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# Challenges in using allylthiourea and chlorate as specific nitrification inhibitors

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## Abstract

Allylthiourea (ATU) and chlorate ( $\text{ClO}_3^-$ ) are often used to selectively inhibit nitrification and nitratation. In this work we identified challenges with use of these compounds in inhibitory assays with filter material from a biological rapid sand filter for groundwater treatment. Inhibition was investigated in continuous-flow lab-scale columns, packed with filter material from a full-scale filter and supplied with  $\text{NH}_4^+$  or  $\text{NO}_2^-$ . ATU concentrations of 0.1-0.5 mM interfered with the indophenol blue method for  $\text{NH}_4^+$  quantification leading to underestimation of the measured  $\text{NH}_4^+$  concentration. Interference was stronger at higher ATU levels and resulted in no  $\text{NH}_4^+$  detection at 0.5 mM ATU.  $\text{ClO}_3^-$  at typical concentrations for inhibition assays (1-10 mM) inhibited nitratation by less than 6%, while nitrification was instead inhibited by 91% when  $\text{NH}_4^+$  was supplied. On the other hand, nitratation was inhibited by 67-71% at 10-20 mM  $\text{ClO}_3^-$  when  $\text{NO}_2^-$  was supplied, suggesting significant nitratation inhibition at higher  $\text{NO}_2^-$  concentrations. No chlorite ( $\text{ClO}_2^-$ ) was detected in the effluent, and thus we could not confirm that nitratation inhibition was caused by  $\text{ClO}_3^-$  reduction to  $\text{ClO}_2^-$ . In conclusion, ATU and  $\text{ClO}_3^-$  should be used with caution in inhibition assays, because analytical interference

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25 and poor selectivity for the targeted process may affect the experimental outcome  
26 and compromise result interpretation.

27 *Keywords:*

28 drinking water, ammonium, nitrite, ATU, chlorate, inhibition

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## 29 **1. Introduction**

30 Aerobic nitrification is a two-step process consisting of the oxidation of am-  
31 monium to nitrite (nitritation) and of nitrite to nitrate (nitrataion). Ammonium  
32 oxidizing bacteria (AOB) and ammonium oxidizing archaea (AOA) are respon-  
33 sible for nitritation (Martens-Habbena et al., 2009; Martens-Habbena and Stahl,  
34 2011; Prosser, 1989; Prosser and Nicol, 2008), whereas nitrite oxidizing bacteria  
35 (NOB) oxidize nitrite to nitrate (Lees and Simpson, 1957). The two nitrification  
36 steps are linked and take place simultaneously as nitrataion uses the product of  
37 nitritation, but can be uncoupled and investigated individually using compounds  
38 that inhibit one of the two steps. Inhibition is the result of blockage or inactivation  
39 of the normal catalytic cycle of the enzyme responsible for a specific function, i.e.  
40 nitritation or nitrataion (McCarty, 1999).

41 Allylthiourea (ATU) is commonly used to inhibit nitritation, by targeting the  
42 ammonia monooxygenase action and chelating the copper in the active site, ulti-  
43 mately hindering its function (Bedard and Knowles, 1989). Nitritation inhibition  
44 has been used in micropollutant biodegradability studies (Batt et al., 2006; Falas  
45 et al., 2012; Shi et al., 2004; Zhou and Oleszkiewicz, 2010; Rattier et al., 2014)  
46 and in studies investigating nitritation kinetics (Munz et al., 2010) and activity  
47 (Dapena-Mora et al., 2007).

48 Chlorate ( $\text{ClO}_3^-$ ) has been used to inhibit nitrite oxidation and inhibition is

49 presumably a result of chlorate reduction to chlorite ( $\text{ClO}_2^-$ ) (Hynes and Knowles,  
50 1983). This reduction is catalyzed by nitrate reductase, which is actually the same  
51 enzyme that is responsible for nitrite oxidation, operating in the reverse direction  
52 (Hynes and Knowles, 1983). As a result, chlorate inhibition is assumed to be  
53 specific for nitrification. Chlorate inhibition has also been widely used when quan-  
54 tifying the ammonium oxidation potential of biomass (Belser and Mays, 1980;  
55 ISO, 2012).

56 Specific inhibition by ATU and chlorate has been used for decades in a variety  
57 of environmental systems, ranging from soils to activated sludge, marine sedi-  
58 ments and pure cultures. Although similar behavior with other oligotrophic sys-  
59 tems was expected, we experienced challenges with the use of these compounds in  
60 inhibition assays with filter material from biological rapid sand filters for ground-  
61 water treatment. The aim of this work was therefore to investigate, address and  
62 report these challenges to avoid the potential occurrence of experimental artifacts  
63 in future work.

