

## Incorporation of the sulfur cycle in sustainable nitrogen removal systems - a review

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### Abstract

In wastewater treatment systems, sulfur (S) removal processes are generally based on heterotrophic sulfate ( $\text{SO}_4^{2-}$ ) reduction by sulfate reducing bacteria and S-dependent autotrophic denitrification by sulfur oxidizing bacteria. A combination of either two cycles (N and S) or three cycles (N, S and C) appears to be a viable approach to sustainable wastewater treatment, resulting in energy savings and reduction of sludge production. This review shows how the S cycle can be coupled with the other cycles in single systems for efficient N and S removal. Operating conditions, advantages, limitations and challenges of such systems are described. S removal processes are generally based on heterotrophic sulfate ( $\text{SO}_4^{2-}$ ) reduction by sulfate reducing bacteria and S-dependent autotrophic denitrification by sulfur oxidizing bacteria. In terms of pH and temperature, the optimum conditions are determined by the narrowest ranges for heterotrophic  $\text{SO}_4^{2-}$  reduction (pH of 7-7.6,  $T = 28-30^\circ\text{C}$ ). The combined processes allow for almost complete N removal, while the efficiency of  $\text{SO}_4^{2-}$  removal can reach up to 75%. Among all the processes linking the N, S and C cycles, SANI (sulfate reduction, autotrophic denitrification and nitrification integrated) has been best recognized. Recently, the growing attention has been paid to the novel sulfammox process, which involves  $\text{SO}_4^{2-}$  dependent, anaerobic ammonia oxidizing bacteria. Numerous systems have been developed to combine  $\text{SO}_4^{2-}$  reduction, S-dependent autotrophic denitrification and partial nitrification/anammox processes. The coexistence of several bacterial groups and their competition for the substrates is thus a key issue to be considered. Specific inhibitors for each bacterial group also need to be recognized before full-scale implementations. Moreover, modeling the transformations of S

compounds has been incorporated with respect to all the processes responsible for those transformations.

**Keywords:** sulfur-dependent autotrophic denitrification; heterotrophic sulfate reduction; sulfamox; sulfur cycle; microbial community; mechanistic model

## 1           **1. Introduction**

2           High concentrations of ammonium ( $\text{NH}_4\text{-N}$ ) lead to eutrophication of surface waters  
3 and pose a threat to the aquatic life and human health (Qin et al., 2021).  $\text{NH}_4\text{-N}$  can effectively  
4 be converted to nitrogen gas by combined nitrification-denitrification, but this method has a  
5 few important disadvantages, including a high demand of energy and carbon, and high sludge  
6 production. On the other hand, sulfate ( $\text{SO}_4^{2-}$ ) is a type of the secondary pollutant because  
7 reduction of sulfide ( $\text{S}^{2-}$ ) under anaerobic conditions is harmful for the aquatic environment  
8 (Hao et al. 2014). S compounds have not been widely used as substrates in wastewater treatment  
9 processes. Simultaneous removal of these two compounds (N and S) from wastewater, with or  
10 without involving the carbon (C) cycle, can be a viable approach to the sustainable wastewater  
11 management. In particular, this approach may be an effective alternative in the case of many  
12 types of industrial wastewater, which are characterized by high concentrations of pollutants,  
13 such as  $\text{NH}_4\text{-N}$ ,  $\text{SO}_4^{2-}$  (>1000 mg/L of both N and S) and chemical oxygen demand (COD) (>  
14 60,000 mg COD/L) (Rikmann et al., 2016, Jarvis and Younger 2000, Chapman, 1992).

15           A viable sustainable approach to biological wastewater treatment comprises a  
16 combination of nitrogen (N), sulfur (S) and carbon (C) removal. Lower operating costs result  
17 from the use of some products in one process as the substrates in other processes and the use  
18 of shared reactors. Moreover, no carbon is needed for S-dependent autotrophic denitrification,  
19 less sludge is generated, and the environmentally neutral compounds, such as nitrogen gas  
20 ( $\text{N}_2$ ) and elemental sulfur ( $\text{S}^0$ ), are the final products of biochemical reactions (Lin et al.,  
21 2018).

22           Conventional nitrification/denitrification for N removal is now being replaced by more  
23 sustainable N-shortcut processes, such as “nitrite shunt” or deammonification. In the case of S  
24 compounds, biological removal is based on heterotrophic  $\text{SO}_4^{2-}$  reduction by sulfate reducing



25 bacteria (SRB) and S-dependent autotrophic denitrification by sulfur oxidizing bacteria  
26 (SOB). Recently, the growing attention has been paid to the novel sulfate reducing ammonia  
27 oxidizing (sulfammox) process, which involves anaerobic ammonium oxidizing bacteria  
28 (AAOB). These bacteria use  $\text{SO}_4^{2-}$ , instead of nitrite nitrogen ( $\text{NO}_2^-$ -N), as an electron  
29 acceptor to oxidize  $\text{NH}_4^+$ -N under anaerobic conditions.

30         The growing importance of using the combined N, S and C cycles in biological  
31 wastewater treatment processes has been confirmed by the increasing number of review  
32 papers on various aspects of S transformations. According to Web of Science database, 15, 12  
33 and 3 review papers have been published specifically on S-dependent autotrophic  
34 denitrification, heterotrophic reduction of  $\text{SO}_4^{2-}$  and sulfammox (–see Figure S1 in the  
35 Supporting Information (SI)). Several papers focused on particular issues, including a detailed  
36 description of mechanisms of the individual processes, responsible microorganisms, reactors  
37 used, optimal operational conditions or inhibiting factors in S-dependent autotrophic  
38 denitrification (Wu et al., 2021, Cui et al., 2019, Lin et al., 2018), heterotrophic sulfate  
39 reduction (Sinharoy et al., 2020b) and sulfammox (Liu et al., 2021, Grubba et al., 2021).

40         However, only a combination of either two cycles – (N and S) or three cycles (N, S  
41 and C) would be the rational approach to wastewater treatment in order to save energy and the  
42 amount of sludge generated, especially for  $\text{NH}_4^+$ -N and  $\text{SO}_4^{2-}$  rich industrial wastewater. Due  
43 to the variety of N, S and C removal processes, the research interests have been shifting to the  
44 use of single- and multi-stage systems based on the combination of several processes, such as  
45 heterotrophic sulfate reduction, S-dependent autotrophic denitrification, nitrification,  
46 denitrification, anaerobic ammonia oxidation (anammox) and sulfammox (Wu et al., 2020,  
47 Yuan et al., 2020, Sun et al., 2018, Liu et al., 2017, Qian et al., 2015a, b, c, Jiang et al., 2013,  
48 Wang et al., 2009b).



49 Only two review papers (Hao et al., 2014, Show et al., 2013) described simultaneously  
50 S-dependent autotrophic denitrification and heterotrophic sulfate reduction. Hao et al. (2014)  
51 described a relationship between the N, S, C and P cycles in biological wastewater treatment  
52 systems. These authors focused on the acceptors and electrons used in the transformations of  
53 S compounds, key microorganisms, developed technologies, factors influencing the process  
54 performance, and achieved  $\text{SO}_4^{2-}$  reduction efficiencies. In the review of Show et al. (2013),  
55 existing models of the transformations of S compounds were additionally described (see –  
56 Table S1 in SI).

57 The present review provides updated results of research on S transformations, which  
58 have been revised and extended with new understanding and discoveries. A novel aspect is  
59 the inclusion of sulfamox in these transformations as no paper has synthesized autotrophic  
60 S-dependent denitrification, heterotrophic sulfate reduction and the sulfamox process in one  
61 review. In addition, the present study describes how sulfamox can increase the efficiency of  
62 N and S removal. Various process configurations and technologies, which are based on the  
63 three (N-S-C) cycles, are described and compared in terms of their efficiency. Moreover,  
64 modeling the transformations of N, S and C compounds has been incorporated with respect to  
65 all processes responsible for those transformations. Such a review provides a deeper insight  
66 into the conversions of S in biochemical processes, including sulfamox.

## 67 **2. Single S-dependent biochemical processes integrating N, S and C** 68 **conversions**

69 There are three known processes combining sulfur and nitrogen conversions: S-dependent  
70 autotrophic denitrification, heterotrophic sulfate reduction and autotrophic sulfamox. The  
71 detailed description of those processes, including the metabolic mechanisms, biochemical  
72 reactions, influencing environmental factors can be found in the SI (S1-S3).

73 S-dependent autotrophic denitrification consists of oxidation of S compounds,  
74 including  $S^{2-}$ ,  $S^0$ , thiosulfate ( $S_2O_3^{2-}$ ) and sulfite ( $SO_3^{2-}$ ), coupled with reduction of  $NO_3^-$ -N  
75 and/or  $NO_2^-$ -N. *T. denitrificans*, *Thiomicrospira denitrificans*, *Thiobacillus versutus*,  
76 *Thiosphaera pantotropha* and *P. denitrificans* are the known microorganisms responsible for  
77 that process. *P. denitrificans* is the chemotrophic  $\alpha$ -proteobacteria which can grow on organic  
78 monocarbon compounds (methanol, methylamine) while using reduced forms of S and  
79 hydrogen as electron donors in denitrification (Baker et al., 1998). *T. denitrificans* belongs to  
80  $\beta$ -proteobacteria that can use  $S_2O_3^{2-}$  and thiocyanates under aerobic conditions, and  
81 additionally  $S^{2-}$  and  $S^0$  under anaerobic conditions. *Sulfurimonas denitrificans* belongs to the  
82  $\epsilon$ -proteobacteria and is capable of oxidizing  $SO_3^{2-}$ ,  $S_2O_3^{2-}$  and  $S^0$ , while both  $NO_3^-$ -N and  
83 oxygen are used as electron acceptors. *T. thioparus* is one of the representatives of autotrophic  
84 denitrifiers that reduce  $NO_3^-$ -N to  $NO_2^-$ -N by oxidation of  $S^{2-}$  (Tang et al., 2009). Although  
85 autotrophic denitrifying bacteria are chemolithotrophic, there are many denitrifying bacteria  
86 capable of adapting to autotrophic, heterotrophic and even mixotrophic growth (*P. versutus*,  
87 *P. denitrificans*, *Beggiatoa* sp.) (Pokorna and Zabranska, 2015).

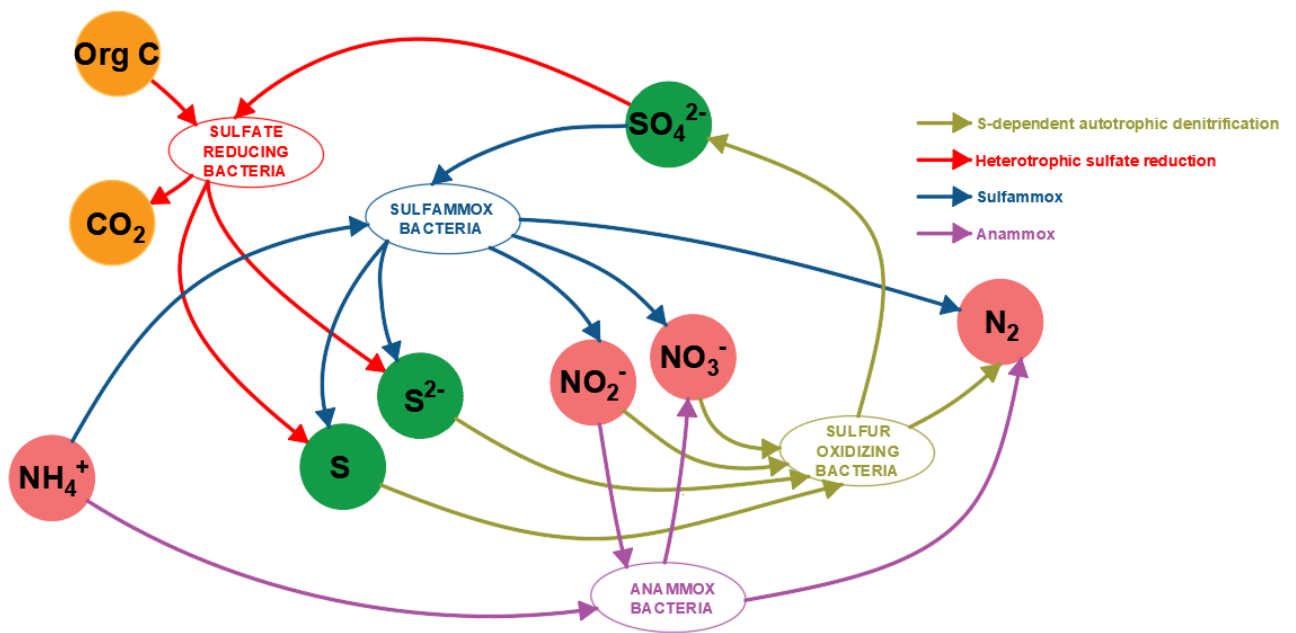
88 Heterotrophic sulfate reduction is  $SO_4^{2-}$  reduction which takes place in two  
89 independent different paths. The first is the use of organic electron donors, which are also the  
90 carbon source for the SRB. The second is the use of inorganic electron donors, which must be  
91 supplemented with a carbon source, such as  $CO_2$  (Sinharoy et al., 2020a). The SRB can be  
92 divided into 7 phylogenetic lines, including five for bacteria and two for archaea. Most of the  
93 SRB found in sulfate reduction reactors belong to 23 genera within *Deltaproteobacteria*  
94 (*Desulfovibrio*, *Desulfobacteraceae*, *Desulfobulbaceae*, *Syntrophobacteraceae*,  
95 *Desulfomicrobium*, *Desulfohalobium*). Another SRB belong to the gram-positive genera  
96 *Clostridia* (*Desulfotomaculum*, *Desulfosporosinus* and *Desulfosporomusa*). Three lineages,  
97 *Nitrospirae* (*Thermodesulfovibrio*), *Thermodesulfobacteria* (*Thermodesulfobacterium*) and



98 *Thermodesulfobiaceae* (*Thermodesulfobium*), contain only thermophilic  $\text{SO}_4^{2-}$  reducing  
99 agents. Archaeal SRB are *Euryarchaeota* and *Crenarchaeota* (Muyzer and Stams, 2008).

100 In a novel sulfammox process,  $\text{NH}_4^+$ -N is oxidized to  $\text{N}_2$ , whereas  $\text{SO}_4^{2-}$  plays the role  
101 of an electron acceptor which is reduced to  $\text{S}^0$  under anaerobic conditions. *Brocadia*  
102 *Anammoxoglobus Sulfate* (Liu et al., 2008) is a functional microorganism responsible for  
103 simultaneous removal of  $\text{NH}_4^+$ -N and  $\text{SO}_4^{2-}$  and ended the conversion of  $\text{NH}_4^+$ -N and  $\text{SO}_4^{2-}$   
104 by producing  $\text{NO}_2^-$ -N as an intermediate. The second isolated species, *Bacillus Benzoevorans*,  
105 is responsible for carrying out the entire sulfammox reaction (Cai et al., 2010).  
106 *Verrucomicrobia* has also been reported to be involved in the sulfammox process (Rikmann et  
107 al., 2016). Some *Proteobacteria*, which may potentially perform sulfammox, include the  
108 following species: *Sulfurimonas*, *Desulfuromonadales*, *Desulfovibrio*, *Desulfuromonas*,  
109 *Desulfobulbus*, *norank Rhodobacteraceae* and *Thiobacillus* (Rios-Del Toro et al., 2018, Wang  
110 et al. 2017).

111 The key issues and challenges of S-dependent autotrophic denitrification,  
112 heterotrophic sulfate reduction and sulfammox are presented in Table 1. Figure 1 below  
113 shows the interactions between S-dependent autotrophic denitrification, heterotrophic sulfate  
114 reduction and sulfammox process.



115

116 Figure 1. Interactions between S-dependent autotrophic denitrification, heterotrophic sulfate  
 117 reduction, anammox and sulfamnox process

118

### 119 3. Operational conditions and performances of single S-dependent processes

120 Each of the discussed processes (S-dependent autotrophic denitrification, heterotrophic sulfate  
 121 reduction and sulfamnox) can be carried out independently, as evidenced by numerous  
 122 studies (Tables 2 and 3). However, the challenge is to combine these processes, in either  
 123 single- or multi-stage systems, in order to make biological wastewater treatment systems  
 124 more efficient.

#### 125 3.1. S-dependent autotrophic denitrification

126 In S-dependent autotrophic denitrification, the most frequently used electron donors are S<sup>0</sup>  
 127 and S<sup>2-</sup> (Table 2). The experiments were mainly carried out in packed bed reactors, but several  
 128 other types of reactors were also used. The reported rates of denitrification varied in a wide  
 129 range - from 0.03 to 8.13 kg N/m<sup>3</sup>/d, depending mainly on the temperature and influent NO<sub>3</sub><sup>-</sup>-



130 N concentrations. The effects of pH in the investigated range (6.0-9.0) and S concentrations  
131 were less significant. For a detailed description of previous research related to S-dependent  
132 autotrophic denitrification, see the SI (S1). This process allowed for the efficient (>90%)  
133 removal of N and  $S^{2-}$  (Yang et al., 2016, Jing et al., 2010) with the  $NO_3^-$ -N concentration in  
134 the range of 20-1230 mg N/L (Zhu et al., 2019, Zou et al., 2016, Kim et al., 2004).

135 During S-dependent autotrophic denitrification,  $SO_4^{2-}$  can be produced from different  
136 electron donors. Frequently, the S balance in the process is not 1/1 for the removed electron  
137 donor to  $SO_4^{2-}$  produced (Zou et al., 2016). In Table 2, the initial donor concentrations and the  
138 amount of  $SO_4^{2-}$  produced are similar. The observed imbalances result from the production of  
139 other S intermediates. The most common electron acceptor is  $NO_3^-$ -N, but several studies  
140 comparing  $NO_3^-$ -N and  $NO_2^-$ -N have been reported (Sun and Nemati, 2012, Moraes et al.,  
141 2012, Jing et al 2010).

