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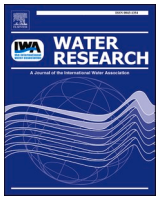

Bacterial dealkylation of benzalkonium chlorides in wastewater produces benzyldimethylamine, a potent *N*-nitrosodimethylamine precursor

Ibrahim Abusallout [a,b,c], Mingrui Song [d], Aron Chan [d,e], Elizabeth McKenna [d,f], Jean Van Buren [g], Seth Mccoy [d], Zachary Ledvina [h], Christopher Jeffrey [h], Daniel L. McCurry [g], David Hanigan [d,*]

[a] *Fraunhofer USA, Inc., Center Midwest, East Lansing, MI 48824, USA*
[b] *Department of Electrical and Computer Engineering, Michigan State University, East Lansing, MI 48824, USA*
[c] *CDM Smith, 75 State Street, Suite 701, Boston, MA 02109, USA*
[d] *Department of Civil and Environmental Engineering, University of Nevada, Reno, NV 89557-0258, USA*
[e] *Nevada Department of Transportation, Carson City, NV 89712, USA*
[f] *Brown and Caldwell, Lakewood, CO 80401, USA*
[g] *Department of Civil and Environmental Engineering, University of Southern California, Los Angeles, CA 90089, USA*
[h] *Department of Chemistry, University of Nevada, Reno, NV 89557-0258, USA*

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ABSTRACT

N-nitrosodimethylamine (NDMA) is a carcinogenic disinfection byproduct that forms during chloramine disinfection of municipal wastewater effluents which are increasingly used to augment drinking water supplies due to growing water scarcity. Knowledge of wastewater NDMA precursors is limited and the known pool of NDMA precursors has not closed the mass balance between precursor loading, precursor NDMA yield, and formed NDMA. Benzalkonium chlorides (BACs) are the most prevalent quaternary ammonium surfactants and have antimicrobial properties. The extensive utilization of BACs in household, commercial and industrial products has resulted in their detection in wastewater at elevated concentrations. We report the formation of a potent NDMA precursor, benzyldimethylamine (BDMA) from the biodegradation of BACs during activated sludge treatment. BDMA formation and NDMA formation potential (FP) were functions of BAC and mixed liquor suspended solids concentration at circumneutral pH, and the microbial community source. Sustained exposure to microorganisms reduced NDMA FP through successive dealkylation of BDMA to less potent precursors. BAC alkyl chain length (C_8 – C_{16}) had little impact on NDMA FP and BDMA formation because chain cleavage occurred at the C–N bond. Wastewater effluents collected from three facilities contained BDMA from 15 to 106 ng/L, accounting for an estimated 4 to 38 % of the NDMA precursor pool.

1. Introduction

Disinfectants reduce pathogenic infections in drinking water, but disinfected water consumption is linked to higher risk of bladder cancer. (Villanueva et al., 2003) *N*-nitrosodimethylamine (NDMA) is a carcinogenic byproduct that forms during chloramine disinfection of treated water. (Chuang and Mitch, 2017; Krasner et al., 2018; McCurry, Krasner, and Mitch, 2016; Mitch, Gerecke, and Sedlak, 2003; Zhang et al., 2016; Krasner et al., 2013) Dichloramine reacts with organic nitrogen (N) precursors, and through several intermediates, forms NDMA. The precursors of NDMA that enter surface water drinking water treatment facilities are believed to predominantly emanate from wastewater sources. These precursors exhibit poor removal efficiency within wastewater treatment processes, thereby allowing them to potentially traverse downstream to drinking water facilities. However, the precise composition and dynamics of the NDMA precursor pool remain inadequately understood. (Krasner et al., 2008; T. Zeng et al., 2016; Song et al., 2023) Because NDMA and NDMA precursors generally pass through drinking water treatment plants unchanged, NDMA occurrence and associated health risk due to NDMA exposure are also expected to increase. (Mitch, Gerecke, and Sedlak, 2003) The identification of wastewater-derived NDMA precursors is therefore critical to minimize

* Corresponding author
E-mail address: dhanigan@unr.edu (D. Hanigan).

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NDMA formation and protect the public from the adverse health effects of NDMA exposure.

Various precursors contribute to the formation of NDMA in wastewater, including dimethylamine (DMA), pharmaceuticals, personal care products, cationic amine-based water treatment polymers containing DMA moieties, and unidentified precursors. (Sedlak et al., 2005; Mitch and Sedlak, 2004; Padhye et al., 2011; Wang, Li, and He, 2014) DMA is a prominent NDMA precursor in untreated wastewater. However, the concentration of DMA and its NDMA yield do not fully account for all the measured NDMA formation potential (FP) of wastewater. (Wang, Li, and He, 2014) The presence of pharmaceuticals and personal care products as NDMA precursors in wastewater is also well-established. For instance, ranitidine, a histamine antagonist used in peptic ulcer treatment, has been identified as a significant source of NDMA with yields exceeding 60 %. (Le Roux, Gallard, and Croué, 2011) Similarly, methadone has been recognized as a contributor to NDMA formation in surface waters and wastewaters during chloramination with NDMA yields ranging from 23 % to 70 %. (Hanigan et al., 2015) Moreover, cationic amine-based water treatment polymers containing DMA moieties have been identified as NDMA precursors, forming NDMA during chlorination or chloramination. (Padhye et al., 2011) Finally, a subgroup of tertiary amines with β-aromatic substituents exhibited the highest NDMA yields of the functional groups tested. (T. Zeng et al., 2016; T. Zeng et al., 2016) However, knowledge of the organic N precursors of NDMA is still relatively limited. Researchers have yet to define the complete pool of NDMA precursors and have not closed the mass balance between precursor loading, precursor NDMA yield, and formed NDMA. This highlights the importance of unidentified NDMA precursors, necessitating further research for effective identification and management.

