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Challenges in using allylthiourea and chlorate as specific nitrification inhibitors

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Abstract

Allylthiourea (ATU) and chlorate (ClO_3^-) are often used to selectively inhibit nitrification and nitrataion. 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 NH_4^+ or NO_2^- . ATU concentrations of 0.1-0.5 mM interfered with the indophenol blue method for NH_4^+ quantification leading to underestimation of the measured NH_4^+ concentration. Interference was stronger at higher ATU levels and resulted in no NH_4^+ detection at 0.5 mM ATU. ClO_3^- at typical concentrations for inhibition assays (1-10 mM) inhibited nitrataion by less than 6%, while nitrification was instead inhibited by 91% when NH_4^+ was supplied. On the other hand, nitrataion was inhibited by 67-71% at 10-20 mM ClO_3^- when NO_2^- was supplied, suggesting significant nitrataion inhibition at higher NO_2^- concentrations. No chlorite (ClO_2^-) was detected in the effluent, and thus we could not confirm that nitrification inhibition was caused by ClO_3^- reduction to ClO_2^- . In conclusion, ATU and 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

29 **1. Introduction**

30 Aerobic nitrification is a two-step process consisting of the oxidation of am-
31 monium to nitrite (nitrification) and of nitrite to nitrate (nitrification). Ammonium
32 oxidizing bacteria (AOB) and ammonium oxidizing archaea (AOA) are respon-
33 sible for nitrification (Martens-Habben et al., 2009; Martens-Habben 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 nitrification uses the product of
37 nitrification, 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 nitrification or nitrification (McCarty, 1999).

41 Allylthiourea (ATU) is commonly used to inhibit nitrification, 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). Nitrification 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 nitrification kinetics (Munz et al., 2010) and activity
47 (Dapena-Mora et al., 2007).

48 Chlorate (ClO_3^-) has been used to inhibit nitrite oxidation and inhibition is

presumably a result of chlorate reduction to chlorite (ClO_2^-) (Hynes and Knowles, 1983). This reduction is catalyzed by nitrate reductase, which is actually the same enzyme that is responsible for nitrite oxidation, operating in the reverse direction (Hynes and Knowles, 1983). As a result, chlorate inhibition is assumed to be specific for nitrification. Chlorate inhibition has also been widely used when quantifying the ammonium oxidation potential of biomass (Belser and Mays, 1980; ISO, 2012).

Specific inhibition by ATU and chlorate has been used for decades in a variety of environmental systems, ranging from soils to activated sludge, marine sediments and pure cultures. Although similar behavior with other oligotrophic systems was expected, we experienced challenges with the use of these compounds in inhibition assays with filter material from biological rapid sand filters for groundwater treatment. The aim of this work was therefore to investigate, address and report these challenges to avoid the potential occurrence of experimental artifacts in future work.

2. Materials & Methods

2.1. Investigated rapid sand filter and filter material sampling

A rapid sand filter at Islevbro waterworks (Copenhagen, Denmark operated by Hofo A/S) was used for the experimental investigations. The filter had been operating for 30 years prior to the experiments without filter material replacement. Filter influent contained on average 0.13 mg/L $\text{NH}_4^+\text{-N}$, which was completely nitrified to NO_3^- , 9.25 mg/L O_2 , 1.93 mg/L NVO, and 0.1 mg/L Fe^{+2} (Tatari et al., 2013). Filter material was collected from the top 5 cm using a sterilized 1 L stainless steel container attached to an extendable aluminum rod. Three random

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

2.2. *Inhibition assays*

The inhibitory effect of ATU and ClO_3^- was investigated by monitoring the nitrification activity of the biomass on the collected filter material in a lab-scale assay. Due to the long operating time of the sampled full-scale filter, the filter material had an already established active nitrifying community, with AOB densities of 31 cells/m³ filter material and a nitrification capacity above 223 g $\text{NH}_4^+\text{-N/m}^3$ filter material/d (Tatari et al., 2016). The experimental system employed small plexiglas columns (5 cm bed height, 2.6 cm inner diameter), packed with the collected filter material and operated continuously (Tatari et al., 2013). Effluent water from the waterworks was supplemented with 1-2 mg/L $\text{NH}_4^+\text{-N}$ as NH_4Cl (Sigma-Aldrich, 254134) or 1 mg/L $\text{NO}_2^-\text{-N}$ as NaNO_2 (Sigma-Aldrich, S2252) and the inhibitory compounds at concentrations described later, and was fed at the inlet of the columns. Alkalinity in the influent water was high (5.4 meq/L as HCO_3^-), so no additional alkalinity was added to the substrate water. The influent flowrate was constant at 39 mL/h giving a hydraulic retention time (HRT) of 2.3 h in the system, as determined experimentally by salt (NaCl) tracer tests. Column effluent was recirculated at a ratio of 50 to 1 to impose complete mixing in the bulk phase in the system (Tatari et al., 2013).