142 Different aspects of S-dependent autotrophic denitrification have been addressed in  
143 several reviews (Wu et al. 2021, Cui et al. 2019, Lin et al. 2018, Sabba et al. 2016). Wu et al.  
144 (2021) summarized all types of biofilm denitrification in terms of the reactor configuration,  
145 microbial transformations, factors influencing the process, and especially focused on  $N_2O$   
146 emissions. The coexistence of S-dependent denitrification with anammox was also reported  
147 and S-driven denitrifiers were identified, including *Thiobacillus denitrificans* and  
148 *Thiobacillus thioparus*.

149 Cui et al. (2019) described S-dependent autotrophic denitrification in terms of the  
150 functional enzymes, electron donors, types of reactors, and operational factors. They also  
151 emphasized a significant advantage regarding S-dependent autotrophic denitrification  
152 compared to heterotrophic denitrification with respect to  $N_2O$  emissions. It was shown that



153 autotrophic denitrification mediated by S compounds ( $S^0$ ,  $S^{2-}$ ) emitted significantly less  $N_2O$   
154 than heterotrophic denitrification with methanol, ethanol or acetate.

155 Sabba et al. (2016) focused mainly on  $SO_3^{2-}$  and its occurrence in the environment,  
156 chemistry, microbiology, and the role in denitrification. It was emphasized that  $SO_3^{2-}$  is an  
157 intermediate in the S oxidation pathway and should be chosen as the most economical  
158 electron donor. Lin et al. (2018) focused primarily on S oxidation, including biological gas  
159 desulfurization, phototrophic  $S^{2-}$  oxidation, S-dependent autotrophic denitrification, biological  
160 sulfur oxidation associated phosphorous removal, dye treatment. They also indicated viable  
161 applications of the products, such as Li batteries, production of S concrete by mixing  $S^0$  with  
162 aggregates, biologically produced S fertilizer, oxidation of  $S^{2-}$  in microbiological fuel cells,  
163 and reclamation of metals from sewage sludge.

### 164 **3.2. Heterotrophic sulfate reduction**

165 Table 3 presents the diversity of research carried out so far on heterotrophic  $SO_4^{2-}$  reduction  
166 in terms of the electron donor, type of reactor and operating conditions. Most studies have  
167 been carried out in the gas lift reactor and fluidized-bed reactor. Both organic and inorganic  
168 donors were used, including carbon monoxide, methane, methanol, ethanol, hydrogen, crab  
169 shell, compost and many others. The use of different donors resulted in a different  $SO_4^{2-}$   
170 reduction efficiency. A detailed description of the research can be found in SI (S2). The use of  
171 different electron donors and  $SO_4^{2-}$  content resulted in a wide range of  $SO_4^{2-}$  removal  
172 efficiencies (51-98%) and rates (0-3400 mg  $SO_4^{2-}$ /L/d). Nielsen et al. (2019) used methanol  
173 and ethylene glycol which resulted in reduction of  $SO_4^{2-}$  by 71.2% and 36.9%, respectively.  
174 The decrease of  $SO_4^{2-}$  concentration was limited to 13.8 and 5.3%, respectively, with the use  
175 of peat and straw. Low temperatures (below 10°C) significantly affected the  $SO_4^{2-}$  removal  
176 rates. For example, Virpiranta et al. (2019) carried out studies at various temperatures (22°C,

177 16°C, 6°C) and found gradually decreasing  $\text{SO}_4^{2-}$  removal rates, i.e. 169, 98 and 13-42 mg  
178  $\text{SO}_4^{2-}/\text{L}/\text{d}$ , respectively.

179 Sulfate reduction is less popular compared to S-dependent autotrophic denitrification,  
180 but that process has also been addressed in several reviews (Kumar et al. 2021, Costa et al.,  
181 2020, Sinharoy et al., 2020b, Serrano et al., 2019, Van den Brand et al., 2015). Kumar et al.  
182 (2021) and Costa et al. (2020) focused on the use of  $\text{SO}_4^{2-}$  reduction for treatment of metal-  
183 rich wastewater and recovery of these metals, showing a high degree of  $\text{SO}_4^{2-}$  reduction (>  
184 90%) along with the efficient (> 99%) recovery of metals (Fe, Zn, Cd, Cu).

185 Similarly, Sinharoy et al. (2020b) described treatment of acid mine drainage (AMD)  
186 with biological reduction of  $\text{SO}_4^{2-}$ . Heavy metals present in AMD can be removed by  $\text{S}^{2-}$   
187 precipitation. The review discussed various gaseous substrates, such as  $\text{H}_2$ ,  $\text{CO}$ ,  $\text{CH}_4$ , as  
188 electron donors that could be used in this process. It was emphasized that only the  
189 microorganisms capable of using gaseous substrates are appropriate for the AMD treatment  
190 systems.

191 Serrano et al. (2019) focused on the optimum conditions for SRB. They presented the  
192 recommended conditions for biomass, electron donor and acceptor and an experimental setup  
193 of three SRB tests: (1) to assess the activity of SRB culture, (2) to determine the reduction  
194 potential of an electron donor, and (3) to determine the possibility of using various sources of  
195  $\text{SO}_4^{2-}$  as an electron acceptor. They collected methodologies and results from many  
196 publications and recommended setup and monitoring conditions to increase the comparability  
197 and reproducibility of the SRB tests. Sodium sulfate and lactate were used as an electron  
198 acceptor and electron donor, respectively.

199 Van den Brand et al. (2015) analyzed important parameters, such as pH, organic  
200 substrates,  $\text{COD}/\text{SO}_4^{2-}$  ratio, substrate composition,  $\text{SO}_4^{2-}$ , salt, temperature and DO. They



201 found that the presence of SRB reduced pathogens, heavy metals and sludge produced.  
202 Sulfate reduction, autotrophic denitrification and nitrification integrated (SANI) was  
203 identified as a process combining the advantages of SRB and S-dependent autotrophic  
204 denitrification. However, they indicated that in order to ensure the benefits of using SRB, a  
205 sufficient  $\text{SO}_4^{2-}$  concentration in the influent wastewater would be required to maintain the  
206 COD/  $\text{SO}_4^{2-}$  ratio below 0.67.

### 207 **3.3. Sulfammox**

208 Sulfammox is a new process that has been addressed in the literature, especially review  
209 papers, only very recently. Sulfammox has mainly been carried out in an upflow anaerobic  
210 sludge bed reactor and circulating flow reactor (Table 3). The obtained  $\text{SO}_4^{2-}$  removal  
211 efficiencies are normally much lower compared to heterotrophic sulfate reduction. However,  
212 sulfammox is an important process linking the N and S cycles, therefore the effect of  
213 sulfammox on the overall reduction of  $\text{SO}_4^{2-}$  and  $\text{NH}_4^+$ -N should not be neglected. In the  
214 studied systems, the typical influent concentrations of  $\text{SO}_4^{2-}$  ranged from 80 to 360 mg/L (Qin  
215 et al., 2021, Zhang et al., 2019b) and the highest obtained  $\text{SO}_4^{2-}$  removal efficiency was 45%  
216 (Zhang et al., 2019a). A detailed description of the research can be found in the SI (S3).

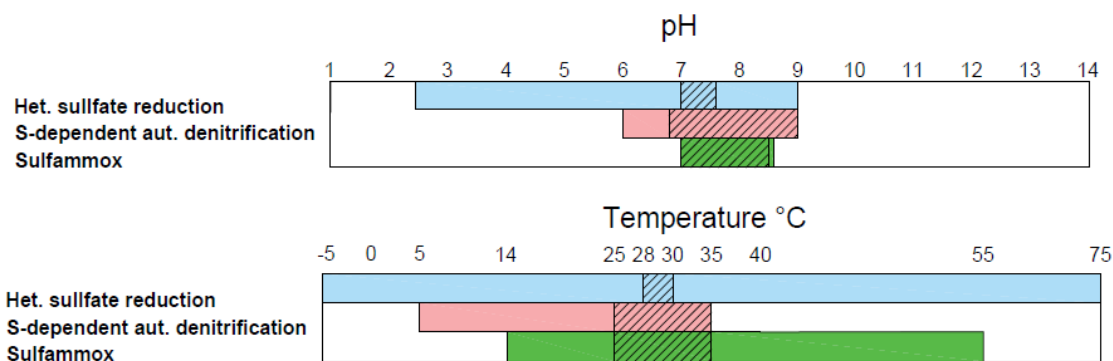
217 Liu et al. (2021) summarized the current understanding of sulfammox, including the  
218 mechanisms, responsible microorganisms and factors influencing the process. It was  
219 emphasized that the understanding of sulfammox has improved significantly in recent years,  
220 but more attention should be paid to recognizing the microbial community and its metabolic  
221 pathways. In addition, a variety of sulfammox end products were described that could be  
222 substrates for various N and S (anammox, S-dependent autotrophic denitrification) processes  
223 and coexist together in wastewater treatment systems. However, a challenge for the process is  
224 to ensure optimal environmental factors, such as temperature, pH, DO, for its practical

225 applications. It was also emphasized that residual organic carbon could have a significant  
226 positive effect on sulfamox, but this requires further research. A significant limitation of  
227 sulfamox is that the process was mostly investigated under laboratory scale. Practical  
228 applications should focus on implementations at low temperatures in full-size reactors.

229 In order to increase the efficiency of S removal in the sulfamox process, it is important to  
230 maintain the optimal pH of 8.5 and temperature of 30°C (Cai et al., 2010). The N/S ratio is  
231 also an important factor affecting that efficiency. When increasing the influent  $\text{NH}_4^+\text{-N}$   
232 concentration from 166-666 mg N/L to 1000-2000 mg N/L, then the  $\text{SO}_4^{2-}$  removal efficiency  
233 increased from 64% to 71%. However, after increasing the influent  $\text{NH}_4^+\text{-N}$  concentration  
234 further to >3000 mg/L, the  $\text{SO}_4^{2-}$  reduction efficiency decreased to 28% (Wang et al., 2017).  
235 Also, reducing the concentration of  $\text{SO}_4^{2-}$  from 223 to 154 mg/L had a positive effect on the  
236 removal of  $\text{SO}_4^{2-}$  in the sulfamox process (Zhang et al., 2020). The N/S ratio also influenced  
237 the  $\text{SO}_4^{2-}$  removal efficiency, as the  $\text{SO}_4^{2-}$  removal efficiency at N/S = 2:1 and 4:1 was 38.8%  
238 and 30.5%, respectively (Zhang et al., 2019a).

### 239 ***3.4. Optimal conditions for S-dependent autotrophic denitrification, heterotrophic sulfate*** 240 ***reduction and the sulfamox process***

241 Figure 2 shows a summary of the reported pH and temperature ranges and their optimal  
242 values for the three S-dependent processes. The overall optimum conditions are explicitly  
243 determined by the narrowest ranges for heterotrophic sulfate reduction, which are 7-7.6 and  
244 28-30°C for pH and temperature, respectively. The processes of S-dependent autotrophic  
245 denitrification, heterotrophic sulfate reduction and sulfamox can occur simultaneously with  
246 deammonification or its component processes, i.e. partial nitrification and anammox.



247

248

249 Figure 2. Ranges of pH and temperatures and their optimal values (“[ ▨ ]” – optimum  
 250 conditions) reported in literature for the S-dependent processes

251

252 For comparison, for partial nitrification, the optimal ranges were 25-35°C for  
 253 temperature (Zhu et al. 2008, Kanders et al., 2014) and 7-8.6 for pH, with the optimal value of  
 254 8 (Jaroszyński et al., 2011). On the contrary, too low temperatures (10 - 15°C) cause the  
 255 excessive activity of NOB (Kouba et al. 2017), which can grow faster than AOB under such  
 256 conditions (Hellings et al. 1998). The optimal pH range for NOB is 6 - 7.5, with the  
 257 maximum at 7 (Yin et al., 2016). For the anammox process, the optimal temperature and pH  
 258 is respectively 35-40°C (Dosta et al., 2008) and 6.7-8.3 (Jetten et al., 2001). The  
 259 recommended ranges for efficient deammonification are as follows: T = 20 - 35°C (Kanders  
 260 et al. 2014) and pH of 7.5-8 (Oshiki et al., 2011).

261 When coupling sulfamnox with S-dependent autotrophic denitrification and heterotrophic  
 262 SO<sub>4</sub><sup>2-</sup> reduction to increase the efficiency of S removal, it is important to keep the optimal  
 263 temperature of 28-30°C and pH of 7-7.6. The N/S ratio should be adjusted based on the  
 264 stoichiometry of all the processes involved, so that products of one process can be the  
 265 substrates for another process. Deviations from the optimal ratio can cause either production

266 of unwanted residues or bacterial competition for the substrates. SRB can compete with  
267 sulfamox bacteria for  $\text{SO}_4^{2-}$ . Moreover, heterotrophic  $\text{SO}_4^{2-}$  reduction and sulfamox  
268 contribute to formation of  $\text{S}^{2-}$  and/or  $\text{S}^0$ , which is the substrate for S-dependent autotrophic  
269 denitrification. Too intensive production of  $\text{S}^{2-}$  may lead to the persistence of this toxic  
270 compound in the effluent. The presence of carbon in heterotrophic  $\text{SO}_4^{2-}$  reduction may also  
271 contribute to the development of heterotrophic bacteria responsible for heterotrophic  
272 denitrification. Then  $\text{NO}_3^-$ -N and/or  $\text{NO}_2^-$ -N may become limited due to their use in both  
273 autotrophic and heterotrophic denitrification. In such a case, it is recommended to use full or  
274 partial nitrification to produce  $\text{NO}_3^-$ -N and/or  $\text{NO}_2^-$ -N. The competition and interactions of  
275 microorganisms participating in the aforementioned processes are shown in Figure 1.

#### 276 **4. Wastewater treatment systems integrating the N-S-C cycles**

##### 277 ***4.1. Systems integrating the sulfur cycle with nitrification-denitrification - Sulfate reduction,*** 278 ***Autotrophic denitrification and Nitrification Integrated (SANI) and its modifications***

279 Biological  $\text{SO}_4^{2-}$  reduction along with biological oxidation of S in the form of  $\text{SO}_3^{2-}$ ,  $\text{S}^0$  or  
280  $\text{S}_2\text{O}_3^{2-}$  are two main pathways responsible for S conversions in wastewater treatment systems  
281 (Cardoso et al., 2006). An integrated process for  $\text{SO}_4^{2-}$  reduction, autotrophic denitrification  
282 and nitrification (SANI) was aimed to primarily remove organic compounds and N (Wang et  
283 al., 2009b). This process was originally developed for saline wastewater in Hong Kong and  
284 demonstrated there in full-scale (Wu et al., 2016, Wang et al., 2009b).

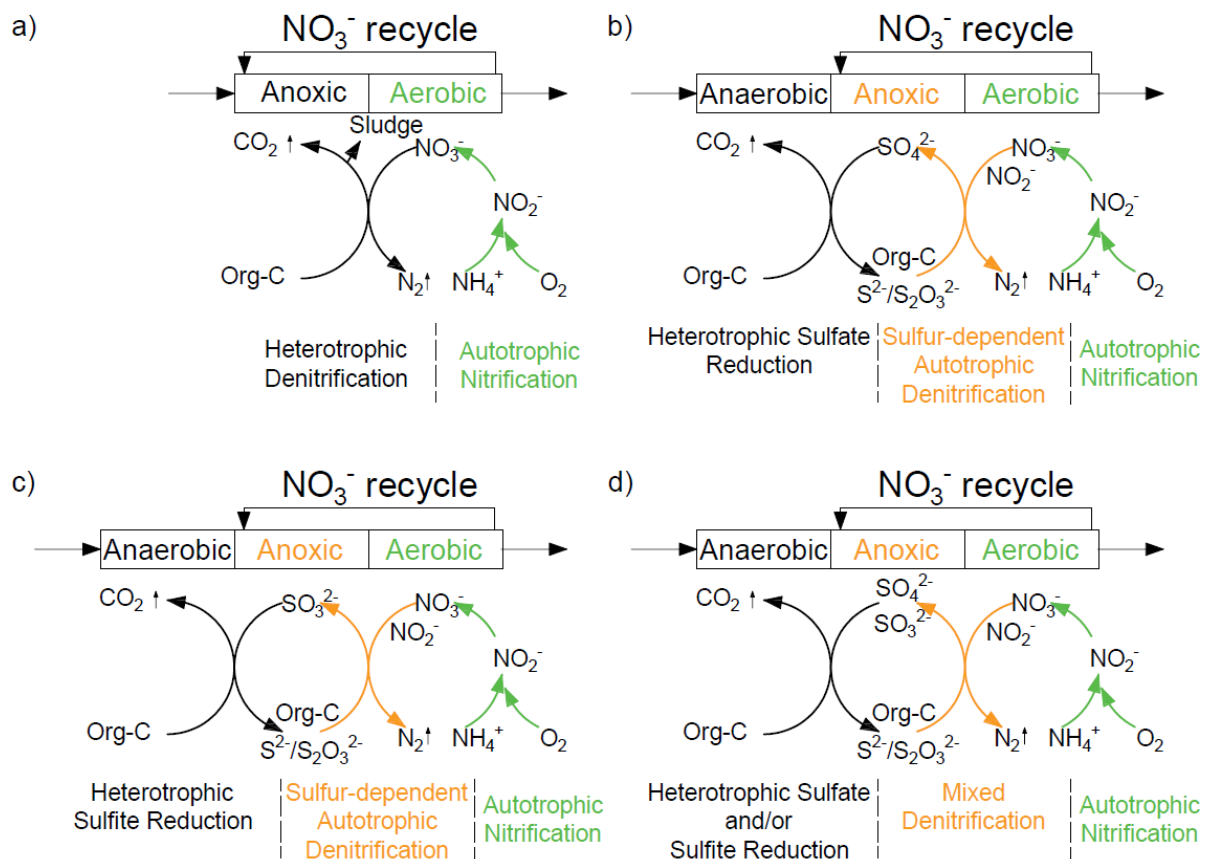
285 With that innovative approach, the conventional wastewater treatment, incorporating  
286 C and N cycles, can be extended with the S cycle, as shown in Figure 3. In the first anaerobic  
287 zone, COD is removed by SRB, which results in  $\text{SO}_4^{2-}$  reduction to  $\text{S}^{2-}$ . In the second anoxic  
288 zone, autotrophic reduction of  $\text{NO}_3^-$ -N occurs with dissolved  $\text{S}^{2-}$  formed in the first zone. In  
289 the third aerobic zone,  $\text{NH}_4^+$ -N is oxidized to  $\text{NO}_3^-$ -N, which is then recirculated to the second  
290 anoxic zone (Wang et al., 2009b). The SANI process and its modifications combine the

291 advantages of energy saving, reduced sludge production and smaller footprint. Wang et al.  
292 (2009b) noted that the total cost reduction for SANI would be >50% for a WWTP with an  
293 influent flow rate of 10,000 m<sup>3</sup>/d.

