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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 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, NH_4^+ can be oxidized by NO_2^- into N_2 . As reported previously, over 40% of 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 NO_2^- , SO_4^{2-} can also be used to oxidize NH_4^+ in anammox, namely SO_4^{2-} -dependent anammox
42 process (Rios-Del Toro et al., 2018). In this process, NH_4^+ is oxidized to N_2 coupled to the reduction of
43 SO_4^{2-} to S^0 . Autotrophic denitrification is another important N transformation pathway. The autotrophic
44 denitrification uses sulfur-compounds, such as elemental sulfur (S^0), S_2O_3^- , and S^{2-} or FeS , as the electron
45 donor to convert NO_3^- or NO_2^- into N_2 . Other electron donors like hydrogen (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 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 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 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 ^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

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 m³/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 N₂ 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 (CH_3COONa), ammonium chloride (NH_4Cl), sodium nitrite
112 (NaNO_2), sodium nitrate (NaNO_3), sodium sulfide (Na_2S) and potassium sulfate (K_2SO_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 ₃ COONa	NH ₄ Cl	NaNO ₂	NaNO ₃	Na ₂ S	K ₂ SO 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

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), NH₄⁺, NO₂⁻, NO₃⁻, 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[®] 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 μ L) for PCR
139 contained: 10 ng of template DNA, 0.8 μ L of each primer (5 μ M), 4 μ L of 5-fold Fast*Pfu* Buffer, 2 μ L of
140 dNTPs (2.5 mM), 0.4 μ L of Fast *Pfu* Polymerase and ultrapure H₂O. 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[™] -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), 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 CH₄ and CO₂ 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 CH₄ and CO₂ 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 NO₂⁻,
156 NO₃⁻, and SO₄²⁻ 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 S²⁻
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 (ρ) 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 NO_2^- generation and

176 obtained slight removal (34.05% of generation to 10.98% of removal, $p=0.12$), and higher NO_3^- removal

177 (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

178 TOC dosage. However, increasing TOC dosages had no effects on the removal of NH_4^+ (1.10% to 1.66%,

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

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 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

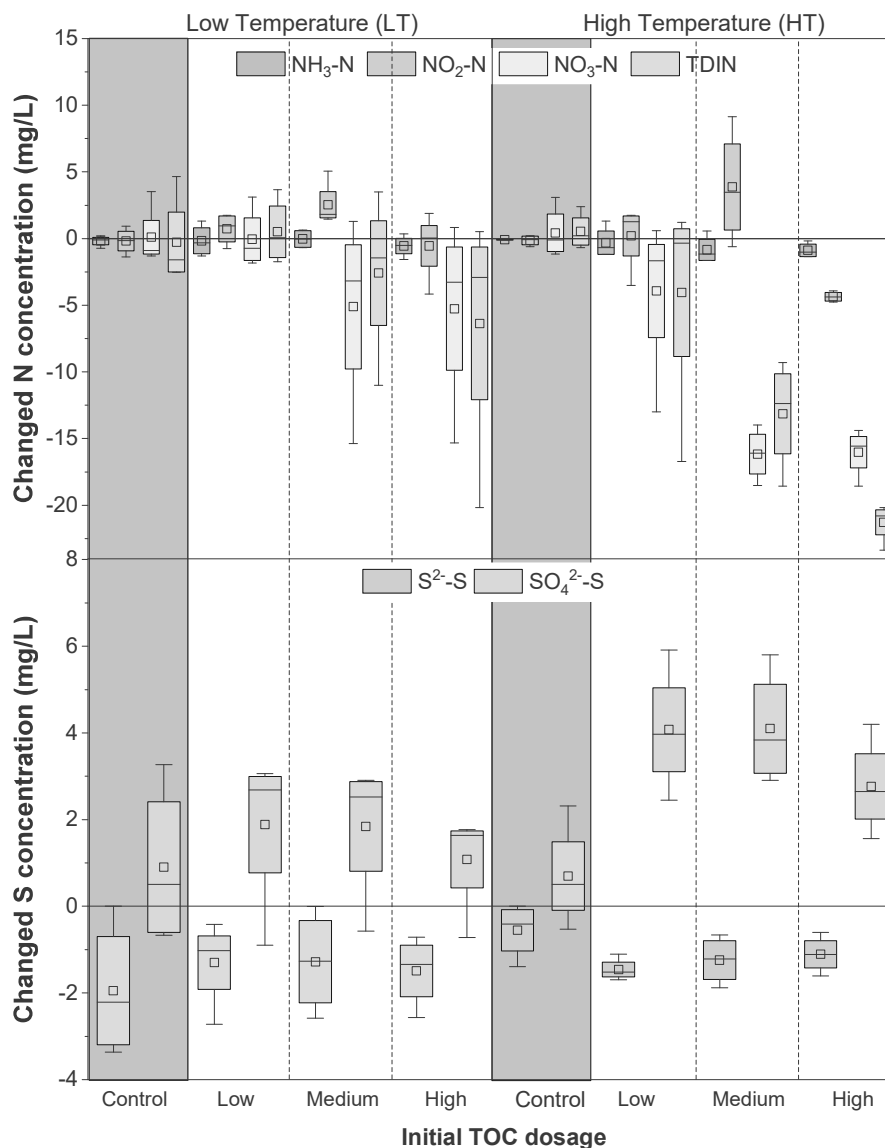
183 TOC dosages significantly promote both NO_2^- transformation from 77.33% of generation to 87.23% of