## 64 **2. Materials & Methods**

### 65 *2.1. Investigated rapid sand filter and filter material sampling*

66 A rapid sand filter at Islevbro waterworks (Copenhagen, Denmark operated  
67 by Høfor A/S) was used for the experimental investigations. The filter had been  
68 operating for 30 years prior to the experiments without filter material replacement.  
69 Filter influent contained on average 0.13 mg/L  $\text{NH}_4^+$ -N, which was completely  
70 nitrified to  $\text{NO}_3^-$ , 9.25 mg/L  $\text{O}_2$ , 1.93 mg/L NVOOC, and 0.1 mg/L  $\text{Fe}^{+2}$  (Tatari  
71 et al., 2013). Filter material was collected from the top 5 cm using a sterilized 1 L  
72 stainless steel container attached to an extendable aluminum rod. Three random

73 horizontal locations were sampled and the collected filter material was mixed to  
74 form a composite sample. After sampling, the sand was stored wet at 4 °C for less  
75 than 7 days before the inhibition assays.

## 76 2.2. *Inhibition assays*

77 The inhibitory effect of ATU and  $\text{ClO}_3^-$  was investigated by monitoring the  
78 nitrification activity of the biomass on the collected filter material in a lab-scale  
79 assay. Due to the long operating time of the sampled full-scale filter, the filter ma-  
80 terial had an already established active nitrifying community, with AOB densities  
81 of 31 cells/m<sup>3</sup> filter material and a nitrification capacity above 223 g  $\text{NH}_4^+\text{-N/m}^3$   
82 filter material/d (Tatari et al., 2016). The experimental system employed small  
83 plexiglas columns (5 cm bed height, 2.6 cm inner diameter), packed with the col-  
84 lected filter material and operated continuously (Tatari et al., 2013). Effluent water  
85 from the waterworks was supplemented with 1-2 mg/L  $\text{NH}_4^+\text{-N}$  as  $\text{NH}_4\text{Cl}$  (Sigma-  
86 Aldrich, 254134) or 1 mg/L  $\text{NO}_2^-\text{-N}$  as  $\text{NaNO}_2$  (Sigma-Aldrich, S2252) and the  
87 inhibitory compounds at concentrations described later, and was fed at the inlet  
88 of the columns. Alkalinity in the influent water was high (5.4 meq/L as  $\text{HCO}_3^-$ ),  
89 so no additional alkalinity was added to the substrate water. The influent flowrate  
90 was constant at 39 mL/h giving a hydraulic retention time (HRT) of 2.3 h in the  
91 system, as determined experimentally by salt ( $\text{NaCl}$ ) tracer tests. Column effluent  
92 was recirculated at a ratio of 50 to 1 to impose complete mixing in the bulk phase  
93 in the system (Tatari et al., 2013).

94 The columns were packed and started-up with only the N-substrate in the in-  
95 fluent, running therefore as controls for a day. On the second day of operation,  
96 the column effluents were sampled twice (with 3-4 h in between) and were an-  
97 alyzed for the  $\text{NH}_4^+$ ,  $\text{NO}_2^-$  and  $\text{NO}_3^-$  concentrations. After sampling of the con-

98 trols, ATU (N-Allylthiourea, Merck chemicals, 808158) or  $\text{ClO}_3^-$  ( $\text{KClO}_3 \geq 99\%$ ,  
99 Sigma-Aldrich, 12634) were added in the influents and continuously supplied with  
100 the N-substrates. ATU was only added in columns supplied with  $\text{NH}_4^+$ .  $\text{ClO}_3^-$   
101 was added in columns supplied with  $\text{NO}_2^-$  to verify nitrification inhibition, and in  
102 columns supplied with  $\text{NH}_4^+$  to assess the selectivity of the nitrification inhibition.  
103 Concentrations of the two compounds and combinations with the N-substrates are  
104 reported in Table 1. The effluents were sampled twice (with 3-4 h in between),  
105 18 h after the addition of the inhibiting compound (at least 8 HRT after onset of  
106 application) and were analyzed for  $\text{NH}_4^+$ ,  $\text{NO}_2^-$  and  $\text{NO}_3^-$  concentrations. Columns  
107 were then emptied and cleaned by high water flow (390 mL/h) for 3-4 h before  
108 re-packing with new filter material for the next experiment, unless specified oth-  
109 erwise in Table 1.

