5-2015

# Biofilm-related Materials as Total N-nitrosamine (TONO) Precursors and Hydroxylamine-based Interferences in TONO and N-nitrosodimethylamine (NDMA) Measurements

David Allen Meints
*University of Arkansas, Fayetteville*

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A thesis submitted in partial fulfillment
of the requirements for the degree of
Master of Science in Civil Engineering

by

David Meints II
University of Arkansas
Bachelor of Science in Civil Engineering, 2013

May 2015
University of Arkansas

Thesis is approved for recommendation to the Graduate Council

Dr. Julian Fairey
Thesis Director

Dr. Wen Zhang
Committee Member

Dr. Ashley Pifer
Committee Member

**Abstract**

In this study, a chemiluminescence-based total N-nitrosamine (TONO) assay was adapted to include a solid-phase extraction (SPE) step to assess the role of biologically derived materials as N-nitrosamine precursors. Methanol was determined to be a suitable solvent for the SPE-TONO assay, with N-nitrosodimethylamine (NDMA) losses estimated to be 30% by SPE alone and an additional 15% from concentration of the methanol extracts by $N_2$-gas blowdown. Three biofilm-derived materials – poly-N-acetylglucosamine (PNAG), *Pseudomonas aeruginosa*, and tryptophan – were individually chloraminated and dose-response relationships were observed with the SPE-TONO assay, indicating biofilm are potential N-nitrosamine precursors. The role of hydroxylamine – a nitrification intermediate – was assessed as a function of five sample treatments related to the TONO assay (*Untreated*, $HgCl_2$ only, sulfanilamide [*SAA*] only, $HgCl_2$ + *SAA*, and *HCl*) in terms of aqueous phase TONO, SPE-TONO and NDMA by GC-FID. A series of batch reactor experiments were performed with various combinations of 3.52 mM hydroxylamine, 35.2 mM dimethylamine (a known NDMA precursor) and 3.52 mM monochloramine. However, several analytical interferences were discovered, associated with excess hydroxylamine in the samples, which obscured results from the TONO assay, GC-FID (for NDMA), and ion chromatography (for nitrite). In the aqueous phase with dimethylamine present, hydroxylamine was catalyzed by (1) $HgCl_2$ to nitrite and NDMA and (2) $HgCl_2$ + *SAA* to NDMA only, as any nitrite formed was removed by SAA. In the methanol phase, hydroxylamine and dimethylamine were catalyzed to NDMA on the activated carbon in the SPE cartridges. However, these experiments revealed a previously unconsidered NDMA formation pathway, in which hydroxylamine is catalyzed to peroxynitrite ($ONOO^-$) in the presence of dissolved oxygen and subsequently reacts with dimethylamine to form NDMA.

Recommendations are provided to guide the design of N-nitrosamine formation pathway experiments.

**Acknowledgements**

This research was sponsored by the Arkansas Water Resources Center (AWRC) and the National Science Foundation (NSF) through CBET Award Number 1254350 to Dr. Julian Fairey.

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# I. INTRODUCTION

The objective of this project was to investigate the hypothesis that biofilm in chloraminated drinking water distribution systems are potential N-nitrosamine precursors. N-nitrosamines are a highly toxic and non-halogenated group of disinfection byproducts (DBPs) that form at ng $L^{-1}$ levels, primarily in chloramination systems.[1] Biofilm contain extracellular polymeric substances (EPS) and release intracellular organic materials (IOM) that are comprised of chemical moieties similar to known N-nitrosamine precursors (e.g., secondary amines),[2] but the contribution of these biological entities to N-nitrosamine formation remains unknown. This may be especially relevant given widespread prevalence of biofilm in drinking water distribution systems.[3]

Many water utilities have switched to chloramines as a secondary (or distribution system) disinfectant[4] to curb formation of regulated DBPs.[5] However, chloramination can increase the formation of unregulated DBPs, including N-nitrosodimethylamine (NDMA),[6] the most widely occurring of the seven EPA Method 521 ($EPA_{7N}$) N-nitrosamines[7] and member of the EPA *Contaminant Candidate List 4*. Known NDMA precursors include quaternary amine-containing coagulants, anion exchange resins, and wastewater-impacted source waters containing pharmaceuticals and personal care products.[1] However, N-nitrosamines, as a group, are comprised of over 150 individual chemical species,[8] and thus it is plausible that occurrence studies to date – which have focused on the $EPA_{7N}$ exclusively – have not captured the complete picture. To this end, another research group has developed the TONO assay,[9] which quantifies all N-nitrosamine species in aggregate and demonstrated the $EPA_{7N}$ species comprised only ~5% of the total N-nitrosamines in drinking water systems.[10] Further, they showed that algal-derived organic matter was a negligible precursor to the $EPA_{7N}$ species, but contributed significantly to the TONO precursor pool. This result implies that other important N-nitrosamine precursors may have been

overlooked. The prospect that biofilm constitute an important source of N-nitrosamine precursors is particularly troubling given the ubiquitous nature of biofilm in distribution systems, that many water utilities have switched to chloramines to curb formation of regulated DBPs, and the long contact times (up to ~8 days) available for these reactions to occur.

Reactions between chloramines and various biofilm organic materials (e.g., exopolysaccharides and intra- and extra-cellular proteins) may yield N-nitrosamines because of amine-like moieties in their chemical structures. The poly-N-acetyl glucosamine (PNAG) polymer is required for bacterial adherence and biofilm formation in some bacterial species[11] and contains substituted amines. Wozniak et al.[12] showed that *P. aeruginosa* is comprised of many carbohydrate structures, including PNAG, and is a commonly encountered pathogen in water systems.[13] Similarly, tryptophan, an aromatic amino acid, can be found in water distribution systems[14] and is a DBP precursor,[15] and inhibits biofilm growth of *P. aeruginosa* and could cause biofilm disassembly.[16]

In addition to biofilm, nitrification episodes are prevalent in chloraminated distribution systems[17] and may exacerbate N-nitrosamine formation through the production of hydroxylamine, a key intermediate. In the nitrification process, biological ammonia oxidation to nitrite occurs in two steps: (1) the ammonia monooxygenase enzyme catalyzes ammonia oxidation to hydroxylamine ($NH_2OH$) and (2) the hydroxylamine oxidoreductase enzyme catalyzes hydroxylamine oxidation to nitrite.[18] Hydroxylamine is known to react with dimethylamine, $(CH_3)_2NH$, to form unsymmetrical dimethylhydrazine (UDMH),[19] which in turn can react with dissolved oxygen to form NDMA.[20] Hydroxylamine has been implicated in NDMA formation during ozonation.[21] Furthermore, if biological ammonia oxidation occurs during chloramination, biologically produced hydroxylamine may react with monochloramine[22,23] leading to production

of peroxynitrite ($ONOO^-$) if oxygen is present. Peroxynitrite is a versatile oxidant and known nitrosating agent,[24, 25] but its role in N-nitrosamine formation under conditions related to nitrification in chloraminated systems remains unknown.

