

Sulfate reducing ammonium oxidation (SULFAMMOX) process under anaerobic conditions

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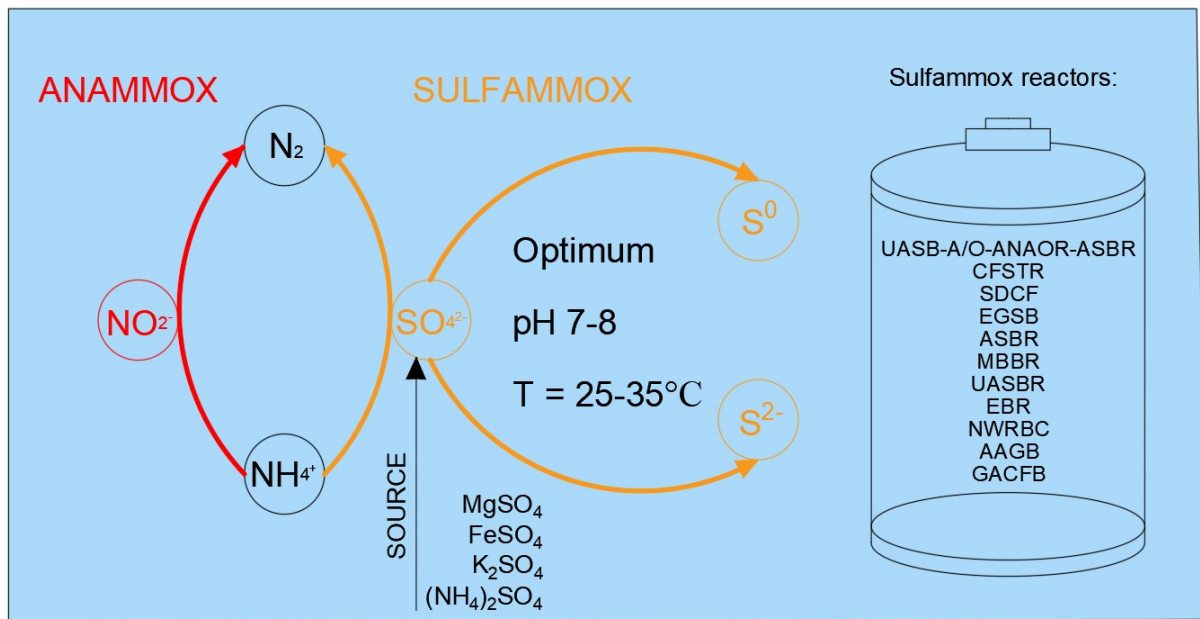
Highlights

- Sulfate may be an additional electron acceptor in the anaerobic ammonium oxidation.
- *Bacillus Benzoevorans* and *Brocadia Anammoxoglobus Sulfate* can perform sulfammox.
- The optimal conditions for the sulfammox is 25°C -35°C and pH - 7.0 – 8.
- Sulfammox is a viable option for specific industrial wastewater with high NH₄⁺ and SO₄²⁻.
- Sulfammox has been studied in suspended growth, biofilm, granular and hybrid reactors.

Abstract

Sulfate (SO₄²⁻) can be an electron acceptor for ammonium nitrogen (NH₄⁺) oxidation under anaerobic conditions. The process is known as sulfammox and can be a viable alternative to conventional, nitrite (NO₂⁻) dependent, anammox. Two bacterial species, including *Bacillus Benzoevorans* and *Brocadia Anammoxoglobus Sulfate*, can perform that process. With sulfammox, an economically inefficient pre-nitration step (due to aeration) is not required. There are more than 10 different systems in which sulfammox has been studied, including suspended growth, biofilm, granular and hybrid reactors. A combination of anammox and sulfur related processes (sulfammox and autotrophic denitrification) would especially be appropriate for specific industrial wastewater with high content of nitrogen compounds and SO₄²⁻. The results of recent studies suggest that very high removal efficiencies could simultaneously be achieved with respect to both NH₄⁺ (92-99%) and SO₄²⁻ (53-60%).

27 Graphical abstract



28

29 **Keywords:** sulfamnox, anamnox, autotrophic denitrification, sulfate, sulfur cycle

30 **Funding:** This work was supported by the Narodowe Centrum Nauki (National Science
31 Center) [UMO-2019/03/X/ST10/01127].

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35 1. Introduction

36 Nitrification and denitrification are the most common processes responsible for nitrogen (N)
37 conversions in wastewater treatment systems. A viable alternative to that pathway of nitrogen removal
38 is the “anaerobic” ammonium oxidation (anammox) process. "Anaerobic" because it is actually an
39 anoxic process due to the presence of nitrite (NO_2^-). It is generally accepted that anaerobic ammonia
40 oxidizing bacteria (AAOB) oxidize ammonia (NH_4^+) to N_2 with NO_2^- as an electron acceptor. In fact,
41 however, AAOB have a more comprehensive metabolism than initially assumed and other phenomena
42 of “anaerobic” NH_4^+ oxidation have been discovered (Kartal et al. 2012; Liu et al. 2008). In addition to
43 NO_2^- , there may be other electron acceptors, including sulfate (SO_4^{2-}), for NH_4^+ oxidation under
44 “anaerobic” conditions (Zandt et al. 2018). This process is known as sulfate reducing ammonium
45 oxidation (SRAO) or sulfammox (Bi et al. 2020).

46 In addition to anaerobic sludge digester liquors, the sulfammox process may especially be appropriate
47 for treatment of some industrial wastewater, containing high concentrations (>1000 mg/l) of both
48 NH_4^+ and SO_4^{2-} . Such characteristics are typical for the effluents from seafood, chemical, textile,
49 paper, fermentation and sugar production (Rikmann et al. 2016).

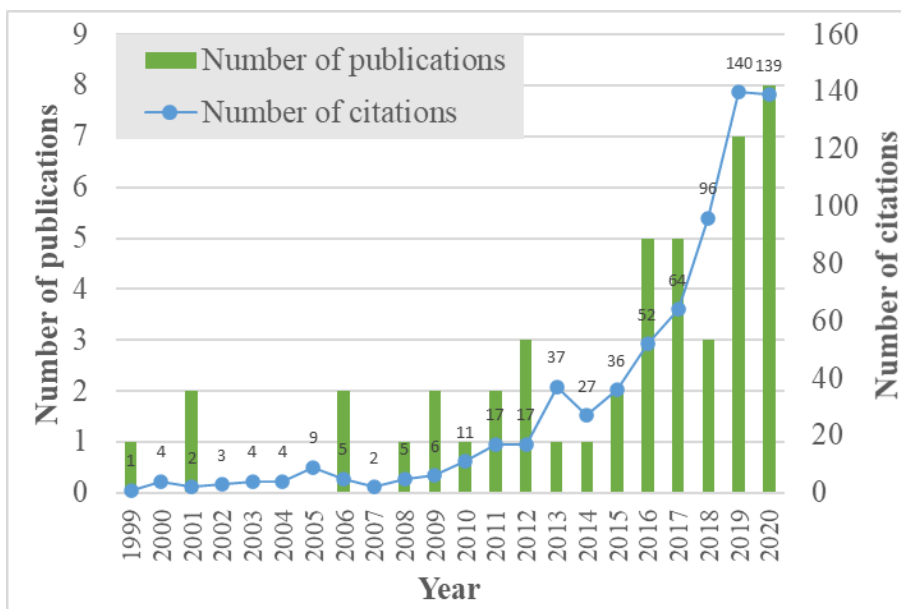
50 In comparison with the conventional anammox, sulfammox is easier to control as nitritation becomes
51 unnecessary (SO_4^{2-} instead of NO_2^- serves as the electron acceptor) (Zhang et al. 2009). Besides, as a
52 reducing process of SO_4^{2-} , it is also free of secondary pollution caused by sulphide (S^{2-}), which is toxic
53 and harmful to human health and aquatic ecosystems (Zhang et al. 2019a). Sulfammox can also
54 prevent interference with the conventional anammox process caused by inhibition of S^{2-} (Xu et al.
55 2020) or hydrogen sulfide (H_2S) (Wiśniewski et al. 2019). Moreover, elemental sulfur (S^0) is formed
56 and its recovery provides a valuable by-product (Rios-Del Toro and Cervantes 2019). The recovered
57 S^0 could be used as electron donor for autotrophic denitrification as reported by Ucar et al. (2020).
58 Moreover, recovering S^0 from wastewater is also essential to reuse it as fertilizer or to re-enter
59 production lines in other industries. The combination of the technology based on the anammox process
60 with the technology based on the sulfammox process would enable a balanced approach to the