184 removal ($p=0.009 < 0.01$), and NO_3^- removal increased from 31.06% to 92.36% ($p=0.008 < 0.01$). Besides,

185 the generation of 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 NH_4^+ dosages had no significant effects on the NH_4^+
198 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%,
199 $p=0.94$; 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
200 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
201 both LT and HT season. The 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 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 NH_4^+ (LT: 2.66% to 4.89%,
205 $p=0.85$; 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%,
206 $p=0.82$), 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^-
207 (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
208 18.64%, $p=0.91$; HT:24.98% to 35.49%, $p=0.51$) at different NH_4^+ dosages had no significant differences.

209 (2) NO_2^-

210 Effects of 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 NO_2^- dosages did not affect NH_4^+ removal (LT:
212 $p=0.80$; HT: $p=0.08$). Besides, the NO_2^- accumulation (LT: $p=0.78$; HT: $p=0.29$) and NO_3^- removal (LT:
213 $p=0.83$; 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-}
214 generation (LT: $p=0.63$; HT: $p=0.65$) were also stable at various initial NO_2^- dosages.

215 Adding organic carbon to $\text{TOC}=25\text{mgC/L}$ led to higher NO_3^- removal and NO_2^- accumulation (Figure
216 2, S3). However, increasing initial 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%, $p=0.75$), NH_4^+ (LT: $p=0.91$; HT: $p=0.12$) and NO_3^-
218 removal (LT: $p=0.96$; HT: $p=0.91$) and 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.