In this study, the TONO assay[10] was adapted to include a solid-phase extraction (SPE) step, which was intended to replace the continuous liquid-liquid extraction (CLLE) procedure developed by Mitch and colleagues.[9] Potential advantages of SPE over CLLE include higher sample throughput and larger recoveries of N-nitrosamines. However, N-nitrosamines are known to form in SPE cartridges by catalysis reactions on the surfaces of the activated carbon,[26] and thus concerns with interferences exist. Various organic solvents were tested for the SPE and NDMA mass recoveries were measured and compared to the CLLE results of others. Next, biofilm-derived materials, which included PNAG, *P. aeruginosa*, and tryptophan, were investigated as potential sources of N-nitrosamine precursors using the SPE-TONO assay. Lastly, abiotic experiments were completed to assess the role of hydroxylamine in NDMA formation, as related to nitrification episodes in chloraminated distribution systems. These results unexpectedly revealed the presence of multiple interference pathways associated with SPE-TONO assay that could be used to guide methodological improvements and help explore alternative NDMA formation pathways.

## II. MATERIALS AND METHODS

### A. Chemicals

Accustandard N-nitrosamine mix for EPA Method 521 and N-nitrosodimethylamine; Alfa Aesar sulfanilamide (98%) and iodine (99.8%); Amresco mercuric chloride (ACS Grade), sodium hydroxide (Reagent Grade), and sodium sulfate (ACS Grade); BDH sodium bicarbonate (ACS Grade), glacial acetic acid (99.7%); Difco BD LB broth; EMD acetone (99.5%), acetonitrile (99.8%), dichloromethane (99.8%), dimethylamine (40% aqueous solution), hydrochloric acid

(ACS Grade) and methanol (99.8%); Fisher Chemical ammonium chloride (99.6%), potassium iodide (99.8%), and sodium phosphate dibasic (99.2%); Hach Company Monochlor F reagent packs; J.T. Baker potassium phosphate monobasic (100%); Macron ethylene glycol; Ricca Chemical Company sodium hypochlorite solution; Migula ATCC 10145 (Schroeter) *Pseudomonas aeruginosa.* Sigma Aldrich hydroxylamine hydrochloride (98.0%) and poly-N-acetyl glucosamine (PNAG); Spectrum Chemical Manufacturing L- $\alpha$ -Amino-3-indolepropionic acid, tryptophan (98.5%); and Restek EPA Method 521 activated carbon solid-phase extraction columns.

**B. Preparation of blanks, spikes, and standards**

Experiments were performed to determine an appropriate extraction solvent for the SPE-TONO assay along with the associated NDMA recovery efficiency. Four extraction solvents were tested, including dichloromethane (recommended in EPA Method 521), and three others – chosen based on the findings of Plumlee et al.[27] – which included acetonitrile, methanol, acetone, and an equal-volume mixture of these three solvents. For each, a blank (15 mL of solvent only) and two spikes (100 and 1,000 ng of NDMA in 15 mL of solvent) were prepared and concentrated to 1 mL under nitrogen gas blow-down. An additional spike (5,000 ng of NDMA in 15 mL of solvent) was prepared and concentrated for acetonitrile and methanol only. For all samples in Table 1, total N-nitrosamines were quantified in the 1 mL extracts prior to quenching interfering compounds ("No Pretreatment") and following quenching to remove S-nitrosothiols ("$HgCl_2$") and nitrite ("Sulfanilamide"), as described previously.[9] To quantify total N-nitrosamines, a 1 ng $\mu L^{-1}$ as NDMA primary standard was prepared in each solvent and used to generate a seven point standard curve, consisting of 1-, 3-, 5-, 10-, 25-, 50-, and 100 µL injections and quantified as detailed in the TONO Analysis section ($r^2 > 0.99$). To measure the recovery efficiency of the SPE-TONO assay,

500 mL aliquots of Milli-Q water buffered with 20 mM sodium bicarbonate were titrated to pH 7.0 and amended with 1,000 ng $L^{-1}$ of NDMA. These samples were extracted and measured for total N-nitrosamines as described in the next sections.

**C. Preparation of the biofilm-related samples**

Biofilm-derived materials (e.g., PNAG, tryptophan, and a pure culture of suspended *P. aeruginosa* cells) were added to Milli-Q water, buffered with 20 mM sodium bicarbonate, filled headspace-free in 500 mL amber glass bottles, and sealed with polytetrafluoroethylene (PTFE) lined caps. *P. aeruginosa* (Schroeter) Migula (ATCC 10145) was grown in nutrient broth (Difco BD) at 37°C. Densely populated cells were harvested after 2 days of growth and used in the SPE-TONO experiments. The samples were titrated to pH 7.0 and dosed with preformed monochloramine at a concentration of 250 mg $L^{-1}$ as $Cl_2$ as described elsewhere.[28] In addition, the biofilm-derived materials were prepared without monochloramine to assess the response of these precursor materials alone relative to the chloraminated samples. The samples were tumbled end-over-end at 7 rpm for 10 days at room temperature (20-22°C). Following this period, monochloramine and total chlorine were measured on a UV/Vis Spectrophotometer using Hach monochloramine powder pillows (Hach Method 10200) and DPD total chlorine powder pillows (Hach Method 8167), respectively. For the remaining sample, the residual was quenched with 500 mg of an ascorbic acid-based dry mix with a weight ratio of 0.9-1-39 (0.9 g ascorbic acid, 1 g $KH_2PO_4$, and 39 g $Na_2HPO_4$) to stop further N-nitrosamine formation reactions. The majority of the samples were extracted immediately for total N-nitrosamines as described in the next section; however, due to delays in acquiring the SPE columns, the PNAG samples were stored following quenching in the aqueous phase at 4°C for 78 days prior to extraction. N-nitrosamines are likely

to be stable over this period of time, as shown by Tate et al. [29] who found that NDMA was resistant to degradation in lake water over a 3.5-month observation period at room temperature.