61 problem of specific industrial wastewater with high content of nitrogen compounds and SO_4^{2-} through
62 their co-treatment in combined processes.

63 The sulfammox process may occur either independently or in conjunction with the conventional
64 anammox process. The combination of both processes can increase the overall nitrogen removal
65 efficiency. Recent studies (Zhang et al. 2019a; Wu et al. 2020) have shown a high degree of
66 simultaneous removal of NH_4^+ and SO_4^{2-} , i.e. in the range 92-99% and 53-60%, respectively, with
67 NO_2^- and SO_4^{2-} as electron acceptors.

68 The sulfammox process has briefly been addressed in reviews on anammox in marine environments
69 (Rios-Del Toro and Cervantes 2019) and in the state of anammox research in China (Ali et al. 2013).

70 There are still a few publications on this process and finding them is not straightforward, as
71 sulfammox also appears as SRAO or sulfate-dependent anammox. Based on the Web of
72 Science database, using the keywords "sulfate", "anammox" and "wastewater", a number of
73 publications and their citations appearing in 1999-2020 years are presented in Fig. 1.



74
75 **Fig 1** Number of publications based on keywords "sulfate", "anammox" and "wastewater" and their citations in
76 the Web of Science database in 1999-2020

77 Based on the data in Fig. 1, it can be projected that the number of publications on sulfamox and their
78 citations will be increasing fast over the next few years. Very recently, Liu et al. (2021) have
79 published the first review paper especially dedicated to sulfamox. However, the paper does not
80 incorporate a few studies, Zhang et al. (2019a) or Wang et al. (2017a), that have a significant effect on
81 the process understanding.. In particular, the latter paper describes the $\text{NH}_4^+/\text{SO}_4^{2-}$ ratio which plays a
82 key role in the sulfamox process. Other issues, omitted or not sufficiently addressed in the study of
83 Liu et a. (2021), comprised the spontaneity of the process, effect of COD on sulfamox, and feeding
84 options (NO_2^- and SO_4^{2-} together and separately).

85 In the present study, the combination of several processes influencing removal of NH_4^+ and SO_4^{2-} from
86 wastewater was addressed, including sulfamox, anammox, sulfide-dependent autotrophic
87 denitrification, sulfur-dependent autotrophic denitrification, nitrification, denitrification, and
88 heterotrophic sulfate reduction. A special attention was given to linking the sulfamox process with
89 sulfur-dependent autotrophic denitrification. Moreover, a wide variety of sulfamox reactors was
90 presented and discussed in terms of the operating conditions and performance efficiency.

91 Both soil, air and water are exposed to the influence of toxic sulfur compounds - H_2S and S^{2-} .
92 Recognition of the sulfamox process may lead to the development of research on this process, and
93 hence to environmental protection, thanks to the decomposition of these compounds into S^0 and
94 reduction of energy consumption by limiting two separate processes of removing NH_4^+ and SO_4^{2-} to
95 one co-treatment. Therefore, the aim of this mini review is to characterize the sulfamox process,
96 indicate the operational conditions in which it can be carried out, and compare the examined
97 sulfamox reactors.

98 **2. The characteristics of the sulfamox process**

99 Sulfamox was first reported by Fdz-Polanco et al. (2001b) in a granular activated carbon anaerobic
100 fluidized bed reactor treating vinasse from an ethanol distillery of sugar beet molasses. The authors
101 observed that approximately 80% of SO_4^{2-} was converted to S^0 with simultaneous oxidation of NH_4^+ to
102 N_2 . The combined process for removal of NH_4^+ and SO_4^{2-} was described as follows:



104 In the follow-up studies, Liu et al. (2008) and Yang et al. (2009) identified SO_4^{2-} as a potential electron
105 acceptor as it was the feed component. They investigated the process of simultaneous removal of
106 NH_4^+ and SO_4^{2-} under anaerobic conditions. The ratio of NH_4^+ to NO_2^- consumption was
107 approximately 1.1 : 1 and 1 : 1.15, respectively, in a non-woven rotating biological contactor (NRBC)
108 and upflow anaerobic sludge blanket reactor (UASBR) (see: Table 2). These values were significantly
109 higher in comparison with 1 : 1.32, which is the theoretical ratio for the conventional, NO_2^- -dependent,
110 anammox process (Xie et al. 2017).

111 Based on the literature (Strous et al. 2006; Zhang et al. 2009; Schrum et al. 2009), formation of HS^- in
112 the sulfammox process may also be considered:



