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Application of effect-directed analysis to identify mutagenic nitrogenous disinfection by-products of advanced oxidation drinking water treatment

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Abstract Advanced oxidation processes are important barriers for organic micropollutants in (drinking) water treatment. It is however known that medium pressure UV/H_2O_2 treatment may lead to mutagenicity in the Ames test, which is no longer present after granulated activated carbon (GAC) filtration. Many nitrogen-containing disinfection by-products (N-DBPs) result from the reaction of photolysis products of nitrate with (photolysis products of) natural organic material (NOM) during medium pressure UV treatment of water. Identification of the N-DBPs and the application of effect-directed analysis to combine chemical screening results with biological activity would provide more insight into the relation of specific N-DBPs with the observed mutagenicity and was the subject of this study. To this end, fractions of medium pressure UV-treated and untreated water extracts were prepared using preparative HPLC and tested using the Ames fluctuation test. In addition, high-resolution mass spectrometry was performed on all fractions to assess the presence of N-DBPs. Based on toxicity data and read across analysis, we could identify five N-DBPs that are potentially genotoxic and were present in relatively high concentrations in the fractions in which mutagenicity was observed. The results of this study offer opportunities to further evaluate the identity and potential health concern of N-DBPs formed during advanced oxidation UV drinking water treatment.

Keywords Disinfection by-products · Advanced oxidation processes · Medium pressure ultraviolet technology · Nitrate photolysis · Effect-directed analysis · Fractionation · High-resolution mass spectrometry · Ames fluctuation test

Introduction

Advanced oxidation processes serve the dual purpose of disinfection of drinking water and removal of chemical contaminants present in source water (Homem and Santos 2011; Yang et al. 2013). In light of an increasing emission and detection of organic micropollutants in the aquatic environment (Fawell 2012; Richardson and Kimura 2016), these technologies are valuable tools to sustain the production of drinking water of high quality. Advanced oxidation processes are however known to produce potentially harmful disinfection by-products (DBPs) (Heringa et al. 2011; Hofman-Caris et al. 2015; Hughes 2013; Martijn and Kruithof 2012; Richardson and Kimura 2016). Many efforts are made to determine the formation, identities, occurrence, and behavior during drinking water treatment, and human health risks of DBPs (Grellier et al. 2015; Richardson et al. 2007). Such research is challenging, though, due to the vast number of DBPs that can result from each combination of source water and disinfection method(s) under various process conditions and the lack of

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toxicity data. The identities and potential health risks of DBPs are therefore only partly known.

Nitrogenous DBPs (N-DBPs) constitute a recently identified subset of DBPs that have been reported to be of higher human health relevance than the long known chlorinated DBPs (Bond et al. 2011; Kundu et al. 2004; Richardson et al. 2007; Shah and Mitch 2012). Earlier research, in which we used an innovative approach combining stable isotope-labeled nitrate with high-resolution mass spectrometry, has shown that medium pressure (MP) UV treatment of artificial water containing natural organic matter (NOM) and nitrate causes formation of multiple nitrogen containing substances (Kolkman et al. 2015). A total of 84 N-DBPs were detected, of which the chemical identities of three were confirmed. Screening for the 84 N-DBPs in water samples from a full-scale drinking water treatment plant based on MP UV/H_2O_2 treatment led to the detection of 22 of the N-DBPs. The magnitude of the chemical screening results and the responses observed in mutagenicity testing of the full-scale water samples was comparable. It was concluded that the 22 N-DBPs may (partly) be responsible for the positive response that has previously been observed for concentrated extracts of treated water in the Ames fluctuation test using *Salmonella* strains TA98 and TA100 (Heringa et al. 2011; Hofman-Caris et al. 2015; Kolkman et al. 2015). After GAC filtration, this genotoxic response is no longer present in the water. Substance-specific health risk assessment of DBPs can only be performed when both identities and toxic potencies are known. The aim of the present study was to (i) further identify the N-DBPs formed during MP UV water treatment and evaluate their genotoxic potential and (ii) indicate which of the N-DBPs may contribute to the mutagenic response observed after MP UV water treatment.

