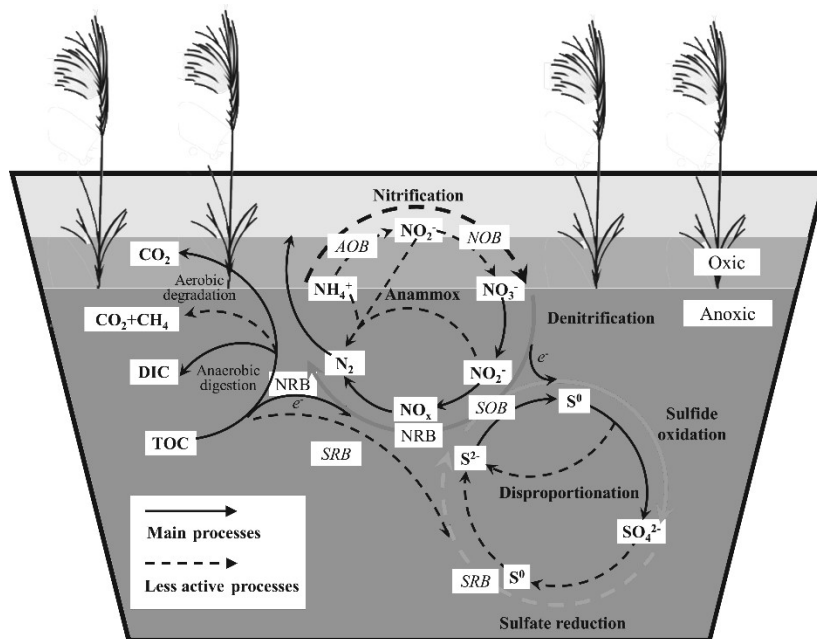
## 388 **5. Coupling transformation of carbon, nitrogen, and sulfur compounds in the long-term operated**

389 **CW**

390 Based on the results, the coupling transformation of carbon, nitrogen, and sulfur compounds in the  
391 long-term operated HSFW was shown in Figure 6. The batch experiment, electron balance analysis, as  
392 well as the microbial community analysis all showed that the main N removal pathway in the system was  
393 heterotrophic denitrification, coupling  $\text{NO}_3^-$  removal and TOC removal. At the same time, sulfur-based  
394 autotrophic denitrification was also present. The contribution of sulfur-based autotrophic denitrification  
395 to TN removal was about 15.2%, similar to the result from other studies ranging from 7% to 16% (Chen  
396 et al., 2016). This process coupled the reduction of  $\text{NO}_3^-$  to  $\text{NO}_2^-$ , and further to  $\text{N}_2$ , with the oxidation of  
397  $\text{S}^{2-}$  to  $\text{SO}_4^{2-}$ . The bacteria mediating ammonia oxidation, nitrification, anammox, dissimilatory DNRA,  
398 and dissimilatory sulfate reduction were also found in the studied CW. However, the results of the batch  
399 experiment showed that these processes did not significantly contribute to carbon, nitrogen, and sulfur  
400 transformation.

401

402



403

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

407

## 408 6. Conclusion

409 In conclusion, the key processes of carbon, nitrogen, and sulfur transformation in the HFSW of the  
 410 studied hybrid CW were denitrification. Based on the electron balance analysis, both heterotrophic  
 411 denitrification and autotrophic denitrification were observed concurrently in the HFSW. The autotrophic  
 412 denitrification used  $\text{NO}_3^-$  as the electron acceptor and  $\text{S}^{2-}$  as the electron donor. Increasing initial  $\text{S}^{2-}$  could  
 413 promote the sulfur-based autotrophic denitrification. The heterotrophic denitrification was the main  
 414 pathway for N removal, while the sulfur-based autotrophic denitrification was not inhibited by the  
 415 heterotrophic denitrification. This research improved the understanding of microbial mechanisms behind  
 416 the pollutant removal in the CWs in practice. The results of this study could help to understand the high  
 417 N removal efficiency in CWs, and promote the understanding of potential carbon, nitrogen, and sulfur

418 coupling transformation.

419

## 420 **Acknowledgment**

421 This research was financially supported by National Natural Science Foundation of China (No.  
422 51878093). The authors appreciated the help from the staff in the wastewater treatment plants during  
423 the sampling.

424

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