114 Alternatively, formation of HS^- may be associated with oxidation of NH_4^+ to NO_3^- (Schrum et al.
115 2009):

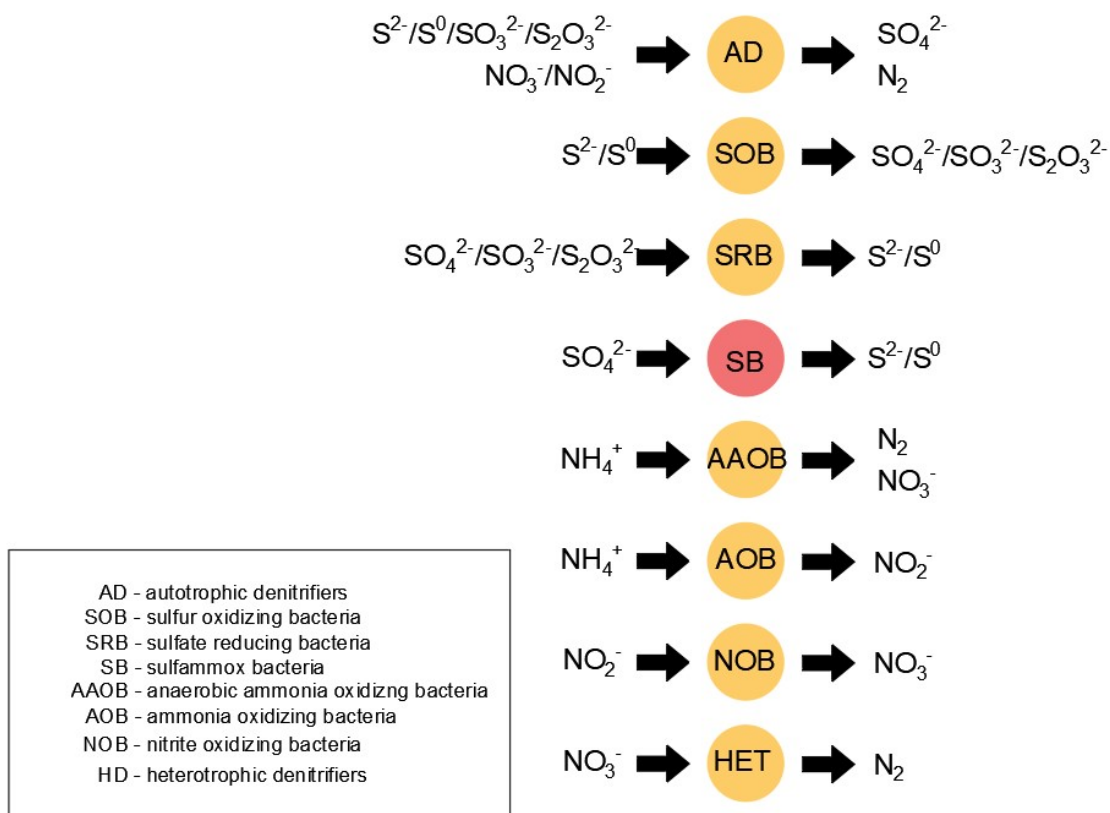


117 The consumption rate of SO_4^{2-} can be estimated based on the corresponding consumption rate of N-
118 NH_4^+ and the theoretical stoichiometric consumption ratio (= 2) of NH_4^+ to SO_4^{2-} in sulfammox (see:
119 reaction 1). An inadequate influent ratio of $\text{NH}_4^+/\text{SO}_4^{2-}$ - different than 2 (see: reaction 2,3), may result
120 in the formation of HS^- in the sulfammox process according to reactions (2,3). The $\text{NH}_4^+/\text{SO}_4^{2-}$ ratio of
121 2 was indeed found in the studies of Zhang et al. (2009), Yang et al. (2009) and Cai et al. (2010). In
122 other studies, the reported ratios were lower, i.e. 1.71 - 1.75 (Liu et al. 2008) and 1.65 (Bi et al. 2020).

123 Rikmann et al. (2012) noted that the stoichiometric ratio of NH_4^+ moles consumed per mole of reduced
124 SO_4^{2-} was higher than could be expected from the amount of SO_4^{2-} reduced. This implicitly indicated
125 the presence of additional electron acceptors, other than SO_4^{2-} (like humic matter) coupled with NH_4^+
126 oxidation or reoxidation of reduced sulfur compounds into SO_4^{2-} . The high efficiency of NH_4^+ removal
127 may result from complex interactions between organic compounds, nitrogen and sulfur like

128 sulfamnox, anammox, autotrophic denitrification, heterotrophic denitrification (Rikmann et al. 2012,
 129 2014, 2016).

130 The newly discovered, SO_4^{2-} dependent, AAOB species have been found to be responsible for carrying
 131 out the above reactions (2-4). The first one was *Brocadia Anammoxoglobus Sulfate* (Liu et al. 2008),
 132 which was a functional microorganism in the simultaneous removal of NH_4^+ and SO_4^{2-} and ended the
 133 conversion of NH_4^+ and SO_4^{2-} by producing NO_2^- as an intermediate. The second isolated species,
 134 *Bacillus Benzoevorans* (Cai et al. 2010), was responsible for carrying out the entire sulfamnox
 135 reaction. In the study of Liu et al. (2015b), the dominant bacteria changed from *Candidatus Brocadia*
 136 to *Bacillus Benzoevorans* when the process transformed from the conventional anammox to
 137 sulfamnox. Sulfamnox bacteria and AAOB combine the N and S cycles, increasing the range of N-S
 138 transformations as shown in Fig. 2.



139
 140 **Fig 2** Bacteria responsible for the specific N and S transformations

141

142 The SO_4^{2-} dependent AAOB are rodshaped with flagellum and spore, having a size of $(0.7-1.0) \times (2.4-$
143 $3.5) \mu\text{m}$. The colony on the plate was round with a diameter of about 1 mm with a light yellow color,
144 and its surface was smooth and wet. The cultivated biomass was dominated by chains of bacilli and
145 cocci. Cocci generally had a diameter of $0.9 \mu\text{m}$, whereas bacilli varied around $0.8 \mu\text{m}$ and $1-1.2 \mu\text{m}$ in
146 width and length, respectively (Zhang et al. 2009; Cai et al. 2010; Ali et al. 2013).

147 Some Proteobacteria, which may potentially perform sulfamox, include the following species: *Sulfu-*
148 *rimonas*, *Desulfuromonadales*, *Desulfovibrio*, *Desulfuromonas*, *Desulfobulbus*, *norank Rhodobacter-*
149 *aceae* and *Thiobacillus* (Rios-Del Toro et al. 2017; Wang et al. 2017a; Rios-Del Toro et al. 2018).

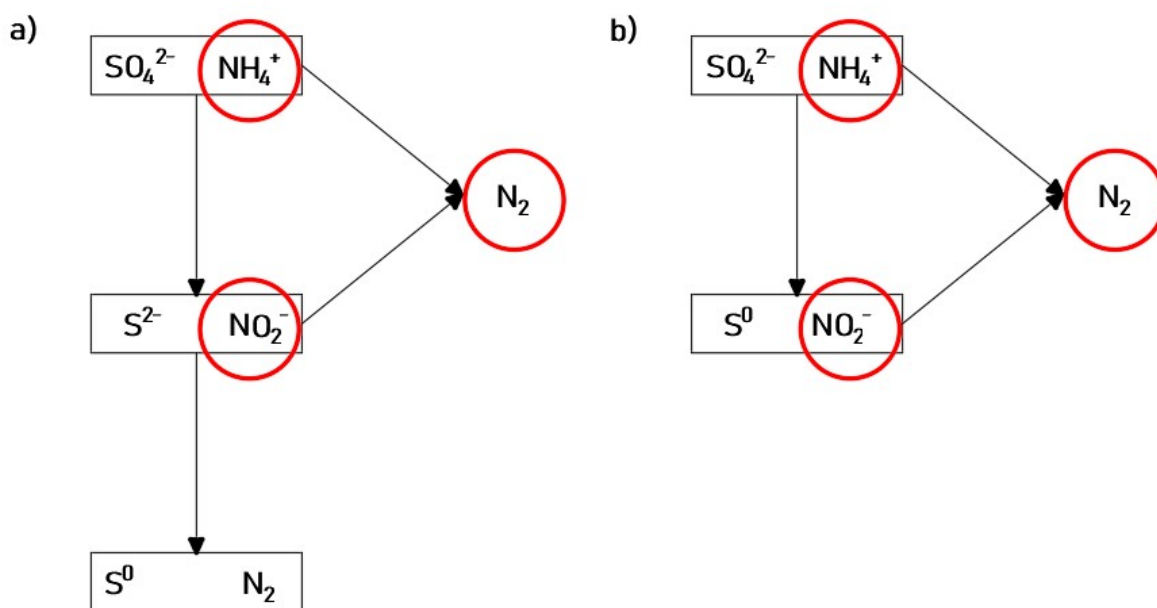
150 A syntrophic relationship between ammonia oxidizing bacteria (AOB), sulfate reducing bacteria
151 (SRB) and AAOB could make thermodynamically not favorable oxidation of NH_4^+ to NO_2^- coupled
152 with a possible reduction of SO_4^{2-} to S^0 (Rikmann et al. 2014). A pure chemical reaction between NH_4^+
153 and SO_4^{2-} without microorganisms is not possible (Yang et al. 2009).