To address the second goal, fractionation of the water extract by preparative chromatography was conducted to reduce the complexity of the mixture of DBPs that results from MP UV treatment. Next, chemical screening of the previously detected N-DBPs was performed in each fraction using high-resolution mass spectrometry, and mutagenicity testing using *Salmonella* strains TA98 and TA100, similar to earlier research, was used to identify the fractions of interest. Combination of the results of these analyses allows the identification of potentially mutagenic N-DBPs. This methodology is known as effect-directed analysis (EDA) (Brack 2003). There is increasing interest in the application of EDA for water quality monitoring, since this approach offers opportunities to reveal and prioritize micropollutants with potential health impact that are currently not included in monitoring programs (Brack et al. 2016). A number of studies have been published in which mutagenic water contaminants were identified by integrating chemical screening and the Ames mutagenicity test (Gallampois et al. 2013, 2015; Higley et al. 2012; Liu et al. 2015).

In order to elaborate on the previous research results (Kolkman et al. 2015), artificial water was used in the current experiments. Fractions of untreated and treated water extracts were prepared using preparative HPLC based on polarity and analyzed by both Orbitrap mass spectrometry and the Ames fluctuation test. Using this approach, five potentially genotoxic N-DBPs were found to be present in relatively high concentrations in the fractions in which mutagenicity was observed. This study shows that EDA can be applied to discover the source of mutagenicity observed after application of advanced oxidation processes in drinking water treatment and serves as a next step in the identification of mutagenic N-DPBs.

Material and methods

Chemicals

Pony Lake NOM was obtained from the International Humic Substances Society. Potassium nitrate (KNO_3) was obtained from Sigma-Aldrich. All solvents used were of analytical grade quality. Methanol (ultra resi-analyzed) and acetonitrile (ultra-gradient HPLC grade) were purchased from Avantor Performance Materials B.V. (Deventer, the Netherlands). Solid phase extraction (SPE) columns (200 mg Oasis HLB 6-mL glass cartridges) were supplied by Waters (Milford, MA, USA). Dimethyl-sulfoxide (DMSO) (99.9 %) was obtained from Acros Organics (Geel, Belgium). The N-DBPs were purchased from Sigma-Aldrich (Steinheim, Germany), Santa Cruz Biotechnology (Dallas, TX, USA), TCI Europe (Zwijndrecht, Belgium), Vitas-M laboratory (Moscow, Russia), Matrix Scientific (Columbia, SC, USA), and Ryan Scientific (Mount Pleasant, SC, USA) (see Electronic Supplementary Material Table SIV for the supplier list for the N-DBPs). The internal standards atrazine-d_5 and bentazone-d_6 were purchased from CDN isotopes (Pointe-Claire, Canada) and LGC Standards (Wesen, Germany), respectively. Ultrapure water was obtained from a Milli-Q system (Millipore, Bedford, MA).

Ames fluctuation test bacterial strains, culture media, and S9 from phenobarbital/ β-naphtoflavone-exposed rats were purchased from Xenometrix GmbH (Allschwil, Switzerland). Histidine, Nutrient Broth No. 2 Oxoid, 2-aminoanthracene (2-AA), $MgCl_2{\cdot}6H_2O$, $NaH_2PO_4{\cdot}H_2O$, and $Na_2HPO_4{\cdot}2H_2O$ were obtained in analytical grade from Boom (Meppel, the Netherlands). NaCl and KCl were purchased from Avantor Performance Materials B.V. (Deventer, the Netherlands). 4-Nitro-*o*-phenylenediamine (4-NOPD), 4-nitroquinoline (4-NQO), nitrofurantoin (NF), D-glucose-6-phosphate, NADP, and ampicillin were purchased from Sigma-Aldrich (Zwijndrecht, the Netherlands). The 24- and 96-well plates were obtained from

Greiner Bio-one (Alphen a/d/ Rijn, the Netherlands) and the Corning 384-well plates from Sigma-Aldrich.