294 The SANI process can be used for treatment of SO<sub>4</sub><sup>2-</sup>-poor wastewater provided that  
295 low-cost and S-rich sources are available. For example, wet flue gas desulfurisation (FGD)  
296 systems used in boilers, coal-fired furnaces and power plants, can be reduced to alkaline flue  
297 gas sorption for production of liquid waste containing SO<sub>4</sub><sup>2-</sup> and SO<sub>3</sub><sup>2-</sup> (Srivastava and  
298 Jozewicz, 2001). Such a waste stream can be co-treated in the main wastewater stream in wet  
299 FGD-SANI after removing suspended solids and heavy metals (Qian et al., 2013).

300 The Mixed Denitrification (MD) - SANI process has also been proposed (Qian  
301 2015a,b,c). MD-SANI generates S<sub>2</sub>O<sub>3</sub><sup>2-</sup>, S<sup>2-</sup>, and some volatile fatty acids (VFA), which are  
302 subsequently converted in both heterotrophic denitrification (VFA) and autotrophic  
303 denitrification (S<sup>2-</sup> and S<sub>2</sub>O<sub>3</sub><sup>2-</sup>) (Qian et al., 2015a). It should be noted that the latter process is  
304 induced faster by S<sub>2</sub>O<sub>3</sub><sup>2-</sup> than S<sup>2-</sup> (Cardoso et al., 2006). Figure 3b-d shows the SANI, FGD-  
305 SANI and MD-SANI processes depending on the available substrates.





306

307 Figure 3. Biological wastewater treatment systems using a) conventional heterotrophic

308 denitrification with autotrophic nitrification b) SANI c) FGD-SANI d) MD-SANI

309

#### 310 4.2. Systems integrating the S cycle with anammox-based nitrogen removal processes

311 In recent years, the growing attention has been paid to N removal using the anammox process.

312 The anammox process completely eliminates the need for organic C source, reduces the

313 amount of sludge produced by 80% and related energy costs for aeration by 60% compared to

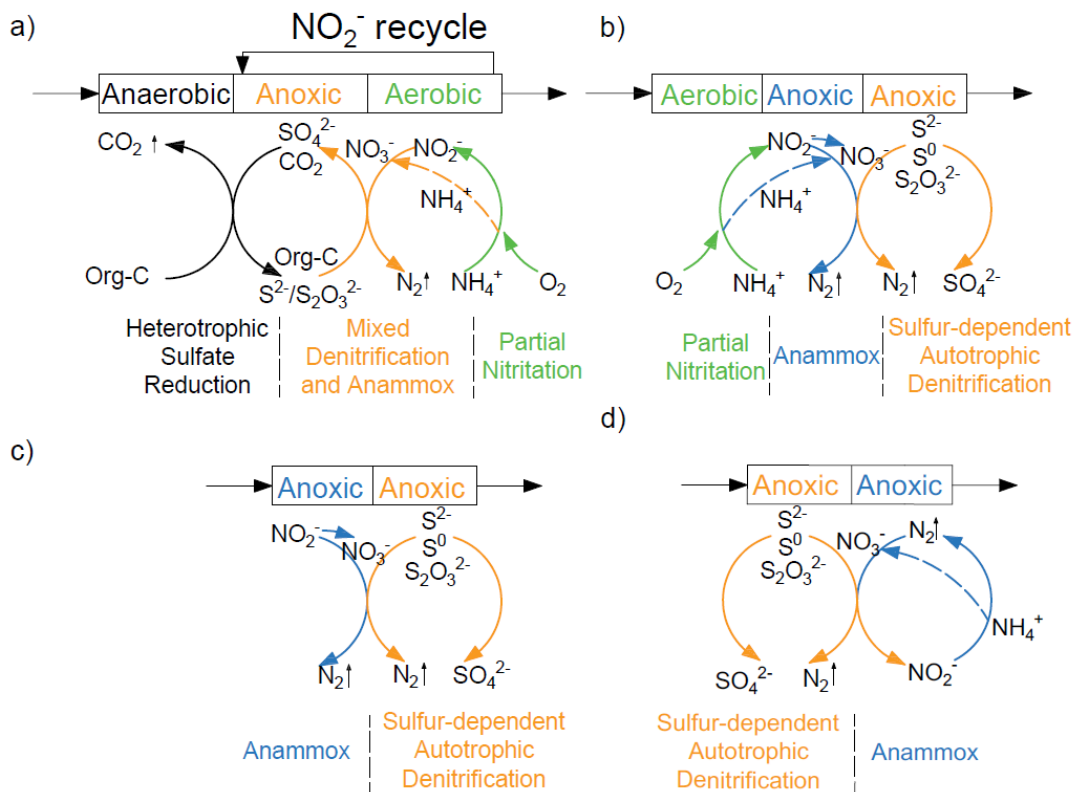
314 conventional nitrification/denitrification. The anammox process also has economic

315 advantages in the context of co-treatment of wastewater containing S compounds, especially

316 S<sup>2-</sup> (Kosugi et al., 2019).

317 The anammox-based systems for combined N and S removal comprise (1) Sulfate  
 318 Reduction, Denitrification/Anammox and Partial Nitrification (SRDAPN), (2) Partial  
 319 Nitrification/Anammox and S-dependent autotrophic Denitrification (PNASD), (3) Anammox  
 320 and S-dependent autotrophic Denitrification (ASD), and (4) S-dependent autotrophic Partial  
 321 Denitrification/Anammox (SPDA).

322 The SRDAPN process is similar to the SANI process, but enhanced with anammox  
 323 (Figure 4a). As a consequence, instead of full nitrification, only PN is needed to produce  $\text{NO}_2^-$   
 324  $-\text{N}$  (Kosugi et al., 2019).



325  
 326 Figure 4. Wastewater treatment systems using the anammox process a) SRDAPN b) PNASD  
 327 c) ASD d) SPDA

328

329 The PNASD process uses PN/A to remove  $\text{NH}_4^+\text{-N}$  under aerobic (PN) – anoxic  
330 (anammox) conditions. With S-dependent autotrophic denitrification, the produced  $\text{NO}_3^-\text{-N}$   
331 can further be reduced to  $\text{N}_2$ , as shown in Figure 4b. The PNASD process has been  
332 implemented as both two-stage (Dasgupta et al., 2017) and one-stage system (Yuan et al.,  
333 2020).

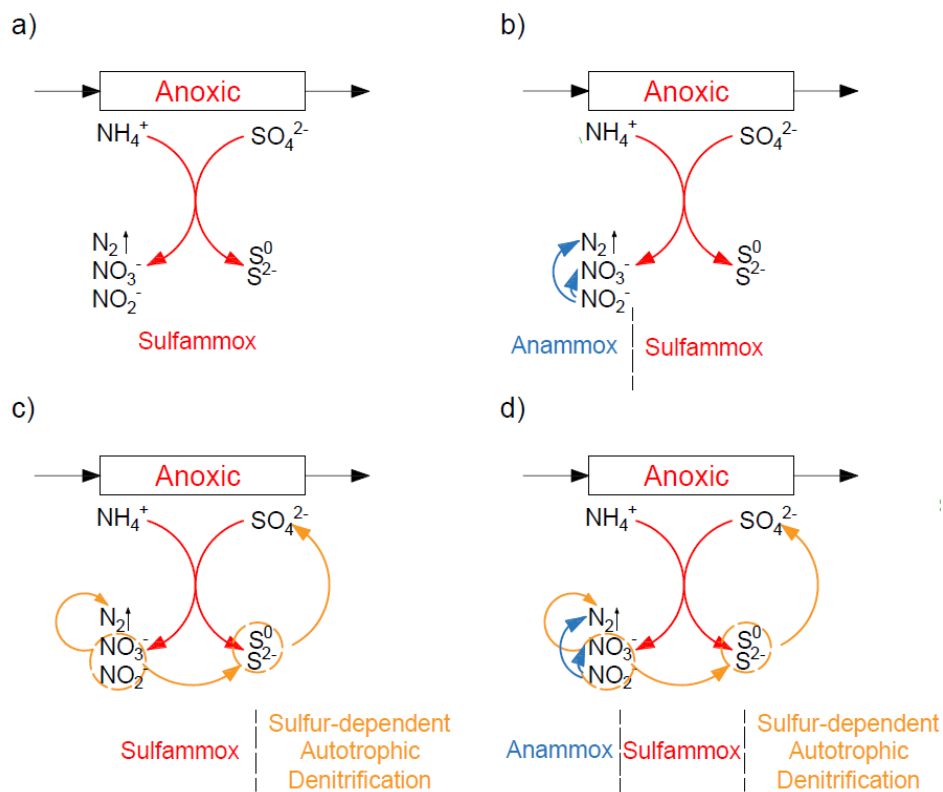
334 The PNASD system can also be limited to an ASD system that ignores the share of  
335 PN, as shown in Figure 4c. Then the  $\text{NO}_2^-\text{-N}$  acceptor for anammox is not obtained from the  
336 conversion of  $\text{NH}_4^+\text{-N}$ , but supplied from external sources. Accordingly, the costs of energy  
337 used to produce  $\text{NO}_2^-\text{-N}$  by AOB in PN are neglected, but the costs of process substrates  
338 increase. The residual  $\text{NO}_3^-\text{-N}$  from anammox can be removed along with S compounds ( $\text{S}^{2-}$ ,  
339  $\text{S}^0$ ,  $\text{S}_2\text{O}_3^{2-}$ ) by S-dependent autotrophic denitrification. The ASD process has been  
340 implemented in both one-stage (Guo et al., 2016) and two-stage (Sun et al., 2018) systems.

341 If  $\text{NO}_2^-\text{-N}$  can be obtained by partial autotrophic denitrification of  $\text{NO}_3^-\text{-N}$  with  
342 oxidation of S compounds ( $\text{S}^{2-}$ ,  $\text{S}^0$ ,  $\text{S}_2\text{O}_3^{2-}$ ), then it can be used as a substrate in the anammox  
343 process. Liu et al. (2017) and Wu et al. (2019) used a UASB reactor to perform S-dependent  
344 denitrification with  $\text{S}^{2-}$  (Liu et al. 2017) and  $\text{S}_2\text{O}_3^{2-}$  (Wu et al., 2019) for  $\text{NH}_4^+\text{-N}$  removal  
345 from wastewater, as shown in Figure 4d.

### 346 ***4.3. Systems including the sulfammox process***

347 Both sulfammox and anammox incorporate “anaerobic” oxidation of  $\text{NH}_4^+\text{-N}$ . The  
348 coexistence of both processes was found in marine sediments (Rios-Del Toro et al., 2018) and  
349 anaerobic sludge (Rikmann et al., 2016). In conventional sulfammox,  $\text{SO}_4^{2-}$  is an electron  
350 acceptor, which is reduced to  $\text{S}^0$  or  $\text{S}^{2-}$ , while  $\text{NH}_4^+\text{-N}$  is oxidized to  $\text{N}_2$ ,  $\text{NO}_2^-\text{-N}$  and/or  $\text{NO}_3^-\text{-N}$ .  
351 Sulfammox may occur on its own, as shown in Figure 5a. Alternatively, the formed  $\text{NO}_2^-\text{-N}$

352 N may be used as an electron acceptor for anammox in the combined Sulfammox/Anammox  
 353 (SA) system (Figure 5b).



354  
 355 Figure 5. Wastewater treatment systems incorporating the sulfammox process a) Sulfammox  
 356 b) SA c) SSD d) SASD  
 357

358 As  $\text{NO}_2^-$ -N and  $\text{NO}_3^-$ -N are generated in sulfammox, the process can be combined with  
 359 autotrophic S-dependent denitrification in an Sulfammox - S-dependent autotrophic  
 360 Denitrification (SSD) system, as shown in Figure 5c (Liu et al., 2021, Grubba et al., 2021).  
 361 The formed  $\text{S}^0$  and  $\text{S}^{2-}$  in sulfammox can be oxidized again to  $\text{SO}_4^{2-}$ , while  $\text{NO}_x$ -N are  
 362 reduced to  $\text{N}_2$ . The SSD system can be expanded with anammox in SASD (Sulfammox –  
 363 Anammox - S-dependent autotrophic denitrification), as shown in Figure 5d. In this case,

364 NO<sub>2</sub><sup>-</sup>-N can be reduced by both AAOB and autotrophic denitrifiers (Liu et al., 2021, Grubba  
365 et al., 2021).

## 366 **5. Operational conditions and performances of the systems integrating the N-S-C** 367 **cycles**

368 The biochemical processes associated with the C, N and S conversions and the  
369 microorganisms responsible for those conversions can be found in the SI (Figure S2).

### 370 **5.1. SANI, FGD-SANI, MD-SANI**

371 The S cycle, which is part of the SANI process, ensures a more efficient use of electrons (Wu  
372 et al., 2020) and eliminates the production of toxic S<sup>2-</sup> (Qian et al., 2015c). In addition, it  
373 reduces sludge production by 90% compared to the conventional biological N removal  
374 processes. This is possible due to very low yield coefficients of the microorganisms  
375 responsible for SO<sub>4</sub><sup>2-</sup> reduction, autotrophic denitrification and nitrification, i.e., 0.02 kg  
376 VSS/kg COD, 0.01 kg VSS/kg NO<sub>3</sub><sup>-</sup>-N and 0.07 kg VSS/kg NH<sub>4</sub><sup>+</sup>-N, respectively (Lu et al.,  
377 2011, Wang et al., 2009b). In addition, there are other significant reductions, including energy  
378 consumption by 35% (Lu et al., 2011), greenhouse gas emission (GHG) by 36% (Lu et al.,  
379 2011), and the space required for the process of wastewater treatment and sludge handling by  
380 30%–40% (Liu et al., 2016).

381 As shown in Table 4, SANI shows a relatively high level of performance compared to  
382 the conventional systems. The efficiencies of SO<sub>4</sub><sup>2-</sup>, total nitrogen (TN) and COD removal  
383 vary in the ranges of 72-98%, 55-74% and 82-97%, respectively (Hao et al., 2015, Lu et al.,  
384 2009). The SANI modifications (FGD-SANI and MD-SANI), which use wastewater streams  
385 from wet flue gas desulphurization, reveal even a greater performance potential (Qian et al.,  
386 2015a, b, Jiang et al., 2013). The biological reduction of SO<sub>3</sub><sup>2-</sup> in FGD-SANI and MD-SANI  
387 provides more energy for bacterial growth, which is associated with a higher sludge efficiency

388 compared to the biological reduction of  $\text{SO}_4^{2-}$  (Jiang et al., 2013). Moreover,  $\text{SO}_3^{2-}$  is an  
389 intermediate in  $\text{SO}_4^{2-}$  reduction, which may result in faster reduction by SRB.

390 Jiang et al. (2013) found that the removal rates of specific organics in the  $\text{SO}_3^{2-}$  and  
391  $\text{SO}_4^{2-}$  reducing reactors were similar. At the extremely low temperatures ( $<10^\circ\text{C}$ ), incomplete  
392 reduction of  $\text{SO}_3^{2-}$  in an anaerobic reactor (Figure 3c) resulted in accumulation of  $\text{S}_2\text{O}_3^{2-}$  and  
393 reduction in the removal rate of organics. However, the anoxic and aerobic reactors (Figure  
394 3c) still provided a high removal efficiency of organics ( $>94\%$ ), while  $\text{NH}_4^+\text{-N}$  and  $\text{NO}_3^-\text{-N}$   
395 were almost completely removed.

396 The MD reaction can lead to a much higher reduction of  $\text{NO}_3^-\text{-N}$  and  $\text{NO}_2^-\text{-N}$   
397 compared to the  $\text{S}^{2-}$  based SANI process (Qian et al., 2015a). Qian et al. (2015b) reported that  
398 the denitrification rate increased sevenfold in MD-SANI compared to SANI. Furthermore, in  
399 comparison with SANI, FGD-SANI shows higher TN and COD removal efficiencies (98%  
400 and 94%). The complete removal of  $\text{SO}_3^{2-}$  and TN was achieved in MD-SANI, while the  
401 COD removal efficiency in that process was 81% (Table 4).