Quaternary ammonium compounds (QACs) are cationic organic surfactants used in a wide variety of commercial products, such as fabric softeners, detergents, personal care products, and others. In addition, QACs are widely used as disinfectants, and their concentrations have increased in households in response to the pandemic. (Hora et al., 2020) The most commonly used QACs are benzalkonium chlorides (BACs or benzalkyl dimethylammonium compounds), chemically characterized by a dimethylamine group one methylene from a benzene, and bonded to an alkyl chain ranging from C_8 to C_{18}. The most frequently detected QACs worldwide are C_{12} and C_{18} BACs, occurring up to mg/L concentrations in wastewater, industrial effluents, and hospital effluents. (Kümmerer et al., 1997; Wieck, Olsson, and Kümmerer, 2018; Kim et al., 2020; Kreuzinger et al., 2007) Another study found up to 6 μg/L C_{12} and 37 μg/L C_{14} in US wastewater influents. (Ferrer and Furlong, 2001) BACs have been evaluated for their potential to form NDMA upon chloramination, including benzyldimethyldodecylammonium chloride (C_{12}-BAC), benzyldimethyltetradecylammonium chloride (C_{14}-BAC), and a mixture of BACs with varying chain length (C_8 to C_{18}). The molar yield of BACs in wastewater effluents was 0.03 – 0.3 %, suggesting that BACs were responsible for a limited fraction of NDMA formation. (Kemper, Walse, and Mitch, 2010) However, it has been shown that, under aerobic conditions, various organisms typically present in wastewater biodegrade BACs. The reaction takes place through cleavage of the N-alkyl bond, resulting in the loss of the BAC and subsequent release of benzyldimethylamine (BDMA), and the alkyl chain as an aldehyde (Figure S1). (; Tezel et al., 2012; Ertekin et al., 2016; Ertekin, Konstantinidis, and Tezel, 2017) In a subsequent step, BDMA can undergo debenzylation resulting in benzoic acid and dimethylamine, the latter of which is mineralized to form ammonium and carbon dioxide. (Patrauchan and Oriel, 2003; Tezel et al., 2012) Alternatively, *N*-demethylation of BDMA can form benzylmethylamine and benzylamine (Figure S2). (Patrauchan and Oriel, 2003) Both are further biodegraded to benzaldehyde and benzoic acid. BDMA is a high yield NDMA precursor (20 – 84 %), (Selbes et al., 2013; Huang, Huang, and McCurry, 2018; Spahr et al., 2017) far higher than dimethylamine (~0.5 %) (Mitch, Gerecke, and Sedlak, 2003; Mitch and Sedlak, 2002; Gerecke and Sedlak, 2003) suggesting that microbial dealkylation of BACs might substantially contribute to wastewater NDMA FP. However, it is currently unclear whether BDMA from BAC biodegradation will be produced and persist at typical wastewater treatment plants, and contribute to the formation of NDMA.

A core part of our curiosity surrounding the potential for BAC to be a primary contributor to wastewater NDMA formation stems from the use of wastewater as a source of NDMA precursors in experiments intended to inform drinking water treatment mitigation approaches (including works by the authors of this manuscript). (McCurry, Krasner, and Mitch, 2016; Mitch and Sedlak, 2004; Hanigan et al., 2012; Zhang et al., 2020; Mulhern, Summers, and Dickenson, 2017; S.W. Krasner et al., 2009; S. W. Krasner et al., 2009) However, BAC is not likely to be a precursor with appreciable abundance given its structure (Selbes et al., 2013), and the biotransformation product BDMA is likely to be reacted to NDMA during wastewater disinfection. Therefore, precursors derived from wastewater may not be appropriate surrogates, limiting the applicability of such experiments. We sought to investigate the conditions under which BACs could be biodegraded to the reactive NDMA precursor BDMA during wastewater treatment. We aimed to provide evidence that bacteria in activated sludge treatment possess the ability to biodegrade BAC and generate relevant quantities of BDMA. Additionally, we sought to examine whether BDMA could persist, reaching the disinfection unit where chloramine is present, and lead to the formation of NDMA. To achieve these objectives, we examined NDMA FP as a function of BAC and biomass concentrations, BAC alkyl chain length, and the microbial source/culture. Finally, we measured BDMA and NDMA FP in three wastewater treatment plant effluents prior to disinfection to estimate the contribution of BAC degradation products to the NDMA precursor pool.

2. Materials and methods

2.1. Reagent

A mixed BAC stock was purchased from Fisher Scientific (Fairlawn, NJ). The stock was made in water with a mixture of C_8 to C_{18} BACs at 49 - 52 % by weight with an average molecular weight of 352.5 g/mole. C_8-, C_{10}-, C_{12}-, C_{14}-, and C_{16}-BAC individual stocks and BDMA stock were purchased from Sigma Aldrich (> 98 % purity, St. Louis, MO). Salts (NH_4NO_3, K_2HPO_4, KH_2PO_4, $CaCl_2$, $MgCl_2$, $MgSO_4$, $FeCl_2$), sodium hypochlorite (5.65 – 6 %), sodium borate, boric acid, ascorbic acid, sodium hydroxide, and sodium sulfite were purchased from Fisher Scientific at the highest purity available (ACS grade or higher). Ammonium chloride was obtained from Sigma Aldrich. Dichloromethane (DCM) was purchased from EMD Chemical (Gibbstown, NJ). Deuterated NDMA (NDMA-d6) was purchased from Cambridge Isotope Laboratories (Andover, MA) and diluted to 100 μg/L before use. Working solutions were prepared with >18.2 MΩ-cm water.

2.2. Wastewater samples

Primary clarifier effluent, mixed liquor suspended solids (MLSS), return activated sludge, secondary clarifier effluent, and filter effluent (from dual media filters prior to disinfection) samples were collected from two local wastewater treatment plants (WWTPs) in Reno, Nevada. The first WWTP (Facility 1) has a traveling screen, aerated grit tank, activated sludge treatment system, tertiary filtration, and chlorine disinfection. The second WWTP (Facility 2) consists of a travelling screen, vortex grit tank (not aerated), enhanced activated sludge with biological phosphorus removal (A/O), nitrifying trickling filters, denitrification with added methanol, reaeration, sand filtration, and disinfection. A dual media filter effluent sample was also collected from a WWTP in Los Angeles, California (Facility 3). The wastewater treatment train in Facility 3 consists of bar screens, an aerated grit tank chamber, primary clarifiers, tricking filters and activated sludge treatment. The WWTPs schematics and design characteristics are presented in Figures S3 – S5 and Table S1, respectively, in the Supporting

Information.

To produce a separate enriched microbial culture acclimated to BACs, a grab sample was taken from Facility 2′s mixed liquor suspended solids one hour prior to enrichment using an autoclaved amber bottle. Mineral salted water (Table S2) was mixed with solids centrifuged from the 20 mL mixed liquor suspended solids sample buffered at pH 7. The microbial culture was dosed with the mixed BAC solution at 50 mg/L every 72 h for >30 days. In the Supporting Information we provided further details on tests confirming increasing bacterial concentrations from BAC addition (Figure S8) within the wastewater inoculum and outline precautions taken to prevent microbial cross-contamination. The enriched sample was essential to ascertain whether BAC could serve as a carbon and energy source for wastewater microbes. Facility 2 was chosen for enrichment due to its larger wastewater treatment capacity, proximity to the laboratory, and broader sewershed characteristics compared to other nearby facilities.

2.3. BAC degradation potential

Six experiments were conducted to evaluate hypotheses about the potential formation of NDMA precursors from BAC biodegradation products in wastewater. A conceptual experiment diagram (Figure S6) is included in the Supporting Information for additional clarification.