154 3. Relationship between sulfamox and sulfide-dependent autotrophic denitrification

155 The overall sulfamox reaction (reaction (1)) has been shown to occur in three consecutive
156 biochemical reactions (reactions (4-6)) (Fdz-Polanco et al. 2001b; Zhang 2019a; Bi et al. 2020):



160 In reaction (4), NH_4^+ reacts with SO_4^{2-} and is oxidized to NO_2^- (intermediate) inside the bacterial cell
161 and SO_4^{2-} is simultaneously deoxygenated to S^{2-} . The NO_2^- produced diffuses outside of the bacterial
162 cell. In reaction (5), part of NO_2^- is reduced with S^{2-} , which leads to production of N_2 and S^0 . Finally,
163 reaction (6) is the conventional anammox process carried out by *Planctomycetes* (Van der Star et al.
164 2007). Yang et al. (2009) described reaction (5) as the denitrification process that occur through
165 reduction of NO_2^- to N_2 with simultaneous oxidation of S^{2-} by autotrophic denitrifiers, where the
166 electron donor is S^{2-} and the electron acceptor is NO_2^- . For better understanding, reactions 4-6 are
167 shown in Figure 3a.



168
169 **Fig 3** Reactions involved in the sulfamnox process as proposed by Yang et al. (2009) (a), and Liu et al. (2008)

170 (b)

171 **It should be emphasized that sulfide-dependent autotrophic denitrification is one of the reactions**
 172 **involved in the overall sulfamnox process, according to Fig 3a. Therefore, it is difficult to**
 173 **distinguish a strict boundary between sulfamnox and sulfide-dependent autotrophic**
 174 **denitrification. In fact, the denitrification reaction is one of the components of sulfamnox and**
 175 **without it sulfamnox cannot occur, as shown in reactions (4-6).** Therefore, some researchers do
 176 not distinguish the efficiency of NH_4^+ removal in the sulfamnox process at all, but only report the total
 177 efficiency of NH_4^+ removal under anaerobic conditions in the presence of SO_4^{2-} (Wu et al 2020; Bi et
 178 al. 2020; Zhang et al 2019a).

179 On the contrary, Liu et al. (2008) explained the sulfamnox process as a combination of two reactions
 180 as shown earlier in Fig. 3b. According to that concept, NH_4^+ would be partially converted to NO_2^- and
 181 coupled with a conversion of SO_4^{2-} (electron acceptor) to S_0 . Then NH_4^+ would be oxidized to N_2 by
 182 NO_2^- in the conventional anammox process. Currently, the exact pathway of sulfamnox remains
 183 largely unknown. More detailed microbiological tests are needed to check which microorganisms and
 184 genes are involved in that process.

185 It should be emphasized that S^{2-} in the sulfammox process (see: reaction 4) can be oxidized to either S^0
186 or SO_4^{2-} , depending on the initial S^{2-} to NO_2^- ratio. Therefore, that ratio must be strictly controlled to
187 avoid re-oxidation to SO_4^{2-} . For sulfammox, it is important to reduce SO_4^{2-} to S^0 . When S^{2-} is oxidized
188 back to SO_4^{2-} , the total reduction of SO_4^{2-} in the sulfammox process decreases.

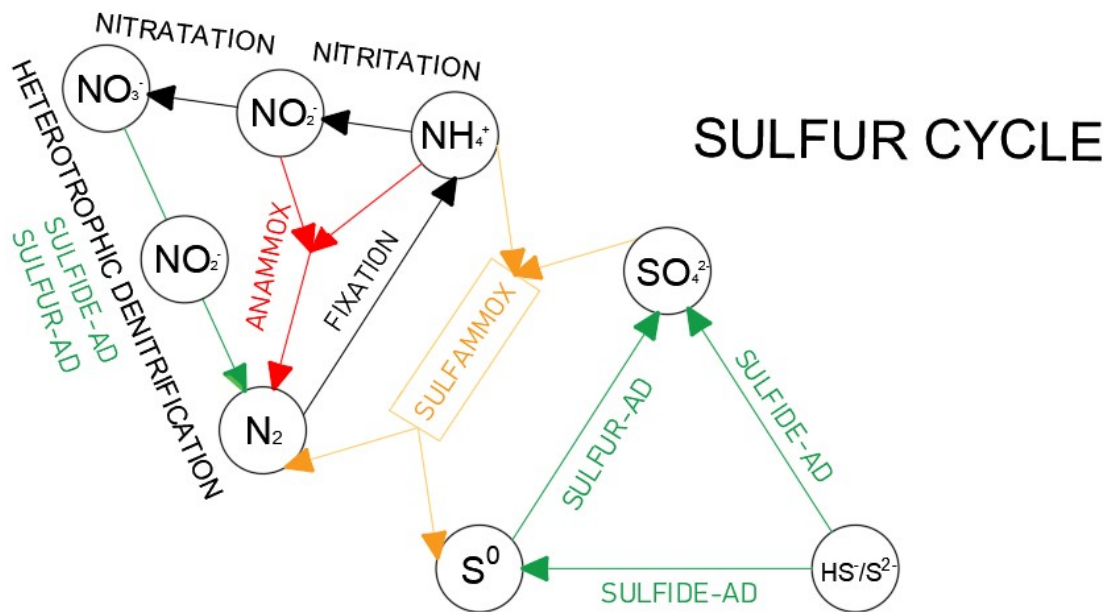
189 When the stoichiometric ratio of NH_4^+ moles consumed per mole of reduced SO_4^{2-} is higher than
190 might be expected from the degree of SO_4^{2-} reduction, this may also be due to the partial reoxidation
191 of S^0 or HS^- to SO_4^{2-} by sulfur-related autotrophic denitrification (Rikmann et al. 2012, 2014; Qin et al.
192 2019; Wang et al. 2020). Some chemolithotrophic denitrifiers, such as *Thiobacillus denitrificans*, are
193 capable of performing sulfur-related autotrophic denitrification.

194 The autotrophic denitrification reactions can occur with S^{2-} , sulphite (SO_3^{2-}), thiosulphate ($S_2O_3^{2-}$) or
195 S^0 as electron donors, and either NO_3^- or NO_2^- as electron acceptors (Guo et al. 2013; Xu et al. 2013;
196 Yu et al. 2013; Wang et al. 2017b; Di Capua et al. 2019). Then, either SO_4^{2-} or S^0 is formed depending
197 on the sulfur to nitrogen ratio (Kalyuzhnyi et al. 2006; Liu et al. 2015a). The following reactions
198 describe those complex phenomena (Li i wsp. 2009; Wang et al. 2017b):



205 A simplified relationship of the sulfammox process with the nitrogen and sulfur cycles is presented in
206 Fig. 4.

NITROGEN CYCLE



207
208 **Fig 4** Coupling the nitrogen and sulfur cycles in sulfamox, sulfur-dependent autotrophic denitrification (sulfur-
209 AD) and sulfide-dependent autotrophic denitrification (sulfide-AD)

4. Environmental factors and operational conditions affecting sulfamox

4.1. Process medium and feeding options

212 Most of the sulfamox studies have been carried out with synthetic wastewater or growth media
213 (Zhao et al. 2006; Liu et al. 2008; Yang et al. 2009; Cai et al. 2010; Bi et al. 2020), but there have also
214 been a few studies using real wastewater (Rikmann et al. 2012, 2014, 2016). Different compounds
215 have been used as the SO_4^{2-} source in the medium (see: Table 1). The inoculum biomass originated
216 from various sources (see: Table 2), including long-term operated anammox reactors and anaerobic
217 digesters. The cultivation experiments have been carried out with three feeding options:

- 218 a) conventional anammox was run at the beginning, and then NO_2^- was replaced with SO_4^{2-} as a new
219 electron acceptor (Yang et al. 2009; Rikmann et al. 2012, 2016; Zhang et al. 2019a, b; Bi et al.
220 2020);
- 221 b) SO_4^{2-} was used since the beginning without any addition of NO_2^- (Zhang et al. 2009; Wang et al.
222 2017a; Zhang et al. 2019b; Bi et al. 2020);

223 c) SO_4^{2-} and NO_2^- were simultaneously used as electron acceptors during the whole study period (Zhao
224 et al. 2006; Liu et al. 2008; Zhang et al. 2019a; Wu et al. 2020).