110 Nitritation inhibition (%) was calculated as the difference in  $\text{NH}_4^+$  removal  
111 during control and test column performance.  $\text{NH}_4^+$  removal (%) was calculated  
112 by subtracting the effluent from the influent  $\text{NH}_4^+$  concentration and normaliz-  
113 ing for the influent  $\text{NH}_4^+$  concentration. Similarly, nitrification inhibition (%) was  
114 calculated as the difference of  $\text{NO}_2^-$  removal during control and test column per-  
115 formance.  $\text{NO}_2^-$  removal (%) was calculated as the difference of effluent and pro-  
116 duced  $\text{NO}_2^-$  concentration, after correction for the  $\text{NO}_2^-$  traces present in the in-  
117 fluent water (0.016 mg/L  $\text{NO}_2^-$ -N) and normalization for the produced  $\text{NO}_2^-$  con-  
118 centration. The  $\text{NO}_2^-$  produced by nitritation was calculated as the difference of  
119 influent and effluent  $\text{NH}_4^+$  concentration.

### 120 2.3. Analytical methods

121  $\text{NH}_4^+$  was quantified colorimetrically with the indophenol blue method (Merk,  
122 Spectroquant test kit, 1.14752) and absorbance was measured at 690 nm with a

**Table 1:** Inhibitory effect of ATU and  $\text{ClO}_3^-$  at different concentration levels on nitritation and nitrataion

Inhibitor	Inhibitor Concentration (mM)	Substrate	Influent Substrate (mg N/L)	% Nitritation Inhibition	% Nitratation Inhibition
ATU	0.10	$\text{NH}_4^+$	1	87	0.0
	0.50 <sup>i</sup>		1	87	0.0
	0.50		2	96	0.0
$\text{ClO}_3^-$	0.01	$\text{NH}_4^+$	1	11	1.1
	0.05		1	15	0.0
	0.10		1	28	0.0
	1.00		1	83	1.9
	5.00		1	80	3.1
	10.0		1	85	5.9
$\text{ClO}_3^-$	0.01	$\text{NO}_2^-$	1	-	5.4
	10.0 <sup>ii</sup>		1	-	67
	20.0 <sup>iii</sup>		1	-	71

<sup>i</sup> Same filter material as in the above experiment (0.1 mM ATU & 1 mg/L  $\text{NH}_4^+$ ). ATU concentration was increased to 0.5 mM at day 3.

<sup>ii</sup> Same filter material as in the above experiment (0.01 mM  $\text{ClO}_3^-$  & 1 mg/L  $\text{NO}_2^-$ ).  $\text{ClO}_3^-$  concentration was increased to 10 mM at day 3.

<sup>iii</sup> Same filter material as in the above experiment (10 mM  $\text{ClO}_3^-$  & 1 mg/L  $\text{NO}_2^-$ ).  $\text{ClO}_3^-$  concentration was increased to 20 mM at day 4.

123 spectrophotometer (Merck, DR 2800). Detection limit of the method was 0.05  
124 mg  $\text{NH}_4^+$ -N. Measured  $\text{NH}_4^+$  concentrations in the influents containing ATU were  
125 lower than the nominal spiked levels, so  $\text{NH}_4^+$  was re-quantified by flow injection  
126 adapting the method from Hall and Aller (1992). In brief, the carrier stream was  
127 10 mM NaOH (Sigma-Aldrich, 30620) with 0.2 M Na-citrate (Sigma-Aldrich,  
128 25116) and the receiving stream was 0.1 mM HCl (Sigma-Aldrich, 30721). Both  
129 streams were fed by a multi-channel pump (LabConco, Model 140250) at a rate  
130 of 6.2 mL/min. 100  $\mu\text{L}$  of sample were manually injected in the carrier stream  
131 through an injection valve.  $\text{NH}_4^+$  in the sample was converted to gaseous  $\text{NH}_3$  due  
132 to the basic pH of the carrier stream. The carrier stream entered the gas exchange  
133 membrane (Teflon tape), through which  $\text{NH}_3$  diffused to the receiving stream and  
134 was converted back to the ionic form ( $\text{NH}_4^+$ ) due to the acidic pH. Conductiv-  
135 ity of the receiving stream was measured by a conductivity meter (VWR Scien-  
136 tific, Model 1054) and was linearly correlated to the  $\text{NH}_4^+$  content of the sample.  
137 Conductivity peaks were retrieved and processed with the Clarity software (Data  
138 Apex, Advanced Chromatography Data Station). Detection limit of the method  
139 was 0.07 mg  $\text{NH}_4^+$ -N.