The columns were packed and started-up with only the N-substrate in the influent, running therefore as controls for a day. On the second day of operation, the column effluents were sampled twice (with 3-4 h in between) and were analyzed for the NH_4^+ , NO_2^- and NO_3^- concentrations. After sampling of the con-

198 trols, ATU (N-Allylthiourea, Merck chemicals, 808158) or ClO_3^- ($\text{KClO}_3 \geq 99\%$,
199 Sigma-Aldrich, 12634) were added in the influents and continuously supplied with
200 the N-substrates. ATU was only added in columns supplied with NH_4^+ . ClO_3^-
201 was added in columns supplied with NO_2^- to verify nitrification inhibition, and in
202 columns supplied with NH_4^+ to assess the selectivity of the nitrification inhibition.
203 Concentrations of the two compounds and combinations with the N-substrates are
204 reported in Table 1. The effluents were sampled twice (with 3-4 h in between),
205 18 h after the addition of the inhibiting compound (at least 8 HRT after onset of
206 application) and were analyzed for NH_4^+ , NO_2^- and NO_3^- concentrations. Columns
207 were then emptied and cleaned by high water flow (390 mL/h) for 3-4 h before
208 re-packing with new filter material for the next experiment, unless specified oth-
209 erwise in Table 1.

210 Nitritation inhibition (%) was calculated as the difference in NH_4^+ removal
211 during control and test column performance. NH_4^+ removal (%) was calculated
212 by subtracting the effluent from the influent NH_4^+ concentration and normaliz-
213 ing for the influent NH_4^+ concentration. Similarly, nitrification inhibition (%) was
214 calculated as the difference of NO_2^- removal during control and test column per-
215 formance. NO_2^- removal (%) was calculated as the difference of effluent and pro-
216 duced NO_2^- concentration, after correction for the NO_2^- traces present in the in-
217 fluent water (0.016 mg/L NO_2^- -N) and normalization for the produced NO_2^- con-
218 centration. The NO_2^- produced by nitritation was calculated as the difference of
219 influent and effluent NH_4^+ concentration.

220 2.3. Analytical methods

221 NH_4^+ was quantified colorimetrically with the indophenol blue method (Merk,
222 Spectroquant test kit, 1.14752) and absorbance was measured at 690 nm with a

Table 1: Inhibitory effect of ATU and ClO_3^- at different concentration levels on nitrification and nitrification

Inhibitor		Influent		%	%
Inhibitor	Concentration (mM)	Substrate	Substrate (mg N/L)	Nitrification Inhibition	Nitrification Inhibition
ATU	0.10	NH_4^+	1	87	0.0
	0.50 ⁱ		1	87	0.0
	0.50		2	96	0.0
ClO_3^-	0.01	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
ClO_3^-	0.01	NO_2^-	1	-	5.4
	10.0 ⁱⁱ		1	-	67
	20.0 ⁱⁱⁱ		1	-	71

ⁱ Same filter material as in the above experiment (0.1 mM ATU & 1 mg/L NH_4^+). ATU concentration was increased to 0.5 mM at day 3.

ⁱⁱ Same filter material as in the above experiment (0.01 mM ClO_3^- & 1 mg/L NO_2^-). ClO_3^- concentration was increased to 10 mM at day 3.

ⁱⁱⁱ Same filter material as in the above experiment (10 mM ClO_3^- & 1 mg/L NO_2^-). 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 NH_4^+ -N. Measured NH_4^+ concentrations in the influents containing ATU were
125 lower than the nominal spiked levels, so 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 μL of sample were manually injected in the carrier stream
131 through an injection valve. NH_4^+ in the sample was converted to gaseous 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 NH_3 diffused to the receiving stream and
134 was converted back to the ionic form (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 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 NH_4^+ -N.

140 NO_3^- , ClO_3^- and 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 Na_2CO_3 (Sigma-Aldrich,
143 31432) and 1.4 mM 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 NO_3^- , 5 min for ClO_3^- and 3.5 min for ClO_2^- .
146 Detection limits were 0.2 mg NO_3^- -N/L, 0.01 mM ClO_3^- and 0.01 mM ClO_2^- .