In the first experiment, we examined NDMA FP in mixed liquor suspended solids samples over five days after spiking BACs at concentrations ranging from 0.01 to 1 mg/L (0.003 – 2.8 μM based on average BAC molecular weight) directly from the 50% w/w mixed BAC stock solution. A maximum of 1 ml was dosed of the BAC stock to achieve the desired BAC concentration in 500 ml wastewater sample volume to prevent further dilution of the wastewater samples and alteration of the wastewater characteristics. The spiked concentration range was selected based on previously reported BAC concentrations in wastewater. Five liters from mixed liquor suspended solids of Facility 1 was collected and transferred separately to 500 mL flasks and agitated with the headspace open to the room air. Agitation was conducted by continuous rapid stirring using magnetic stirrer and dissolved oxygen (DO) was measured continually. Details about the wastewater collection procedure and wastewater characteristics of the facilities and during collection events are described in the Supporting Information and Table S1. BAC was spiked to the 500 mL flasks immediately following collection and transportation to the University of Nevada, Reno (UNR), approximately 1 hour after collection.

To directly link BAC degradation to the formation of NDMA precursors and NDMA FP, [^{15}N]-*N,N*-dimethyl-N-octylbenzenemethanaminium (^{15}N labeled C_8 analogue of BAC), or ^{15}N-BAI) was synthesized by our team. ^{15}N-BAI was added at 1 mg/L into mixed liquor suspended solids sample collected from Facility 1. ^{15}N-NDMA FP was analyzed in duplicate over 48 h. Controls were conducted similar to the other BACs experiments (see below). The synthesis of [^{15}N]-benzamide was completed by benzoylation of [^{15}N] ammonium chloride with benzoyl chloride and NaOH in water and ether (3:2). The [^{15}N]-benzamide was subsequently methylated with methyl iodide using NaH in dimethoxyethane (DME), yielding [^{15}N]-*N,N*-dimethylbenzamide, which was then reduced using conditions developed by Zhu and Pittman using *in-situ* generated $LiBH_4$ (from LiCl and $NaBH_4$) in diglyme at reflux to give [^{15}N]-*N,N*-dimethylbenzylamine. (Zhu et al., 2003) The [^{15}N] *N,N*-dimethylbenzylamine was then quaternized with 1-iodooctane in acetonitrile at elevated temperature resulting in the desired ^{15}N-labeled benzalkonium iodide. More details of the synthesis process are in the Supporting Information. ^{1}H NMR suggested the product was at least 90 % pure (Figure S7). Impurities were also not expected to significantly impact the conclusions of this experiment as only ^{15}N-NDMA was measured after wastewater was spiked with the ^{15}N-benzalkonium iodide, reacted, and then chloraminated. Impurities in the labeled benzalkonium iodide would need to follow the same reaction pathway to incorporate any ^{15}N atom from an impurity into the ^{15}N-NDMA structure and this was thought to be highly unlikely.

The second experiment examined the impact of microbial concentration on NDMA FP over a period of 24 h. A return activated sludge sample was collected from Facility 1 on the day of the experiment, with an initial volatile suspended solids (VSS) concentration of 5500 mg/L. volatile suspended solids was used as surrogate for microbial concentration. Further dilutions of the sample were made to 5000, 4000, 3000, 2000, and 1000 mg VSS/L using Milli-Q water containing mineral salts (salts used and concentrations are provided in Table S2). Mixed BACs were then spiked at 1.0 mg/L, and NDMA FP was measured over 24 h.

The third experiment examined whether varying microbial cultures/sources affected the production of NDMA precursors from BACs over a period of five days. Two samples were collected from aeration basins at Facilities 1 and 2 (980 and 1900 mg VSS/L, respectively). A third sample was prepared from the enriched community fed the mixed BAC stock for >30 days. The sample was prepared by mixing 10 ml of the enriched sample with 500 mL DI and mineral salts, resulting in a volatile suspended solids of 182 mg/L. Optical density at 600 nm was used as a surrogate for microbial growth in the enriched culture (Figure S8). The mixed BAC stock was spiked to 10 mg/L to all three communities, and changes in NDMA FP were measured in parallel over 120 h.

The fourth experiment examined the biodegradation of individual BACs (C_8, C_{10}, C_{12}, C_{14}, and C_{16}) in mixed liquor suspended solids samples collected from Facility 1. Each BAC was introduced separately at 1 mg/L. Samples were conducted in duplicate and collected over 120 h for further analysis including NDMA FP, BAC degradation, and NDMA precursor formation.

In the fifth experiment, we examined the potential formation of NDMA precursors in a bench-scale simulation of a WWTP. Hydraulic retention times were 6, 8, and 5 h, intended to simulate a primary clarifier, aeration tank, and a secondary clarifier, respectively. These values are at the upper end of typical retention times, (Davis, 2010) and were selected conservatively to estimate minimum NDMA precursor contribution, as preliminary experiments indicated that NDMA FP from BAC addition was transient, due to subsequent dealkylation of BDMA to lower yielding NDMA products. Samples taken from the primary clarifier of Facilities 1 and 2 were spiked with the mixed BAC stock to 1.0 mg/L and held with the with the headspace open to the room air, without agitation, to simulate an unaerated primary clarifier. NDMA FP was measured over 6 h. Following, suspended solids from a centrifuged sample of the aeration tank at the same facilities (collected during the initial 6 h period) were spiked. Samples were agitated with the headspace open to the room air to simulate activated sludge treatment. Periodically, DO was measured and exceeded 4 mg/L. Aliquots were taken at fixed intervals for 8 h and NDMA FP was measured. After 8 h, solids were allowed to settle for 5 h and supernatants were treated as secondary clarifier effluent samples and analyzed for NDMA FP. Each experiment was conducted in duplicate without pH adjustment (pH = 7.3 ± 0.5 throughout).

In the sixth experiment, we conducted an analysis for the presence of BDMA in filtered wastewater effluents obtained from Facilities 1, 2, and 3. The samples were collected from WWTPs on specific days when no rain events had occurred. Immediately after collection, containers holding filter effluents from Facilities 1 and 2 were surrounded by ice packs and transferred to a refrigerator at the UNR laboratory. For Facility 3, the filter effluent samples were shipped overnight to the UNR lab with ice packs and promptly placed in a refrigerator upon arrival to preserve the samples.