140  $\text{NO}_3^-$ ,  $\text{ClO}_3^-$  and  $\text{ClO}_2^-$  were quantified by Ion Chromatography (Dionex, ICS  
141 1500) using a guard column (Dionex, AG 22) and an analytical column (Dionex,  
142 ION PAC AS22). The eluent solution contained 4.5 mM  $\text{Na}_2\text{CO}_3$  (Sigma-Aldrich,  
143 31432) and 1.4 mM  $\text{NaHCO}_3$  (Sigma-Aldrich, 71630), and detection was done by  
144 suppressed conductivity (Dionex Thermo Scientific, ASRS 300). Retention times  
145 were approximately 5.7 min for  $\text{NO}_3^-$ , 5 min for  $\text{ClO}_3^-$  and 3.5 min for  $\text{ClO}_2^-$ .  
146 Detection limits were 0.2 mg  $\text{NO}_3^-$ -N/L, 0.01 mM  $\text{ClO}_3^-$  and 0.01 mM  $\text{ClO}_2^-$ .



### 147 **3. Results & Discussion**

#### 148 *3.1. ATU interference in $\text{NH}_4^+$ quantification by the indophenol blue method*

149  $\text{NH}_4^+$  quantification by the indophenol blue method resulted in lower  $\text{NH}_4^+$  val-  
150 ues compared to the concentrations measured by flow injection in all samples  
151 where ATU was present (Figure 1).  $\text{NH}_4^+$  concentration measured by flow injec-  
152 tion in the influent samples matched the nominal spiked concentrations and fitted  
153 the N-balance within the standard error of the analytical methods (data not shown).

154 To exclude a potential matrix effect, 7 standard solutions were prepared by  
155 spiking the effluent water from Islevbro waterworks with  $\text{NH}_4^+$ -N at 0.05-1 mg/L.  
156 The standard solutions were analyzed colorimetrically and  $\text{NH}_4^+$  was retrieved in  
157 all standards within the standard deviation of the method (0.023 mg  $\text{NH}_4^+$ -N/L).  
158 A second series of 4 standard solutions in the same water matrix contained 1  
159 mg/L  $\text{NH}_4^+$ -N and 0.05-0.5 mM ATU, and was also quantified colorimetrically.  
160 Measured  $\text{NH}_4^+$ -N concentrations were between 0 and 0.80 mg/L, confirming the  
161 underestimation of  $\text{NH}_4^+$  concentration by the indophenol blue method in the pres-  
162 ence of ATU.

163 Interference by ATU in the flow injection method was excluded by analyzing  
164 a series of 6 standard solutions (0.07-1.12 mg/L  $\text{NH}_4^+$ -N) without or with 0.5 mM  
165 ATU, where all measured concentrations matched the nominal standard concen-  
166 trations.

167  $\text{NH}_4^+$  analysis by the indophenol blue method did not detect any  $\text{NH}_4^+$  in the in-  
168 fluents spiked with 0.5 mM ATU, although their nominal  $\text{NH}_4^+$  concentration was  
169 1-2 mg/L  $\text{NH}_4^+$ -N (Figure 1). Interference of ATU with the colorimetric method  
170 was stronger at higher ATU concentrations. However, the  $\text{NH}_4^+$  concentration from  
171 colorimetric determination was not correlated with the actual  $\text{NH}_4^+$  concentration