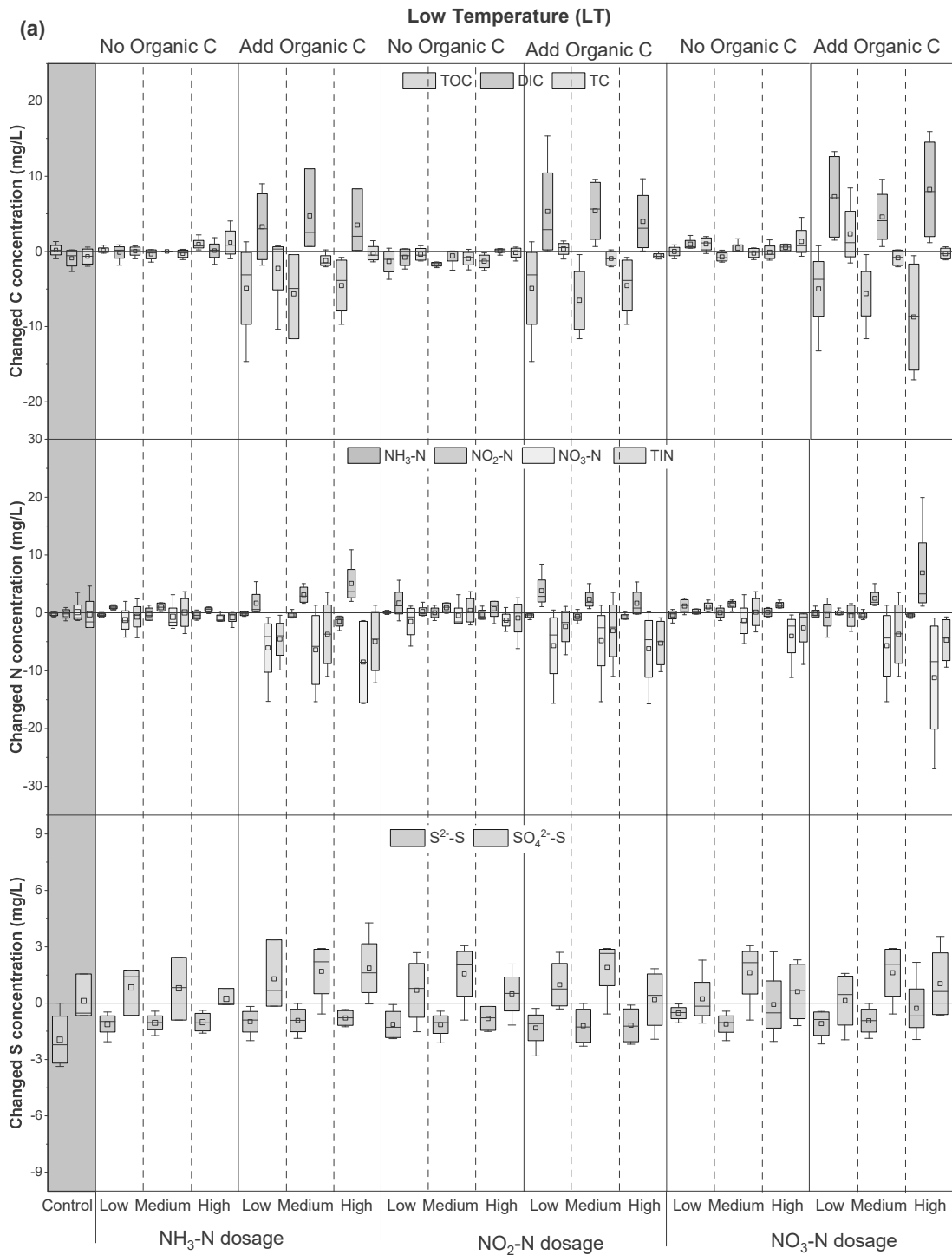
231 At neutral pH used in our experiment, the main form of ammonia nitrogen was NH_4^+ . A high
232 concentration of NH_4^+ was regarded as an inhibitor in anaerobic digestion but the ammonium toxicity
233 was very reversible (Yenigün and Demirel, 2013). The high NH_4^+ concentration is also toxic to 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