In each of the first five stated experiments, four control samples were conducted to accurately calculate NDMA FP from BAC biodegradation products within the collected wastewater samples. One of the controls was conducted in DI water and three in the collected mixed liquor suspended solids samples as follows: 1) DI control with BAC and chloramine additions to account for any NDMA FP that might be generated from BAC or its hydrolysis products, 2) wastewater control with no BAC and no chloramine to account for background NDMA concentration, 3)

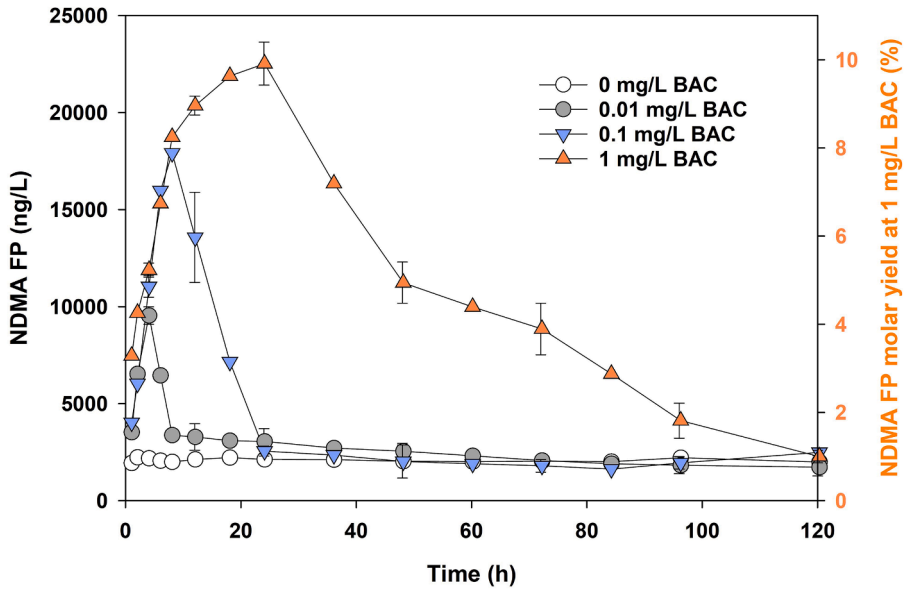

Fig. 1. Exp.# 1 - NDMA FP and calculated molar yield from varying initial BAC concentrations. mixed liquor suspended solids samples were collected from Facility 1 on the day of the experiment. Analysis was conducted at room temperature and at pH 7.3 ± 0.6, O_2 = 5.3 ± 0.7 mg/L and 1934 mg VSS/L. The right axis is relative to the spiked BAC concentration. Molar yield was determined using control subtracted NDMA FP. Error bars represent the standard deviation of triplicate samples.

wastewater control with no BAC but with chloramine to account for NDMA FP from NDMA precursors present in the collected mixed liquor suspended solids, 4) wastewater control with BAC and chloramine in a mixed liquor suspended solids sample treated by sodium azide addition (0.2% w/w) to account for BAC loss in the wastewater matrix in absence of viable microbes. The inhibited mixed liquor suspended solids control also determined if microbes are responsible in biodegradation of BAC and generation of NDMA precursors.

2.4. Analytical methods

40 mL samples were collected at fixed intervals and were centrifuged for 10 min at 22,360 g and filtered with 0.22 μm PTFE filters to remove microorganisms prior to NDMA FP experiments. Samples were then stored at 4 °C for a maximum of 3 days for further analysis. NDMA FP and monochloramine preparation were performed following previous published methods. (Zhang et al., 2016; Hanigan et al., 2012) NDMA FP was measured by adding preformed monochloramine at 18 mg/L as Cl_2 to samples diluted to a total volume of 500 mL (1:50 dilution) buffered with 10 mM borate to pH 8. The chloramine solution was prepared freshly before experiments by mixing 10 mM borate buffered (pH 8) aqueous ammonium chloride and sodium hypochlorite solutions at a Cl_2/N molar ratio of 0.8:1. pH 8 was used to simulate WWTPs in-situ preparation of chloramine and to allow for the predominant species to be monochloramine. Free chlorine from the sodium hypochlorite stock and monochloramine concentrations were standardized with a Hach DR 5000 spectrophotometer (Method 8021). (Hach 2017) After the addition of chloramines, samples were stored at room temperature for 72 h in the dark. Residual chloramines were checked and quenched at the end of the 72 h period using 5 mL of 0.5 M ascorbic acid. Dichloramine was not measured directly but is present in all chloramine solutions and is the reactive chloramine species in the formation of NDMA. (Huang, Huang, and McCurry, 2018; Pham, Wahman, and Fairey, 2021) DO was measured using LDO 101 Hach probe and HQ 40D Hach multimeter. The oxygen probe was calibrated using Winkler method. (Montgomery, Thom, and Cockburn, 1964)

NDMA was extracted from samples according to EPA Method 521. EPA 521 activated carbon solid phase extraction (SPE) cartridges (Restek Corporation, PA) were first conditioned with DCM, methanol and Milli-Q water. Prior to extraction, samples were spiked with 1 mL of 100 μg/L NDMA-d6. Samples were loaded to the cartridges at 5 mL/min by a Dionex Autotrace 280 SPE instrument (Thermo Fisher Scientific, Waltham, MA). Cartridges were dried with ultrahigh-purity nitrogen for 30 min and then eluted with 5 mL DCM. Eluates were dewatered with sodium sulfate drying cartridges (Santa Clara, CA, USA), and further concentrated to 1 mL under a gentle stream of ultrahigh-purity nitrogen at 40 °C. NDMA was quantified via gas chromatography tandem mass spectrometry (Shimadzu GC–MS/MS TQ8040) operated in electron impact (EI) mode, as described previously. (Song et al., 2023) The NDMA detection limit was estimated to be 1 ng/L based on a signal-to-noise ratio of 5. NDMA FP and BDMA molar yield were calculated using Eqs. (1) and (2), respectively, taking into account the appropriate dilution factors:

$$NDMA\ FP\ = (NDMA\ sample\ - NDMA\ controls) \quad (1)$$

$$BDMA\ molar\ yield\ (\%)\ = \left(\frac{NDMA\ FP}{NDMA\ MW} \div \frac{BDMA}{BDMA\ MW}\right) \quad (2)$$

In experiments where BACs and BDMA were expected to be present at high concentration (i.e., spiked BAC experiments, BDMA at ppb range), they were quantified without extraction with a Thermo Scientific Ultimate 3000 UPHLC coupled to a TSQ Vantage Triple Quadrupole Mass Spectrometer (LC-MS/MS). Separation was achieved with an Agilent Zorbax C18 column (2.1 × 50 mm × 1.8 μm). The column temperature was maintained at 30 °C with a mobile phase flow rate of 0.4 mL/min. BDMA, C_8-, C_{10}-, C_{12}-, and C_{14}-BACs were separated with a mobile phase consisting of acetonitrile (solvent A) and 10 mM aqueous ammonium formate (solvent B). The proportion of solvent A in the mobile phase was increased from 50 % to 70 % over 3 min, increased to 80 % at 5 min, to 90 % at 8 min, and to 100 % at 12.2 min and held till 14.9 min. Another mobile phase with methanol (solvent A) and 0.1 % aqueous formate (solvent B) was used to analyze C_{16}-BAC. Solvent A was increased from 50 % to 60 % over 3 min, to 70 % at 6 min, 80 % at 8 min, and 100 % at 12.2 min and held till 14.9 min. Analytes were ionized in ESI+ and product ions for each species were optimized using direct infusion at 1 mg/L into the MS at 5 μL/min. The two most abundant product ions from each analyte were selected for quantification. C_{12}-BAC was used before each run to tune the MS parameters including gas temperature, nozzle voltage, flowrate, nebulizer, and capillary voltage. Data were analyzed in Thermo Xcalibur QuanBrowser.

