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Short Communication

Challenges in using allylthiourea and chlorate as specific nitrification inhibitors

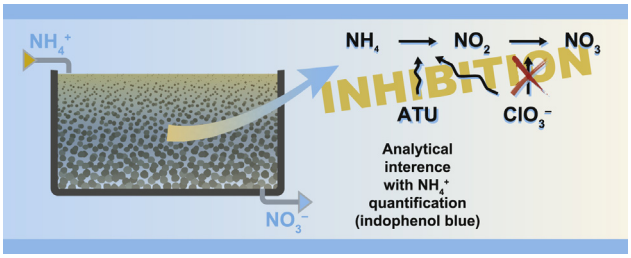
K. Tatari [a], A. Gülay [a], B. Thamdrup [b], H.-J. Albrechtsen [a], B.F. Smets [a, *]

[a] Department of Environmental Engineering, Technical University of Denmark, Miljøvej 113, 2800 Kgs. Lynby, Denmark
[b] Nordic Center for Earth Evolution, Department of Biology, University of Southern Denmark, Campusvej 55, 5230 Odense, Denmark

HIGHLIGHTS

- ATU interfered with the indophenol blue method underestimating the NH_4^+ concentration.
- ClO_3^- inhibited both nitritation and nitratation, depending on the N-substrate supplied.
- Nitratation inhibition by ClO_3^- increased with increasing NO_2^- concentration.
- Response to ClO_3^- may serve as an indicator of the contribution of comammox to nitrification.

GRAPHICAL ABSTRACT



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ABSTRACT

Allylthiourea (ATU) and chlorate (ClO_3^-) are often used to selectively inhibit nitritation 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 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 nitratation by less than 6%, while nitritation was instead inhibited by 91% when NH_4^+ was supplied. On the other hand, nitratation was inhibited by 67–71% at 10–20 mM ClO_3^- when NO_2^- was supplied, suggesting significant nitratation inhibition at higher NO_2^- concentrations. No chlorite (ClO_2^-) was detected in the effluent, and thus we could not confirm that nitritation 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 and poor selectivity for the targeted process may affect the experimental outcome and compromise result interpretation.

1. Introduction

Aerobic nitrification is a two-step process consisting of the oxidation of ammonium to nitrite (nitritation) and of nitrite to nitrate (nitratation). Ammonium oxidizing bacteria (AOB) and ammonium oxidizing archaea (AOA) are responsible for nitritation (Martens-Habbena et al., 2009; Martens-Habbena and Stahl, 2011; Prosser, 1989; Prosser and Nicol, 2008), whereas nitrite oxidizing bacteria (NOB) oxidize nitrite to nitrate (Lees and Simpson, 1957). The two nitrification steps are linked and take place simultaneously

* Corresponding author.
E-mail address: bfsm@env.dtu.dk (B.F. Smets).

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as nitratation uses the product of nitritation, but can be uncoupled and investigated individually using compounds that inhibit one of the two steps. Inhibition is the result of blockage or inactivation of the normal catalytic cycle of the enzyme responsible for a specific function, i.e. nitritation or nitratation (McCarty, 1999).

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

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 mediated by the nitrate reductase activity of nitriteoxidoreductase (NXR), 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 nitritation. 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 Hofor 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 NH_4^+-N, which was completely nitrified to NO_3^-, 9.25 mg/L O_2, 1.93 mg/L NVOC, 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 10^{13} cells/m^3 filter material and a nitrification capacity above 223 g NH_4^+-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 NH_4^+-N as NH_4Cl (Sigma-Aldrich, 254134) or 1 mg/L NO_2^--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 controls, ATU (N-Allylthiourea, Merck chemicals, 808158) or ClO_3^- ($KClO_3 \geq 99\%$, Sigma-Aldrich, 12634) were added in the influents and continuously supplied with the N-substrates. ATU was only added in columns supplied with NH_4^+. ClO_3^- was added in columns supplied with NO_2^- to verify nitratation inhibition, and in columns supplied with NH_4^+ to assess the selectivity of the nitratation inhibition. Concentrations of the two compounds and combinations with the N-substrates are reported in Table 1. The effluents were sampled twice (with 3–4 h in between), 18 h after the addition of the inhibiting compound (at least 8 HRT after onset of application) and were analyzed for NH_4^+, NO_2^- and NO_3^- concentrations. Columns were then emptied and cleaned by high water flow (390 mL/h) for 3–4 h before re-packing with new filter material for the next experiment, unless specified otherwise in Table 1.

Table 1
Inhibitory effect of ATU and ClO_3^- at different concentration levels on nitritation and nitratation.

Inhibitor	Inhibitor Concentration (mM)	Substrate	Influent Substrate (mg N/L)	% Nitritation Inhibition	% Nitratation Inhibition
ATU	0.10	NH_4^+	1	87	0.0
	0.50[a]		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[b]		1	–	67
	20.0[c]		1	–	71

[a] 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.
[b] 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.
[c] 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.