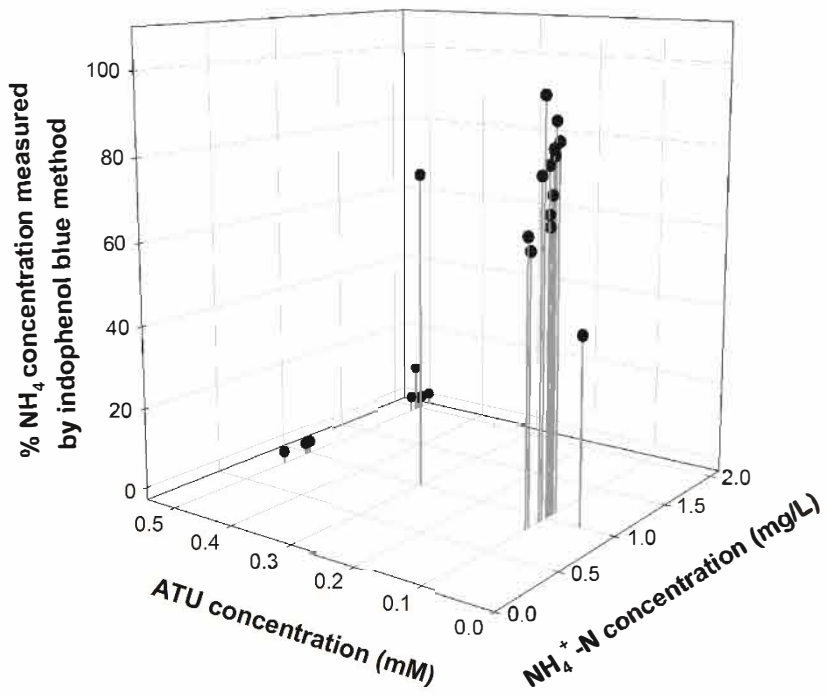


Figure caption

Figure 1: NH<sub>4</sub><sup>+</sup> concentration underestimation by the indophenol blue method when ATU was present in the samples

172 for a given ATU level, so no correction factor could be calculated.

173 Although the indophenol blue method has been used in several works to quan-  
174 tify  $\text{NH}_4^+$  in ATU inhibited samples (Law et al., 1992; Lehtovirta-Morley et al.,  
175 2013; Minzoni et al., 1988), this is the first study to identify and report the analyt-  
176 ical interference of ATU with this method. These previous studies however, used  
177 ATU concentrations between 0.009 and 0.08 mM, which are lower than the range  
178 used in our study. Only Montuelle et al. (2003) using 1.7 mM ATU suggested  
179 a potential interference in the  $\text{NH}_4^+$  quantification, although they did not in-  
180 vestigate nor specifically attribute the interference to the presence of ATU. ATU  
181 concentrations up to 0.08 mM are typically used to inhibit AOB activity, while  
182 higher concentrations up to 0.86 mM are relevant for archaeal (AOA) activity in-  
183 hibition (Santoro and Casciotti, 2011; Hatzenpichler et al., 2008; Taylor et al.,  
184 2010). Therefore, when ATU is used to inhibit AOA activity at concentrations  
185 higher than 0.08 M the use of the indophenol blue method for  $\text{NH}_4^+$  quantifica-  
186 tion should be discouraged. Erroneous conclusions can arise if only effluent  $\text{NH}_4^+$   
187 concentrations are measured as they would be underestimated due to interference,  
188 resulting in overestimation of the ATU inhibitory effect.

### 189 3.2. *Selectivity of $\text{ClO}_3^-$ inhibition for nitrataion*

190 The efficiency and selectivity of  $\text{ClO}_3^-$  in inhibiting nitrataion was evaluated  
191 by experiments with  $\text{NH}_4^+$  or  $\text{NO}_2^-$  as the supplied substrate. During the initial  
192 operation as controls,  $\text{NH}_4^+$  removal in the columns supplied with  $\text{NH}_4^+$  was higher  
193 than 98%, and nitrataion was complete since the effluent  $\text{NO}_2^-$ -N concentration  
194 was below 0.016 mg/L.  $\text{NO}_2^-$  removal during the control operation of the columns  
195 supplied with  $\text{NO}_2^-$  ranged between 88 and 97%.

196 Inhibition observed due to  $\text{ClO}_3^-$  addition is illustrated in Table 1 for the 9

197 combinations of N-substrates and  $\text{ClO}_3^-$  concentrations.  $\text{ClO}_3^-$  at 1-10 mM inhibited  
198 nitritation (80-85%), but not nitrataion (< 6%) in the columns supplied with  
199  $\text{NH}_4^+$  (Table 1). On the other hand, nitrataion was inhibited (67-71%) at 10-20  
200 mM  $\text{ClO}_3^-$  when  $\text{NO}_2^-$  was the sole influent substrate (Table 1). This differential  
201  $\text{ClO}_3^-$  effect on nitrataion can be possibly related to the different  $\text{NO}_2^-$  concentra-  
202 tion in the columns. At 10 mM  $\text{ClO}_3^-$ , the effluent  $\text{NO}_2^-$ -N concentration was 0.82  
203 mg/L in the column supplied with  $\text{NO}_2^-$  and 0.02 mg/L in the column supplied  
204 with  $\text{NH}_4^+$ . These effluent concentrations were equal to the  $\text{NO}_2^-$  concentrations in  
205 the columns because of complete mixing in the system. Hence, this indicates that  
206 nitrataion inhibition was higher at high vs. low  $\text{NO}_2^-$  concentrations. Our results  
207 are in contrast with those of Belser and Mays (1980), who observed a stronger  
208 inhibitory effect at low  $\text{NO}_2^-$  concentrations suggesting a competitive inhibition  
209 mechanism. Our results do not support such a mechanism, but indicate that dif-  
210 ferent NOB communities may respond differently to  $\text{ClO}_3^-$ .