**D. Solid phase extraction and quenching agents**

To lower the TONO method detection limit, the samples were concentrated by SPE and eluted to an organic solvent. The SPE columns were conditioned by sequential rinsing with solvent and water as follows: 3 mL of solvent followed by aspiration (repeated once), 3 mL of solvent and left wet (repeated once), and 3 mL of Milli-Q water and left wet (repeated four times). To load the sample onto the SPE columns, a sample delivery system was used to draw each 500 mL sample through a single column into a waste container at a flow rate of approximately 5 mL $min^{-1}$. This was followed by an aspiration period of 10 minutes of atmospheric air at full vacuum. Next, the waste container was removed and replaced with individual 15 mL glass centrifuge tubes, one for each extraction column. To elute the N-nitrosamines from the SPE columns, 12 mL of solvent was passed through each column drop-wise and collected in a centrifuge tube. The eluted extract was passed through a wetted drying column (rinsed with 6 mL of solvent), which consisted of 6 g sodium sulfate encapsulated by glass fiber frits in a 6 mL glass SPE column and followed by 3 mL of organic solvent in an attempt to purge the drying column of any remaining N-nitrosamines. To remove leached sodium sulfate the 15 mL sample extracts were filtered through a solvent rinsed 6 mL polypropylene SPE column fitted with a polyethylene frit and attached to a 25 mm diameter 0.2 µm nominal pore size PTFE syringe filter. The sample extracts were then concentrated from ~15 mL to precisely 1 mL in a 37°C water bath using an evaporator with a gentle stream of UHP grade nitrogen. The 1 mL sample extracts were transferred to individual 2 mL amber glass vials sealed with PTFE lined screw caps and stored at -20°C.

As described by Kulshrestha et al.[9], interferences caused by S-nitrosothiols and nitrite can produce false-positive signals in the chemiluminescence detector and thus need to be quenched in the sample extracts. S-nitrosothiols were quenched from the 1 mL sample extracts with 100 µL of the mercuric chloride solution (20 g $L^{-1}$ in Milli-Q water) and allowed to sit in the dark for 30 minutes. Next, nitrite was quenched with 100 µL of the sulfanilamide solution (50 g $L^{-1}$ in 1 N HCl) and allowed to sit in the dark for 15 min. Interfering compounds were quenched and N-nitrosamines quantified within 2 weeks of sample extraction.

**E. TONO analysis**

Total N-nitrosamines were quantified in the purified sample extracts using a chemiluminescence NO detector (Eco Physics CLD 88sp detector equipped with an IFNOX RS232 data output circuit board). The relevant chemistry involves an acidic iodine/iodide mixture that forms reducing agents,[9] such as HI and $HI_3$, that release excited state NO* by mediating reactions of the following form:

$$2\ HI + 2\ R_2NNO \rightarrow\ I_2 + 2\ NO^* + 2\ R_2NH$$

The procedure used for the TONO analysis was detailed by Mitch and Dai.[30] Briefly, the two solutions were prepared for use in the reaction chamber (Fig. S1), which were: 5 mL triodide solution (2.7 g potassium iodide and 0.57 g iodine in Milli-Q water, made fresh daily) and 100 mL of 1 M sodium hydroxide. Approximately two hours prior to analysis, two recirculating water/ethylene glycol (30/70% v/v) baths were powered on to reach their set point temperatures – one set at +80°C attached to the custom blown glass reaction chamber, and the other set at -10°C attached to the condenser (Fig. 2a). The base trap was filled with 100 mL of 1 M sodium hydroxide and kept at 0°C by submerging it in a custom-made water/ethylene glycol (30/70% v/v) bath throughout the duration of the experiment (Fig 2b). UHP nitrogen gas was supplied to the reaction

chamber and 15 mL of glacial acetic acid was added, then 1 mL of the triodide solution was added prior to attaching the condenser. Next, the gas flow rate was adjusted such that the bubbles formed were below the neck of the reactor. The condenser outlet was attached to the inlet of the base trap. From there, the gas flowed through a 25 mm diameter 0.2 µm nominal pore size PTFE membrane prior to entering the NO detector. PTFE tubing was used throughout to minimize interferences and atmospheric nitrogen penetration.[30] An appropriate volume of each purified extract (10- to 1,200 µL) was injected into the reaction chamber with a glass-barreled gas tight syringe. Output signals from the chemiluminescence detector were discretized at 0.2 second intervals and captured using a MS Excel macro. These data were then imported into MATLAB R2012a to calculate the area under each sample peak using a summation and baseline subtraction formula. Each sample peak area was then compared to that of the standard curve preceding its respective injection to determine the concentration as NDMA based on the volume of the injection and the initial volume of the sample processed by SPE, if applicable.

**F. Hydroxylamine experiments**

The impact of hydroxylamine on TONO and NDMA formation were assessed in batch reactors at room temperature (20-22°C) with combinations of hydroxylamine (3.52 mM), monochloramine (3.52 mM), and dimethylamine (35.2 mM). Each batch reactor consisted of an amber glass bottle filled with 400 mL of 10 mM sodium borate (prepared in Milli-Q water) and purged with $O_2$ for 10 minutes to achieve ~40 mg $L^{-1}$ dissolved oxygen (DO) (data not shown). Preliminary experiments showed that 10 minutes of purging was sufficient to raise the DO above 40 mg $L^{-1}$ at room temperature in a pH 9.0 sodium borate buffer (results not shown). Each reagent addition was followed by an allotted time prior to other amendments, as follows: sodium borate (10 minutes), hydroxylamine (5 minutes), monochloramine (5 minutes), dimethylamine (5

minutes), and the combined sample (60 minutes). As a result of several unexpected results, various combinations of the TONO sample treatments were investigated to assess potential interferences on aqueous phase aliquots and methanol extracts following SPE. Regardless of the sample phase (i.e., aqueous or methanol), the TONO standard curve was prepared by direct injections of NDMA into methanol, as described previously. The following five treatments were assessed: (1) untreated (i.e., no sample treatment), (2) sulfanilamide only (i.e., samples dosed with 100 μL of 50 g $L^{-1}$ sulfanilamide in 1 N HCl and held in the dark for 15 minutes), (3) mercuric chloride only (i.e., samples dosed with 100 μL of 50 g $L^{-1}$ mercuric chloride and held in the dark for 30 minutes), (4) mercuric chloride and sulfanilamide (i.e., samples dosed with mercuric chloride followed by sulfanilamide in HCl at doses stated previously), and (5) HCl only (i.e., samples dosed with HCl at an equimolar dose equivalent to that added in the sulfanilamide treatment). Aqueous phase samples were measured by the TONO assay following the five sample treatments by direct injection into the reaction chamber (Fig. 2). Between 404-412 mL of each aqueous phase sample was processed by SPE and eluted into 10 mL of methanol, but was not further concentrated using the nitrogen gas blowdown step to avoid further volatile losses. These samples were subjected to the five sample treatments followed by the TONO assay and GC-FID. Details for NDMA analysis by GC-FID are provided in Table 5, and the GC-FID method had an estimated detection limit of 1 μg $L^{-1}$ as NDMA and standard curves with regression coefficients > 0.997.