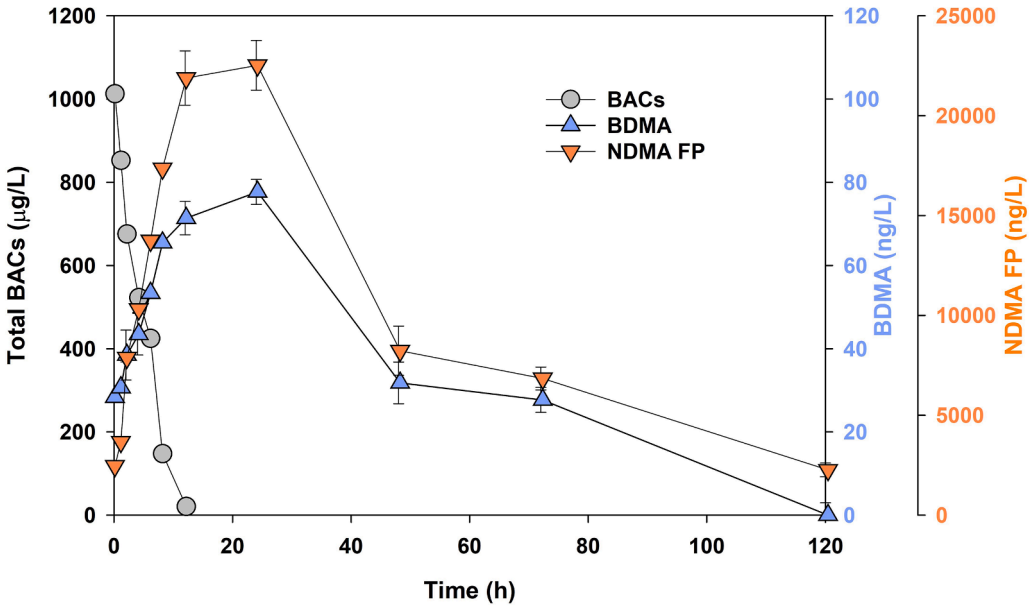

Fig. 2. Exp.# 1 - BAC degradation and formation of BDMA in samples taken from an aeration tank from Facility 1. Five BACs were measured and are reported as the sum of their mass concentrations regardless of each BAC molecular weight. Individual BAC concentrations are presented in Figure S9.

When BDMA was expected to be present at lower concentrations (i.e., experiment six, quantifications of BDMA in WWTP samples, BDMA at ppt range), it was extracted using an Dionex Autotrace 280 SPE instrument (Thermo Fisher Scientific, Waltham, MA) with weak ion exchange mixed-mode sorbent WCX Oasis cartridges (150 mg sorbent per cartridge, Waters Corporation, MA, USA). WCX cartridges were preconditioned with 5 ml of 2 % formic acid in methanol followed by 5 mL of Milli-Q water. 100 mL of samples were spiked to 100 ng/L of the internal standard diphenhydramine-d3, adjusted to pH 5.5 with sulfuric acid, and loaded to the cartridges at 0.5 mL/min, similar to Montes et al. (Montes et al., 2019) (note that in this reference the acid/base rinse/elution appear to be reversed, with the acid being used for elution rather than the base). Cartridges then were dried with nitrogen gas, and eluted with 6 mL of methanol mixed with 5 % ammonium hydroxide. Extracts were then packed and shipped at 4 °C to the University of Southern California for analysis using LC-MS/MS.

Wastewater BDMA extracts were analyzed on a triple quadrupole LC-MS/MS (Agilent 1260/6460). 20 µL injections were separated on an Agilent Zorbax C18 column (2.1 × 50 mm × 1.8 µm) with 0.3 mL/min of a mobile phase consisting of 0.1 % aqueous formic acid (solvent A) and pure methanol (solvent B). The HPLC was operated in gradient mode with an initial composition of 5 % solvent B for 2 min, increasing to 95 % at 7 min, followed by an isocratic hold at 95 % for two min, and finally decreasing to 5 % solvent B at 9.1 min. The ion source was operated in ESI+ mode with a gas temperature of 200 °C, gas flow of 8 LPM, nebulizer pressure of 45 psi, 8 LPM of sheath gas flow at 200 °C, and capillary voltage of 4000 V. Mass transitions and collision energies for BDMA, benzylmethylamine, benzylamine, diphenhydramine, and diphenhydramine-d3 are provided in Table S3. Data were analyzed in Agilent MassHunter software.

3. Results and discussion

3.1. Exp. #1: formation of NDMA from the biodegradation of BACs in aeration basins

To examine the potential for BACs to be biodegraded to NDMA precursors, a mixed liquor suspended solids sample from Facility 1 was spiked with the mixed BAC stock and agitated to provide oxygen to the microbial community, similar to average oxygen concentrations of the aeration basins. NDMA FP increased rapidly after dosing BAC, and maximum NDMA FP increased with increasing BAC dose (Fig. 1). At 1 mg/L BAC, NDMA FP peaked at 22,500 ± 1100 ng/L at 24 h, approximately ten-fold higher than in the two mixed liquor suspended solids controls in which NDMA FP was relatively consistent, averaging 2140 ± 321 ng/L throughout the experiment, consistent with values reported by others in activated sludge systems. (Mitch and Sedlak, 2004; S.W. Krasner et al., 2009) The microbial inhibited controls had concentrations similar to the other two controls in the presence of BAC and chloramines, with an average of 2044 ng/L, suggesting that additional NDMA precursors were not formed in the absence of bacterial activity and demonstrating that microbial activity in mixed liquor suspended solids is responsible for BAC biotransformation to NDMA precursors.

NDMA yield from samples supplemented with BAC was determined by subtracting NDMA FP from the two controls (not spiked with BAC), and peaked at 4, 8 and 24 h for 0.01, 0.1 and 1 mg/L, respectively. Peak yield was approximately 10 % (mol NDMA/mol spiked BAC). In addition, the later yield maxima (Fig. 1) at greater BAC concentrations (although relatively low and not expected to directly stimulate or inhibit substantial bacterial growth (Zhang et al., 2011)) suggests that BAC conversion may be limited by the amount of available biomass.

Peak NDMA FP concentrations in samples spiked with 1 mg/L of BAC were 2.4 times greater than from 0.01 mg/L of BAC, and 1.3 times greater than from 0.1 mg/L of BAC. The modest increase in NDMA FP despite a two order of magnitude increase in BAC concentration suggests that even low BAC concentrations in wastewater could lead to appreciable increases in NDMA FP. However, the observed NDMA FP was not proportional to the concentration of BACs added. This lack of proportionality is attributable to the constraints imposed by biomass concentration and the limitation of bacterial growth kinetics (see Exp.#3). NDMA FP rapidly decreased after 24 h, declining to <18 % of the peak concentration by the end of the experiment, due to further dealkylation of BDMA to products with lower NDMA yield (e.g., benzylmethylamine, DMA). (Patrauchan and Oriel, 2003; Tezel et al., 2012) NDMA FP declined more slowly in samples spiked with higher BAC concentrations, and cumulative NDMA FP (integral of NDMA FP vs time) production was somewhat proportional to the amount of spiked BAC (Table S4).