225 4.2. Temperature

226 The process temperature set point normally ranged from 30°C to 36°C as shown in Table 1. Cai et al.
227 (2010) tested the sulfamox process efficiencies at the following series of temperatures: 15°C, 25°C,
228 30°C, 35°C, 45°C and 55°C. The NH_4^+ and overall SO_4^{2-} removal efficiencies were approximately
229 37.5% and 35%, 36% and 30%, respectively, at $T = 15^\circ\text{C}$ and $T = 55^\circ\text{C}$. The highest NH_4^+ and overall
230 SO_4^{2-} removal efficiencies were observed at $T = 30^\circ\text{C}$, i.e. 44.4% and 40%, respectively. The removal
231 rate of NH_4^+ and SO_4^{2-} at that temperature was 0.168 mg N/l/h ($R^2 = 0.98$) and 0.191 mg S/l/h ($R^2 =$
232 0.95), respectively. The optimal temperature range for the sulfamox process is 25°C -35°C (Cai et
233 al., 2010).

234 The sulfamox process was also studied at lower temperatures, e.g. 20°C (Rikmann et al. 2016) and
235 14-15°C (Wu et al. 2020). In the latter case, despite such a low temperature, the NH_4^+ and overall
236 SO_4^{2-} removal efficiencies remained at a high level, i.e. 98.5% and 52.8%, respectively (Wu et al.
237 2020). Due to the combination of anaerobic-aerobic, continuous and batch processes adopted in this
238 process, the anammox and sulfamox coupled to remove nitrogen. Rikmann et al. (2016) studied two
239 reactors at different temperatures, i.e. MBBR (20°C) and UASBR (36°C). That approach was not
240 clearly explained, but apparently resulted from the use of different sludges in both reactors. In the
241 UASBR, the inoculum originated from an anaerobic reactor for treatment of industrial wastewater
242 (yeast production), whereas the MBBR was inoculated with carriers with a well-deposited anammox
243 biofilm developed in a conventional laboratory-scale anammox reactor. The TN removal efficiencies
244 were in the range 5 - 72% for the MBBR and 10 - 75% for the UASBR, respectively. Despite the use
245 of different temperatures, the TN removal rates were similar, i.e. 0.05 kg N/m³/d for the MBBR and
246 0.04 kg N/m³/d for the UASBR.

247 4.3. pH



248 The optimal pH value is 7.0 - 8.5 for both conventional anammox and sulfammox (Wu et al. 2020),
 249 therefore, many studies on sulfammox have been carried out in that pH range (Yang et al. 2009; Zhang
 250 et al. 2009; Zhang et al. 2019a,b; Bi et al. 2020; Wu et al. 2020). Cai et al. (2010) studied the effect of
 251 pH on the efficiency of NH_4^+ and overall SO_4^{2-} removal. The following pH values were considered:
 252 6.5, 7.5, 8.5, 9.5 and 10.5, and the optimum pH was found at 8.5. On the contrary, Zhao et al. (2006)
 253 found the optimum pH = 7.8.

254 **Tab. 1** Environmental factors and operational conditions in the sulfammox studies

Source of SO_4^{2-}	COD addition	pH [-]	Temperature [°C]	Reference
$\text{MgSO}_4 / \text{FeSO}_4$	No	7.8	35	Bi et al. [2020]
$(\text{NH}_4)_2\text{SO}_4$	No	8.1-8.3	35	Zhang et al. [2019a]
$(\text{NH}_4)_2\text{SO}_4$	No	8.1-8.6	30	Zhang et al. [2019b]
K_2SO_4	No	8.5	30	Cai et al. [2010]
Na_2SO_4	No	7.5-8.5	35	Yang et al. [2009]
Na_2SO_4	No	7.5	30	Zhang et al. [2009]
$(\text{NH}_4)_2\text{SO}_4$	No	8-8.2	35	Liu et al. [2008]
n.a.	Yes	7-8.5	14-15	Wu et al. [2020]
n.a.	Yes	6.9-8.1	36	Wang et al. [2017a]
K_2SO_4	Yes	8.4	20	Rikmann et al. [2016]
K_2SO_4	Yes	8.11	36	Rikmann et al. [2014]
n.a.	Yes	7.8-8.3	36	Fdz-Polanco et al. [2001b]

255 n.a.: not available

256 4.4. COD addition

257 Even though COD is not required for the sulfammox process (Zhang et al. 2009), the experiments
 258 were performed either without COD addition (Liu et al. 2008; Cai et al. 2010; Prachakittikul et al.
 259 2016; Zhang et al. 2019a,b; Bi et al. 2020) or with COD addition (Fdz-Polanco et al. 2001a,b;
 260 Rikmann et al. 2012, 2014, 2016; Wang et al. 2017a; Wu et al., 2020). When COD is present in
 261 wastewater, the sulfammox process can be coupled with subsequent heterotrophic denitrification

262 (Zhang et al. 2019b). In the studies of Zhang et al. (2019b) sulfammox was mainly due to the high
263 proportion of Proteobacteria, but approximately 12.4% of denitrifiers were also found in the sediment.
264 This indicates that nitrification, denitrification and the traditional anammox with sulfammox may
265 simultaneously occur in oxidation of NH_4^+ . This allows for simultaneous removal of NH_4^+ , SO_4^{2-} and
266 COD from wastewater (Wang et al. 2017a). Kosugi et al. (2019) proposed a combined SO_4^{2-} reduction,
267 denitrification/anammox and partial nitrification process in an anaerobic-anoxic reactor. The authors
268 confirmed the coexistence of heterotrophic denitrifying bacteria, sulfur denitrifying bacteria and
269 anammox *Candidatus Brocadia* bacteria. They also recognized that heterotrophic and autotrophic
270 denitrifying bacteria, competing for NO_3^- and NO_2^- , can be used to oxidize S^{2-} to S^0 prior to oxidation
271 of organic carbon.

272 Yin et al. (2017) showed that sulfur-based autotrophic denitrification occurred with heterotrophic
273 denitrification in an anaerobic baffled reactor. The authors also indicated that without addition of S^{2-} a
274 significant amount of NO_3^- was reduced heterotrophically to N_2 (76.6%). However, the addition of S^{2-}
275 stimulated autotrophic denitrification (from 19.7% to 40.8%) and inhibited heterotrophic
276 denitrification (decreased to 46.9%), thereby resulting in a shift (8%) in the NO_3^- reduction pathway
277 from denitrification to dissimilatory NO_3^- reduction to NH_4^+ . The addition of S^{2-} caused a proportional
278 increase in the population of sulfur-oxidizing nitrate-reducing bacteria (mainly *Paracoccus*) from
279 18.6% to 27.2% and suppressed heterotrophic nitrate-reducing bacteria (mainly *Pseudoxanthomonas*
280 and *Pseudomonas*), which caused a decrease (25.5%) in their population.