**G. Ion chromatography**

Anions (nitrate and nitrite) and cations (ammonium) were measured in aqueous phase samples using a Metrohm 850 Ion Chromatography system, equipped with an autosampler, UV/Vis detectors, and operated at a column temperature of 45°C. For anions, a seven point standard curve (0.01- to 5.0 mg $L^{-1}$, Metrohm Custom Mix 3 – REAIC1035) was used to quantify

unknowns ($r^2 > 0.997$). A Metrosep A Supp 7 250/4.0 anion column was used and samples were injected into a 10 μL loop at a flow rate of 0.7 mL $min^{-1}$ with a 3.6 mM sodium carbonate eluent. For cations, an eight point standard curve (0.01- to 10.0 mg $L^{-1}$, Metrohm Custom Mix 2 – REAIC1230) was used to quantify unknowns ($r^2 > 0.999$). A Metrosep C 4 – 150/4.0 cation column was used and samples were injected into a 20 μL loop at a flow rate of 0.9 mL $min^{-1}$ with a 3.5 mM oxalic acid eluent.

### H. Statistical analyses.

In the hydroxylamine experiments, a portion of the N-nitrosamine data were collected in triplicate ($n_i = 3$) and analyzed using Tukey's paired comparison method, following the approach described by Berthouex and Brown.[31] For each of *k* treatments, the sample mean ($\overline{y_i}$) and variance ($s^2$) was calculated and used to determine the pooled variance, $s_{pool}^2 = \frac{(n_1-1)s_1^2+\cdots+(n_k-1)s_k^2}{n_1+\cdots+n_k-k}$. Next, the confidence interval for the difference in two means was calculated, taking into account all possible comparisons of *k* treatments using: $\overline{y_i} - \overline{y_j} \pm \frac{q_{k,v,\alpha/2}}{\sqrt{2}} s_{pool}\sqrt{\frac{1}{n_i}+\frac{1}{n_j}}$ where $q_{k,v,\alpha/2}$ is the upper significance level of the studentized range for *k* means and *v* degrees of freedom in the estimate of the pool variance. Tabulated critical values[32] of $q_{k,v,\alpha/2}$ were used to calculate the two-sided 95% confidence interval. Differences in the treatment means were significant if they were larger than the confidence interval.

## III. RESULTS AND DISCUSSION

### A. Extraction solvent and nitrogen gas blowdown

Table 1 shows the recoveries of NDMA spiked into the five extraction solvents tested. For the blanks (i.e., NDMA spikes of 0 ng), dichloromethane produced an interference signal (equivalent to ~400 ng as NDMA), which was not subsequently quenched with mercuric chloride

or sulfanilamide. As such, dichloromethane was determined to be an inappropriate extraction solvent for the SPE-TONO assay. In contrast, blanks measured using the other solvents had signals that were not detected (Table 1, "ND"), an expected result that indicates acetonitrile, methanol, acetone, and an equal volume mixture of these solvents could be suitable extraction solvents. For acetonitrile and acetone, the NDMA-spiked solvents (100- and 1,000 ng NDMA, Table 1) showed decreased signals following $HgCl_2$ treatment. This is a confounding result considering S-nitrosothiols[9] should not be present in these solvent mixtures. Even though pretreatment was able to quench these compounds, the presence of a possible solvent-derived interference signal was a concern that ruled out their subsequent use in the TONO assay. Logically, the same was true for the equal volume mixture of acetonitrile, methanol, and acetone. Fortunately, no interferences were observed using methanol alone in either the blanks or NDMA-spiked solvents (Table 1), as indicated by the relatively small variation in mass recoveries for the 1 hr blowdown time (23-28 ng as NDMA for the 100 ng spike and 440-445 ng as NDMA for the 1,000 ng spike). For the extraction with methanol, increasing the blowdown time from 1- to 2 hours (see details in Table 1) increased the NDMA mass recoveries for the sulfanilamide-treated extracts from 28- to 86 ng as NDMA and 440- to 720 ng as NDMA, indicating the longer blowdown time reduced losses of NDMA. For the 2-hour blowdown time, the mass recoveries were 86%, 72%, and 59% for 15 mL solvent aliquots spiked with NDMA at 100-, 1000- and 5000 ng, respectively (Table 1), indicating either that the NDMA mass recoveries decreased with increasing mass injected into the reaction chamber or higher NDMA concentrations led to higher volatile losses during the volume reduction step. Regardless, methanol was determined to be the most suitable SPE solvent for the TONO assay.

## B. Recovery of NDMA from SPE-TONO assay

Table 2 shows the recoveries of NDMA spiked into water for the SPE-TONO assay with methanol. Three samples were measured in duplicate, and average recoveries ranged between 54-59%, which compares favorably to 37% average recovery of NDMA in the continuous liquid-liquid extraction process reported by others.[30] Based on findings in Table 1 that showed ~86% recovery of a spike directly to methanol, additional losses of ~30% were incurred in the SPE step, either in loading NDMA onto the SPE cartridges and/or the subsequent elution with methanol. As such, SPE-TONO concentrations measured in unknown samples are likely conservatively low estimates, although the extraction efficiencies with SPE (or any other technique) may vary amongst the various N-nitrosamine species present in a given water sample, presumably based on their polarity. Importantly, the estimated 30% loss of NDMA in the SPE step was applied in this research to compare NDMA concentrations measured in the aqueous phase with that in the methanol extracts.

## C. Biofilm-derived materials as TONO precursors

Based on their relevance to drinking water distribution systems,[18] we examined the TONO formation potential of PNAG, tryptophan, and *P. aeruginosa*, following 10-days of chloramination at pH 7. Fig. 1A shows a dose-response relationship between PNAG (0.25-, 0.50-, 1.0-, and 6.0 g $L^{-1}$) and TONO (7.3-, 12.4-, 21.8-, and 51.1 ng $L^{-1}$ as NDMA, respectively). No TONO was formed in the absence of monochloramine, as expected, for the control and the total nitrogen was proportional to the PNAG dose. Similarly, Fig. 1B shows a dose-response relationship between tryptophan (1-, 10-, and 100 μg $L^{-1}$) and TONO (0.55-, 5.3-, 50.2 μg $L^{-1}$ as NDMA, respectively) and Fig. 1C shows a dose-response relationship between *P. aeruginosa* (10-, 75-, and 125 mL of growth media broth) and TONO (260-, 1300-, and 1450 ng $L^{-1}$ TONO as NDMA). In Fig. 1C,

while the TONO measured in absence of *P. aeruginosa* (i.e., the growth media broth only) was higher for the 10 mL dose (365 ng $L^{-1}$ TONO as NDMA), the growth media broth was an insignificant contributor to TONO at the two higher volumes added (82.7- and 17.3 ng $L^{-1}$ TONO as NDMA, for the 75- and 125 mL doses). On balance, the data in Fig. 1 indicate that biofilm-derived materials are N-nitrosamine precursors; however, their relevance in chloraminated distribution systems has not been systematically assessed.