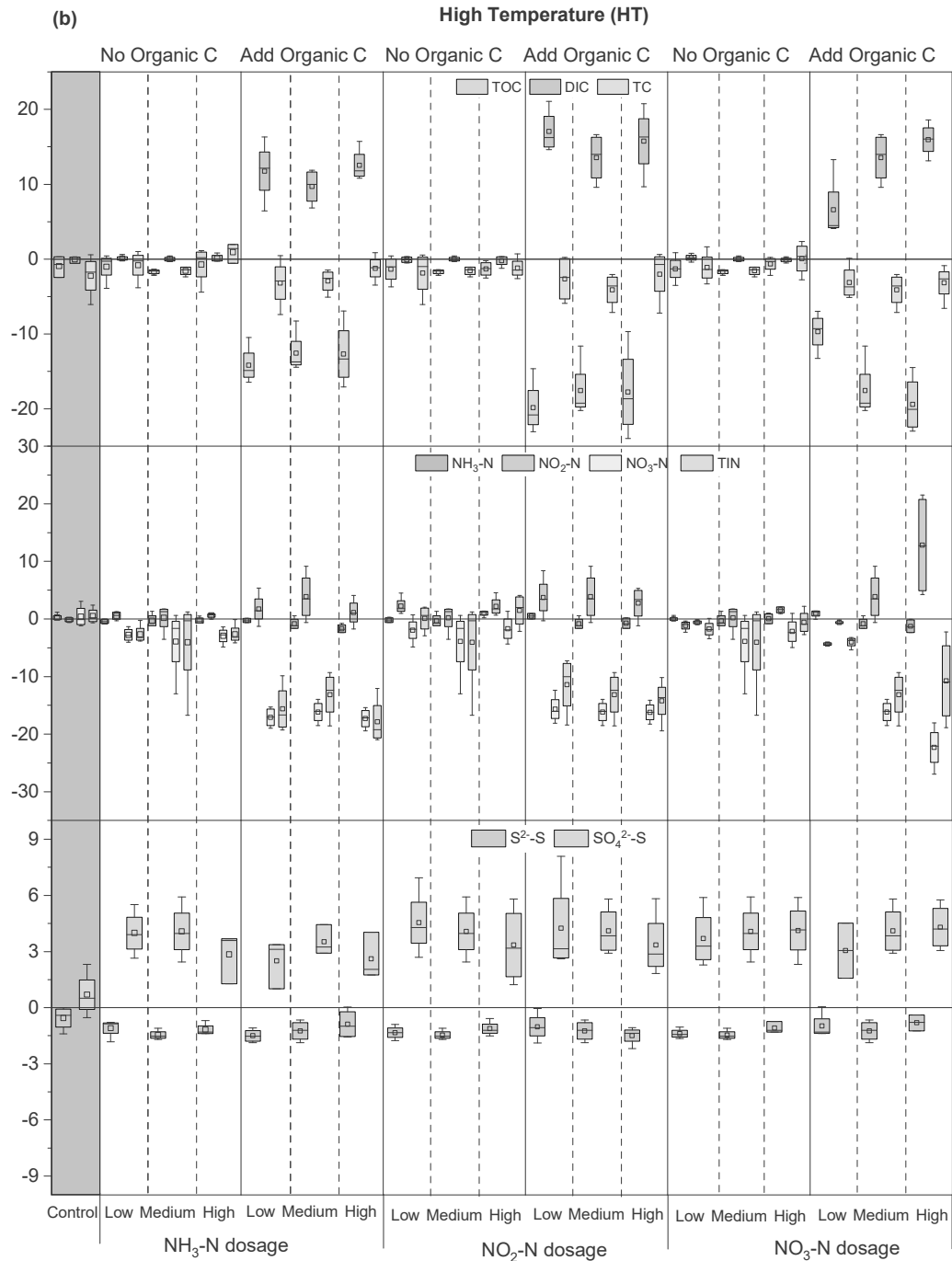
241 (Glass et al., 1997; Tang et al., 2020). In our experiment, increasing NO_2^- dosage showed no obvious

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: [NH₄⁺]₀=0 mgN/L, [NO₂⁻]₀=0 mgN/L, [NO₃⁻]₀=0**

249 **mgN/L; Medium: [NH₄⁺]₀=15 mgN/L, [NO₂⁻]₀=5 mgN/L, [NO₃⁻]₀=10 mgN/L; High: [NH₄⁺]₀=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