281 On the contrary, Zhao et al. (2006) found that more efficient removal of NH_4^+ was obtained when the
282 COD concentration was lower. In the studies of Wu et al. (2020), organic matter (300 mg COD/l in the
283 influent) negatively affected conventional anammox, but sulfammox was not affected. As a
284 consequence, the concentration of the dominant potential sulfammox bacteria (*Sulfurimonas*,
285 *Desulfovibrio*, *Desulfuromonas*, *Desulfobulbus*, norank *Rhodobacteraceae* and *Thiobacillus*) was
286 higher than the concentration of *Candidatus Kuenenia* performing conventional anammox.

287 4.5. Spontaneity and oxidation-reduction potential

288 Zhang et al. (2009) described the spontaneity of the sulfamnox reaction. ΔG^0 of the sulfamnox is
289 -45.35 kJ/mol. The reaction is obviously more difficult to proceed than conventional anammox, which
290 has $\Delta G^0 = -357$ kJ/mol.

291 As the SO_4^{2-} dependent AAOB are obligate anaerobic bacteria, high substrate concentrations and a low
292 oxidation-reduction potential (ORP) (< -100 mV) can intensify the sulfamnox process (Zhang et al.
293 2009; Ali et al. 2013). Fdz-Polanco et al. (2001a) found that the calculated values of redox potential
294 for the half reactions of reduction of N_2 to NH_4^+ and SO_4^{2-} to S^0 at $\text{pH} = 8$ was in the narrow range
295 from -330 to -360 mV. Those results suggested that SO_4^{2-} reduction and NH_4^+ oxidation could coexist
296 together under anaerobic conditions. Similar to conventional anammox, hydrazine injections have also
297 been reported to improve the sulfamnox activity (Rikmann et al. 2012, 2014, 2016).

298 4.6. Other factors influencing the sulfamnox process

299 In contrast, there are also several factors that may negatively affect the sulfamnox process. Wu et al.
300 (2020) found that DO levels $>0.3 - 0.5$ mg/l could have a negative effect on sulfamnox, as this leads
301 to partial nitrification and the production of NO_2^- . DO inhibits the enrichment of the dominant bacteria
302 of both sulfamnox and anammox and leads to the growth of AOB, competing with AAOB for NH_4^+ .
303 High concentrations of NO_2^- and NO_3^- also favor SO_4^{2-} resynthesis as a result of sulfur-related
304 autotrophic denitrification. Rikmann et al. (2016) also pointed out that NO_2^- and HCO_3^- concentrations
305 exceeding 10 mg N/l and 1000 mg/l, respectively, disrupted sulfamnox. - the latter because it
306 affected TN removal efficiency.

307 Yang et al. (2009) noted that NH_4^+ and SO_4^{2-} removal efficiencies could negatively be affected by the
308 presence of H_2S and S^{2-} . However, the authors did not provide the exact thresholds at which
309 sulfamnox could be inhibited.

310 In the study of Zhao et al. (2006), the obtained efficiencies of $\text{NH}_4^+ = 43\%$ (low) and $\text{SO}_4^{2-} = 59\%$
311 (high) implied a competition between SRB and not identified microorganisms responsible for
312 simultaneous removal of NH_4^+ and SO_4^{2-} . Therefore, it is worth of paying attention to the participation

313 of SRB in the sulfamox process, as they are responsible for the reduction of SO_4^{2-} to S^{2-} under
314 anaerobic conditions. High COD concentration increases the growth of SRB.

315 **5. Sulfamox based reactors and reported efficiencies**

316 Until now, the sulfamox process has been studied in different reactors in terms of the flow
317 conditions and biomass retention method (see: Table 2).

318

Tab. 2 Sulfamnox based reactors and efficiency of NH_4^+ , SO_4^{2-} removal

Reactor	Origin of biomass	Influent NH_4^+ [mg/l]	Influent SO_4^{2-} [mg/l]	NH_4^+ removal efficiency [%]	SO_4^{2-} removal efficiency [%]	Highlights of the study	Reference
Combining system: Upflow Anaerobic Sludge Blanket (UASB), Anoxic/Oxic Reactor (A/O), Anammox and Sulfamnox Reactor (ANAOR), Anaerobic Sequencing Batch Reactor (ASBR)	landfill leachate	610-700	1870-1920	ca. 98	ca. 53	Landfill leachate was used as a substrate. The tests were carried out at a low temperature (14-15°C). The relative abundances of dominant sulfamnox bacteria were 10-20 times higher than that of <i>Candidatus</i> Kuenenia (anammox). Reduction of SO_4^{2-} and NH_4^+ was considered as a combination of anammox, sulfamnox, nitrification and denitrification processes.	Wu et al. [2020]
Continuous Flow Stirred Tank Reactor (CFSTR)	long-term operation anammox up-flow reactor	110 60 60	0-110 90 90	ca. 40 ca. 30 ca. 55	ca. 0 ca. 10 ca. 0	SRAO occurred only in the cases of high amounts of inoculum biomass at $\text{DO} = 0.2 - 0.5$ mg/L. When $\text{DO} < 0.2$ mg/L, the process was not observed. SRAO was considered as a combination of aerobic ammonium oxidation, anammox, and heterotrophic sulfate reduction processes.	Bi et al. [2020]
Self-Designed Circulating Flowreactor (SDCF)	n.a.	120 160 110 80 120 160 160 90	183 216 116 100 183 216 216 133	ca. 30 ca. 55 ca. 75 ca. 100 30 11 ca. 15 ca. 100	ca. 40 ca. 0 ca. 30 ca. 45 40 11 ca. 25 ca. 70	NH_4^+ oxidization and SO_4^{2-} reduction efficiencies increased in the presence of NO_2^- and NO_3^- . <i>Proteobacteria</i> , <i>Chloroflexi</i> , <i>Bacteroidetes</i> , <i>Chlorobi</i> , <i>Acidobacteria</i> , <i>Planctomycetes</i> and <i>Nitrospirae</i> were detected. <i>Proteobacteria</i> were the dominant functional microorganisms removing nitrogen. These results showed that nitrogen was converted by nitrification, denitrification, and conventional anammox, simultaneously with SRAO. The sulfur-based autotrophic denitrification and denitrification in the reactor were caused by the influent NO_2^- and NO_3^-.	Zhang et al. [2019a]
Self-Designed Circulating Flowreactor (SDCF)	mixed sludge, which consisted anaerobic granular sludge from a municipal wastewater plant and denitrification sludge from a continuous stirred-tank reactor	50 120 180	90 170 360	ca. 40 ca. 90 ca. 20	ca. 30 ca. 30 ca. 5	The increasing ratio of N/S in the influent resulted in higher NO_2^- concentrations in the effluent. The microbial community comprised <i>Proteobacteria</i> , <i>Chloroflexi</i> , <i>Bacteroidetes</i> , <i>Chlorobi</i> , <i>Acidobacteria</i> and <i>Planctomycetes</i> . SRAO was mainly due to the high performance of <i>Proteobacteria</i> (12.4% of denitrifying bacteria were found in the biomass). Part of nitrogen was converted by nitrification-denitrification, and conventional anammox, simultaneously with SRAO.	Zhang et al. [2019b]
Expanded Granular Sludge Bed (EGSB)	anaerobic hydrolysis acidification reactor	166-666 1000-2000 >3000	3600	40-58 40-70 10-25	64-71 66-82 28	The removal efficiency of SO_4^{2-} gradually improved as the influent NH_4^+ concentrations increased from 166-666 mg N/l to 1000-2000 mg N/l. At the same time, 71% NH_4^+ was removed. After increasing the NH_4^+ concentration to > 3000 mg N/l, the SO_4^{2-} reduction efficiency was reduced to 28%. SRB and	Wang et al. [2017a]