Identification of N-DBPs

LC-QToF mass spectrometric analysis

In earlier research (Kolkman et al. 2015), 84 N-DBPs were detected of which the identities of three by-products were confirmed and five compounds were identified tentatively using an LTQ-Orbitrap mass spectrometer (Thermo Fisher Scientific, Bremen, Germany). In order to identify additional N-DBPs and to confirm the tentatively identified compounds, a LC-QToF mass spectrometric analysis was employed. First, samples of the original extracts from the aforementioned study were reanalyzed using the QToF mass spectrometer, to obtain MS/MS spectra with an increased number of fragments from the unidentified N-DBPs. Subsequently, potential candidates of the N-DBPs were proposed using the software tools PeakView (AB SCIEX) and MetFrag (Wolf et al. 2010). Reference chemicals of potential candidates were acquired when a suitable supplier was found. Individual stock solutions for these reference compounds were prepared in methanol at a concentration of 100 mg/L and diluted to 0.5 mg/L, and these solutions were analyzed using a LC-QToF. Candidates were confirmed by matching MS spectra, MS/MS spectra, and retention time of the reference standard with the candidate (level 1 identification according to (Schymanski et al. 2014).

The LC system consisted of a LC-30AD binary gradient pump, SIL-30AC auto sampler and a CTO-20AC column oven (Shimadzu Corporation, Kyoto, Japan). The chromatographic separation was performed on a Xbridge C18 XP column (2.1 × 150 mm, 2.5 μm, Waters Corp.). The gradient started with 5 % acetonitrile, 95 % water, and 0.05 % formic acid (*v*/*v*/*v*), increased to 100 % acetonitrile with 0.05 % formic acid in 40 min, and subsequently was held constant for 5 min. The flow rate was 0.3 mL/min, and the column temperature was 21 °C. Ten microliters of sample was used for injection. Detection was performed on a QToF mass spectrometer operated in both positive and negative electrospray ionization (ESI) mode using a DuoSpray ion source (TripleTOF 5600+, AB SCIEX, Concord, Canada). Full scan accurate MS and MS/MS mass spectra were recorded from 100 to 1000 Da and 40 to 1000 Da, respectively, with a resolving power of 30,000 FWHM (at m/z 400). The TurboIonSpray heater was held at 500 °C. The Ion Spray Voltage was set at 5.0 kV and 3.0 kV for the positive and negative ionization mode, respectively. Curtain gas, Gas 1 and Gas 2 were set at 25, 40, and 50 psi, respectively. Fragmentation (MS/MS) was performed by data-dependent acquisition using an inclusion list for the N-DBPs. Eight MS/MS scans of 50 ms per cycle with an collision energy of 20, 35, and 50 eV (spectra were averaged) were recorded. When no N-DBP from the inclusion list was detected, then the most intense ion was fragmented.

Toxicity evaluation

Open-access toxicological (meta)databases were consulted for evaluation of mutagenic potency and other hazardous properties of the identified N-DBPs. Toxicological data were retrieved from International Toxicity Estimates for Risk (ITER) (TERA 2010), Hazardous Substances Data Bank (HSDB) (NLM 2011), Integrated Risk Information System (IRIS) (EPA 2016), registration dossiers of the European Chemicals Agency (ECHA 2015), and the Dutch National Institute for Public Health and the Environment (RIVM 2015). Classifications for carcinogenic properties were derived from International Agency for Research on Cancer monographs (IARC 2016) and the Ministry of Social Affairs and Employment of the Netherlands (SZW) list of carcinogenic, mutagenic, and reprotoxic substances (SZW 2016). Besides, the OECD QSAR Toolbox (OECD 2013) was applied to retrieve measured data from in vitro mutagenicity (Ames gene mutation and micronucleus assay), in vivo mutagenicity (Comet assay), and carcinogenicity studies in rats and mice, if available, and to identify structural alerts that indicate potential mutagenic, genotoxic, and/or carcinogenic properties of substances by read across.