**D. Hydroxylamine, NDMA formation, and the SPE-TONO assay**

Due to the association between chloramination and nitrification in distribution systems,[33] TONO and NDMA formation were assessed in batch reactors containing combinations of hydroxylamine (3.52 mM), dimethylamine (35.2 mM), and monochloramine (3.52 mM). N-nitrosamines were measured in triplicate in (1) aqueous phase aliquots taken prior to SPE (TONO Aqueous, Table 3) and (2) methanol following SPE (TONO Solvent and NDMA by GC-FID, Table 3). For the batch reactors with hydroxylamine or dimethylamine as single reagents, just one TONO measurement was made in the aqueous phase only.

Aqueous TONO data for the batch reactors with hydroxylamine only (Table 3) showed a comparatively large TONO response in the $HgCl_2$-treated aqueous phase sample (57,249 μg $L^{-1}$ as NDMA), which was subsequently removed by treatment with SAA. The corresponding data in Table 4 indicate a high concentration of nitrite in this sample (18,356 μg $L^{-1}$ as N), presumably from mercury-aided catalysis of hydroxylamine reacting with oxygen.[22] This result demonstrates the need to use SAA when applying the TONO assay to waters that do not contain nitrite, such as those with hydroxylamine that could produce an interference signal by $HgCl_2$ catalyzing nitrite formation.

An additional observation from the hydroxylamine only experiments (Table 3) is the apparent production of nitrite during IC analysis from residual hydroxylamine in the sample. $HgCl_2$ treatment presumably removed any remaining hydroxylamine in the sample by catalyzing hydroxylamine's reaction with oxygen, producing nitrite as a product. Also, treatment with SAA (by itself or with $HgCl_2$) should result in complete nitrite removal and the associated TONO response. Therefore, residual hydroxylamine is only expected in the *Untreated* and *SAA*-only treated samples, and nitrite is only expected in the *Untreated* and *$HgCl_2$*-only treated samples, producing an associated TONO response. While the *$HgCl_2$ + SAA* treated sample had an expected non-detectable nitrite (Table 4) and minimal TONO response (<10 μg $L^{-1}$ as NDMA, Table 3), the *SAA*-only treated sample had a measurable nitrite concentration (1,522 μg $L^{-1}$ as N) with a minimal TONO response (<19 μg $L^{-1}$ as NDMA), suggesting formation of nitrite during IC analysis. Taken together, these results indicate that hydroxylamine present in the *SAA*-only treated sample was converted to nitrite during IC analysis.

For the batch reactors with dimethylamine only, there was a TONO response in all four treatments, including after *$HgCl_2$ + SAA* (102 μg $L^{-1}$ as NDMA). Coupled with the results in Table 4 that showed nitrite was low or below detection (method detection limit of ~304 μg $L^{-1}$ nitrite as N), we conclude the dimethylamine solution had a background concentration of ~100 μg $L^{-1}$ as NDMA of total N-nitrosamines. Data interpretation of batch reactor experiments with dimethylamine must take this into account.

For the batch reactors with hydroxylamine and dimethylamine, Tukey's tests were done to compare the triplicate means between treatments. For NDMA and TONO in methanol, there were no statistically significant differences between sample treatments, indicating potentially interfering compounds (e.g., nitrite) were not present in the methanol following SPE or created by the

treatment (e.g., $HgCl_2$). For TONO in the aqueous phase, the comparatively high TONO response in the *$HgCl_2$*-treated sample (62,134 μg $L^{-1}$ as NDMA) was attributed to nitrite (15,455 μg $L^{-1}$ as N, Table 4) and NDMA, presumably from mercury catalyzing the reaction of the residual hydroxylamine in the presence of dissolved oxygen. As in the hydroxylamine only experiments, residual hydroxylamine may have resulted in nitrite production during IC analysis as the *SAA*-only treatment had a nitrite concentration (1,350 μg $L^{-1}$ as N) without a correspondingly large TONO response (77 μg $L^{-1}$ as NDMA). In contrast, the *$HgCl_2$ + SAA* treatment showed an undetectable nitrite concentration and a large TONO response (15,834 μg $L^{-1}$ as NDMA). This suggests that hydroxylamine and dimethylamine reacted to form UDMH, which subsequently reacted with dissolved oxygen catalyzed by mercury to form NDMA. In sum, two interferences were apparent in the aqueous phase batch reactors with hydroxylamine and dimethylamine: (1) nitrite and NDMA interferences produced by $HgCl_2$ treatment and (2) an NDMA interference produced by *$HgCl_2$ + SAA* treatment.

For the batch reactors with hydroxylamine, dimethylamine, and monochloramine, Tukey's tests were done to compare the triplicate means between treatments. For NDMA and the TONO samples in methanol (Table 3), there were no statistically significant differences between sample treatments, indicating potentially interfering compounds (e.g., nitrite) were not present in the methanol following SPE. In contrast, for the aqueous phase TONO samples, statistically significant differences were found between the *Untreated* sample and the other treatments, which was attributed to the formation and quenching of nitrite in the presence of hydroxylamine by $HgCl_2$ and SAA, respectively (Table 4). Interestingly, HCl treatment resulted in a comparatively low TONO response in the aqueous phase (141 ± 18 μg $L^{-1}$ as NDMA), suggesting hydroxylamine in its acidic form ($NH_3OH^+$, $pK_a \approx 6$) does not react with dimethylamine to form UDMH. This result

is in agreement with Zhang et al.[21] that found the reaction between hydroxylamine and dimethylamine to form UDMH was pH dependent.

Comparing the batch reactors containing hydroxylamine, dimethylamine, and monochloramine with those containing hydroxylamine and dimethylamine indicated that in the (1) aqueous phase, hydroxylamine reacted in the $HgCl_2$-treatment, and (2) solvent phase, hydroxylamine reacted with the activated carbon in the SPE cartridges. NDMA formed at over one order of magnitude greater in the batch reactors with hydroxylamine and dimethylamine compared to those with monochloramine (Table 3). This suggests that excess hydroxylamine was present in the batch reactors without monochloramine and reacted with dimethylamine and dissolved oxygen in the SPE cartridges to form NDMA.

Padhye et al.[26] showed that N-nitrosamines formed from secondary amines by nitrogen fixation on activated carbon. Additionally, the solvent TONO and NDMA results support the assertion that any nitrite present or formed does not elute from the SPE process, as both were insensitive to treatment type (i.e., *SAA* only or *$HgCl_2$ + SAA* should have quenched nitrite and reduced the TONO and NDMA, but Tukey's tests for the solvent phase showed no difference amongst any treatments). Therefore, following SPE, hydroxylamine and monochloramine are not present and only NDMA and dimethylamine remained.