## 402 ***5.2. SRDAPN and PNASD - challenges resulting from the combination of aerobic and*** 403 ***anaerobic conditions***

404 The presence of  $\text{S}^{2-}$  in the influent wastewater imposes a significant risk of inhibition of the  
405 AAOB responsible for anammox. Threshold levels of  $\text{S}^{2-}$  inhibiting AAOB were found in the  
406 range of  $<1$  to  $64$  mg S/l (Jin et al., 2013, Carvajal-Arroyo et al., 2013, Dapena-Mora et al.,  
407 2007). The study by Wiśniewski et al. (2019) determined the half maximal inhibitory  
408 concentration ( $\text{IC}_{50}$ ) under two different  $\text{S}^{2-}$  conditions. The  $\text{IC}_{50}$  was  $4.25$  mg  $\text{H}_2\text{S-S/L}$  at a  
409 constant  $\text{S}^{2-}$  concentration of  $11$  mg TS-S/L and pH in the range  $7-7.9$  vs.  $4.67$  mg  $\text{H}_2\text{S-S/L}$  at  
410 a varying concentration of  $\text{S}^{2-}$  ranging from  $1$  to  $15$  mg TS-S/L and a constant pH of  $7$ . The  
411 decrease in AAOB activity was due to the pH-dependent non-ionized form of  $\text{H}_2\text{S}$ . In

412 addition, heterotrophic bacteria may coexistence with AAOB but also outcompete AAOB at  
413 high influent C/N ratios (Chamchoi et al., 2008).

414 The PNASD process has been implemented in both one- and two-stage systems. The  
415 two stage-systems are easier to maintain and allow to avoid the negative impact of  $S^{2-}$  on  
416 AAOB and the competition between AOB and SOB for DO (Sahinkaya and Kilic, 2014a).

417 Zhang et al. (2020) used  $S^0$  for denitrification and observed only a small effect, when  
418 DO was kept at the level of 0.4-0.8 mg/L. When the DO concentration increased to 1.2 mg  
419  $O_2/L$ , the concentrations of  $NO_3^-$ -N and  $SO_4^{2-}$  also increased. This indicates excessive  
420 oxidation of  $S^{2-}$  or its reduced compounds in aerobic systems. Under non-limited DO  
421 conditions, autotrophic SOB can readily utilize oxygen, which leads to accumulation of  $SO_4^{2-}$ .  
422 On the other hand, too low DO concentrations in the PNASD process can reduce the  $NO_2^-$ -N  
423 production rate in PN.

### 424 **5.3. ASD, SPDA and sulfammox systems - coexistence of AAOB and denitrifiers**

425 Under anaerobic conditions, the combination of anammox process and S-dependent  
426 autotrophic denitrification can work with high removal efficiencies of TN (88-96%) and S  
427 (90-100%) (Table 4). AAOB and *T. denitrificans* can assist in the combined N and S removal  
428 without inhibition by  $S^{2-}$  (Guo et al., 2016). In that study, most of  $S^{2-}$  was oxidized to  $S^0$  at the  
429 influent ratios of  $NH_4^+$ -N/ $S^{2-}$  and  $NO_2^-$ -N/ $S^{2-}$  at 1.74 and 2.2-2.27, respectively. Two S forms  
430 can accumulate depending on the S/N ratio in the reactor, i.e.,  $SO_4^{2-}$  (at S/N ratio <1) or  $S^0$  (at  
431 S/N ratio >1) (Cardoso et al., 2006).

432 When  $NO_2^-$ -N is fed to the anammox process, S-dependent autotrophic denitrification  
433 may occur. When both  $NO_2^-$ -N (anammox substrate) and  $NO_3^-$ -N (anammox product) are  
434 simultaneously present in the influent, the latter form is the preferred electron acceptor for

435 denitrification (Guo et al., 2016). However, a small portion of  $\text{NO}_2^-$ -N can also be used by T.  
436 *denitrificans* and increase the overall efficiency of N and S removal.

437         Instead of complete denitrification, partial reduction to  $\text{NO}_2^-$ -N can be achieved. This  
438 approach is advantageous for the Partial Denitrification/Anammox (PD/A) systems by  
439 continuously producing  $\text{NO}_2^-$ -N for anammox (Wu et al., 2019). In addition, the consumption  
440 of electron donors can be reduced in comparison with the conventional biological nitrogen  
441 removal processes. The reported TN removal efficiencies exceeded 90% in SPDA (Table 4).

442         The novel sulfamnox process has been applied in  $\text{SO}_4^{2-}$  and  $\text{NH}_4^+$ -N-rich wastewater  
443 treatment systems. One of the intermediates in the sulfamnox reaction is  $\text{NO}_2^-$ -N, which can  
444 be used by either AAOB or S-dependent autotrophic denitrification along with the residual  
445  $\text{NO}_3^-$ -N from anammox. Wu et al. (2020) combined sulfamnox and anammox and obtained  
446 high removal efficiencies of  $\text{NH}_4^+$ -N (98.5%) and  $\text{SO}_4^{2-}$  (53%). Furthermore, the sulfamnox  
447 and anammox processes can also be combined with S-dependent autotrophic denitrification  
448 (Rios-Del Toro et al., 2018).

## 449 **6. Modeling N, S and C conversions in wastewater treatment systems**

450 Modeling has been proven to be an effective tool to understand complex, interrelated N, S and  
451 C transformations (Show et al., 2013). In principle, two modeling approaches are possible,  
452 including empirical models, such as artificial neural networks (ANNs), and mechanistic  
453 models based on the Activated Sludge Model (ASM) family.

### 454 **6.1. Artificial neural networks (ANNs)**

455 The ANN model does not require a detailed process description, and it can be established by  
456 simple input and output parameters. Therefore, the ANN has been known for a long time as a  
457 tool in setting control mechanisms and performance models of biological wastewater  
458 treatment processes (Choi and Park, 2001). Wang et al (2009a) developed an ANN model to



459 monitor a denitrifying  $S^{2-}$  removal (DSR) process. The proposed model revealed that the  
460 comparative influences of four input factors on DSR performance were as follows: hydraulic  
461 retention time (HRT) >  $S^{2-}$  concentration > C/S ratio > N/S ratio. Even though the ANN  
462 model is capable of predicting an intricate function between input and output parameters, it  
463 cannot help in understanding mechanisms of the complex biochemical processes.

## 464 **6.2. Mechanistic models**

465 The International Water Association Activated Sludge Models (ASMs) No. 1, 2, 2d and 3  
466 (Henze et al., 2000) describe conversions of organic C and N compounds (ASM1 and ASM3),  
467 and additionally P compounds (ASM2 and ASM2d). However, to simplify the model  
468 structure, all the ASMs only considered  $NO_3^-$ -N reduction as a one-step heterotrophic process  
469 using readily biodegradable organic compounds as electron donors. Moreover, one-step  
470  $NH_4^+$ -N oxidation to  $NO_3^-$ -N was the only autotrophic N transformation.

471 S-dependent autotrophic denitrification and the synergistic and competitive  
472 relationships among microorganisms were subsequently integrated with the ASMs. On one  
473 hand, developing realistic models is essential for practical applications in simultaneous N, C  
474 and S removal systems. On the other hand, due to the complex interactions between  
475 autotrophic and heterotrophic denitrifiers, developing an exhaustive model and appropriate  
476 control strategy becomes challenging. The existing models (Table 5) have been used in  
477 bench-scale reactors to predict the process involving intricate metabolic pathways with  
478 synthetic substrates. However, further work is still necessary to confirm the models in  
479 practical applications with real wastewater.

480 A detailed description of the mechanistic models can be found in SI (S4).

481 **7. Implications of combining the N, S and C cycles in wastewater treatment**  
482 **systems**

483 **7.1. Processes application opportunities**

484  $S^0$  and  $S^{2-}$  are considered good alternatives to organic matter in the denitrification process due  
485 to the absence of organic residues in the treated wastewater. It is thus strongly recommended  
486 to use S-dependent autotrophic denitrification instead of heterotrophic denitrification,  
487 especially for wastewater with a low organic content. Attention should also be paid to the  
488 water-insoluble  $S^0$ , which can physically be removed from wastewater and reused for  
489 production of sulfuric acid, pesticides, fertilizers, in construction (Lin et al., 2018). It is  
490 economic, effective and readily available source of electrons. On the other hand,  $S_2O_3^{2-}$  is  
491 readily bioavailable and may mediate a higher rate of denitrification compared to  $S^0$  and  $H_2S$ .  
492  $S^{2-}$  is often used in municipal and industrial areas requiring desulphurization. Depending on  
493 the local conditions, S-dependent autotrophic denitrification can occur with a wide spectrum  
494 of S compounds. Moreover, it can get them from the initial  $SO_4^{2-}$  reduction stage in the  
495 integrated systems combining N-S-C cycles.

496 Biological SRB-based methods are a sustainable way of treating AMD compared to  
497 physico-chemical methods (Sinharoy et al., 2020b). SRB are capable of using toxic metals in  
498 their metabolism, thus reducing environmental and human health problems. SRB can grow in  
499 a wide range of environmental conditions, which provides many opportunities for the  
500 development of technologies based on their metabolism, with  $SO_4^{2-}$  reduction being  
501 recognized as a key step in all S- dependent processes (Hao et al., 2014).

502 Among the various gaseous substrates for  $SO_4^{2-}$  reduction,  $H_2$  is most energetic for  
503 SRB. The resources that can be recovered from this process are metal sulfides and  $S^0$ , which  
504 has also been identified by Kumar and Pakshirajan (2020) as a potential substrate for S-  
505 dependent autotrophic denitrification.



506 The combination of the N, S and C cycles could lead to the development of  
507 economically feasible and sustainable wastewater treatment systems that produce less sludge  
508 and reduce carbon footprint compared to the existing systems. The SANI process has already  
509 been used in several full-scale wastewater treatment installations in Hong Kong due to the  
510 practice of flushing toilets with seawater (Jiang et al., 2013). The process can also be applied  
511 to freshwater wastewater, even in cold inland areas that do not contain enough  $\text{SO}_4^{2-}$  or  $\text{SO}_3^{2-}$   
512 rich wet flue gas desulphurization (Qian et al., 2015a, b, Jiang et al., 2013). It can also be  
513 adapted to treat industrial wastewater by adding  $\text{SO}_4^{2-}$ , seawater or some  $\text{SO}_4^{2-}$ -rich  
514 wastewater. Lu et al. (2009, 2012) suggested that the SANI process could be a good solution  
515 in densely populated cities to treat saline wastewater as an economic source in terms of water  
516 scarcity and wastewater treatment in water-poor coastal areas.

517 Other technologies that include anammox and SANI processes have discovered the  
518 advantages of AAOB coexisting with SRB, SOB, and AOB. In addition, compared to the  
519 SANI process, the combination of  $\text{SO}_4^{2-}$  reduction, denitrification/anammox and partial  
520 nitrification will further reduce aeration energy consumption due to the lack of full  
521 nitrification required for  $\text{NO}_3\text{-N}$  production. The presence of anammox in the SRDAPN  
522 process resulted in an increased  $\text{NO}_2\text{-N}$  removal efficiency by over 30% (Kosugi et al.,  
523 2019).

524 For wastewater with a low organic content, PNASD can be considered a viable option.  
525 The two-step PNASD system was more efficient for N and S removal, and easier to maintain  
526 than the one-step system (where bacteria competed for DO) (Dasgupta et al., 2017).  
527 Moreover, it has also been proven that the process can be applied in a single reactor under  
528 mainstream conditions (Yuan et al., 2020).

529 Instead of combining the heterotrophic  $\text{SO}_4^{2-}$  reduction with anammox, sulfammox  
530 can replace or accompany both processes by using a  $\text{SO}_4^{2-}$  dependent AAOB. Recent studies



531 have proposed the use of sulfammox based on the combined reduction of  $\text{NH}_4^+\text{-N}$  and  $\text{SO}_4^{2-}$ .  
532 If  $\text{SO}_4^{2-}$  was reduced to  $\text{S}^{2-}$  or  $\text{S}^0$  with organic compounds, this process would be replaced  
533 with sulfammox, while eliminating the addition of external carbon. Another suggested  
534 solution is to combine the sulfammox process with heterotrophic  $\text{SO}_4^{2-}$  reduction in order to  
535 increase the reduction rate of  $\text{SO}_4^{2-}$ . Moreover, if sulfammox is used upstream of an S-  
536 dependent autotrophic denitrification reactor, it contributes to oxidation of  $\text{NH}_4^+\text{-N}$  to  $\text{N}_2$   
537 (which increases the overall efficiency of  $\text{NH}_4^+\text{-N}$  removal) or  $\text{NO}_2^-\text{-N}$  and  $\text{NO}_3^-\text{-N}$  (which  
538 can be used in S- dependent autotrophic denitrification). By combining sulfammox and  
539 anammox, the efficiency of  $\text{NH}_4^+\text{-N}$  removal and  $\text{SO}_4^{2-}$  reduction to  $\text{S}^0$  can be simultaneously  
540 increased (Liu et al., 2021, Grubba et al., 2021).

## 541 *7.2. Advantages and disadvantages of two cycles or three cycles in wastewater treatment*

542 The advantages and disadvantages of the systems based on the N-S-C cycles and their  
543 coupling are summarized below.

### 544 **Advantages:**

- 545 1. Approximately 35% reduction in energy consumption and up to 90% reduction in sludge  
546 production compared to full nitrification-denitrification.
- 547 2. Reduction or even no external carbon dosing for S-dependent autotrophic denitrification.
- 548 3. For the combined processes, almost complete N and  $\text{S}^{2-}$  removal and up to 75% efficiency  
549 of  $\text{SO}_4^{2-}$  removal.
- 550 4. Products of one process used as the substrates for another process.
- 551 5. When replacing heterotrophic denitrification with S-dependent autotrophic denitrification,  
552 carbon consumption is reduced by 100%. If heterotrophic  $\text{SO}_4^{2-}$  reduction is replaced by  
553 sulfammox, carbon consumption is also reduced by 100%.
- 554 6. Removal of a few harmful compounds ( $\text{NH}_4^+$ ,  $\text{NO}_2^-$ ,  $\text{SO}_4^{2-}$ ,  $\text{S}^{2-}$ ) in one system.
- 555 7. Approximately 30–40% reduction of volumes required for wastewater and sludge treatment  
556 processes.
- 557 8. Reduction of GHG emissions by 36% compared to conventional nitrification-  
558 denitrification.

559

560 **Disadvantages:**

- 561 1. Limited use in cold regions due to the high optimal temperature range (28-30°C).  
562 2. Complex interactions and competition for substrates between the functional  
563 microorganisms.  
564 3. Greater complexity of the systems potentially resulting in higher investment costs.  
565 4. The operating conditions must be compatible with all the N-S-C processes.  
566 5. Some substrates/products involved in one process may be inhibitors for other processes,  
567 e.g.  $S^{2-}$ .

568 **7.3. Processes application limitations**

569 One of the most important limitations of technologies combining N, S and C cycle processes  
570 is the narrow optimal range of temperature (28-30°C) and pH (7-7.6). Thus, cold weather in  
571 inland areas also restricts the use of coupled systems.

572 An important factor that should be considered when implementing technologies  
573 containing the S-dependent autotrophic denitrification process is the inhibition of this process  
574 caused by  $S^{2-}$  (Cardoso et al., 2006) as well as  $NO_2^-$ -N,  $NO_3^-$ -N and free nitric acid (FNA)  
575 (Cui et al., 2019). Even though  $S^0$  is an inexpensive and non-toxic electron donor, but it  
576 provides a low denitrification rate due to its low solubility. The use of smaller S granules with  
577 a larger surface area improves the reaction efficiency, however it can cause low porosity and  
578 clogging and fouling of the reactors due to small S grain size or cracking (Wu et al., 2021).  
579 Moreover, as  $S^0$  and  $H_2S$  reveal a much lower rate of  $NO_3^-$ -N reduction, mainly the use of  
580  $S_2O_3^{2-}$  is recommended in the process. However, its natural content of wastewater is rather  
581 limited due to its instability (Cui et al., 2019).

582 In the case of heterotrophic  $SO_4^{2-}$  reduction, the presence of DO,  $NO_3^-$ -N and  $NO_2^-$ -N  
583 inhibits reduction of  $SO_4^{2-}$  and enhances oxidation of  $S^{2-}$  to  $S^0$  or  $SO_4^{2-}$  (Mohanakrishnan et  
584 al., 2009). Moreover, the activity of SRB is inhibited by heavy metals, including Pb and Cd

585 (Sinharoy and Pakshirajan, 2019b). The toxicity of heavy metals depends mainly on the type  
586 of metal, responsible microorganisms, presence of other pollutants, and process conditions  
587 (Mal et al., 2016). Therefore, the systems based on heterotrophic  $\text{SO}_4^{2-}$  reduction cannot be  
588 used for wastewater rich in heavy metals. Moreover, a significant limitation is the limited  
589 number of microorganisms that are able to carry out  $\text{SO}_4^{2-}$  reduction with the use of gaseous  
590 substrates. Moreover, the low gas-liquid mass transfer also makes it difficult to scale-up the  
591 process.

592 A significant limitation in the implementation of integrated systems connecting N-S-C  
593 cycles is also the insufficient knowledge about the mechanism of sulfamox and responsible  
594 microorganisms. Until now, there has been no genomic evidence to support the ability of  
595 AAOB to use  $\text{SO}_4^{2-}$  as an electron acceptor. The growth rate of potential functional bacteria is  
596 also low, which limits their unambiguous identification (Liu et al., 2021). Moreover, the  
597 organic matter present in the wastewater stimulates the survival of heterotrophic bacteria,  
598 including denitrifiers. This leads to a competition between these bacteria and the sulfamox  
599 bacteria, thus destroying the sulfamox process.

#### 600 ***7.4. Processes application challenges***

601 Using specific N, S and C removal processes independently of each other is much easier to  
602 maintain than the processes combining these cycles. To link those processes in the combined  
603 technologies as presented in this review, it is important to recognize the effects of  $\text{S}^{2-}$  on N  
604 removal processes, such as autotrophic/heterotrophic denitrification and anammox, as well as  
605 the competition between AOB and SOB for DO.  $\text{S}^{2-}$  and organic matter, which are fed to an  
606 anaerobic compartment, can inhibit AAOB in anammox-coupled systems (Kosugi et al.,  
607 2019). Chen et al. (2018) showed that DO can react with  $\text{S}^{2-}$  while reducing the  $\text{NO}_3^-$ -N  
608 removal rate. In addition,  $\text{S}^{2-}$  was reduced to  $\text{S}^0$  and then converted to  $\text{SO}_4^{2-g}$  due to the

609 presence of DO. These findings highlight the challenges faced by single-stage integrated  
610 systems.