Effect-directed analysis

Experimental design

The experimental part of the effect-directed analysis is presented in Fig. 1. Both an untreated and MP UV-treated artificial water sample (i.e., reconstituted water containing NOM and nitrate) were concentrated by SPE (see below). The extracts were then split: Part of the extract was directly tested in the Ames fluctuation test and used to prepare a dilution series that was tested in the Ames test as well. Although fractionation will result in the presence of specific subsets of substances in each fraction, rather than a lower concentration of all components, the dilution series was used to indicate whether an Ames test response above the detection limit could still be expected after fractionation and to decide on the feasible fraction size. The other part of the extract was fractionated followed by testing all fractions using the Ames fluctuation test. Finally, target screening using mass spectrometry was performed in both the unfractionated extract and all fractions.

Preparation of artificial water

Forty liters of artificial water was prepared in a stainless steel container and consisted of ultrapure water, Pony Lake NOM (2.5 mg/L C), and nitrate (10.4 mg/L), which was quantified

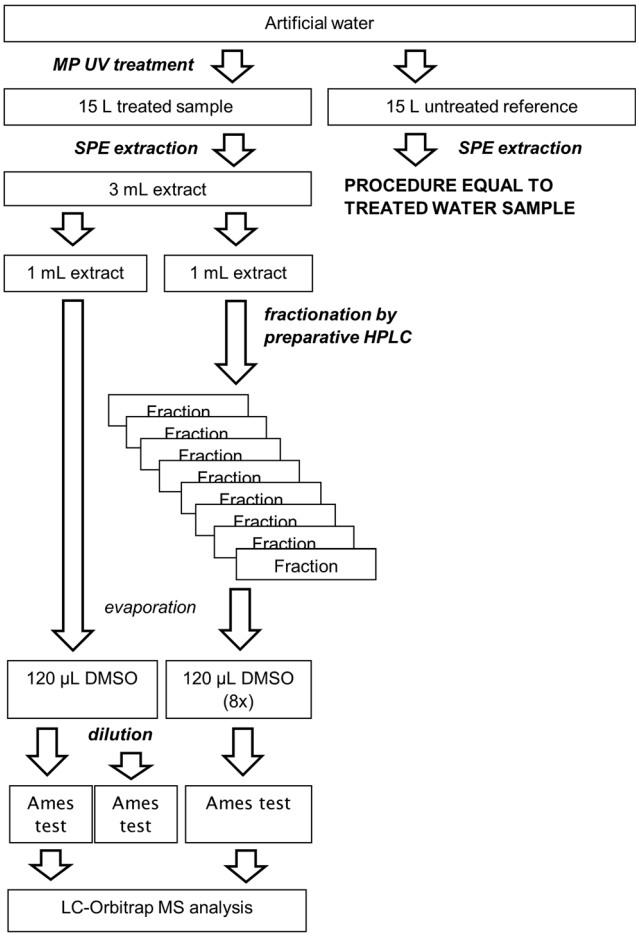

Fig. 1 Schematic representation of the experimental design of the effect-directed analysis

by dissolved organic carbon (DOC) and nitrate analysis. The artificial water was filtered using a 12–25-μm filter for the removal of undissolved particles and homogenized by stirring extensively for 4 h. After the homogenization, 30 samples were collected in 1-L glass bottles and stored at 1–5 °C until MP UV treatment and/or sample pretreatment.

MP UV treatment

Fifteen liters of artificial water was treated with MP UV using a collimated beam (CB) setup. Portions of 55 mL were treated in a 60 × 35-mm crystallizing dish using a CB apparatus equipped with a 3-kW medium pressure Hg lamp. The UV dose delivered to the solution was calculated according to Bolton and Linden 2003. UV intensity was measured using a radiometer with an unfiltered sensor (International Light Inc. (IL 2000)). The irradiation path length was 19.5 mm.