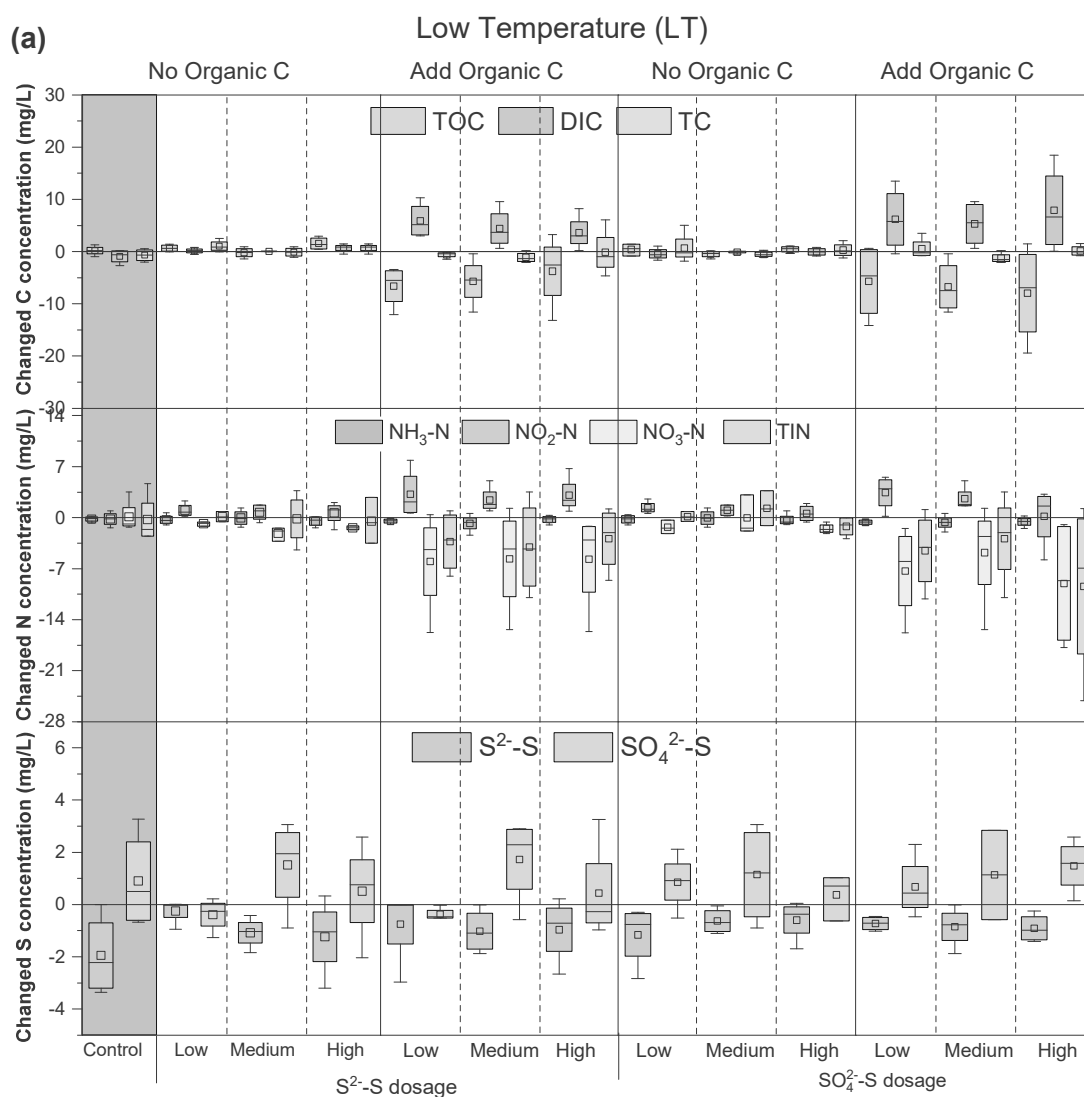
254 (1) S^{2-}

255 In Batch 5, various initial 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 S^{2-} dosages led to higher NO_3^- removal and NO_2^-
257 generation (Figure 3, S4) but the differences were not significant. The removal efficiency of 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 S^{2-} removal (LT: $p=0.19$, HT: $p=0.19$) increased but SO_4^{2-} generation decreased (LT:
260 $p=0.14$, HT: $p=0.09$). Adding organic carbon improved NO_3^- removal and NO_2^- generation (Figure 3, S4),
261 but increasing initial S^{2-} dosages resulted in no significant differences in NO_3^- removal (LT: $p=0.99$, HT:
262 $p=0.95$) and NO_2^- generation (LT: $p=0.90$, HT: $p=0.96$). As to the S compounds, higher initial S^{2-} dosages
263 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: $p=0.27$).