611 In order to avoid the inhibition of  $\text{SO}_4^{2-}$  reduction by heavy metals, it is recommended  
612 to use an upstream reactor in order to remove metals from AMD using  $\text{S}^{2-}$ . In order to use  
613  $\text{SO}_4^{2-}$  reduction coupling systems, it is also necessary to consider selection of the appropriate  
614 type of reactor, use of resistant microorganisms, and presence of other pollutants. Designing  
615 novel reactor configurations with high gas-liquid mass transfer can also help in applying the  
616 process in full scale. Moreover, instead of obtaining pure gases, a cost-effective solution  
617 would be production of gaseous substrates by thermochemical or biochemical methods from  
618 various compounds (e.g. waste) (Sinharoy et al., 2020b).

619 In the case of sulfamox, more research is needed to identify potential applications  
620 and integration with other systems. The key enzymes involved in the metabolism of  $\text{NH}_4^+\text{-N}$   
621 and  $\text{SO}_4^{2-}$  should also be investigated. For this purpose, it is important to develop appropriate  
622 reactor configurations and create operational conditions that can enrich functional bacteria  
623 and allow for simultaneous removal of  $\text{NH}_4^+\text{-N}$  and  $\text{SO}_4^{2-}$ . Under non-limited  $\text{NO}_3^-\text{-N}$   
624 conditions, the  $\text{SO}_4^{2-}$  concentration may increase due to S-dependent autotrophic  
625 denitrification. The role of organic matter also requires further investigation with regard to the  
626 existence of the sulfamox process.

627 The combination of anammox, S-dependent autotrophic denitrification and sulfamox  
628 processes is challenging due to the different requirements of the microorganisms responsible  
629 for each process. The S-dependent autotrophic denitrification process may result in the  
630 production of  $\text{SO}_4^{2-}$  from  $\text{S}^{2-}$  or  $\text{S}^0$ , which negatively affects sulfamox, where  $\text{SO}_4^{2-}$  must be  
631 reduced to  $\text{S}^0$  (Liu et al., 2021). More focused research on the coexistence of sulfamox with  
632 other bacteria and the development of a mechanistic model are needed to better understand  
633 and predict N and S dynamics. Moreover, the S/N ratio also plays an important role in



634 determining the S-dependent autotrophic denitrification end products, requiring a closer look  
635 at the N and S dynamics. On the other hand, in order to avoid fouling and clogging of the  
636 reactors due to the presence of  $S^0$ , it is important to search for the appropriate sulfur grain  
637 size.

638 Wang et al. (2009b) identified three main challenges for the SANI process. First of all,  
639 it is the low efficiency of both  $SO_4^{2-}$  reduction during heterotrophic and S-dependent  
640 autotrophic denitrification reduction. Secondly, high concentrations of  $SO_4^{2-}$  are required,  
641 which may increase residual  $S^{2-}$  in the treated wastewater. Thirdly, transfer of  $NO_3^-$ -N from  
642 the nitrification reactor to the S-dependent autotrophic denitrification reactor can also be  
643 difficult.

## 644 **8. Conclusions**

645 In terms of sustainability, the combination of N-S-C cycles processes has a few important  
646 benefits, including energy savings and lower sludge production. The combined processes  
647 allow for almost complete N and  $S^{2-}$  removal, while the efficiency of  $SO_4^{2-}$  removal can reach  
648 up to 75%.

649 Among all the processes linking the N-S-C cycles, SANI has been best recognized,  
650 but is rather not applicable in the case of wastewater with low organic content. Instead, it is  
651 worth of considering the sulfammox process that can reduce  $SO_4^{2-}$  and increase  $NH_4^+$ -N  
652 removal rate under anoxic conditions without the addition of external carbon.

653 Practical applications of the reviewed systems still face many challenges, especially in  
654 the single-stage configurations. In particular, the coexistence of several bacterial groups  
655 (AOB, AAOB, sulfammox bacteria, SOB, SRB) and their competition for the substrates is a  
656 key issue to be considered. Moreover, practical applications of the coupled S and N/C cycles  
657 require realistic models. However, due to the complex interactions between autotrophic and



658 heterotrophic denitrifiers, development of a mechanistic model and appropriate control  
659 strategy becomes challenging.

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Table 1. Key issues and challenges of S-dependent autotrophic denitrification, heterotrophic sulfate reduction and sulfamox

Topic	Process		
	S-dependent autotrophic denitrification	Heterotrophic sulfate reduction	Sulfamox
<b>Key issues</b>	consists of oxidation of S compounds, including $S^{2-}$ , $S^0$ , thiosulfate ( $S_2O_3^{2-}$ ) and sulfite ( $SO_3^{2-}$ ), coupled with reduction of $NO_3^-$ -N and/or $NO_2^-$ -N	$SO_4^{2-}$ reduction, which involves the use of organic electron donors or inorganic electron donors, which must be supplemented with a carbon source	$NH_4^+$ -N is oxidized to $N_2$ , whereas $SO_4^{2-}$ plays the role of an electron acceptor and is reduced to $S^0$ under anaerobic conditions
<b>Challenges and opportunities</b>	<ul style="list-style-type: none"> <li>a) a good alternative to heterotrophic denitrification due to the lack of carbon dosing;</li> <li>b) reduction of toxic <math>S^{2-}</math>;</li> <li>c) the possibility of treating wastewater poor in organic content;</li> <li>d) residual <math>SO_4^{2-}</math> in wastewater;</li> <li>e) a long incubation time is needed before a fully adapted culture is obtained;</li> <li>f) precise control strategy (from <math>S^{2-}</math> to <math>S^0</math>) and novel <math>S^0</math> recovery technology at the source;</li> <li>g) acclimation and adjustment of microorganisms: the concentration of <math>S^{2-}</math> should be controlled; maintaining the denitrification efficiency of autotrophic denitrification systems at low temperatures; alkalinity and pH control is necessary to prevent the formation of <math>NO_2^-</math>-N; influence of the N/S ratio on the reactions and bioproducts, the optimal N/S ratio = 0.5-0.9 for S oxidation and <math>NO_3^-</math>-N reduction (see Eqs. 1-8 in the SI);</li> <li>h) when the dissolved oxygen (DO) concentration is <math>&gt; 1.6</math> mg <math>O_2</math>/L, denitrification is completely inhibited</li> </ul>	<ul style="list-style-type: none"> <li>a) <math>SO_4^{2-}</math> reduction, especially in <math>SO_4^{2-}</math> rich industrial wastewater;</li> <li>b) use of wastewater rich in organic compounds;</li> <li>c) high concentrations of <math>SO_4^{2-}</math> inhibit SRB activity;</li> <li>d) elevated levels of heavy metals may reduce or terminate SRB activity</li> </ul>	<ul style="list-style-type: none"> <li>a) anaerobic oxidation of <math>NH_4</math>-N without carbon addition;</li> <li>b) <math>SO_4^{2-}</math> reduction in wastewater;</li> <li>c) knowledge of microorganisms, mechanisms and their metabolic pathway is still limited;</li> <li>d) temperature, DO and pH would influence its practical applications;</li> <li>e) inhibition of sulfamox activity due to <math>S^{2-}</math> accumulation;</li> <li>f) with a high concentration of <math>NO_3^-</math>-N, <math>SO_4^{2-}</math> concentration may increase due to autotrophic denitrification</li> </ul>



Table 2. Process conditions and observed  $\text{NO}_3^-$  utilization rates during S-dependent autotrophic denitrification in different types of reactors

Reactor type	Electron donor	Temperature	pH	S-compound	Initial $\text{NO}_3^-$ -N concentration	$\text{SO}_4^{2-}$ production	Denitrification rate	References
		(°C)	(-)	(mg S/L)	(mg N/L)	(mg S/L)	(kg N/m <sup>3</sup> /d)	
Fluidized-bed reactor	$\text{S}_2\text{O}_3^{2-}$	20-30	7	184-2260	100-1230	150-320	1.24-3.25	Zou et al., 2016
Fluidized-bed reactor	$\text{S}^0$	28-30	7.2-9	na	25-75	100-600	0.07-0.2	Sahinkaya and Dursun 2015
Fluidized-bed reactor	$\text{S}^0/\text{S}_2\text{O}_3^{2-}$	20	6.8-8.2	na	20-700	na	2.53-3.37	Kim et al., 2004
Packed-bed reactor	$\text{S}^0$	28-30	6-8	na	50-75	200-600	0.07-0.1	Sahinkaya and Kilic, 2014a
Packed-bed reactor	$\text{S}^0$	10-26	6-8	na	30-60	191-483	0.03-0.24	Sahinkaya et al., 2014b
Packed-bed reactor	$\text{S}^0$	15.2-29	6.7-8.4	592.42-5924.17	20-25	640	0.2	Kimura et al. 2002
Packed-bed reactor	$\text{S}^0$	20-25	8.3-8.7	na	60-251	na	0.27-0.87	Koenig and Liu 2002
Packed-bed reactor	$\text{S}^0$	20-25	na	na	60-400	na	0.48-0.77	Koenig and Liu 2001
Up-flow continuous reactor	$\text{S}^{2-}$	29-31	7	160-1000	30.4-169.6	na	0.15-0.61	Jing et al. 2010
Up-flow anaerobic sludge blanket	$\text{S}^{2-}$	30	7.5	0.62 <sup>a</sup>	0.33 <sup>a</sup>	na	0.09-0.31	Yang et al. 2016
Up-flow column reactor	$\text{S}^0$	30	7.3	na	20	6.15 - 7.92 <sup>b</sup>	0.22	Zhu et al., 2019
Vertical fixed-bed reactors	$\text{S}^{2-}$	30	7-7.5	49.3	20	20	na	Moraes et al. 2012

<sup>a</sup>kg/m<sup>3</sup>/d

<sup>b</sup> g/g N<sub>removal</sub>



Table. 3. Reactor types, operational (environmental) conditions, influent S concentrations and efficiency of heterotrophic  $\text{SO}_4^{2-}$  reduction and sulfamox

Reactor type	Electron donor	Temperature	pH	$\text{SO}_4^{2-}$	$\text{SO}_4^{2-}$ -removal efficiency or rate	References
		(°C)		concentration (mg/L)		
<b>HETEROTROPHIC SULFATE REDUCTION</b>						
<b>Gas lift reactor</b>	Carbon monoxide	30	7	250–1000	62.5–97.5%	Sinharoy et al., 2020a
<b>Moving bed biofilm reactor</b>	Carbon monoxide	30	7	250–1000	67.1–95.2%	Sinharoy et al., 2019a
<b>Batch</b>	Succinic acid, yeast extract	22	-	1700	169 mg $\text{SO}_4^{2-}$ /L/d	Virpiranta et al., 2019
		16			98 mg $\text{SO}_4^{2-}$ /L/d	
		6			13–42 mg $\text{SO}_4^{2-}$ /L/d	
<b>Batch</b>	Methanol	5	7	-	26.7 mg $\text{SO}_4^{2-}$ /L/d	Nielsen et al., 2019
<b>Packed bed reactor</b>	Ethylene glycol	30	7	250–1000	4.1 mg $\text{SO}_4^{2-}$ /L/d	Kumar et al., 2018
<b>Inverse fluidized bed reactor</b>	Scourer	30	7	700	34 mg $\text{SO}_4^{2-}$ /gVSS/d	Reyes-Alvarado et al., 2018
	Cork				6.1 mg $\text{SO}_4^{2-}$ /gVSS/d	
<b>Packed bed reactor</b>	Molasses	4–8	6.5–7.1	287–548.2	0–22 mg $\text{SO}_4^{2-}$ /L/d	Nielsen et al., 2018
	Crab shell				721–738	
<b>Batch</b>	Potato	30	7	764–766	22–34 mg $\text{SO}_4^{2-}$ /gVSS/d	Reyes-Alvarado et al., 2017
	Filter paper			752–823	50–65 mg $\text{SO}_4^{2-}$ /gVSS/d	
<b>Fluidized-bed reactor</b>	Glycerol	23	5.5–8.5	2000–3000	167 mg $\text{SO}_4^{2-}$ /gVSS/d	Bertolino et al., 2014
<b>Stirred tank reactor</b>	Hydrogen + carbon dioxide	30	6.95–7.05	-	3400 mg $\text{SO}_4^{2-}$ /L/d	Sáez-Navarrete et al., 2012
<b>Fluidized-bed reactor</b>	Ethanol	35	7.5	-	211 mg $\text{SO}_4^{2-}$ /gVSS/d	Nevatalo et al., 2010
	Ethanol + lactate				2016 mg $\text{SO}_4^{2-}$ /gVSS/d	
<b>Gas lift reactor</b>	Hydrogen	30–35	7–7.5	5000–30000	7080 kg $\text{SO}_4^{2-}$ /d	Van Houten et al., 2009
	Ethanol, spent manure				961–1345 mg $\text{SO}_4^{2-}$ /L/d	
<b>Anaerobic filter</b>	Methanol, spent manure	6	2.5–4.3	900	1057–1441 mg $\text{SO}_4^{2-}$ /L/d	Tsukamoto et al., 2004
<b>SULFAMMOX</b>						
<b>Upflow anaerobic sludge bed reactor</b>	Ammonium nitrogen	35	7.9–8.3	80	8.18 mg S/L/d	Qin et al., 2021
<b>Circulating flow completely anaerobic reactor</b>	Ammonium nitrogen	30	8.1–8.6	88	2–27%	Zhang et al., 2020
				223	2–27%	
				154	18–64%	
<b>Self-designed circulating flow reactor</b>	Ammonium nitrogen	35	8.1–8.3	183	approx. 40%	Zhang et al., 2019a
				216	approx. 0%	



				116	approx. 30%	
				100	approx. 45%	
				90	approx. 30%	
<b>Self-designed circulating flow reactor</b>	Ammonium nitrogen	30	8.1-8.6	170	approx. 30%	Zhang et al., 2019b
				360	approx. 5%	
<b>Sequencing batch reactor</b>	Ammonium nitrogen	-	-	261	19%	Prachakittikul et al., 2016
<b>Batch</b>	Ammonium nitrogen	30	8.5	163	40%	Cai et al., 2010
<b>Upflow anaerobic sludge blanket reactor</b>	Ammonium nitrogen	35	7.5-8.5	240	30%	Yang et al., 2009
<b>Non-woven rotating biological contactor</b>	Ammonium nitrogen	35	8-8.2	-	-	Liu et al., 2008

Table 4. Technologies for integrated S, N, COD removal and the observed removal efficiencies for S, N and COD

Process	Reactor type	S removal efficiency	N removal efficiency (N form)	COD removal efficiency	References
<b>Sulfate reduction, Autotrophic denitrification and Nitrification Integrated (SANI)</b>	Up-flow anaerobic sludge bed, an anoxic filter, an aerobic filter	16-68 mg S <sup>2-</sup> /L	74% (TN)	95%	Wang et al., 2009b
<b>SANI</b>	Up-flow sludge bed reactor, an anoxic reactor and an aerobic reactor	98 % S <sup>2-</sup>	55% (TN)	87%	Lu et al., 2012
<b>SANI</b>	Up-flow anaerobic sludge bed, an anoxic filter and an aerobic filter	97 % S <sup>2-</sup>	74% (TN)	97%	Lu et al., 2009
<b>SANI</b>	Sulfate-reducing up-flow sludge bed	75% SO <sub>4</sub> <sup>2-</sup>	-	90%	Hao et al., 2013
<b>SANI</b>	Sulfate-reducing up-flow sludge bed	72% SO <sub>4</sub> <sup>2-</sup>	-	82%	Hao et al., 2015
<b>Flue gas desulfurization - Sulfate reduction, Autotrophic denitrification and Nitrification Integrated (FGD-SANI)</b>	Sulfite-reducing upflow anaerobic sludge bed	~54 % S <sup>2-</sup>	~98% (TN)	94%	Jiang et al., 2013
<b>Mixed Denitrification - Sulfate reduction, Autotrophic denitrification and Nitrification Integrated (MD-SANI)</b>	Sulfate/sulfite reducing up-flow sludge bed and anoxic up-flow sludge bed	-	100% (NO <sub>3</sub> <sup>-</sup> -N)	80%	Qian et al., 2015a
<b>MD-SANI</b>	Sulfur-reducing upflow sludge bed and the anoxic upflow sludge bed	~100% SO <sub>3</sub> <sup>2-</sup>	100% (TN)	81%	Qian et al., 2015b
<b>Sulfate reduction, denitrification/anammox and partial nitrification (SRDAPN)</b>	Laboratory scale up-flow anaerobic-anoxic biological filter reactor	400-500 mg S <sup>2-</sup> /d	79% (TN)	500-2300 mg/d	Kosugi et al., 2019
<b>Partial Nitrification/Anammox and S-dependent autotrophic Denitrification (PNASD)</b>	PN/A reactor and an elemental sulfur-supported packed bed autotrophic denitrification	-	97% (TN)	-	Dasgupta et al., 2017
<b>PNASD</b>		~100% S <sup>2-</sup>	84% (TN)	-	Yuan et al., 2020



	Single reactor under main-stream conditions				
<b>Anammox and S-dependent autotrophic Denitrification (ASD)</b>	Expanded granular sludge bed	90-100% $S_2O_3^{2-}$	98% (TN)	-	Sun et al., 2018
<b>ASD</b>	Up-flow anaerobic sludge blanket reactor	99.6% $S^{2-}$ , 330 mg $S^{2-}$ /L	88% (TN), 252 mg $NH_4^+$ -N/L	-	Guo et al., 2016
<b>S-dependent autotrophic Partial Denitrification and Anammox (SPDA)</b>	Up-flow anaerobic sludge blanket reactor	~100% $S_2O_3^{2-}$	>90% (TN)	-	Wu et al., 2019
<b>SPDA</b>	Up-flow anaerobic sludge blanket reactor	70% $S^0$	90% ( $NO_2^-$ -N)	-	Liu et al., 2017
<b>Sulfamnox/Anammox (SA) with COD</b>	Moving Bed Biofilm Reactor	10% $SO_4^{2-}$	30% ( $NH_4^+$ -N)	-	Rikmann et al., 2016