Anaerobic Sequencing Batch Reactor (ASBR)	activated sludge from the aerobic tank of digested liquor	97	261	ca. 88	ca. 19	denitrifying bacteria were mainly responsible for SO₄²⁻ and nitrogen removal.		
Moving Bed Biofilm Reactor (MBBR)	well-established attached anammox biofilm withdrawn from a lab-scale conventional anammox reactor treating reject water	69	ca. 70	ca. 30	ca. 10	The presence of Planctomycetes revealed that anammox was a highly involved pathway in NH₄⁺ removal, even without NO₂⁻ in the feed. Other autotrophic denitrifying bacteria, related to species the <i>Paracoccus Denitrificans</i>, were also present. These bacteria utilize S⁰ as an electron donor and produce SO₄²⁻, and competitively use NO₂⁻ with anammox.	Pra-chakittikul et al.. [2016]	
Upflow Anaerobic Sludge Blanket Reactor (UASBR)	anaerobic sludge from a yeast factory wastewater treating facility (Salutaguse, Estonia)	69	ca. 70	ca. 25	ca. 10	SRAO tests were performed in MBBR at 20°C and UASBR at 36°C. Very similar results of NH₄⁺ and overall SO₄²⁻ removal were obtained in both reactors. The SRAO process took place as one reaction of the multiple complex interactions between N-compounds, S-compounds, and organics (primarily humic matter) resulting in a significantly higher removal ratio of NH₄⁺ than the SRAO stoichiometry predicts. It was postulated that the phylum Verrucomicrobia could also be involved in sulfamnox.	Rikmann et al.. [2016]	
Upflow Anaerobic Sludge Blanket Reactor (UASBR)	reject water from anaerobic digestion of municipal wastewater sludge	221	193	ca. 30	ca. 20	Sulfamnox and anammox tests were carried out at 36°C and 20°C, respectively. NO₂⁻ was proved to be a more efficient electron acceptor than SO₄²⁻. The reduction of SO₄²⁻ and NH₄⁺ was considered as a combination of sulfamnox and denitrification processes.	Rikmann et al.. [2014]	
Expanded Bed Reactor (EBR)	lab-scale reactor treating N-NH ₄ ⁺ and SO ₄ -S simultaneously for more than two years	229	163	ca. 44	40	<i>Bacillus Benzoevorans</i> was isolated. Its optimum pH and temperature were 8.5 and 30°C, respectively. The reduction of SO₄²⁻ and NH₄⁺ was considered as as sulfamnox only.	Cai et al.. [2010]	
Upflow Anaerobic Sludge Blanket Reactor (UASBR)	nitrifying sludge in a municipal wastewater treatment plant	60	240	40	30	Sulfamnox was successfully performed by changing NO₂⁻ into SO₄²⁻ as an electron acceptor. The reduction of SO₄²⁻ and NH₄⁺ was considered as sulfamnox only.	Yang et al.. [2009]	
Expanded Bed Reactor (EBR)	anaerobic digester in a municipal wastewater treatment plant	84-270 30-90	450-740 80-200	ca. 40 ca. 55	ca. 10 ca. 43	Sulfate-dependent anaerobic ammonium oxidation occurs with acclimated anaerobic digested sludge in the absence of organic matter. Anaerobic ammonium oxidation with sulfate does not tend to occur spontaneously due to its low ΔG^o value. The experiment demonstrated that high substrate concentrations and low ORP may be favorable for sulfamnox. The reduction of SO₄²⁻ and NH₄⁺ was as a sulfamnox only.	Zhang et al.. [2009]	
Non-Woven Rotating Biological Contactor (NWRBC)	long-term operation anammox up-flow reactor	ca. 198	ca. 528	ca. 100	ca. 70	Bacteria belonging to <i>Planctomycetales</i>, especially the new species '<i>Anammoxoglobus Sulfate</i>, were identified as the functional community. The reduction of SO₄²⁻ and NH₄⁺ was considered as a sulfamnox only.	Liu et al.. [2008]	
Anaerobic Attached-Growth Bioreactor (AAGB)	anaerobic activated sludge collected from an anaerobic continuous stirred tank reactor	50	57	ca. 43	ca. 59	Low removal of NH₄⁺ was obtained with high removal of SO₄²⁻, implying the existence of competition between SRB) and microorganisms responsible for using SO₄²⁻ and NH₄⁺. Low COD, high SO₄²⁻ and high NH₄⁺ loadings at pH = 7.8 could	Zhao et al.. [2006]	

						promoted sulfamox. The reduction of SO_4^{2-} and NH_4^+ was considered as a sulfamox only.	
Granular Activated Carbon Fluidized-Bed (GACFB)	diluted vinasse originating from an ethanol distillery plant processing beet sugar molasses	<10	1000	50	80	The first report on the sulfamox process. The "anomalous" NH_4^+ removal was obtained in a granular activated carbon (GAC) anaerobic fluidized-bed reactor. The reactor treated vinasse from an ethanol distillery of sugar beet molasses. About 50% of the influent nitrogen load was removed from the liquid phase appearing as N_2 in the gas phase. Simultaneously, only 20% of the SO_4^{2-} initially present in the influent appears as S^{2-} in the effluent or H_2S in the biogas, indicating that 80% of the sulfur was removed in sulfamox. The reduction of SO_4^{2-} and NH_4^+ was considered as a combination of sulfamox and denitrification processes.	Fdz-Polanco et al.. [2001b]

268 Zhang et al. (2019a) studied the effects of NO_2^- and NO_3^- on sulfamox and found that the removal
269 efficiencies of both NH_4^+ and SO_4^{2-} increased from 30% to 100% and from 40% to 70%, respectively,
270 while increasing NO_x concentrations. Autotrophic denitrification had a large share in the removal.
271 With the influent NH_4^+ concentration of 80 mg N/l, SO_4^{2-} of 100 mg S/l and NO_2^- of 28 mg N/l, the
272 NH_4^+ removal efficiency reached almost 100%, while the overall SO_4^{2-} removal efficiency was only
273 45%. Similarly, with the influent NH_4^+ concentration of 90 mg N/l and SO_4^{2-} of 133 mg S/l and NO_3^-
274 of 90 mg N/l, the NH_4^+ and overall SO_4^{2-} removal efficiencies were approximately 100% and 70%,
275 respectively.

276 On the contrary, there have been studies indicating a lower efficiency of sulfamox for reject water,
277 i.e. approximately 30% and 10% for NH_4^+ and SO_4^{2-} , respectively (Rikmann et al. 2016). The influent
278 ratio of $\text{NH}_4^+/\text{SO}_4^{2-}$ was implicitly a key factor as studied by Wang et al. (2017a). When the SO_4^{2-}
279 $/\text{NH}_4^+$ ratio was close to 2, the process efficiency was highest, while too low or too high ratios resulted
280 in lower efficiencies.

281 In an Expanded Granular Sludge Bed Reactor (EGSBR) performing sulfamox (Wang et al. 2017a),
282 the removal efficiency of SO_4^{2-} and organic compounds gradually improved from 64% to 71% and
283 66% to 82%, respectively, as the influent NH_4^+ concentrations increased from 166-666 mg N/l
284 ($\text{NH}_4^+/\text{SO}_4^{2-} = 0.25-0.99$) to 1000-2000 mg N/l ($\text{NH}_4^+/\text{SO}_4^{2-} = 1.48-2.96$). At the same time,
285 approximately 71% NH_4^+ was removed. However, after increasing the NH_4^+ concentration to >3000
286 mg N/l ($\text{NH}_4^+/\text{SO}_4^{2-} > 4.44$), the SO_4^{2-} reduction efficiency was reduced to approximately 28%. Zhao
287 et al. (2006) also reported that the volumetric NH_4^+ removal rates could reach the highest level when
288 the concentration of NH_4^+ was 450 mg N/l (37.5 g N/m³/d), compared to 50 mg N/l (4.17 g N/m³/d)
289 and 250 mg N/l (20.8 g N/m³/d).