SPE

Pretreatment of 15 × 1 L of untreated artificial water and 15 × 1 L of MP UV-treated artificial water was performed using solid phase extraction (SPE). For the sample pretreatment, 1 L of sample was acidified to pH 2.3 using HCl and loaded on a 200-mg OASIS HLB glass SPE cartridge (Milford, MA, USA). After loading the sample, the SPE column was dried for 1 h by air. Elution was performed with 7.5 mL of 8:2 (*v*/*v*) acetonitrile/methanol. The eluate was evaporated (60 °C) under a gentle stream of nitrogen until a volume of 500 μL was reached. Next, all the MP UV-untreated extracts were pooled, as well as the MP UV-treated extracts. Two extracts (untreated and MP UV-treated) of each 7.5 mL remained. In both extracts, precipitation was visible. In order to dissolve this precipitation, additional methanol was added to a volume of 15 mL. The samples were then evaporated (60 °C) again using nitrogen to a volume of 3 mL (methanol), which resulted in clear extracts. A blank of 15 mL methanol that was evaporated to 3 mL was prepared as well to include in the Ames test analysis. One milliliter of both SPE extracts were evaporated (60 °C) under a gentle stream of nitrogen to a volume of approximately 100 μL. Then, 120 μL of DMSO was added as a keeper, and the remaining acetonitrile was evaporated under a gentle stream of nitrogen at 65 °C for 10 min. DMSO volume was subsequently corrected by replenishing to 120 μL by weight. The remaining extracts and methanol blank were stored at −25 °C until fractionation, and the Ames fluctuation test was performed. An overview of final concentration factors and performed analyses per untreated and MP UV-treated sample is provided in Electronic Supplementary Material Tables SII and SIII, respectively.

Fractionation

Fractionation of the SPE extracts was performed using a HPLC UV system, which consisted of a ASPEC XL autosampler and a 202 fraction collector (Gilson), a 2996 photodiode Array Detector, and a 600 HPLC pump (Waters). The chromatographic separation was performed on an XSelect CSH C18 Prep Column (130 Å, 5 μm, 10 mm × 250 mm, Waters). The gradient started with 10 % acetonitrile, 90 % water, and 0.05 % formic acid (*v*/*v*/*v*), increased to 100 % acetonitrile with 0.05 % formic acid in 80 min, and subsequently was held constant for 20 min. The flow rate was 1 mL/min, and the column temperature was 30 °C. The fractionation was conducted for 1-mL untreated and 1-mL MP UV-treated extract. To prevent potential overloading, only 200 μL of extract was injected each time. After sample injection, eight fractions with a fraction range of 10 min and a fraction volume of 10 mL were collected. The first 10 min was not collected. Each extract was injected five times, and the resulting fractions from each injection (fractions 1 from injections 1, 2, 3, 4, and 5; fractions 2 from injections 1, 2, 3, 4, and 5; and so on) were combined which resulted in a total volume of 50 mL per fraction.

After fractionation, the collected fractions consisted mainly of mobile phase of which the composition depends on the gradient and the fraction range. For the Ames test and Orbitrap analysis, the fractions needed to be reconstituted in 120 μL DMSO. Due to the high percentage of (ultrapure) water in the fractions, evaporation with nitrogen was not feasible. Because acetonitrile forms an azeotrope with water at 15 % water (or lower) in acetonitrile at atmospheric pressure, the water could be removed with a distillation setup. The water was removed as follows: 250 mL of acetonitrile was added to 50 mL of each fraction. Then, the sample was concentrated to 50 mL by distillation. Subsequently, 100 mL of acetonitrile was added to the sample, which was distilled again until 5 mL of acetonitrile remained. Next, the samples were evaporated (60 °C) under a gentle stream of nitrogen, reconstituted in 125 μL of DMSO, and stored at −25 °C until the Ames test and Orbitrap analysis.