Table 5. Overview of the reported mechanistic models linking C, S and N transformations

No.	Reactor type	Substrate	Influent concentrations			Model structure			References	
			Organic (mg COD/L)	S <sup>2-</sup> (mg S <sup>2-</sup> -S/L)	NO <sup>3-</sup> -N (mg -N/L)	No. of processes	No. of components	No. of parameters		S and N involved processes
1	Bench-scale EGSB reactor	Synthetic wastewater	200-800	200-800	75-275	7	10	18	Hydrolysis: Particulate N → Organic N Ammonification: Organic N → NH <sub>4</sub> <sup>+</sup> -N Heterotrophic: NO <sub>3</sub> <sup>-</sup> -N → N <sub>2</sub> Autotrophic: NO <sub>3</sub> <sup>-</sup> -N → N <sub>2</sub>	Wang et al., 2010
2	Bench-scale EGSB reactor	Synthetic wastewater	275-2300 mg C/L	156-1490	100-800	6	8	31	Autotrophic: S <sup>2-</sup> → S <sub>0</sub> → SO <sub>4</sub> <sup>2-</sup> Autotrophic: NO <sub>3</sub> <sup>-</sup> -N → NO <sub>2</sub> <sup>-</sup> -N → N <sub>2</sub> Heterotrophic: NO <sub>3</sub> <sup>-</sup> -N → NO <sub>2</sub> <sup>-</sup> -N → N <sub>2</sub>	Xu et al., 2014
3	Bench-scale SBR	Synthetic wastewater	-	194 145	321 202	4	5	9	Autotrophic: S <sup>2-</sup> → S <sub>0</sub> → SO <sub>4</sub> <sup>2-</sup> Autotrophic: NO <sub>3</sub> <sup>-</sup> -N → NO <sub>2</sub> <sup>-</sup> -N → N <sub>2</sub>	Xu et al., 2016
4	Bench-scale EGSB reactor	Synthetic wastewater	2700	1000 mg SO <sub>4</sub> <sup>2-</sup> -S/L	200-700	14	15	38	Autotrophic: S <sup>2-</sup> → S <sub>0</sub> Autotrophic: NO <sub>3</sub> <sup>-</sup> -N → NO <sub>2</sub> <sup>-</sup> -N Heterotrophic: NO <sub>3</sub> <sup>-</sup> -N → NO <sub>2</sub> <sup>-</sup> -N → N <sub>2</sub> Heterotrophic: SO <sub>4</sub> <sup>2-</sup> → S <sup>2-</sup>	Xu et al., 2017
5	MBfR	Anaerobic digestion liquor	50-100	30	50-1000	18	17	60	Autotrophic: NH <sub>4</sub> <sup>+</sup> -N → NO <sub>2</sub> <sup>-</sup> -N → NO <sub>3</sub> <sup>-</sup> -N Autotrophic: NH <sub>4</sub> <sup>+</sup> -N, NO <sub>2</sub> <sup>-</sup> -N → N <sub>2</sub> , NO <sub>3</sub> <sup>-</sup> -N Heterotrophic: NO <sub>3</sub> <sup>-</sup> -N → N <sub>2</sub> Autotrophic: S <sup>2-</sup> → S <sup>0</sup> → SO <sub>4</sub> <sup>2-</sup> Autotrophic: CH <sub>4</sub> → CO <sub>2</sub>	Chen et al., 2016
6	Coastal upwelling system	Sea water	-	0.1 mmol S/m <sup>3</sup>	0.1 mmol N/m <sup>3</sup>	9	14	46	Autotrophic: NH <sub>4</sub> <sup>+</sup> -N → NO <sub>2</sub> <sup>-</sup> -N → NO <sub>3</sub> <sup>-</sup> -N Heterotrophic: NO <sub>3</sub> <sup>-</sup> -N → NO <sub>2</sub> <sup>-</sup> -N → N <sub>2</sub> Heterotrophic: SO <sub>4</sub> <sup>2-</sup> → S <sup>2-</sup> Autotrophic: S <sup>2-</sup> → SO <sub>4</sub> <sup>2-</sup>	Azhar et al., 2014

SBR: sequencing batch reactor, EGSB: expanded granular sludge bed, MBfR: membrane biofilm reactor.

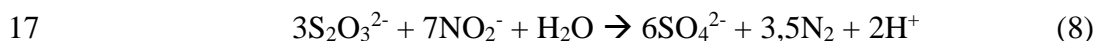
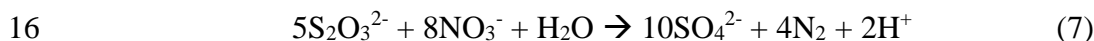
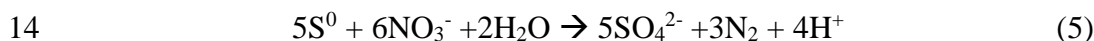
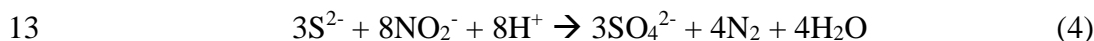
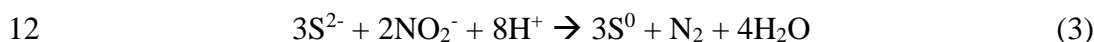
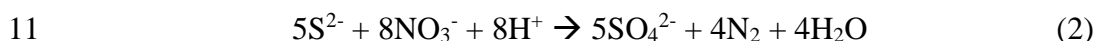


## 1 **Supporting information (SI)**

### 2 *Sulfur-dependent autotrophic denitrification (SI)*

#### 3 *Mechanism of the process*

4 The process of autotrophic sulfur (S)-dependent denitrification is used for the treatment of  
5 domestic and industrial wastewater (Shao et al., 2010), landfill leachate (Koenig and Liu,  
6 1996), groundwater (Wan et al., 2009) and salt water (Wang et al., 2009). Process reactions  
7 are illustrated by the equations 1-8 (Huang et al., 2019, Cui et al., 2019, Lin et al., 2018,  
8 Pokorna and Zabranska, 2015, Sun and Nemati 2012):



18 Compared to heterotrophic denitrification, this process has many advantages, such as  
19 no addition of organic substrate, reduction of biomass (the biomass yield coefficient is 0,15-  
20 0,57 g for autotrophic biomass production, and 0,71–1,2 g for heterotrophic biomass  
21 production per 1 g of denitrified  $\text{NO}_3^-$ -N and  $\text{NO}_2^-$ -N) and a decrease in  $\text{N}_2\text{O}$  emissions  
22 (Huang et al., 2019, Sun and Nemati, 2012, Yang et al., 2016).



23 Thus, the process of autotrophic S-dependent denitrification can be used successfully  
24 in major carbon-deficient wastewater treatment systems (Cui et al., 2019). This also explains  
25 the use of this process for biogas desulfurization, cleaning of crude oil tanks before  
26 acidification, and anti-corrosion treatment of sewage systems (Qian et al., 2015, Park et al.,  
27 2014). To date, most of the research done on the S-dependent autotrophic denitrification  
28 process has been carried out with flocculent sludge (Cui et al., 2019).

29 Zhou et al. (2016) indicated that  $S_2O_3^{2-}$  is more efficient in S-dependent autotrophic  
30 denitrification than  $S^0$  or  $S^{2-}$ . Similarly, Park et al. (2015) obtained  $NO_3^-$ -N removal  
31 efficiencies of 96.5% for  $S_2O_3^{2-}$ , 64.1% for  $S^{2-}$ , 58.1% for persulfide (pyrite) and 38.8% for  
32  $S^0$ . Thus, it has frequently been shown that  $S_2O_3^{2-}$  is a suitable S source for the described  
33 process. Cardoso et al. (2006) found that the specific rate of nitrate reduction for  $S_2O_3^{2-}$  was  
34 4.6 and 9.5 times higher than for  $H_2S$  and  $S^0$ , respectively.

35 When S compounds are transformed, intermediate compounds can also be involved in  
36 the process. An example is participation of  $S^0$ , resulting from oxidation of  $S^{2-}$ , in S-dependent  
37 autotrophic denitrification (Xu et al., 2016). Fan et al. (2021) described the effect of  
38 intermediates, such as  $S^{2-}$ , acid volatile sulfide (AVS), and  $S^0$ , in autotrophic denitrification  
39 with  $S_2O_3^{2-}$  as an electron acceptor. When  $S^{2-}$ ,  $S_2O_3^{2-}$ , AVS, and  $S^0$  coexisted in the  
40 autotrophic process of denitrification, their preferences were as follows:  $S^{2-} > S_2O_3^{2-} > AVS \approx$   
41  $S^0$ .

## 42 *Environmental factors influencing the process performance*

### 43 *Temperature*

44 The optimal temperature for most SOB remains under mesophilic conditions, i.e. in the range  
45 of 25-35°C (Fajardo et al., 2014, Shao et al., 2010). When the temperature dropped from 20-  
46 25°C to 5-10°C, the efficiency of N removal decreased from about 99% to 50% (Zhou et al.,  
47 2011). However, there were also studies showing the denitrification rate of approximately 3.3



48 kg N/m<sup>3</sup>·d at 3°C in a fluidized-bed reactor with the HRT of 1h during 98 days (Di Capua et  
49 al., 2017).

#### 50 *pH*

51 The optimal pH for this process has been shown to be in the range of 6.8-8.2 and a decrease in  
52 the activity has been reported at pH <6.5 and >9 (Chen et al., 2016a, Vidal et al., 2002). The  
53 optimal pH values for reduction of NO<sub>2</sub>-N and NO<sub>3</sub>-N were 7.0 and 8.5, respectively, using S<sup>0</sup>  
54 as an electron donor (Chen et al., 2018).

#### 55 *Previous research*

56 Jing et al. (2010) performed the S-dependent autotrophic denitrification process with S<sup>2-</sup> as an  
57 electron donor in a 1.3 L up-flow continuous reactor fed with synthetic wastewater. When the  
58 NO<sub>3</sub><sup>-</sup>-N concentration increased from 30.4 mg N/L to 169.6 mg N/L, the volumetric NO<sub>3</sub><sup>-</sup>-N  
59 removal rate increased from 0.15 kg N/m<sup>3</sup>/d to 0.61 kg N/m<sup>3</sup>/d. However, when the NO<sub>3</sub><sup>-</sup>-N  
60 concentration increased again to 189.7 mg N/L, the volumetric NO<sub>3</sub><sup>-</sup>-N removal rate  
61 decreased to 0.59 kg N/m<sup>3</sup>/d. On the contrary, when the influent concentration of S<sup>2-</sup>  
62 increased from 160 mg/L to 1000 mg/L, the volumetric S<sup>2-</sup> removal rate increased from 0.78  
63 kg/m<sup>3</sup>/d to 4.57 kg/m<sup>3</sup>/d and the efficiency of S<sup>2-</sup> removal was >90%.

64 Similarly, in the experiments of Kim et al. (2004) with two FBRs, the effect of NO<sub>3</sub><sup>-</sup>-N  
65 concentration on the process was explicitly observed. The feed of FBR-1 was prepared from  
66 treated domestic wastewater, while pre-treated leachate from a municipal landfill was used in  
67 FBR-2. FBR-1 was operated for 358 days with NO<sub>3</sub><sup>-</sup>-N of 20 mg N/L, and FBR-2 for 342  
68 days with NO<sub>3</sub><sup>-</sup>-N of 700 mg N/L. The maximum denitrification rates in FBR-1 and FBR-2  
69 were respectively 2.53 kg N/m<sup>3</sup>/d (denitrification efficiency 91.7%) and 3.37 kg N/m<sup>3</sup>/d  
70 (denitrification efficiency 83%).

71 Zou et al. (2016) investigated the S-dependent autotrophic denitrification process in  
72 two FBRs operated at different temperatures, i.e. 20 and 30°C, for 200 days. Oxidation of  
73  $S_2O_3^{2-}$  was particularly unstable until day 54, and then  $S_2O_3^{2-}$  remained below the detection  
74 limit in both FBRs, and  $SO_4^{2-}$  concentration increased sharply to approximately 3700 and  
75 3200 mg/L on days 38 and 33 in FBR-1 and FBR-2, respectively. The temperature had no  
76 significant effect in the course of the process and the denitrification rate remained at the level  
77 of 1.24-3.25 kg N/m<sup>3</sup>/d. Autotrophic denitrification and denitrification with  $S_2O_3^{2-}$  as electron  
78 donors were thus effectively maintained in the two FBRs.

79 In the study by Sahinkaya and Dursun (2015), the influent  $NO_3^-$ -N concentration  
80 increased from 25 to 75 mg N/L in a FBR with  $S^0$  as an electron donor. The start-up period  
81 was relatively short as almost complete  $NO_3^-$ -N and  $NO_2^-$ -N reduction was achieved within  
82 one week. However, the process efficiency decreased during the long-term operation, which  
83 resulted in an increase in  $NO_3^-$ -N and  $NO_2^-$ -N concentrations. The denitrification rate ranged  
84 from 0.07 to 0.2 kg N/m<sup>3</sup>/d.  $SO_4^{2-}$  was produced and reached the level of 100-600 mg S/L in  
85 accordance with the process stoichiometry.

86 Moraes et al. (2012) investigated S-dependent autotrophic denitrification involving  
87 two electron acceptors –  $NO_3^-$ -N and  $NO_2^-$ -N in the absence of  $S^{2-}$ , in excess of  $S^{2-}$  and  
88 according to the stoichiometric equation of the S-dependent autotrophic denitrification  
89 process in vertical FBRs. The results showed that sulfur intermediate compounds ( $S^0$ ) were  
90 mainly formed when excess of  $S^{2-}$  was used, especially for  $NO_3^-$ -N. Moreover,  $NO_2^-$ -N was  
91 more readily consumed than  $NO_3^-$ -N, and higher concentrations of  $S^{2-}$  led to greater formation  
92 of S intermediates. The observed  $NO_3^-$ -N removal efficiencies were 60-15%, 25.5-98.5% and  
93 84%, respectively, in the absence of  $S^{2-}$ , in excess of  $S^{2-}$  and in concentration according to the  
94 stoichiometric equation of the S-dependent autotrophic denitrification process.

95 In the study by Sahinkaya and Kilic (2014a), two parallel column bioreactors were  
96 operated under autotrophic and heterotrophic conditions. For S-dependent autotrophic  
97 denitrification, a packed bed reactor was used. The simultaneous removal of  $\text{NO}_3^-$ -N and  
98 chromate (VI) was achieved under both autotrophic and heterotrophic conditions. Cr (VI)  
99 concentrations up to 0.5 mg/L did not adversely affect the autotrophic denitrification  
100 efficiency and higher concentrations reduced the denitrification potential of the column.  
101 During the entire period, production of  $\text{SO}_4^{2-}$  ranged from 200 to 600 mg S/L and the  
102 denitrification rate was 0.07-0.1 kg N/m<sup>3</sup>/d.

103 Sahinkaya et al. (2014b) performed S-dependent autotrophic denitrification under the  
104 temperature falling from 26 to 10°C. Three identical pilot-scale column bioreactors were  
105 installed, different S to limestone ratios (1/1–3/1) were used and the results were compared  
106 under different loading conditions during the long-term operation. Complete denitrification  
107 was achieved until the  $\text{NO}_3^-$ -N loading was 10 mg N/L/h. When the temperature dropped to  
108 10°C in winter at the load of 18 mg N/L/h, the denitrification efficiency decreased to 60-70%  
109 and the bioreactor with the S/L ratio of 1/1 showed slightly better performance. Throughout  
110 the study, the denitrification rate was 0.03-0.24 kg N/m<sup>3</sup>/d.

111 Yang et al. (2016) conducted S-dependent autotrophic denitrification in an up-flow  
112 anaerobic sludge blanket reactor operated continuously for 600 days. The nitrogen removal  
113 efficiency of 94% and complete removal of  $\text{S}^{2-}$  were achieved. The denitrification rate was  
114 0.09-0.31 kg N/m<sup>3</sup>/d with the HRT of 5 h, and the influent  $\text{NO}_3^-$ -N and  $\text{S}^{2-}$  loads were 0.33  
115 kg-N/m<sup>3</sup>/d and 0.62 kg S/m<sup>3</sup>/d, respectively.



## 116 *Sulfate reduction (S2)*

### 117 *Mechanism of the process*

118 Due to the formation of wastewater streams rich in  $\text{SO}_4^{2-}$  resulting from various anthropogenic  
119 activities, there is a growing interest in the  $\text{SO}_4^{2-}$  reduction process using SRB. Although high  
120 concentrations of  $\text{SO}_4^{2-}$  do not pose a direct threat to the environment and health, they disrupt  
121 the natural S cycle. This imbalance can lead to  $\text{H}_2\text{S}$  formation, metal corrosion, and  $\text{SO}_x$   
122 emissions (Abel et al., 2015).  $\text{H}_2\text{S}$  is often undesirable in wastewater treatment, but  $\text{SO}_4^{2-}$   
123 reduction may be beneficial due to the use of  $\text{S}^{2-}$  for heavy metal removal by precipitation,  
124 autotrophic denitrification or autotrophic phosphorus removal (Rubio -Rincón et al., 2017).