264 (2) SO_4^{2-}

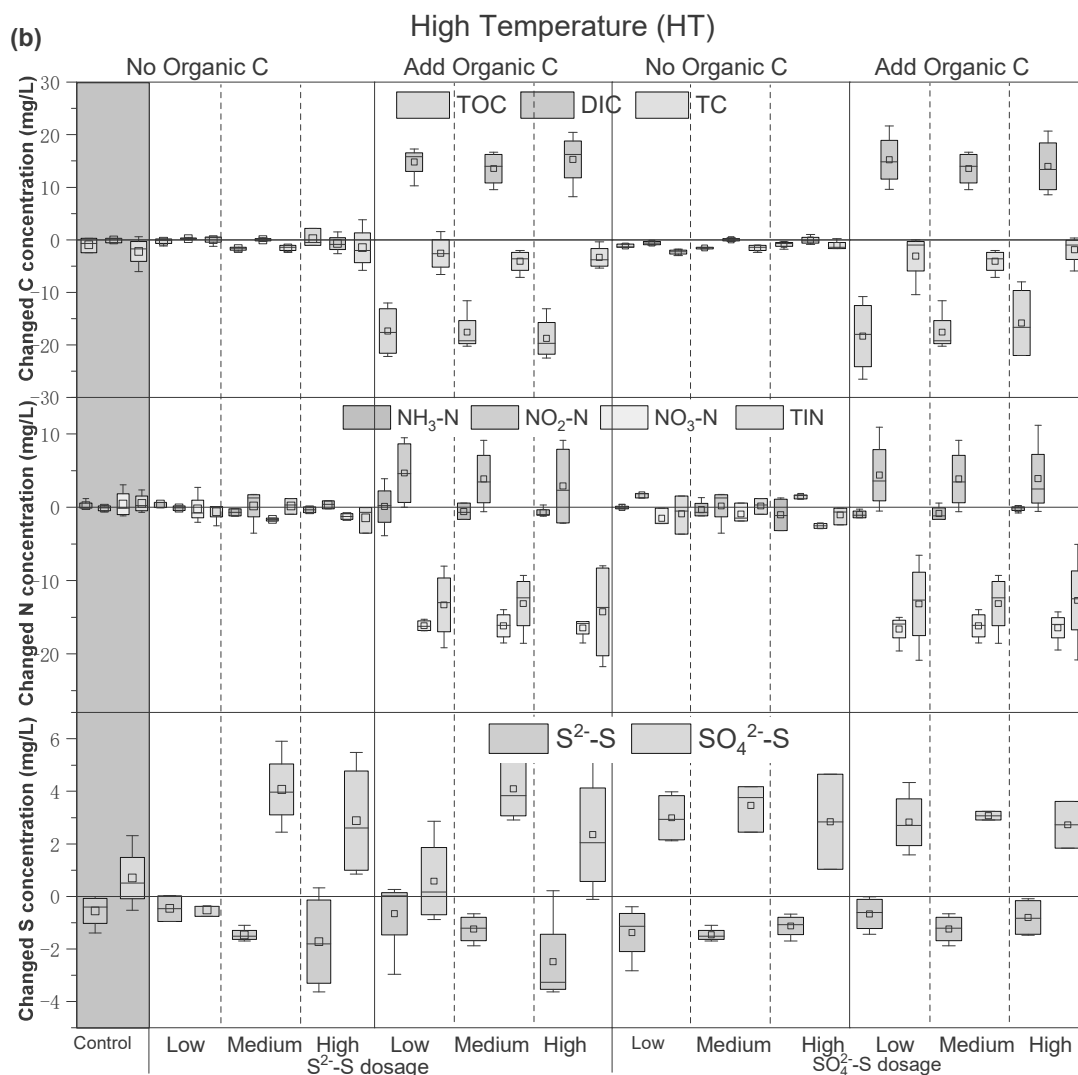
265 In the Batch 6, initial SO_4^{2-} dosages were varied in the experiment (0, 10, 20 mgS/L). When no
266 organic carbon was added, increasing initial SO_4^{2-} dosages had no influence on carbon, nitrogen, and
267 sulfur transformation ($p>0.16$, Figure 3, S4, Table S2). When organic carbon was added to 25 mgC/L
268 (TOC), increasing initial SO_4^{2-} dosages had no influence on TOC removal (LT: $p=0.90$, HT: $p=0.77$),
269 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
270 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
271 (LT: $p=0.69$, HT: $p=0.95$).