Ames fluctuation test

The Ames fluctuation test was performed in the original SPE extracts, a dilution series (10, 15, 20, and 50 times diluted) of these extracts, and the fractions obtained by fractionation of the SPE extracts all reconstituted in DMSO. The Ames fluctuation test was performed as reported previously (Heringa et al. 2011), using strain TA100 instead of TAmix. Each sample was tested in triplicate. Since additional methanol was used for preparation of the SPE extracts, a methanol blank was included besides the negative and positive control when testing the dilution series.

LC-Orbitrap MS analysis

The DMSO extracts were analyzed by liquid chromatography and high-resolution mass spectrometry (LC-Orbitrap MS, analysis was performed in both positive in negative ionization mode) to screen for the 84 known N-DBPs as described by Kolkman (Kolkman et al. 2015). One hundred microliters of acetonitrile was added to 25 μL of DMSO extract. Next, 375 μL of ultrapure water containing the internal standards atrazine-d_5 and bentazone-d_6 was added to the extract. The final concentration of the internal standards atrazine-d_5 and bentazone-d_6 was 0.5 mg/L, corresponding to a concentration of circa 1.0 μg/L in the original sample. The acquired mass spectrometric data were processed with Xcalibur software and were screened for the 84 previously detected N-DBPs (see Electronic Supplementary Material Table SI for the list of 84 N-DBPs). The maximum allowed mass error and retention time deviation for the detected N-DBPs were set at 5 ppm and 0.20 min, respectively. The mass spectra of the N-DBPs were manually checked for possible adduct ions (ammonium and sodium). For the quantification of the N-DBPs, bentazone-d_6 and atrazine-d_5 internal standards were used.

Results

Identification of N-DBPs

LC-QToF mass spectrometric analysis

The results of the QToF analysis for the identification of "unknown" N-DBPs found in our previous work (Kolkman et al. 2015) are shown in Table 1. Fourteen N-DBPs were confirmed by analyzing the reference standards and matching the accurate mass, retention time, and MS/MS fragmentation patterns of the reference standard with the N-DBP in the artificial water samples. A QToF mass spectrometer was used for the structure elucidation instead of the LTQ-Orbitrap, since more informative MS2 spectra are obtained on the QToF compared to the LTQ-Orbitrap (no HCD cell is present in our system). The identities of 4-nitrophenol, 4-nitrocatechol, and 2-methoxy-4,6-dinitrophenol had already been confirmed previously (Kolkman et al. 2015), and the identity of the five tentatively identified compounds, i.e., 2-hydroxy-5-nitrobenzoic acid, 2,4-dinitrophenol, 5-nitrovanillin, 4-nitrobenzenesulfonic acid, and 4-nitrophthalic acid, was now also confirmed. In addition, the identities of six hitherto unidentified N-DBPs were elucidated, namely 4-nitro-1,3-benzenediol, 2-nitrohydroquinone, 4-hydroxy-3-nitrobenzoic acid, 2-hydroxy-3-nitrobenzoic acid, 3,5-dinitrosalicylic acid, and dinoterb.

The N-DBPs 2-hydroxy-3-nitrobenzoic acid and 4-nitro-1,3-benzenediol were not detected in the previous study and were therefore not included in the screenings list of disinfection by-products. Due to the enhanced sensitivity of the QToF mass spectrometer compared with the Orbitrap used in the previous study, and because these two by-products are isomers of other N-DBPs targeted for identification, they were incidentally detected and confirmed by the analysis of reference standards.

Toxicity evaluation

The results of the toxicity evaluation of the identified N-DBPs are presented in Table 2. In general, few toxicity data are available for the identified N-DBPs. None of the substances have been listed as (potential) human mutagen or carcinogen. For only three substances (4-nitrophenol, 2,4-dinitrophenol, 4-nitrobenzenesulfonic acid), in vitro and/or in vivo studies on genotoxicity have been reported, which indicate that these substances yield negative results in the Ames test. For 4-nitrobenzenesulfonic acid, this is supported by the read across analysis, which indicates the absence of genotoxic potential

Table 1 Identities and relevant properties of N-DBPs confirmed by QToF analysis of reference standards