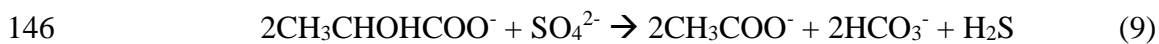
125 Although  $\text{SO}_4^{2-}$  is the main electron acceptor for SRB, they can also use S compounds  
126 such as  $\text{S}_2\text{O}_3^{2-}$ ,  $\text{SO}_3^{2-}$ ,  $\text{S}^0$ . When  $\text{SO}_4^{2-}$  is the electron acceptor, the process takes place in two  
127 stages - first  $\text{SO}_4^{2-}$  is reduced to  $\text{SO}_3^{2-}$  and then to  $\text{S}^{2-}$  (Muyzer and Stams, 2008). When  $\text{SO}_4^{2-}$   
128 decomposes into  $\text{S}^{2-}$ , some of the decomposed  $\text{S}^{2-}$  leaves the reactor along with the biogas as  
129  $\text{H}_2\text{S}$  gas and the remaining  $\text{H}_2\text{S}$  present in the reactor as total dissolved sulfide (TDS). The  
130 components of TDS in the aquatic environment are  $\text{S}^{2-}$ ,  $\text{HS}^-$  and  $\text{H}_2\text{S}$  (aq) (Samarathunga and  
131 Rathnasiri, 2019).

132 Organic electron donors are used for the biological reduction of  $\text{SO}_4^{2-}$ , which at the  
133 same time provide a carbon source for the SRB, as well as inorganic ones that require  
134 supplementation with a carbon source, e.g.  $\text{CO}_2$  (Sinharoy et al., 2020a). Some  $\text{SO}_4^{2-}$  rich  
135 wastewater also contains high concentrations of organic compounds that can be used by SRB  
136 to reduce  $\text{SO}_4^{2-}$ . When they contain no organic compounds, compounds such as sugars  
137 (glucose and sucrose) (Barber and Stuckey, 2000), alcohols (methanol and ethanol)  
138 (Kaksonen et al., 2003), short-chain fatty acids (acetate, propionate, butyrate, lactate,  
139 pyruvate, malate) (Kiran et al., 2017) and aromatics (benzoate, phenol) can support the  
140 biological reduction of  $\text{SO}_4^{2-}$  (Liamleam and Annachatre, 2007).



141 Lactate, and especially sodium lactate, is the most common electron donor in the  
142 biological reduction of  $\text{SO}_4^{2-}$  with SRB. It can be degraded by a wider range of SRB  
143 compared to methanol. However, lactate is more expensive compared to methanol, ethanol or  
144 acetate, making this solution on an industrial scale not economical.

145 Reduction of  $\text{SO}_4^{2-}$  with lactate (9) (Virpiranta et al., 2019):

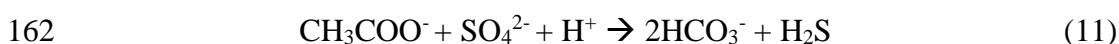
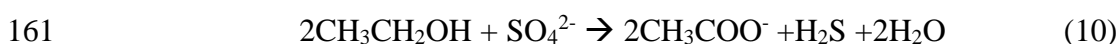


147 Alternative methanol is the cheapest carbon source and is therefore widely used as an  
148 electron donor in biological processes. Compared to other carbon sources, its complete  
149 oxidation to  $\text{CO}_2$  is ensured, while e.g. lactate or ethanol are oxidized only to acetate (Rubio-  
150 Rincón et al., 2017), which reduces treatment costs due to the smaller amount of carbon  
151 source needed to reduce all  $\text{SO}_4^{2-}$ . However, it has been reported that methanogens compete  
152 with SRB for methanol under mesophilic and thermophilic conditions (Kaksonen and  
153 Puhakka, 2007). However, adjusting factors such as pH, temperature,  $\text{S}^{2-}$  concentration and  
154 metal concentration can limit the growth of methanogens in the presence of methanol  
155 (Tsukamoto et al., 2004).

156 Also, ethanol and succinate are widely used as a carbon and electron source as it is a  
157 relatively economical option (Virpiranta et al., 2019).

158 Ethanol reduction occurs in two reactions (10-11), the latter of which is based on  
159 acetate reduction (Virpiranta et al., 2019).

160 Reduction of  $\text{SO}_4^{2-}$  with ethanol (Virpiranta et al., 2019):

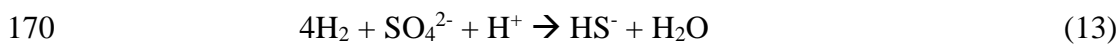


163 Reduction of  $\text{SO}_4^{2-}$  with succinate (12) (Virpiranta et al., 2019):



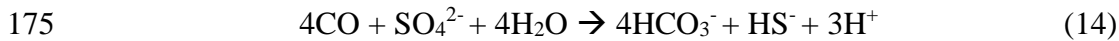
165  $\text{H}_2$ , CO and  $\text{CH}_4$  can also be used as electron donors, as well as their mixtures, which  
166 reduce the cost of the process. Van Houten et al. (1996) have indicated that  $\text{H}_2$  is the best  
167 electron donor for  $\text{SO}_4^{2-}$  reduction when working on a large scale. Often, however,  $\text{CO}_2$  is  
168 required to achieve high process efficiency.

169 Reduction of  $\text{SO}_4^{2-}$  with  $\text{H}_2$  (13) (Sinharoy et al., 2020b):



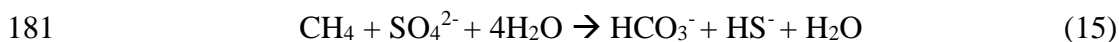
171 There are SRBs that use CO as a carbon and energy source, however, for most of  
172 them, CO is toxic. However, this toxicity can be mitigated by the use of mixed culture  
173 systems or the use of a layered structure of biomass (Sipma et al., 2006).

174 Reduction of  $\text{SO}_4^{2-}$  with CO (14) (Sinharoy et al., 2020b):



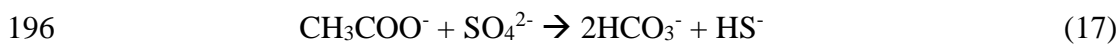
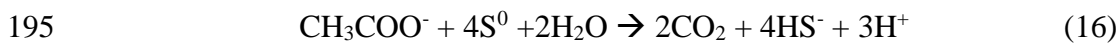
176 The biological reduction of  $\text{SO}_4^{2-}$  can also be combined with the anaerobic oxidation  
177 of  $\text{CH}_4$ .  $\text{CH}_4$  can provide 4 electrons, which is twice as much as  $\text{H}_2$ , so less gas volume is  
178 required to achieve the same  $\text{SO}_4^{2-}$  reduction efficiency. However,  $\text{CH}_4$  can only be used by a  
179 limited number of microorganisms, which is a major disadvantage of this substrate.

180 Reduction of  $\text{SO}_4^{2-}$  with  $\text{CH}_4$  (15) (Sinharoy et al., 2020b):



182 Electron donors were also investigated as manure, sawdust, wood shavings,  
183 lignocellulosic materials (Reyes-Alvadro et al., 2018), agricultural residues (Chang et al.,  
184 2000), sewage sludge, whey or dairy sewage (Wolicka, 2008) and organic sewage (Das et al.,  
185 2013), which unfortunately cause secondary environmental pollution.

186 Sun et al. (2018) proposed a conceptual model for the reduction of  $\text{SO}_4^{2-}$  and  $\text{S}^0$  in the  
187 presence or absence of  $\text{HS}^-$ . When there is no  $\text{HS}^-$  in the system, the production of  $\text{HS}^-$  is  
188 mainly through the reduction of  $\text{SO}_4^{2-}$ , while when  $\text{HS}^-$  is present, polysulfide is formed by  
189 the reaction of  $\text{S}^0$  with  $\text{HS}^-$ . As the concentration of  $\text{HS}^-$  increases, more polysulfide is  
190 formed which stimulates indirect  $\text{S}^0$  reduction. When  $\text{HS}^-$  exceeds a certain level, the indirect  
191 reduction of  $\text{S}^0$  with polysulfide plays a major role in the production of  $\text{HS}^-$ , and the reduction  
192 of  $\text{SO}_4^{2-}$  is almost completely stopped. This is important from the point of view of the process  
193 economy, as the reduction of  $\text{S}^0$  requires  $\frac{1}{4}$  of the organic matter needed to reduce  $\text{SO}_4^{2-}$ ,  
194 according to the (16) and (17) reactions (Sun et al., 2018):



197 In a study by Sun et al. (2018), an effective  $\text{S}^0$  reduction was observed in the  
198 sulfidogenic bioreactor even at 1300 mg S/L of  $\text{SO}_4^{2-}$  in the inlet. 98.5% of  $\text{HS}^-$  was obtained  
199 as a result of  $\text{S}^0$  reduction, and  $\text{SO}_4^{2-}$  was barely consumed in the bioreactor.

200

201 *Environmental factors influencing the proces performance*

202 *Temperature*

203 SRBs are able to tolerate temperatures ranging from  $-5^\circ\text{C}$  to  $75^\circ\text{C}$  and readily adapt to  
204 temperature changes (Cocos et al., 2002). However, the optimal temperature for most SRBs is  
205 in the narrow range of  $28-30^\circ\text{C}$  (Virpiranta et al., 2019), although there are strains for which  
206 the optimum temperature is below  $20^\circ\text{C}$  (Knoblauch et al., 1999). Most of the bacteria that  
207 tolerate cold temperatures are mesophilic (rather than psychrophilic) strains that can grow  
208 under such conditions, termed psychrotolerant bacteria or psychrotrophs. For example, some  
209 *Desulfobulbus* strains can grow at  $6-10^\circ\text{C}$  (Kharrat et al., 2017, Virpiranta et al., 2019).



210 Studies have also been conducted in the range of even lower 4-8°C (Nielsen et al., 2018),  
211 which, however, has extended the adaptation time to such extreme conditions. The presence  
212 of SRB was revealed by the presence of the *Deltaproteobacteria* and *Clostridia*. The study  
213 showed a change in the composition of SRB taxa over time - an increase in the relative  
214 abundance of *Deltaproteobacteria* and a decrease in the relative abundance of members of the  
215 *Clostridia*.

#### 216 *pH*

217 Most of the studies on  $\text{SO}_4^{2-}$  reduction were carried out at pH in the range of 7-7.6, which is  
218 close to the optimal value for SRB. Pagnanelli et al. (2012) found the optimal pH value of 7.6  
219 for the SRB, while Bratkova et al. (2013) observed the maximum  $\text{SO}_4^{2-}$  reduction rate at pH  
220 7.25.

#### 221 *Previous research*

222 Sinharoy et al. (2019, 2020a) desulphurized wastewater containing  $\text{SO}_4^{2-}$  using carbon  
223 monoxide in a moving bed biofilm reactor (2019) and in a gas lift reactor (2020a). The effect  
224 of HRT on the process was investigated and it was found that at the HRT of 72, 48 and 24 h,  
225  $\text{SO}_4^{2-}$  removal was 93.5%, 91.9% and 80.1%, respectively, and CO use was 85% throughout  
226 the study (Sinharoy et al., 2019). These results were improved in a subsequent study  
227 (Sinharoy et al., 2020a). At the HRT of 72 h, the reduction of  $\text{SO}_4^{2-}$  and the use of CO were  
228 97% and 89%, respectively (2020a). The best results in terms of  $\text{SO}_4^{2-}$  reduction (> 80%)  
229 were obtained for low  $\text{SO}_4^{2-}$  loading and high CO loading conditions. The results for both  
230 types of reactors were slightly better for the gas lift reactor.

231 Batch studies of Virpiranta et al. (2019) were performed in sealed vials and incubated  
232 at 3 different temperatures of 6°C, 16°C and 22°C for cold acclimatization and  
233 characterization of SRB consortia enriched from a sample of arctic sediments. Postgate

234 medium was supplemented with lactate, ethanol or succinic acid and the resulting consortia  
235 grew with lactate and succinic acid, but not ethanol. The  $\text{SO}_4^{2-}$  reduction rates at  $22^\circ\text{C}$  were  
236  $169 \text{ mg/L/d}$ , at  $16^\circ\text{C}$  -  $98 \text{ mg/L/d}$ , and for  $6^\circ\text{C}$  the rates ranged from  $13$  to  $42 \text{ mg/L/d}$ .  
237 Temperature had a significant effect on the activity of SRB. However, it is useful to be able to  
238 acclimatize SRB to low temperatures due to the versatile applicability of the process.

239 Nielsen et al. (2019) conducted batch tests to check various carbon sources for  $\text{SO}_4^{2-}$   
240 reduction. Simple organic carbon sources (methanol and ethylene glycol) and complex  
241 organic carbon sources (potato oil, brewing residues, peat and straw) were used to support  
242 SRB growth. After 162 days, all bioreactors showed a decrease in both total organic carbon  
243 and  $\text{SO}_4^{2-}$  concentration at  $5^\circ\text{C}$ . However, a long acclimation period (98–112 days) was  
244 required. The use of methanol and ethylene glycol resulted in  $\text{SO}_4^{2-}$  reduction by 71.2% and  
245 36.9%, respectively. The decrease in  $\text{SO}_4^{2-}$  concentrations was limited to 13.8 and 5.3%,  
246 respectively, when using peat and straw.

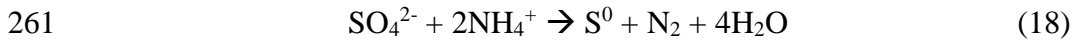
247 Reyes-Alvarado et al. (2017, 2018) used electron donors, such as potato, filter paper,  
248 crab shell (2017) and natural scourer and cork (2018), in their research. Maintaining  $\text{SO}_4^{2-}$   
249 concentration close to  $700\text{--}800 \text{ mg/L}$  in both studies, the following  $\text{SO}_4^{2-}$  removal rates were  
250 obtained: 6-9, 22-34, 50-65, 34,  $6.1 \text{ mg SO}_4^{2-}/\text{gVSS/d}$  for crab shell, potato, filter paper,  
251 scourer, cork, respectively. It was shown that the natural properties of the carbohydrate-based  
252 polymers limit the hydrolysis-fermentation step and thus the  $\text{SO}_4^{2-}$  reduction rate.

### 253 ***Sulfammox process (S3)***

#### 254 *Mechanism of the process*

255 A novel sulfammox biological process has recently been described in which  $\text{NH}_4^+\text{-N}$  is  
256 oxidized to  $\text{N}_2$  and  $\text{SO}_4^{2-}$  plays the role of an electron acceptor and is reduced to  $\text{S}^0$  under  
257 anaerobic conditions. Sulfammox was first reported by Fdz-Polanco et al. (2001b) in a

258 granular activated carbon anaerobic fluidized bed reactor treating vinasse from an ethanol  
259 distillery of sugar beet molasses. The sulfammox process can be most generally represented  
260 by the following equation (18):



262 In the follow-up studies, Liu et al. (2008) and Yang et al. (2009) identified  $\text{SO}_4^{2-}$  as a  
263 potential electron acceptor as it was the feed component. The mechanism of the sulfammox  
264 process is not fully understood yet - it turns out that there are exceptions to the described  
265 process. In addition to  $\text{N}_2$  and  $\text{S}^0$ , sulfammox can lead to the formation of  $\text{NO}_2^-$ -N and  $\text{NO}_3^-$ -N  
266 as well as  $\text{S}^{2-}$ . Moreover, there are two different assumptions about the actual course of the  
267 sulfammox process. The first assumes that  $\text{NH}_4^+$ -N is partially oxidized to  $\text{NO}_2^-$ -N with  $\text{SO}_4^{2-}$   
268 and some of the  $\text{NO}_2^-$ -N produced is reduced to  $\text{N}_2$  by  $\text{S}^{2-}$  and then  $\text{NO}_2^-$ -N and  $\text{NH}_4^+$ -N are  
269 converted to  $\text{N}_2$ . The second is that  $\text{NH}_4^+$ -N is partially oxidized to  $\text{NO}_2^-$ -N by  $\text{SO}_4^{2-}$  and then  
270  $\text{NH}_4^+$ -N is oxidized to  $\text{N}_2$  by  $\text{NO}_2^-$ -N (Yang et al., 2009, Liu et al., 2008).

271 Even though COD is not required for the sulfammox process (Zhang et al., 2009), the  
272 experiments were performed either without COD addition (Bi et al., 2020, Zhang et al.  
273 2019a,b, Prachakittikul et al., 2016, Cai et al., 2010) or with COD addition (Wang et al.,  
274 2017, Rikmann et al., 2016, Fdz-Polanco et al., 2001a,b). When COD is present in  
275 wastewater, the sulfammox process can be coupled with subsequent heterotrophic  
276 denitrification (Zhang et al. 2019b).

277 The differences in the  $\text{NH}_4^+$ -N/ $\text{SO}_4^{2-}$  ratio in the conducted studies with respect to the  
278 stoichiometric ratio may result from the activity of ammonia oxidizing bacteria (AOB), which  
279 oxidizes  $\text{NH}_4^+$ -N, and the reduction of  $\text{SO}_4^{2-}$  by organic compounds, as described by Bi et al.  
280 (2020). In this way, they questioned the presence of the sulfammox process, so it is important  
281 to conduct more detailed research.



282 *Environmental factors influencing the process performance*

283 *Temperature*

284 The sulfammox process has been carried out at the temperatures in the range of 15-55°C  
285 (Zhang et al., 2019a,b, Cai et al., 2010, Yang et al., 2009, Zhao et al., 2006) with the optimal  
286 value in the range of 25-35°C (Cai et al., 2010). However, the sulfammox process could also  
287 be maintained at lower temperatures, e.g. 20°C (Rikmann et al. 2016) and 14-15°C (Wu et al.  
288 2020). In the latter case, the  $\text{NH}_4^+\text{-N}$  and overall  $\text{SO}_4^{2-}$  removal efficiencies still remained  
289 high, i.e. 98.5% and 52.8%, respectively.

290 *pH*

291 Sulfammox studies have mainly been carried out in the pH range of 7.5-8.6, specifically 7.5-  
292 8.5 (Yang et al., 2009), 8-8.2 (Liu et al., 2008) 8.1-8.3 (Zhang et al., 2019a), 8.1-8.6 (Zhang et  
293 al., 2019b) with the best value of 8.5 (Cai et al., 2010). The efficiency of  $\text{NH}_4^+\text{-N}$  and  $\text{SO}_4^{2-}$   
294 removal decreased when the pH was  $< 7.5$  and  $> 9.5$  (Cai et al., 2010).