For the batch reactors with hydroxylamine and dimethylamine, the comparatively low *Untreated* aqueous TONO (1,358 μg $L^{-1}$ as NDMA) further supports the assertion that NDMA formation was catalyzed by the activated carbon in the SPE cartridges. Logically, the majority of this aqueous TONO signal was associated with nitrite (2,689 μg $L^{-1}$ as N, Table 4) that subsequently reacted with *$HgCl_2$ + SAA* to form N-nitrosamines (15,834 μg $L^{-1}$ as NDMA, Table 3, but nitrite was not detected, Table 4). In this case, an alternative NDMA formation mechanism

is also plausible, one that does not involve UDMH. Here, hydroxylamine is catalyzed to peroxynitrite ($ONOO^-$) in the presence of dissolved oxygen by $HgCl_2$[34], and $ONOO^-$ subsequently reacts with dimethylamine to form NDMA.[25]

In summary, the presence of hydroxylamine presents two problems in assessing total N-nitrosamine formation: (1) in the aqueous phase, hydroxylamine is catalyzed by $HgCl_2$ to nitrite and NDMA, and (2) in the solvent phase, hydroxylamine reacts with dimethylamine and is catalyzed to NDMA on the surfaces of the activated carbon in the SPE cartridges. While the data in Tables 3 and 4 illustrate the potential importance of hydroxylamine in N-nitrosamine formation pathways, interferences in the TONO assay, the SPE process, and possibly IC analysis must be overcome to elucidate hydroxylamine's role.

## E. Future Work

More extensive studies with biofilm are warranted under chloramination conditions, and should include measurement of TONO and N-nitrosamine species. Additional experiments should be done to assess the role of hydroxylamine in N-nitrosamine formation at lower molar ratios and longer reaction times to ensure no unreacted hydroxylamine is present in the batch reactors prior to measurement of N-nitrosamines by TONO and GC-FID and anions by IC. Further, batch experiments with UDMH will help elucidate other potential NDMA reaction pathways, similar to the one proposed involving peroxynitrite. Further examination of extraction techniques and quenching agents are necessary to eliminate method-derived interferences from the TONO assay and GC-FID measurement of NDMA.

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**Table 1**. N-nitrosodimethylamine (NDMA) recovered following concentration of four organic solvents by nitrogen gas blowdown (BD) to 1 mL for blanks and NDMA-spiked solvents.

| Extraction Solvent | Solvent Volume mL | NDMA Spike ng | Total N-nitrosamines Recovered ng as NDMA | | | |
|---|---|---|---|---|---|---|
| | | | No Pretreatment [c] | $HgCl_2$ [a, c] | Sulfanilamide [a, b] | |
| | | | | | 1 h BD [c] | 2 h BD [d] |
| Dichloromethane | 15 | 0 | 398 | 470 | 364 | NM |
| | | 100 | 376 | 553 | 404 | NM |
| | | 1,000 | 980 | 1,018 | 1,090 | NM |
| Acetonitrile | 15 | 0 | ND | ND | ND | ND |
| | | 100 | 52 | 29 | 23 | 70 |
| | | 1,000 | 702 | 415 | 487 | 650 |
| | | 5,000 | NM | NM | NM | 2,897 |
| Methanol | 15 | 0 | ND | ND | ND | ND |
| | | 100 | 25 | 23 | 28 | 86 |
| | | 1,000 | 440 | 442 | 445 | 720 |
| | | 5,000 | NM | NM | NM | 2,955 |
| Acetone | 15 | 0 | ND | ND | ND | NM |
| | | 100 | 86 | 36 | 33 | NM |
| | | 1,000 | 659 | 355 | 424 | NM |
| Acetonitrile/ Methanol/ Acetone | 5/5/5 | 0 | ND | ND | ND | NM |
| | | 100 | 64 | 16 | 16 | NM |
| | | 1,000 | 574 | 311 | 354 | NM |

[a] quenched S-nitrosothiols with 100 µL of the mercuric chloride solution
[b] quenched nitrite with 100 µL of the sulfanilamide solution after $HgCl_2$
[c] 1 hr nitrogen gas blowdown at a gas flowrate of 0.67 L $min^{-1}$ in a 60°C water bath
[d] 2 hr nitrogen gas blowdown at a gas flowrate of 0.33 L $min^{-1}$ in a 37°C water bath
ND – not detected
NM – not measured

**Table 2**. Recoveries of N-nitrosodimethylamine (NDMA) from the solid phase extraction total N-nitrosamine (SPE-TONO) assay following nitrogen gas blowdown.

| Sample [a] | NDMA Mass Injected ng [b] | Total N-nitrosamines Recovered ng $L^{-1}$ as NDMA | Average Recovery % [c] |
|---|---|---|---|
| 1 | 10.6 | 531 | 54 |
| | 11.0 | 549 | |
| 2 | 12.4 | 617 | 59 |
| | 11.4 | 569 | |
| 3 | 11.2 | 565 | 55 |
| | 10.7 | 536 | |

[a] 479 $\pm$ 2 mL of NDMA-spiked water samples in 20 mM sodium bicarbonate at pH 7.0 was drawn through SPE activated carbon cartridge at 5.7-5.9 mL $min^{-1}$ under vacuum

[b] 50 µL injections of methanol extract into the reaction chamber; extract was made by reducing ~15 mL of methanol to precisely 1 mL under a 2-hr nitrogen gas blowdown at 37°C and quenched for S-nitrosothiols and nitrite[9]

[c] Recoveries correspond to water samples spiked with 1,000 ng $L^{-1}$ of NDMA

**Table 3**. Total N-nitrosamines and N-nitrosodimethylamine formed from reactions with 3.52 mM hydroxylamine ($NH_2OH$), 3.52 mM monochloramine ($NH_2Cl$), and 35.2 mM dimethylamine ($(CH_3)_2NH$) for various sample treatments

| Reagents | Treatment | Total N-nitrosamine Assay (TONO) | | NDMA | Nitrite Equivalent TONO [a] |
|---|---|---|---|---|---|
| | | μg $L^{-1}$ as NDMA Average ± 95% confidence interval | | | |
| | | Aqueous [b] | Solvent [c] | Solvent [c] | μg $L^{-1}$ as NDMA |
| $NH_2OH$ + $(CH_3)_2NH$ + $NH_2Cl$ | Untreated | 3,230 ± 534 | 527 ± 55 | 420 ± 23 | NA |
| | SAA | 133 ± 22 | 332 ± 97 | 200 ± 55 | NA |
| | $HgCl_2$ | 6,243 ± 209 | 521 ± 48 | 489 ± 49 | 7,561 |
| | $HgCl_2$ + SAA | 765 ± 218 | 326 ± 112 | 393 ± 234 | NA |
| | HCl | 141 ± 18 | 407 ± 17 | 323 ± 100 | NA |
| $NH_2OH$ + $(CH_3)_2NH$ | Untreated | 1,358 ± 1,237 | 13,087 ± 1,298 | 9,028 ± 771 | 13,835 |
| | SAA | 77 ± 48 | 10,623 ± 3,721 | 9,558 ± 1,039 | 9,009 |
| | $HgCl_2$ | 62,134 ± 4,187 | 12,145 ± 892 | 8,935 ± 1,015 | 54,534 |
| | $HgCl_2$ + SAA | 15,834 ± 2,866 | 11,882 ± 1,149 | 7,930 ± 1,487 | NA |
| | HCl | 100 ± 108 | 12,222 ± 957 | 6,283 ± 909 | 9,330 |
| $NH_2OH$ | Untreated | 118 | NM | NM | 25,578 |
| | SAA | < 19 | NM | NM | 9,330 |
| | $HgCl_2$ | 57,249 | NM | NM | 70,622 |
| | $HgCl_2$ + SAA | < 10 | NM | NM | NA |
| $(CH_3)_2NH$ | Untreated | 125 | NM | NM | NA |
| | SAA | 49 | NM | NM | NA |
| | $HgCl_2$ | 326 | NM | NM | NA |
| | $HgCl_2$ + SAA | 102 | NM | NM | NA |