These findings indicate that reaction time plays a pivotal role in the formation of BDMA through the biodegradation of BACs in the mixed liquor suspended solids samples collected. However, it is imperative to

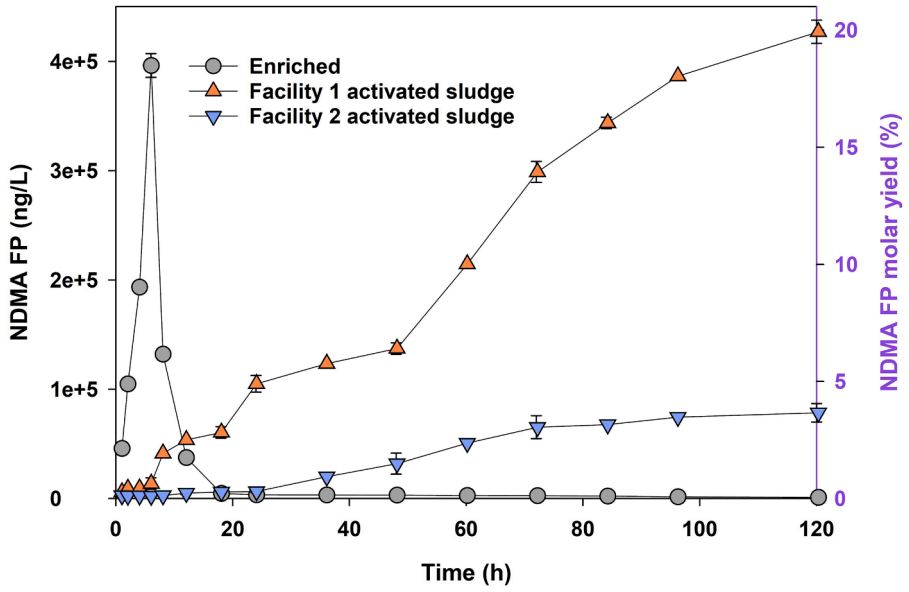

Fig. 3. Exp.# 2 - NDMA FP and control subtracted yield resulting from BAC degradation by different microbial cultures. Analysis was conducted at room temperature and at pH 7.1 ± 0.9 and O_2 = 5.3 ± 0.3 mg/L. Error bars represent the standard deviation of triplicate samples. The NDMA FP molar yield values for the three data sets are presented on the secondary axis.

clarify that the term 'reaction time' as used herein does not equate to solids retention time (SRT) in the activated sludge treatment unit because sludge sorption of BAC is imperfect. (Ismail, Tezel, and Pavlostathis, 2010)

To evaluate our hypothesis that BDMA is the principal NDMA precursor produced by BAC degradation, we again dosed mixed liquor suspended solids samples from Facility 1 with 1 mg/L mixed BACs and measured BAC decay, BDMA, and NDMA FP. LC-MS/MS analysis of 1 mg/L of the BAC mixture stock indicated the presence of five individual BACs with varying alkyl chain lengths: C_8, C_{10}, C_{12}, C_{14}, and C_{16} at concentrations of 147, 235, 323, 212 and 96 μg/L, respectively. Within 12 h of being spiked to the aeration basin samples, all five BACs were no longer detectable (Fig. 2, Figure S9). No clear relationship between alkyl chain length and degradation rate was observed. The least abundant BAC in the stock (C_{16}) was completely degraded within 4 h, whereas the most abundant (C_{12}) remained detectable until 12 h after dosing.

In the same samples, the concentration of BDMA rapidly increased after BAC was spiked and peaked at 78 ng/L after 24 h (Fig. 2). BDMA was not detected in either of the two control samples collected throughout the experimental period. Subsequently, BDMA was degraded and was eliminated within 120 h. The increase and decrease in BDMA concentration over time closely follows that of NDMA FP, consistent with BDMA serving as the principal NDMA precursor produced by BAC degradation. NDMA yields from BDMA (calculated from blank corrected NDMA FP and BDMA measurements) ranged from 16 to 55 % (Figure S10), consistent with prior experiments in buffered deionized water reporting molar yields of NDMA from BDMA of 20 – 84 %. (Selbes et al., 2013; Huang, Huang, and McCurry, 2018; Spahr et al., 2017) Efficient conversion of BDMA to NDMA during chloramination of wastewater suggests even trace amounts of BDMA (e.g., low-to-modest ng/L levels) might significantly contribute to the NDMA precursor pool in aerobically treated wastewater containing BACs. For example, 1.8 μg/L of BAC would produce 100 ng/L NDMA, assuming the biodegradation and subsequent chloramination of the product results in a 25 % yield, and BACs are typically present in wastewater at concentrations in the μg/L to mg/L range. (Kümmerer et al., 1997; Wieck, Olsson, and Kümmerer, 2018; Kim et al., 2020; Kreuzinger et al., 2007)

Direct correlation between BAC lost, BDMA formation, and NDMA FP could not be determined from this experiment because a mixture of five unlabeled BACs was used as the BAC source, and BDMA is formed from all of the BACs, independent of alkyl chain length (See Exp #4 and associated Figure S13). However, in one additional experiment to demonstrate the direct relationship between BAC biodegradation and NDMA FP, we synthesized C_8 ^{15}N-benzalkonium iodide (^{15}N-BAI). ^{15}N-BAI was spiked at a concentration of 1 mg/L to mixed liquor suspended solids from Facility 1 and ^{15}N-NDMA FP was measured over 48-hours after chloramination. ^{15}N-NDMA FP results exhibited the same trend as the NDMA FP from the biodegradation of the mixed BAC stock (Figure S11), although at substantially lower NDMA FP concentrations/yields. Reduced formation of NDMA can be attributed to fast consumption of chloramines by iodide (~0.1 mg/L, 2.4 x 10^3 M^{-1} s^{-1}) (Bichsel and Von Gunten, 1999), forming hypoiodous acid (HOI), which reacts with organic compounds much faster (up to 1 x 10^6 M^{-1} s^{-1}) (Rose and Roberts, 2019; Bichsel and von Gunten, 2000; Zhao, Ma, and von Gunten, 2017) than the dichloramine reaction with NDMA precursors (0.01 – 0.09 M^{-1} s^{-1}) (Zhang et al., 2016), thus reducing overall NDMA formation. While the iodine could have been ion exchanged for chlorine and the experiment repeated, we found these results satisfactory in demonstrating that the source of NDMA formed is from biodegraded benzalkonium, and, together, these multiple lines of evidence clearly indicate that BAC biodegradation occurs in mixed liquor suspended solids, leading to the formation of the NDMA precursor BDMA.