211 Nitritation inhibition by  $\text{ClO}_3^-$  has been observed previously and has been at-  
212 tributed to reduction of  $\text{ClO}_3^-$  to  $\text{ClO}_2^-$  by NOB (Hynes and Knowles, 1983). We  
213 did not detect  $\text{ClO}_2^-$  in the column effluents (LOD 0.02 mM), while the  $\text{ClO}_3^-$  added  
214 to the influents was retrieved in the effluents (data not shown). We therefore could  
215 not confirm the hypothesized reduction of  $\text{ClO}_3^-$  to  $\text{ClO}_2^-$  or the reduction to  $\text{Cl}^-$  as  
216 suggested by Xu et al. (2011). This, however, does not exclude the possibility that  
217  $\text{ClO}_3^-$  was temporarily reduced and oxidized in a cyclic pattern, producing inter-  
218 mediates that inhibited nitritation. According to Hynes and Knowles (1983), AOB  
219 are presumably much more sensitive than NOB to  $\text{ClO}_2^-$ , meaning that even low  
220 concentrations may inhibit nitritation. These observations ultimately suggest that  
221 selectivity of  $\text{ClO}_3^-$  inhibition towards nitrataion may be compromised by specific

222 experimental factors, such as the  $\text{NO}_2^-$  concentration and the type of active NOB.  
223 Therefore, the  $\text{ClO}_3^-$  inhibition method to estimate nitrification potential should  
224 be tested before use to exclude an eventual significant nitritation inhibition during  
225 the assay that may lead to experimental artifacts .

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## Addendum

Since the original submission of this article, we have discovered – using 16S rRNA targeted amplicon sequencing and qPCR - that the microbial community on the sand material from the examined rapid sand filters is highly enriched in members of the *Nitrospira* over the *Nitrosomonas* genus (ca 100/1, (Gülay et al., 2016; Tatari et al., 2016)) We subsequently discovered – via metagenomics – that the *Nitrospira* population was dominated by types with the genetic capacity for complete ammonium oxidation (comammox) (Palomo et al., 2016). Our recent work – using stable isotope probing – reveals that comammox *Nitrospira* drive ammonium oxidation in the RSF samples under the examined conditions (Gülay et al., unpublished). Hence, our observations on the behavior of  $\text{ClO}_3^-$  as a strong inhibitor of nitrification can be explained: even though  $\text{ClO}_3^-$  is only converted to the toxic  $\text{ClO}_2^-$  in cells that have an active nitrite oxidoreductase enzyme (NXR, i.e. perform nitrification), the majority of these cells are likely comammox *Nitrospira*, and the toxic  $\text{ClO}_2^-$  would then not only inhibit most cells in their nitrite oxidation capacity (nitrification) *but also* in their ammonium oxidation capacity (nitrification). The observed ‘lack of nitrification inhibition’ under ammonium-fed conditions can also be explained: as nitrification inhibition is not 100% complete, there are some cells that are not affected by  $\text{ClO}_3^-$ ; those cells’ nitrification capacity would then neither be affected, limited-to-no  $\text{NO}_2^-$  accumulation would be observed, and absence of nitrification inhibition would be inferred. Yet, we note that  $\text{ClO}_3^-$  causes strong nitrification inhibition when  $\text{NO}_2^-$  is provided as sole substrate: this is consistent with the normal mode of action of  $\text{ClO}_3^-$ , either by inhibiting comammox *Nitrospira* or other NOBs that oxidize extracellular  $\text{NO}_2^-$ . Clearly, our further observations on the abundance of comammox *Nitrospira* strengthen and refine the conclusion: the selectivity of  $\text{ClO}_3^-$  as inhibitor of nitrification may be compromised by the type of NOB and is abolished when comammox *Nitrospira* dominate ammonium oxidation in the system. These observations, furthermore, suggests that the response to  $\text{ClO}_3^-$  may serve as an indicator of the contribution of comammox to nitrification.

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Tatari K., Smets B.F. and Albrechtsen H.J., Depth investigation of rapid sand filters for drinking water production reveals strong stratification in nitrification biokinetic behavior, *Water Res.* 101, 2016, 402–410