[a] Theoretical response from nitrite in TONO assay based on 1:1 molar yield and 100% efficiency
[b] Sample processed in aqueous phase without solid-phase extraction
[c] Sample concentrated by solid-phase extraction and eluted into methanol; values corrected for the estimated NDMA extraction efficiency (70%, see text)
HCl – treated with 100 μL of 1 N HCl
$HgCl_2$ – treated with 100 μL of 20 g $L^{-1}$ mercuric chloride and held in the dark 30 minutes
$HgCl_2$ + SAA – treated with mercuric chloride followed by sulfanilamide
NA – not applicable
ND – not detected
NM – not measured
SAA – treated with 100 μL of 50 g $L^{-1}$ sulfanilamide in 1 N HCl and held in the dark 15 minutes

**Table 4**. Inorganic nitrogen formed from reactions with 3.52 mM hydroxylamine ($NH_2OH$), 3.52 mM monochloramine ($NH_2Cl$), and 35.2 mM dimethylamine ($(CH_3)_2NH$) for various sample treatments

| Reagents | Treatment | Aqueous phase concentrations, µg $L^{-1}$ as N Average ± 95% confidence interval | | |
|---|---|---|---|---|
| | | Nitrite | Nitrate | Ammonium |
| $NH_2OH$ + $(CH_3)_2NH$ + $NH_2Cl$ | Untreated | 1,126 ± 210 | 1,644 ± 1,441 | 46,267 ± 1,931 |
| | SAA | ND | 2,667 ± 168 | 49,262 ± 9,516 |
| | $HgCl_2$ | 1,806 ± 211 | 2,048 ± 53 | 7,200 * |
| | $HgCl_2$ + SAA | ND | 2,033 ± 179 | 40,122 ± 232 |
| | HCl | ND | 2,620 ± 77 | 41,542 ± 2,075 |
| $NH_2OH$ + $(CH_3)_2NH$ | Untreated | 2,689 ± 130 | 512 ± 431 | 4,983 ± 483 |
| | SAA | 1,350 ± 143 | 4,593 ± 691 | ND |
| | $HgCl_2$ | 15,455 ± 224 | ND | 5,300 * |
| | $HgCl_2$ + SAA | ND | ND | ND |
| | HCl | 1,421 ± 293 | 5,015 ± 271 | ND |
| $NH_2OH$ | Untreated | 2,709 | 5,783 | 3,021 |
| | SAA | 1,522 | 5,128 | ND |
| | $HgCl_2$ | 18,356 | 497 | 3,486 |
| | $HgCl_2$ + SAA | ND | ND | ND |
| $(CH_3)_2NH$ | Untreated | BDL | BDL | 3,873 |
| | SAA | ND | ND | ND |
| | $HgCl_2$ | ND | ND | 2,634 |
| | $HgCl_2$ + SAA | ND | ND | ND |

BDL – below detection limit
HCl – treated with 100 µL of 1 N HCl
$HgCl_2$ – treated with 100 µL of 20 g $L^{-1}$ mercuric chloride and held in the dark 30 minutes
$HgCl_2$ + SAA – treated with mercuric chloride followed by sulfanilamide
ND – not detected
SAA – treated with 100 µL of 50 g $L^{-1}$ sulfanilamide in 1 N HCl and held in the dark 15 minutes
* One of three samples had detectable concentrations
Method detection limits for nitrite, nitrate, and ammonium were respectively 304-, 226- and 775 µg $L^{-1}$ as N