272 It is notable that the amount of S forming SO_4^{2-} was higher than the detected S^{2-} converted. This is
 273 because S^{2-} was easily form precipitations like FeS and retained in solid form, leading the total amount
 274 of S^{2-} is higher than the amount in the liquid phase. In our experiments, the detected S^{2-} initial
 275 concentration was 1.31 ± 0.25 mg S/L, lower than the 2.5 mg S/L supplied by adding Na_2S (Table 2).
 276 The solid 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 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: $[S^{2-}]_0=0$ mgS/L, $[SO_4^{2-}]_0=0$ mgS/L; Medium: $[S^{2-}]_0=2.5$ mgS/L,
 285 $[SO_4^{2-}]_0=10$ mgS/L; High: $[S^{2-}]_0=5$ mgS/L, $[SO_4^{2-}]_0=20$ mgS/L. The positive values represent generation
 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

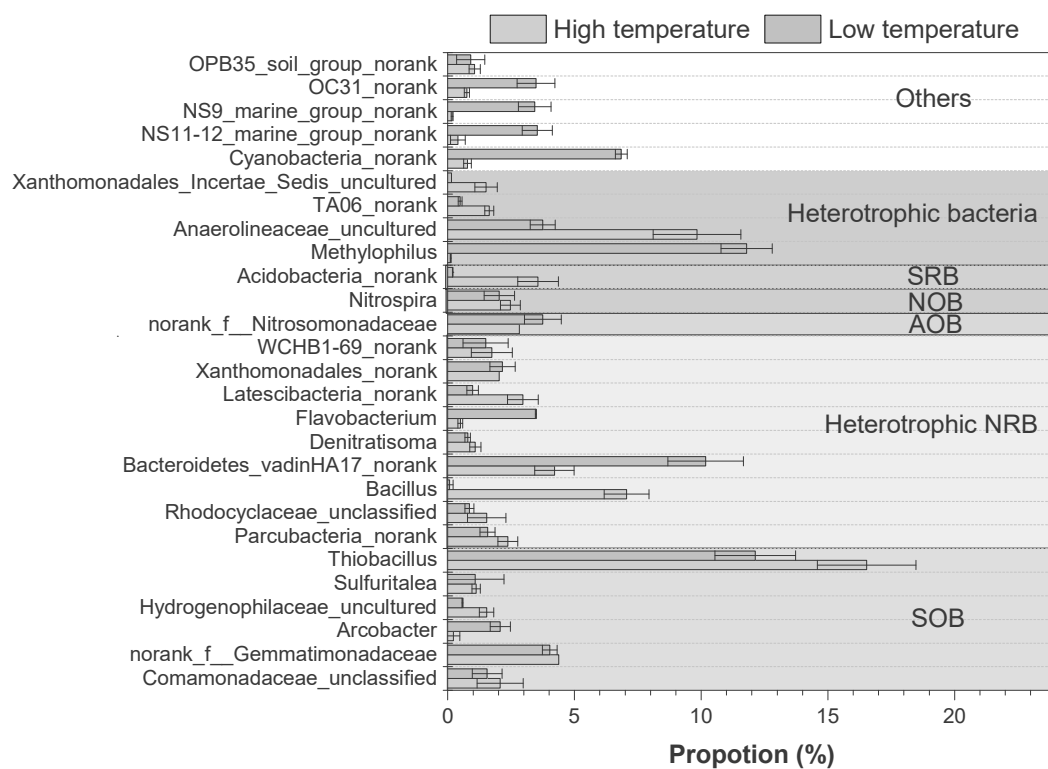
334 As shown in Figure 4, the dominant bacteria (proportion > 1% in the genus level) were categorized