3.2. *Exp. #2: impact of biomass source and concentration on NDMA FP*

To assess BAC biodegradation and NDMA precursor formation across different microbial communities/sources, NDMA FP was measured in samples from aeration tanks in Facilities 1 and 2, along with Milli-Q water containing an enriched microbial community. All samples, spiked with a higher concentration of mixed BAC stock (10 mg/L), were subjected to analysis. The elevated BAC concentration aimed to facilitate NDMA FP assessment, as enriched microbes rapidly consumed reactive products in prior experiments, making it challenging to measure NDMA FP at lower BAC concentrations.

After 120 h, NDMA FP was 6.3 times higher in the sample from Facility 1 compared to Facility 2 (Fig. 3), attributable to the higher volatile suspended solids concentration in Facility 1 (1990 mg VSS/L, compared to Facility 2's 980 mg VSS/L). Consistent with previous observations, reduced BAC degradation in wastewater with lower biomass concentrations aligns with the understanding that degradation rates are

Table 1
Observed rate constants for the degradation of BACs, and observed formation rates of BDMA, and NDMA FP upon exposure to mixed liquor suspended solids.

BAC	k_{BAC} (h^{-1})	$t_{1/2}$ (min)	R^2	R_{BDMA} (μg/h)	R^2	$R_{NDMA\ FP}$ (ng/h)	R^2	Average NDMA yield (%)
C_8	0.223 ± 0.053	19.3 ± 4.6	0.943	5.5 ± 0.5	0.928	1177 ± 111	0.999	39.1
C_{10}	0.226 ± 0.018	19.6 ± 1.6	0.923	5.3 ± 0.1	0.928	1130 ± 68	0.994	38.9
C_{12}	0.263 ± 0.088	22.8 ± 7.6	0.921	5.4 ± 0.1	0.866	959 ± 107	0.975	32.4
C_{14}	0.228 ± 0.023	19.7 ± 2.0	0.913	5.3 ± 0.6	0.989	1059 ± 96	0.96	36.5
C_{16}	0.234 ± 0.035	20.3 ± 3.0	0.829	6.3 ± 0.2	0.954	912 ± 26	0.976	26.4

a. k: degradation rate constant, R: formation rate constant.
b. Conditions: activated sludge from Facility 1, VSS = 1943 mg VSS/L, pH = 7.1 ± 0.24, Temperature = 20 °C, O_2 = 4.68 ± 0.8 mg/L.
c. Formation rate constants were calculated using the data points prior to any observed degradation for BDMA and NDMA FP.
d. k_{BAC} was calculated from first order linear regression of the individual logarithmic BAC degradation kinetic points, where R_{BDMA} and $R_{NDMA\ FP}$ were calculated from linear regression of the individual formation kinetics points for each BAC separately.
e. Control NDMA FP was subtracted when calculated R for NDMA.
f. Rate constants show the average value and 95 % confidence intervals from linear regression of the individual logarithmic kinetic points.

influenced by biomass abundance. NDMA FP consistently increased over 120 h in both aeration tank samples, possibly due to the 10 mg/L BAC dose chosen. In contrast, production of NDMA FP by the BAC-enriched community (VSS = 182 mg/L) occurred at a much faster rate, peaking after 6 h and disappearing after 14 h, demonstrating that organisms acclimated to BAC exhibit more rapid degradation.

3.3. Exp. #3: impact of bacteria concentration on NDMA FP

To further demonstrate that transformation of BACs to NDMA precursors is biomass-limited under typical activated sludge conditions, we measured the production of NDMA FP from 1 mg/L BAC at a range of volatile suspended solids concentrations (1000 - 5000 mg VSS/L) (Figure S12). NDMA FP formation after 24 h was 44 % lower at 1000 mg VSS/L than 2000 mg VSS/L, however, NDMA FP continued to form over the next three days in the 1000 mg VSS/L sample, reaching similar values to those of 2000 mg VSS/L, while the latter significantly decreased after reaching its initial peak after 24 h. At higher biomass concentrations (3000 and 4000 mg VSS/L), NDMA FP peaked quickly, 4 h post BAC addition, then declined. These results highlight that biomass concentration is a major contributor and a critical parameter to control NDMA FP from BACs biodegradation in activated sludge. The cumulative amount of NDMA precursors formed (area under NDMA FP vs time curve [Table S5]) were again similar between biomass concentrations that resulted in a complete NDMA FP curve (head and tail near zero) further demonstrating the rate limitation of biomass concentration.

3.4. Exp. #4: biodegradation of individual BACs and formation rates of BDMA and NDMA

To evaluate whether BAC alkyl chain length affects BAC conversion to BDMA and NDMA FP, five individual BACs (C_8, C_{10}, C_{12}, C_{14}, and C_{16}) were added at 1 mg/L each separately to a mixed liquor suspended solids sample from Facility 1. Similar NDMA FP formation was observed for all BACs and peaked at 24 h at an average of 24,100 ± 1300 ng/L, followed by a rapid decline (Figure S13). Similar trends were observed for BDMA formation (Figure S14), consistent with BDMA serving as the principal NDMA precursor among metabolites of each BAC. Calculated degradation half-lives of each BAC and formation rates of NDMA and BDMA were similar (Table 1). The degradation of the five BACs were first-order relative to BAC concentration, with observed rate constants ranging from 0.223 to 0.263 h^{-1}, and an average half-life of 20.3 ± 1.3 min. NDMA FP and BDMA formation from BAC appeared zeroth-order, potentially due to formation of intermediate(s) between BACs and BDMA, or reflecting the difference in microbial kinetics between BAC dealkylation forming BMDA, and BDMA debenzylation. The formation rate of BDMA averaged 5.6 ± 0.4 μg/h and yielded NDMA at an average of 1048 ± 100 ng/h, with NDMA molar yields from 26.4 to 39%.

Similar BAC degradation and BDMA formation rates among all the BACs evaluated suggests that BAC alkyl chain length does not affect BDMA and NDMA FP production, and that dealkylation occurs at the C—N bond between the quaternary nitrogen and first alkyl methylene of the BAC. While the rates reported in Table 1 are dependent on the microbial concentration during the experiment, and thus we expect different rates at different microbial concentrations, the ~2000 mg VSS/L used in this experiment is within the typical range in wastewater treatment aeration basins.