290 Wu et al. (2020) investigated the sulfamox process in a system consisting of four types of reactors
291 connected in series, including a UASBR, an anoxic/oxic reactor (A/O), an Anammox and Sulfamox
292 reactor (ANAOR), and an ASBR. In the first reactor (UASBR), the NH_4^+ concentration decreased
293 mainly due to dilution, while NO_2^- and NO_3^- (from nitrification solution recycle) were reduced by
294 denitrification. Partial nitrification was carried out at the A/O reactor, while anammox and sulfamox



295 were performed in the ANAOR and ASBR. In the ANAOR, NH_4^+ was removed by anammox (38 mg
296 NH_4^+ /l) and sulfammox (148 mg NH_4^+ /l). Those results indicated that the sulfammox share in the
297 NH_4^+ removal was more than 3 times higher than conventional anammox. Moreover, relatively high
298 amounts of NH_4^+ and SO_4^{2-} were removed in the ANAOR compared to other reactors. These amounts
299 were 187 mg N/l, 52 mg N/l and 35 mg N/l of NH_4^+ in the ANAOR, A/O and ASBR respectively. The
300 corresponding amounts for SO_4^{2-} were 393 mg S/l, 73.5 mg S/l and 42.3 mg S/l. The mass balance
301 calculations revealed that the combined system allowed to achieve the NH_4^+ removal efficiency at
302 98.5%, including 44.2% removed by sulfammox, whereas the overall SO_4^{2-} removal efficiency was
303 52.8%.

304 Rikmann et al. (2012) found that changing the electron acceptor from NO_2^- to SO_4^{2-} resulted in
305 reduction of the anammox efficiency. The efficiency of TN removal with NO_2^- was 85%, whereas after
306 changing to SO_4^{2-} , the average TN removal efficiency was only 23-24% in two different reactors
307 (MBBR and UASBR).

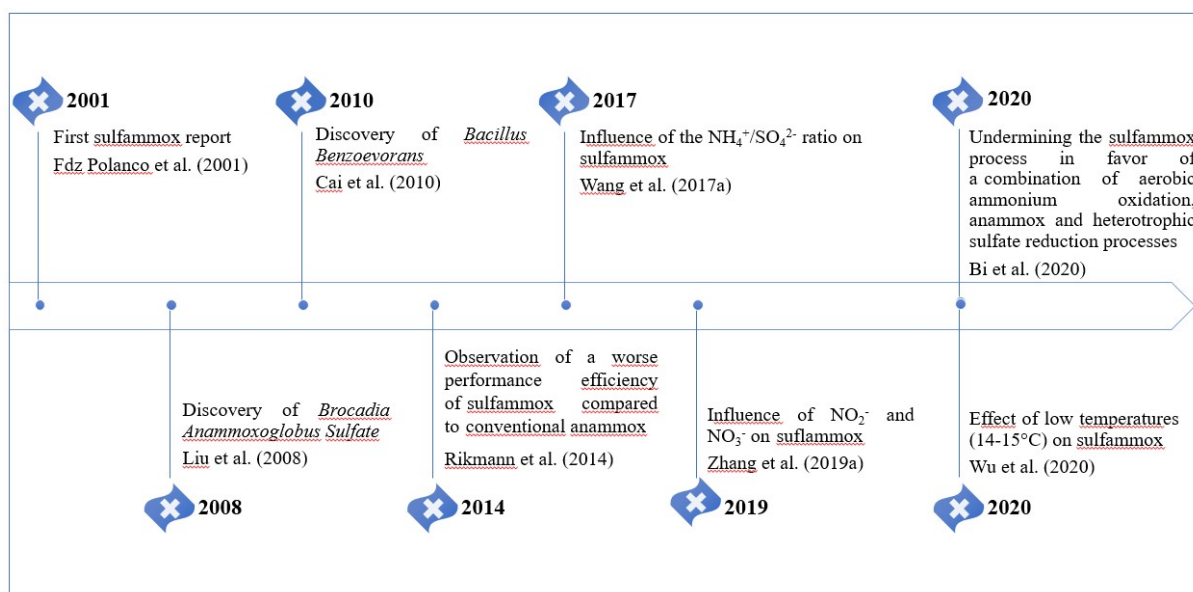
308 In order to compare sulfammox and conventional anammox, these processes were run in two parallel
309 UASBRs (Rikmann et al. 2014). It was assumed that a higher temperature could promote sulfammox,
310 partially compensating for its thermodynamic deficiency. Therefore, sulfammox and anammox
311 reactors were carried out at 36°C and 20°C, respectively. The use of NO_2^- as an electron acceptor was
312 still much more efficient than SO_4^{2-} as evidenced by the TN removal efficiency, i.e. 75% (conventional
313 anammox) and 17% (sulfammox), despite the significant temperature difference.

314 In the most recent study of Rikmann et al. (2016), sulfammox was carried out in a MBBR at 20°C and
315 a UASBR at 36°C. Very similar NH_4^+ and overall SO_4^{2-} removal efficiencies were obtained in both
316 reactors, i.e. 30% and 25% for NH_4^+ in the MBBR and UASBR, respectively, and 10% for SO_4^{2-} in
317 both reactors.

318 One of the principal drawbacks of sulfammox is the start-up time of the process. The sulfammox
319 reactor start-up takes even more time than conventional anammox due to the fact that the growth rate
320 of SO_4^{2-} dependent AAOB is very slow (Ali et al. 2013). For example, Zhang et al. (2009) found that
321 the cultivated sludge became capable of sulfammox reaction after 3 years of the operation under



322 anaerobic conditions. This makes sulfamnox impossible to implement in the mainstream reactor. In
 323 addition, when undesirable process disturbances occur, slow growth causes a long period of bacterial
 324 regeneration. However, this disadvantage (slow start-up) can be partially overcome by enriching the
 325 reactor by sulfamnox consortia from marine sediments (Ali et al. 2013). Figure 5 summarizes all the
 326 major research and discoveries related to the development of the sulfamnox process.



327
 328 **Fig 5** Sulfamnox process development timeline

329 6. Perspectives and conclusions

330 The conventional anammox process appears to be more advantageous than sulfamnox for treatment of
 331 nitrogen rich wastewater. With sulfamnox, however, an economically inefficient pre-nitration step
 332 (due to aeration) is not required and formation of toxic sulphide (S^{2-}) could be avoided. The main
 333 disadvantage of SO_4^{2-} dependent AAOB, which is a very slow doubling time, could partially be
 334 overcome by enriching inoculum biomass with marine sediments.

335 A combination of anammox and sulfur related processes (sulfamnox and autotrophic denitrification)
 336 would be a viable option for specific industrial wastewater with high content of nitrogen compounds
 337 and SO_4^{2-} . There are more than 10 different novel systems in which sulfamnox has been studied,
 338 including suspended growth, biofilm, granular and hybrid reactors. Evidence suggests that high
 339 removal efficiencies could be achieved with respect to both NH_4^+ (>90%) and SO_4^{2-} (>50%).

340

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