147 3. Results & Discussion

148 3.1. ATU interference in NH_4^+ quantification by the indophenol blue method

149 NH_4^+ quantification by the indophenol blue method resulted in lower NH_4^+ val-
150 ues compared to the concentrations measured by flow injection in all samples
151 where ATU was present (Figure 1). 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 NH_4^+ -N at 0.05-1 mg/L.
156 The standard solutions were analyzed colorimetrically and NH_4^+ was retrieved in
157 all standards within the standard deviation of the method (0.023 mg NH_4^+ -N/L).
158 A second series of 4 standard solutions in the same water matrix contained 1
159 mg/L NH_4^+ -N and 0.05-0.5 mM ATU, and was also quantified colorimetrically.
160 Measured NH_4^+ -N concentrations were between 0 and 0.80 mg/L, confirming the
161 underestimation of 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 NH_4^+ -N) without or with 0.5 mM
165 ATU, where all measured concentrations matched the nominal standard concen-
166 trations.

167 NH_4^+ analysis by the indophenol blue method did not detect any NH_4^+ in the in-
168 fluents spiked with 0.5 mM ATU, although their nominal NH_4^+ concentration was
169 1-2 mg/L NH_4^+ -N (Figure 1). Interference of ATU with the colorimetric method
170 was stronger at higher ATU concentrations. However, the NH_4^+ concentration from
171 colorimetric determination was not correlated with the actual NH_4^+ concentration

Figure caption

Figure 1: NH_4^+ 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 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 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 NH_4^+ quantifica-
186 tion should be discouraged. Erroneous conclusions can arise if only effluent 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 ClO_3^- inhibition for nitrataion

190 The efficiency and selectivity of ClO_3^- in inhibiting nitrataion was evaluated
191 by experiments with NH_4^+ or NO_2^- as the supplied substrate. During the initial
192 operation as controls, NH_4^+ removal in the columns supplied with NH_4^+ was higher
193 than 98%, and nitrataion was complete since the effluent NO_2^- -N concentration
194 was below 0.016 mg/L. NO_2^- removal during the control operation of the columns
195 supplied with NO_2^- ranged between 88 and 97%.

196 Inhibition observed due to ClO_3^- addition is illustrated in Table 1 for the 9

197 combinations of N-substrates and ClO_3^- concentrations. ClO_3^- at 1-10 mM inhibited
 198 nitritation (80-85%), but not nitrataion ($< 6\%$) in the columns supplied with
 199 NH_4^+ (Table 1). On the other hand, nitrataion was inhibited (67-71%) at 10-20
 200 mM ClO_3^- when NO_2^- was the sole influent substrate (Table 1). This differential
 201 ClO_3^- effect on nitrataion can be possibly related to the different NO_2^- concentra-
 202 tion in the columns. At 10 mM ClO_3^- , the effluent NO_2^- -N concentration was 0.82
 203 mg/L in the column supplied with NO_2^- and 0.02 mg/L in the column supplied
 204 with NH_4^+ . These effluent concentrations were equal to the 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 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 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 ClO_3^- .

211 Nitritation inhibition by ClO_3^- has been observed previously and has been at-
 212 tributed to reduction of ClO_3^- to ClO_2^- by NOB (Hynes and Knowles, 1983). We
 213 did not detect ClO_2^- in the column effluents (LOD 0.02 mM), while the 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 ClO_3^- to ClO_2^- or the reduction to Cl^- as
 216 suggested by Xu et al. (2011). This, however, does not exclude the possibility that
 217 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 ClO_2^- , meaning that even low
 220 concentrations may inhibit nitritation. These observations ultimately suggest that
 221 selectivity of ClO_3^- inhibition towards nitrataion may be compromised by specific

222 experimental factors, such as the NO_2^- concentration and the type of active NOB.
223 Therefore, the 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 .

226 **4. Acknowledgements**

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228 project DW-Biofilters. The authors acknowledge the Urban Water Technology
229 Research School and the waterworks involved in this study.

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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 ClO_3^- as a strong inhibitor of nitrification can be explained: even though ClO_3^- is only converted to the toxic 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 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 ClO_3^- ; those cells’ nitrification capacity would then neither be affected, limited-to-no NO_2^- accumulation would be observed, and absence of nitrification inhibition would be inferred. Yet, we note that ClO_3^- causes strong nitrification inhibition when NO_2^- is provided as sole substrate: this is consistent with the normal mode of action of ClO_3^- , either by inhibiting comammox *Nitrospira* or other NOBs that oxidize extracellular NO_2^- . Clearly, our further observations on the abundance of comammox *Nitrospira* strengthen and refine the conclusion: the selectivity of 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 ClO_3^- may serve as an indicator of the contribution of comammox to nitrification.

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