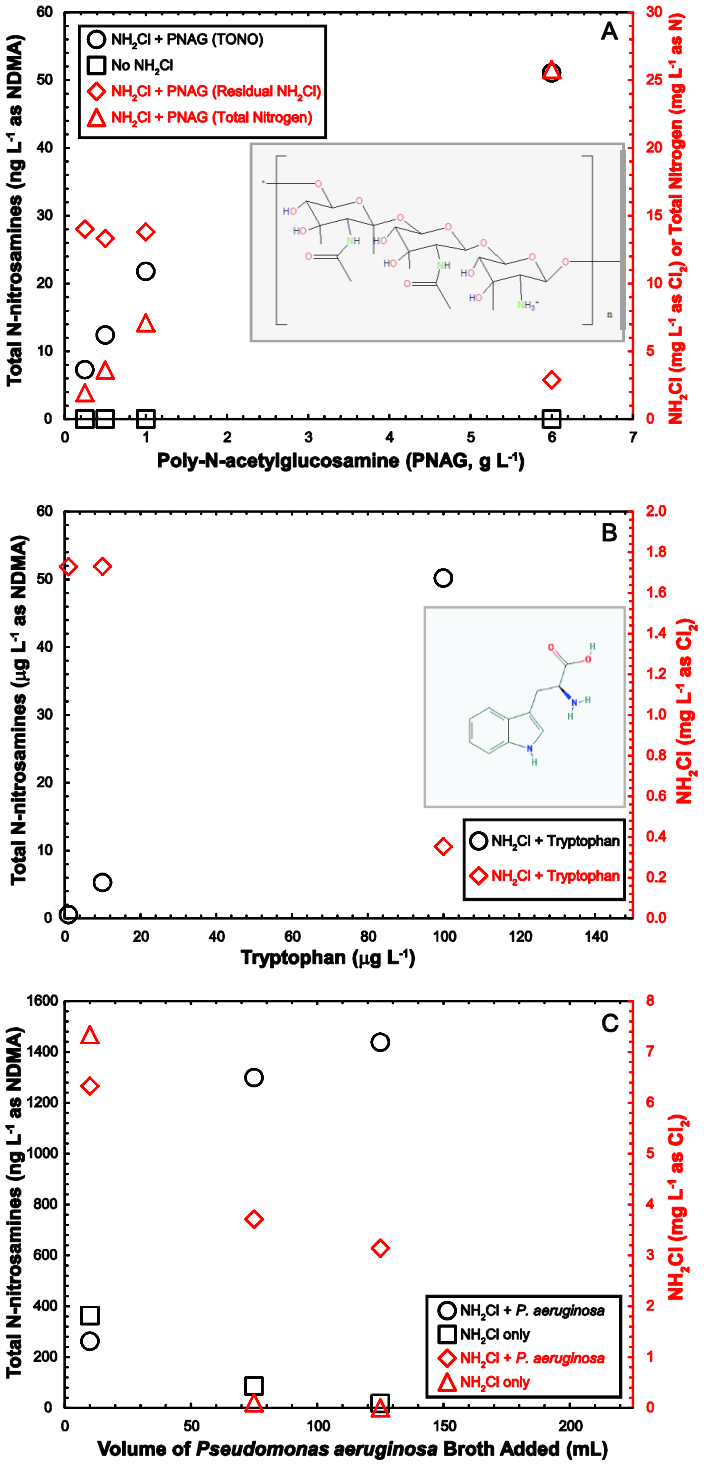


**Figure 1**. Total N-nitrosamines (TONO) formed from chloramination of (A) poly-N-acetylglucosamine (PNAG) with chemical structure shown in inset, (B) Tryptophan with chemical structure of L-α-Amino-3-indolepropionic acid shown in inset, and (C) *Pseudomonas aeruginosa*. Each sample was dosed at a monochloramine concentration of 250 mg $L^{-1}$ as $Cl_2$ at pH 7.0 in 20 mM carbonate buffer. TONO and monochloramine residuals were measured after 10 days. TONO was not adjusted to account for losses by SPE and $N_2$-gas blowdown.

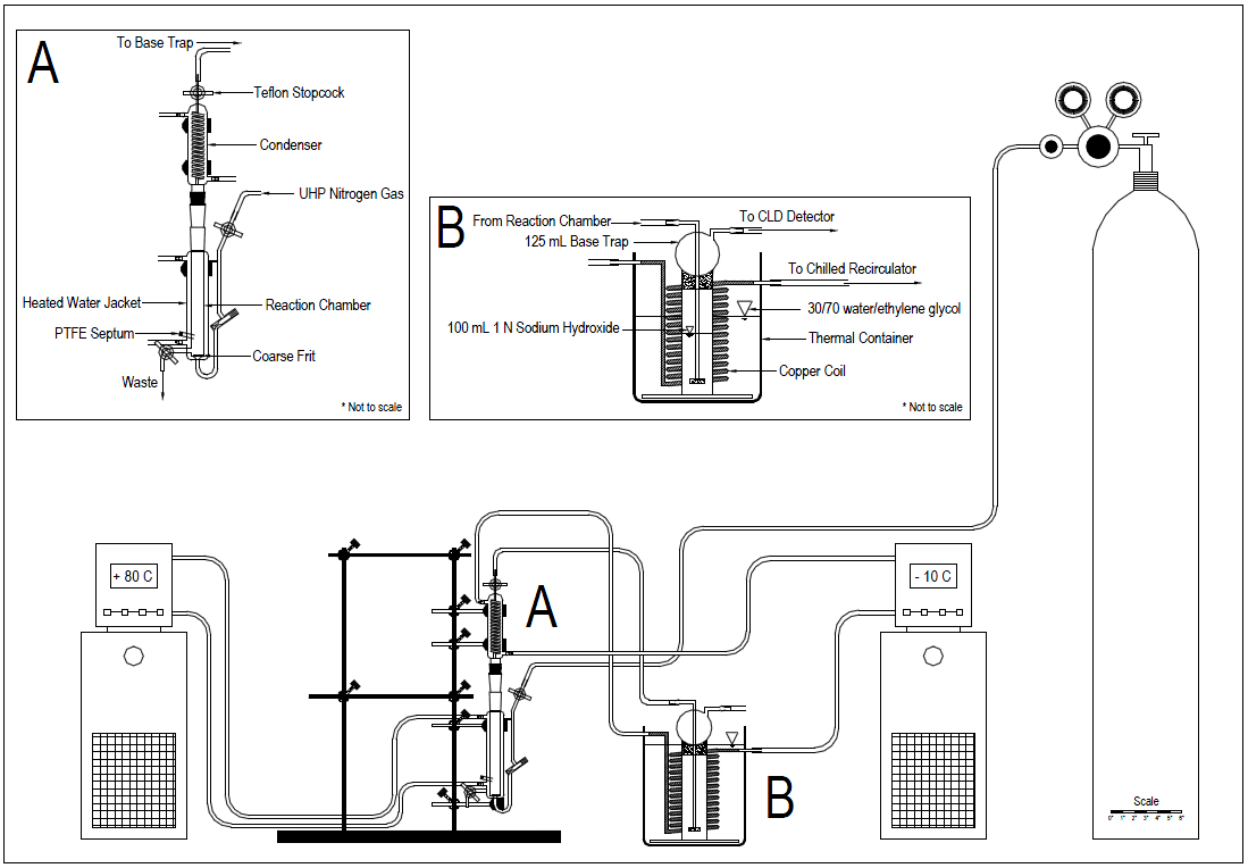


**Figure 2.** Schematic of the total N-nitrosamine (TONO) extraction apparatus. (A) Inset of the TONO reaction chamber and condenser for cleaving N-N bonds and (B) inset of the thermal container for keeping the 1 M NaOH between 0-4°C to ensure complete removal of interfering $NO_x$ species other than NO*.

**Table 5**. GC-FID conditions for the quantification of N-nitrosodimethylamine (NDMA).

| **Gas Chromatograph** | **Shimadzu GC-2010** |
|---|---|
| **Injector** | Splitless |
| **Injection Volume** | 1 µL |
| **Injector Temperature** | 250°C |
| **Sampling Time** | 1 minute |
| **Carrier Gas** | Helium |
| **Flow Control Mode** | Linear Velocity |
| **Pressure** | 85.4 kPa |
| **Total Flow** | 17.2 mL $min^{-1}$ |
| **Column Flow** | 0.89 mL $min^{-1}$ |
| **Linear Velocity** | 24.4 cm $sec^{-1}$ |
| **Purge Flow** | 3 mL $min^{-1}$ |
| **Split Ratio** | 15 |
| **Analytical Column** | RTX® - 5 Amine 30 m x 0.250 mm x 1 µm |
| **Oven Temperature** | 75°C hold 10 min, then 10°C $min^{-1}$ to 150°C, then 20°C $min^{-1}$ to 250°C hold 15 min (37.5 minute runtime) |
| **Detector** | Flame Ionization Detection (FID) |
| **FID Temperature** | 275°C |
| **FID Sampling Rate** | 40 msec |
| **FID Makeup Gas** | Helium |
| **FID Makeup Flow** | 30 mL $min^{-1}$ |
| **FID $H_2$ Flow** | 45 mL $min^{-1}$ |
| **FID Air Flow** | 450 mL $min^{-1}$ |