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



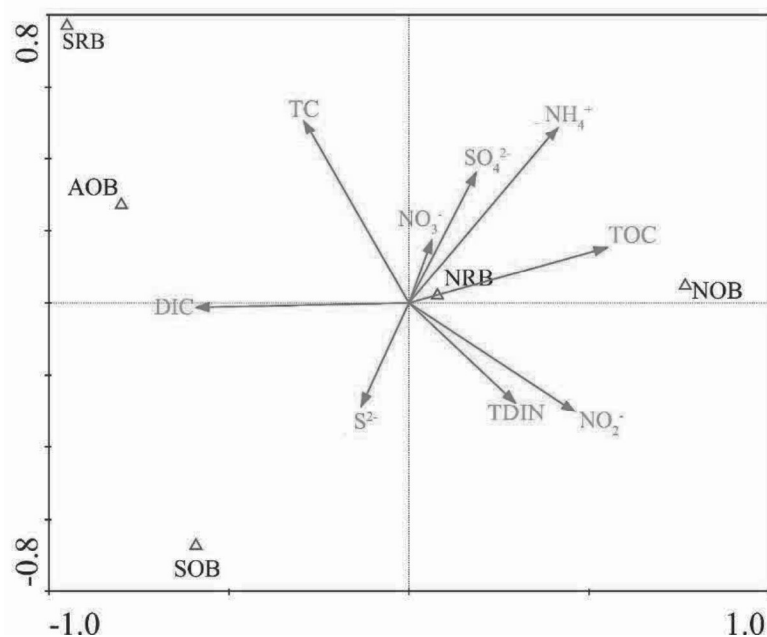
366

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 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 (ρ , Table S4) and canonical correlation analysis
376 (CCA, Figure 5). The results clearly showed that NO_3^- removal was closely related to TOC removal
377 ($\rho=0.786$, $p=0.04$) and 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

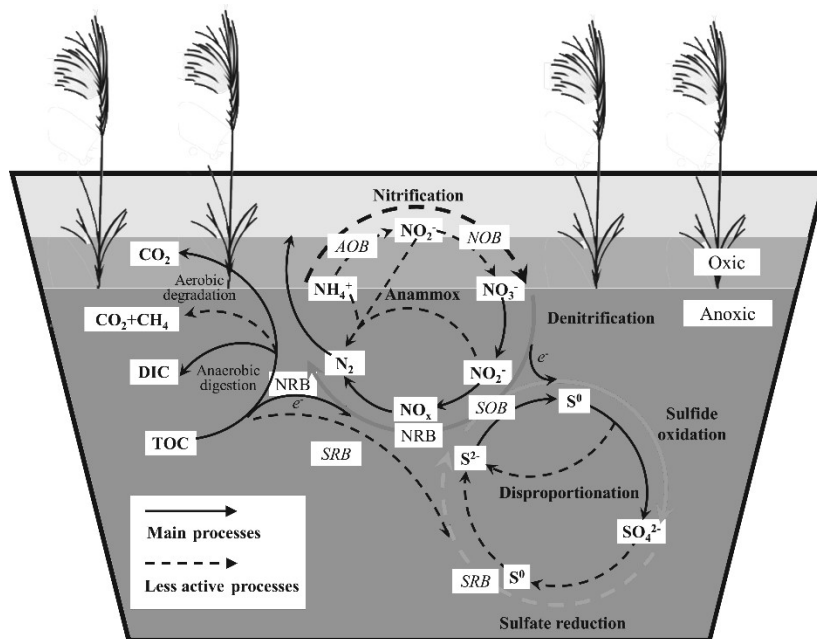
388 **5. Coupling transformation of carbon, nitrogen, and sulfur compounds in the long-term operated**

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 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 NO_3^- to NO_2^- , and further to N_2 , with the oxidation of
397 S^{2-} to 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 NO_3^- as the electron acceptor and S^{2-} as the electron donor. Increasing initial 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

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423 the sampling.

424

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