3.5. Exp. #5: formation of BDMA and NDMA FP in a bench-scale simulated wastewater treatment plant

In a fifth experiment to assess the fate of BACs within typical wastewater treatment plants, as well as validate the production of BDMA from BAC biodegradation in our bench-scale experiments, we measured NDMA FP and BDMA from BACs across a bench-scale treatment sequence intending to simulate primary clarification, aeration, and settling using spiked biomass from Facilities 1 and 2 (Figure S15). 1 mg/L of the mixed BAC solution was spiked to a sample taken from the primary clarifiers and produced 13,300 ng/L and 4100 ng/L of NDMA FP during the hold period, for samples from Facility 1 and 2, respectively. Volatile suspended solids concentration in the primary clarifier were slightly greater at Facility 1 (285 mg VSS/L) compared to Facility 2 (211 mg VSS/L) explaining the greater precursor formation. After spiking additional biomass collected from the aeration tanks to make volatile suspended solids concentrations of 2023 mg/L and 1124 mg/L in Facility 1 and Facility 2 samples, respectively, NDMA FP reached its maximum value of 27,200 ng/L and 16,600 ng/L in the same samples. Following the removal of the volatile suspended solids through quiescent sedimentation, NDMA FP was relatively unchanged in samples representing both facilities, indicating no further biodegradation occurred at low microbial abundance. NDMA precursor formation was consistent with BDMA formation across all of the simulated treatment processes, and demonstrated that activated sludge and bacterial concentrations are the key steps during wastewater treatment leading to BDMA and NDMA FP formation from BACs.

3.6. Exp. #6: BDMA in treated wastewater effluents

Our bench-scale experiments demonstrated that BDMA is a byproduct formed from BAC degradation in aeration tanks, even at low BAC concentrations. Because BACs have been detected in wastewater at various concentrations, we expected BDMA to also be present in full-scale treated wastewater effluents, potentially explaining a portion of NDMA formation during disinfection of wastewater effluent. The experiments conducted above (Fig. 2) indicate that in microbe-free environments (after filtration of mixed liquor suspended solids for BDMA analysis), BDMA exhibits high stability and does not undergo biodegradation. This is in contrast to scenarios with high microbial activity, as observed in mixed liquor suspended solids samples, where BDMA experienced an average biodegradation of 41.6 % within 24 h

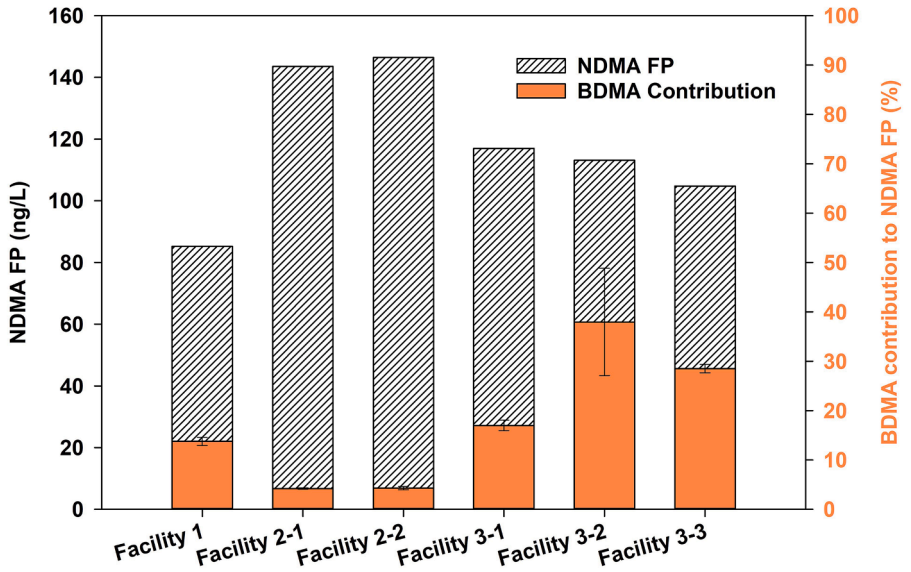

Fig. 4. BDMA concentrations and NDMA FP in filter effluent samples (prior to disinfection) from three facilities on multiple occasions. X-axis represent number of facility followed by a numerical representing the replication.

(Figure S14). Therefore, it is anticipated that if BDMA can endure in secondary clarifiers until it reaches filter effluents, where microbial concentrations are low, BDMA will remain available during the disinfection process at WWTPs and contribute to NDMA FP. To investigate the occurrence of BDMA and its impact on NDMA FP in actual wastewater effluents, we conducted two tests. The first test aimed to establish the NDMA molar yield by introducing spiked BDMA into the effluent from dual media filters collected from Facility 2. In the second test, we measured the concentrations of BDMA and NDMA FP in filter effluents collected from Facilities 1, 2, and 3.

To estimate the contribution of BDMA to NDMA FP, and the NDMA yield from BDMA in filter effluent (as opposed to prior studies in buffered deionized water) was measured. NDMA FP was measured after spiking a range (0 to 1000 ng/L) of BDMA in the filter effluent from Facility 2. The in-matrix NDMA molar yield from BDMA in filter effluent, calculated from the slope of NDMA FP with respect to spiked BDMA levels, was 73.5 % (Figure S16), consistent with prior experiments in clean systems, and indicating efficient conversion to NDMA even in the presence of potentially interfering compounds in the matrix including dissolved organic matter, naturally present ions (nitrate, bromide, iodide, etc.) and emerging contaminants (i.e., pharmaceuticals).

Measured BDMA concentrations were compared to NDMA FP in the effluents of the three sampled facilities, including two separate sampling events at Facility 2 and three events at Facility 3, to account for potential temporal variability. BDMA concentrations ranged from 15 to 106 ng/L, and NDMA FP was ~100 ng/L at each facility (Fig. 4). Facility 2 effluent contained the lowest BDMA concentration at an average of 15 ng/L across two sampling events, however, its average NDMA FP was the greatest among all samples collected (145 ng/L). Estimated contributions of BDMA to NDMA FP, produced by multiplying measured BDMA concentrations by the measured in-matrix NDMA yield, were 14 % at Facility 1, approximately 4 % at Facility 2 in both sampling events, and ranged from 17 to 38 % at Facility 3. These results suggest that BDMA, likely produced by bacterial dealkylation of BACs in sewage, can serve as a significant NDMA precursor in wastewater effluents. Because BDMA is likely to be reacted during wastewater disinfection and is relatively biodegradable under aerobic conditions, it is unlikely to persist in the environment and contribute to downstream drinking water NDMA precursor loading. Thus, wastewater and drinking water precursors may be fundamentally different and extrapolating the results from experiments conducted using wastewater-derived NDMA precursors to other water sources should be done with caution.

CRediT authorship contribution statement

Ibrahim Abusallout: Writing – review & editing, Writing – original draft, Visualization, Validation, Project administration, Methodology, Investigation, Formal analysis, Conceptualization. **Mingrui Song:** Investigation, Data curation. **Aron Chan:** Investigation. **Elizabeth McKenna:** Investigation. **Jean Van Buren:** Methodology, Investigation. **Seth Mccoy:** Investigation. **Zachary Ledvina:** Investigation. **Christopher Jeffrey:** Investigation. **Daniel L. McCurry:** Writing – review & editing, Supervision, Funding acquisition, Conceptualization. **David Hanigan:** Writing – review & editing, Validation, Supervision, Resources, Project administration, Funding acquisition, Data curation, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.watres.2024.121945.

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