295 *Previous research*

296 Qin et al. (2021) conducted a sulfammox process in an upflow anaerobic sludge bed reactor  
297 for more than one year. The influent  $\text{NH}_4^+\text{-N}$  concentration was 70 mg N/L and the  $\text{NO}_2^-$ :  
298  $\text{NO}_3^-$ :  $\text{SO}_4^{2-}$  molar ratio were 1:0.2:0.2, 0.5: 0.1: 0.3 and 0: 0:0.5, respectively, in stages I, II  
299 and III. The  $\text{NH}_4^+\text{-N}$  and  $\text{SO}_4^{2-}$  removal rates were 31 mg N/L/d and 8.18 mg S/L/d,  
300 respectively. The excessive conversion of  $\text{NH}_4^+\text{-N}$  in stage III was mainly attributed to the  
301 sulfammox reaction due to the high removal rate ratio of  $\text{NH}_4^+\text{-N}$ :  $\text{SO}_4^{2-}$  (8.67: 1),  
302 accumulation of  $\text{S}^{2-}$  and decreased pH, as well as a changed structure of the microbial  
303 communities.

304 The results of Zhang et al. (2019a) in a circulating flow reactor showed that the  
305 efficiency of  $\text{NH}_4^+\text{-N}$  oxidation and  $\text{SO}_4^{2-}$  reduction increased in the presence of  $\text{NO}_2^-$ -N and



306  $\text{NO}_3^-$ -N. Nitrogen has been converted by nitrification, denitrification and conventional  
307 anammox, simultaneously with the sulfammox process. The  $\text{SO}_4^{2-}$  removal efficiency reached  
308 the maximum of 45%.

309 In the batch tests of Cai et al. (2010), reduction of  $\text{SO}_4^{2-}$  (40%) and  $\text{NH}_4^+$ -N (44%)  
310 was considered exclusively due to sulfammox. Similarly, Yang et al. (2009) successfully  
311 performed sulfammox in an upflow anaerobic sludge blanket reactor. Only sulfammox was  
312 assumed to be responsible for the reduction of  $\text{NH}_4^+$ -N and  $\text{SO}_4^{2-}$  with the  $\text{SO}_4^{2-}$  removal  
313 efficiency of 30%.

#### 314 ***Modeling N, S and C transformations in wastewater treatment systems (S4)***

315 Koenig and Liu (2001) established a half-order kinetic model for biofilms to describe  
316 autotrophic denitrification by *Thiobacillus denitrificans* in an upflow S packed-bed reactor  
317 fed with synthetic wastewater. The half-order reaction rate constants for autotrophic  
318 denitrification using  $\text{S}^0$  were approximately one order of magnitude lower than those of  
319 heterotrophic denitrification. However, the model was validated with pure substrate and pure  
320 bacteria in a biofilm under autotrophic conditions, which largely limited its application to  
321 other complex systems.

322 An et al. (2011) investigated the kinetics of two-step heterotrophic denitrification  
323 (reduction of  $\text{NO}_3^-$ -N to  $\text{NO}_2^-$ -N, and subsequently to N oxides and  $\text{N}_2$  gas) using an oil  
324 reservoir culture, which was capable of functioning under both autotrophic and heterotrophic  
325 conditions. The developed kinetic model predicted the experimental results of batch and  
326 continuous systems in terms of simultaneous removal of  $\text{S}^{2-}$ ,  $\text{NO}_3^-$ -N,  $\text{NO}_2^-$ -N and organic  
327 compounds.

328 Wang et al (2010) proposed a kinetic model to monitor a denitrifying  $\text{S}^{2-}$  removal  
329 (DSR) process with the ASM1 as a core model. By establishing inhibition and switch  
330 functions, the competition between autotrophic and heterotrophic denitrifiers was described,



331 including the effect of  $S^{2-}$  inhibition on heterotrophic denitrification. The calibrated model  
332 was used to quantify the impact of the influent C/S ratio and  $S^{2-}$  levels on the performance of  
333 a bench-scale EGSB reactor. Model predictions indicated that the DSR reactor would operate  
334 efficiently when the influent C/S ratio was kept in the range of 0.5-3.0, and the  $S^{2-}$   
335 concentration remained below 1000 mg S/L.

336 Xu et al (2014) developed a model to describe simultaneous removal of  $S^{2-}$ ,  $NO_3^-$ -N  
337 and acetate (sole organic substrate) under denitrifying  $S^{2-}$  removal conditions in a continuous  
338 flow reactor. The kinetic parameters were estimated via data fitting while considering the  
339 effects of initial  $S^{2-}$  concentration,  $S^{2-}/NO_3^-$ -N ratio and acetate/ $NO_3^-$ -N ratio. The proposed  
340 model accurately described the performance of DSR over wide ranges of the parameters.  
341 Model predictions suggested that the adjustment of HRT would be an efficient way to  
342 mitigate high  $S^{2-}$  loadings. Despite accurate predictions for a pure substrate (acetate), the  
343 model might not be applicable for actual industrial wastewater with complex characteristics.

344 Xu et al (2016) developed an autotrophic denitrification kinetic model to describe  $S^{2-}$   
345 oxidation and  $NO_2^-$ -N removal in a bench-scale sequencing batch reactor (SBR). The model  
346 parameters were estimated by data fitting from two studies with different combinations of  $S^{2-}$ ,  
347  $S^0$ ,  $SO_4^{2-}$ ,  $NO_3^-$ -N and  $NO_2^-$ -N. The final products of  $S^{2-}$  oxidation ( $S^0$  and  $SO_4^{2-}$ ) and their  
348 concentrations could be accurately predicted, providing a strategy to control the effluent  
349  $SO_4^{2-}$  concentration or recover  $S^0$  as the main end product from  $S^{2-}$  oxidation.

350 Xu et al (2017) further developed a complex model for C-N-S removal by combining  
351 the ASMs and ADM1 (Anaerobic Digestion Model No.1), extended with oxygen/ $NO_3^-$ -N  
352 driven  $S^{2-}$  oxidation processes. The proposed model was also capable of simulating S relevant  
353 processes, such as  $SO_4^{2-}$  reduction,  $S^{2-}$  oxidation and denitrifying  $S^{2-}$  removal process. Due to  
354 some simplifications in the model structure and parameter uncertainty, that model would not  
355 yet serve as a precise and quantitative tool in various full-scale applications.



356 Mechanistic models may also be useful in understanding the mechanisms of  
357 sulfamnox and its interactions with co-existing biochemical processes. However, due to the  
358 complexity of the interactions, it still lacks research reports about the model development for  
359 the sulfamnox process. Chen et al (2016b) established a S-involved anammox model to  
360 simulate the coexistence of AOB, nitrate oxidizing bacteria (NOB), anaerobic ammonia  
361 oxidizing bacteria (AAOB), denitrification anaerobic methane oxidation (DAMO) bacteria,  
362 and SOB in a membrane biofilm reactor (MBfR). The model described removal of  $\text{NH}_4^+\text{-N}$ ,  
363 dissolved  $\text{CH}_4$ , and  $\text{S}^{2-}$  from sidestream anaerobic sludge digestion liquors. However, other  
364 potential processes, such as endogenous heterotrophic denitrification, S-dependent  
365 autotrophic denitrification and  $\text{SO}_4^{2-}/\text{S}$  reduction, were not incorporated in that model, and  $\text{S}^{2-}$   
366 inhibition was also neglected.

367 Azhar et al (2014) modeled the coupled N and S cycles in a coastal upwelling system.  
368 In the S cycle,  $\text{S}^{2-}$ -driven denitrification,  $\text{SO}_4^{2-}$  reduction, and  $\text{S}^{2-}$  oxidation by  $\text{NO}_3^-\text{-N}$   
369 (chemolithoautotrophic  $\text{NO}_3^-\text{-N}$  reduction) were considered. However, as the experimental  
370 study was conducted in a natural costal water column and sediment, the reaction formulations,  
371 process rates and kinetic parameters might not be directly applicable in wastewater treatment  
372 systems. Moreover, some reactions, such as nitrogen fixation and remineralization, might be  
373 neglected in those systems.

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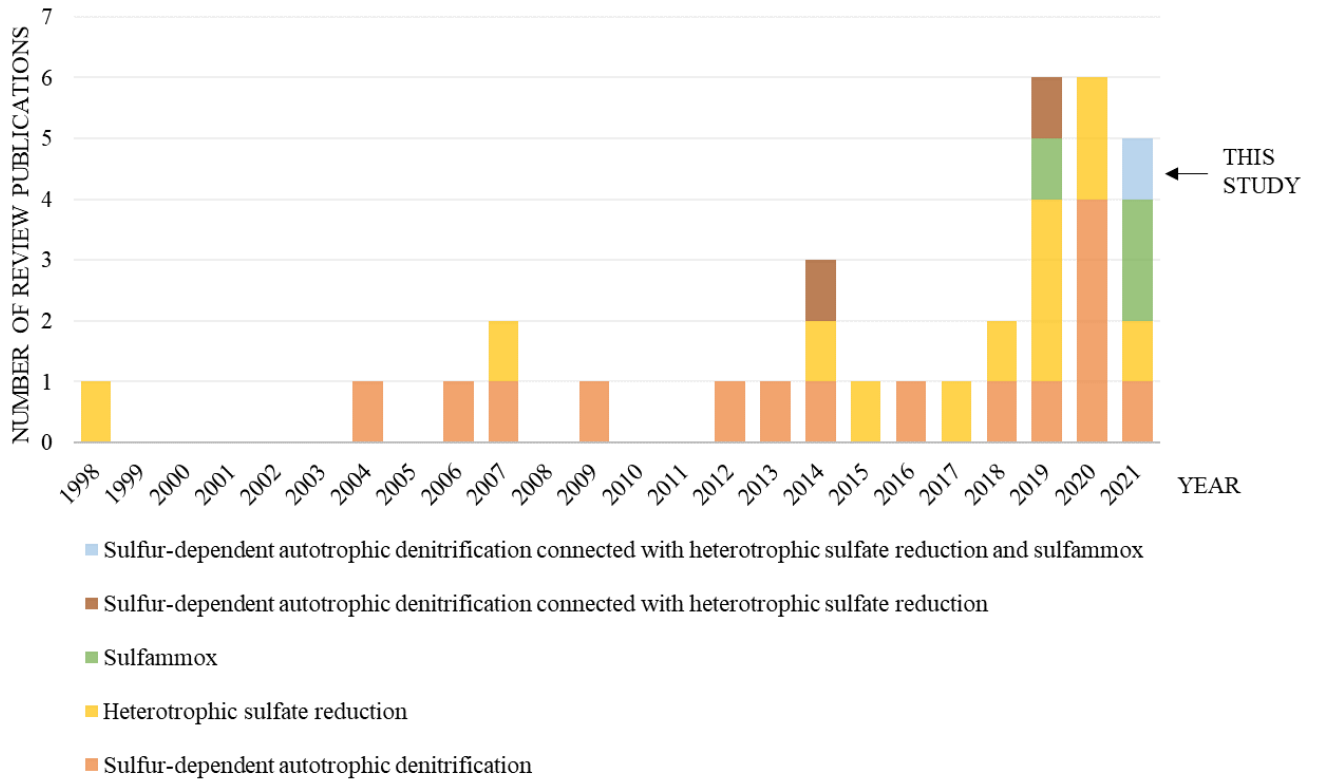
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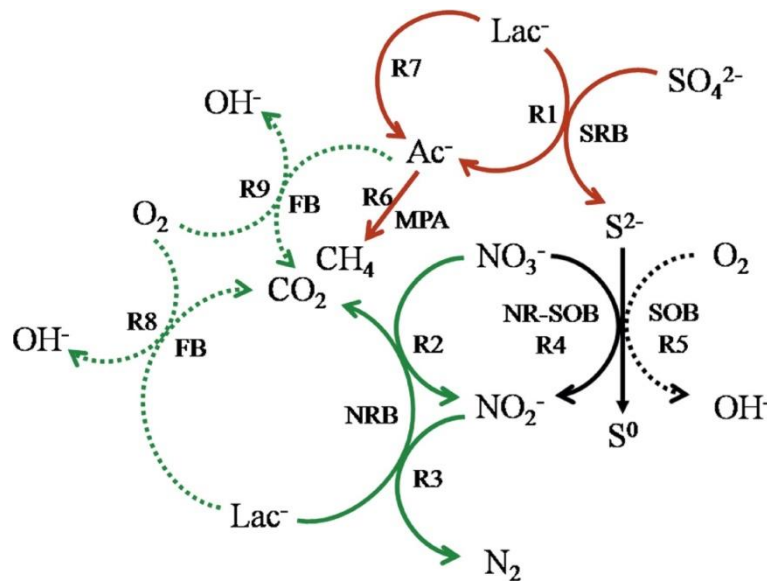




379

380 Figure S1. Number of review papers on S-dependent biochemical processes, including  
 381 autotrophic denitrification, heterotrophic sulfate reduction and sulfamox

382



383

384 Figure S2. Simplified representation of the biochemical process associated with C, N and S  
 385 conversions (Xu et al., 2017).

386



Process/System	Issue	Cui et al., 2019	Tian and Yu, 2020	Hu et al., 2020	Ma et al., 2020	Wang et al., 2020	Park and Yoo, 2009	Lin et al., 2018	Wu et al., 2021	Show et al., 2013	Costa et al., 2020	Serrano et al., 2019	Lens et al., 1998	Xu and Chen, 2020	Kumar et al., 2021	Hao et al., 2014	van den Brand et al., 2015	Rios-Del Toro and Cervantes, 2021	Grubba et al., 2020	This study	
<b>S - DEPENDENT AUTOTROPHIC DENITRIFICATION</b>	recent advances in S-dependent autotrophic denitrification	✓	✓	✓	✓	✓	✓	✓	✓	✓										updated	
	potential use of S-dependent autotrophic denitrification	✓	✓	✓	✓	✓	✓	✓	✓	✓						✓	✓				updated
	microbiological reactions and microorganisms involved	✓		✓	✓	✓	✓	✓	✓	✓											✓
	factors influencing the operation of S-dependent autotrophic denitrification	✓		✓	✓	✓			✓	✓								✓			✓
	comparison of greenhouse gas emissions from autotrophic and heterotrophic denitrification	✓					✓			✓							✓				
	reactor configurations										✓										✓
	comparison of different electron donors (S compounds) for autotrophic denitrification	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓										✓
	cost comparison of reduced-S species and organic substrate as an electron donor	✓					✓	✓													
	recent works on the hydrogen and/or iron- and/or methane- and/or carbon- S-dependent autotrophic denitrification	✓	✓		✓	✓	✓	✓		✓											
	mixotrophic denitrification		✓					✓		✓	✓										✓
natural occurrence of S-dependent autotrophic denitrification				✓																	



	characteristics and effects of water pollution by $\text{SO}_4^{2-}$				✓							
	treatment of acid mine drainage (AMD)	✓							✓			
	heavy metal removal	✓			✓	✓	✓					
	methods for enhancement of SRB activity				✓				✓			
	sulphide forms in aqueous medium at different pH values	✓		✓								
	prospects, limitations and future research needs	✓	✓	✓	✓	✓	✓		updated			
SULFAMMOX	recent advances in sulfamox process							✓	✓	✓	updated	
	characteristics of sulfamox							✓	✓	✓	✓	
	potential use of sulfamox process							✓	✓	✓	updated	
	characteristics of $\text{NH}_4\text{-N}$ anaerobic oxidation processes with different electron acceptors							✓				
	spontaneity and oxidation-reduction potential									✓		
	functional bacteria								✓	✓	✓	
	possible interactions with other bacteria								✓	✓	✓	
	bacteria responsible for the specific N and S transformations								✓	✓	✓	
	factors influencing the sulfamox process								✓	✓	✓	
	the occurrence of sulfamox in the environment								✓		✓	
	source of $\text{SO}_4^{2-}$									✓		
	reactors types								✓	✓	✓	✓
	co-existence of anammox, S-dependent autotrophic denitrification and sulfamox									✓	✓	✓

	prospects, limitations and future re- search needs			✓	✓	✓	✓
<b>SANI PROCESS</b>	SANI process characteristics	✓	✓				✓
	diagram of the SANI reactor sys- tem	✓	✓				✓
	SANI key-parameters		✓				✓
	recent advances in SANI process						✓
	FGD-SANI and MD-SANI charac- teristics						✓
	diagram of the FGD-SANI and MD-SANI reactors system						✓
	FGD-SANI and MD-SANI key-pa- rameters						✓
<b>S CYCE WITH ANAMMOX</b>	Sulfate Reduction, Denitrifica- tion/Anammox and Partial Nitrifi- cation (SRDAPN) characteristics						✓
	Partial Nitrification/Anammox and S-dependent autotrophic Denitrifi- cation (PNASD) characteristics						✓
	Anammox and S-dependent auto- trophic Denitrification (ASD) char- acteristics						✓
	S-dependent autotrophic Partial Denitrification/Anammox (SPDA) characteristics						✓
	diagram of SRDAPN, PNASD, ASD nad SPDA						✓
	recent advances in SRDAPN, PNASD, ASD nad SPDA						✓
	SRDAPN, PNASD, ASD nad SPDA key-parameters						✓

<b>SYSTEMS WITH SULFAMMOX</b>	Sulfamnox/Anammox (SA) characteristics	✓
	Sulfamnox - S-dependent autotrophic Denitrification (SSD) characteristics	✓
	Sulfamnox – Anammox - S-dependent autotrophic denitrification (SASD) characteristics	✓
	diagram of the SA, SSD and SASD	✓
	recent advances in SA, SSD and SASD	✓
	SA, SSD and SASD key parameters	✓

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