Compound	CAS no.	Formula	Theoretical accurate mass $[M-H]^-$ (m/z)[d]	RT sample (min)	RT standard (min)	MS/MS fragment ions (m/z)
4-Nitrophenol[a]	100-02-7	$C_6H_5NO_3$	138.0197	12.89	12.87	108.0220, 92.0268
4-Nitrocatechol[a]	3316-09-4	$C_6H_5NO_4$	154.0146	10.38	10.36	123.0082, 95.0119
4-Nitro-1,3-benzenediol[c]	3163-07-3	$C_6H_5NO_4$	154.0146	13.00	13.00	137.0112, 107.0136
2-Nitrohydroquinone	16090-33-8	$C_6H_5NO_4$	154.0146	11.20	11.16	123.0081, 79.0187
2-Hydroxy-5-nitrobenzoic acid[b]	96-97-9	$C_7H_5NO_5$	182.0095	12.57	12.53	108.0221, 138.0199
4-Hydroxy-3-nitrobenzoic acid	616-82-0	$C_7H_5NO_5$	182.0095	11.24	11.25	108.0218, 138.0193
2-Hydroxy-3-nitrobenzoic acid[c]	85-38-1	$C_7H_5NO_5$	182.0095	10.21	10.23	138.0188, 108.0211
2,4-Dinitrophenol[b]	51-28-5	$C_6H_4N_2O_5$	183.0047	15.02	15.02	109.0167, 123.0081
5-Nitrovanillin[b]	6635-20-7	$C_8H_7NO_5$	196.0252	12.62	12.62	181.0018, 135.0085
4-Nitrobenzenesulfonic acid[b]	138-42-1	$C_6H_5NO_5S$	201.9816	5.85	5.88	138.0189, 155.9875
4-Nitrophthalic acid[b]	610-27-5	$C_8H_5NO_6$	210.0044	8.11	8.17	122.0239, 166.0119
2-Methoxy-4,6-dinitrophenol[a]	4097-63-6	$C_7H_6N_2O_6$	213.0153	15.39	15.36	197.9921, 124.0037
3,5-Dinitrosalicylic acid	609-99-4	$C_7H_4N_2O_7$	226.9946	13.76	13.78	183.0048, 123.0082
Dinoterb	1420-07-1	$C_{10}H_{12}O_5N_2$	239.0673	26.68	26.67	207.0419, 177.0430

[a] Already confirmed in previous study

[b] Tentatively confirmed in previous study

[c] N-DBP not detected during Orbitrap analysis

[d] Measured accurate mass falls within a 5 ppm mass range of the theoretical mass

for only this compound. For all other compounds, DNA binding and/or genotoxic potential was predicted based on structural characteristics. For most of these substances (because of structural similarity), the OCED QSAR Toolbox indicated a potential for DNA binding through production of ROS and/or nitrenium ions and a structural alert for genotoxic carcinogenicity. Although these observations may indicate mutagenic potency, these substances will not necessarily yield positive responses in the Ames test. This is illustrated by 2,4-dinitrophenol, for which similar read across results were obtained but which was shown to have a negative response in the Ames test (NLM 2011; TERA 2010). For two of the substances for which read across analysis predicted DNA binding and genotoxicity, ECHA has reported negative results in the Ames test based on QSAR prediction (ECHA 2015). For eight compounds (indicated in bold in Table 2), potential mutagenicity in the Ames test based on read across analysis cannot be excluded in the absence of measured mutagenicity testing data. From these, dinoterb is additionally known to cause developmental and aquatic toxicity (ECHA 2015; SZW 2016) and is listed as a substance of high concern (RIVM 2015).

For none of the identified compounds, statutory or provisional drinking water guidelines have been reported to our knowledge, except for 4-nitrophenol. The US EPA has included a reference dose (RfD) for 4-nitrophenol of 0.008 mg/kg body weight/d and a drinking water equivalent level (DWEL) of 0.3 mg/L (assuming 100 % exposure via drinking water) (EPA 2012). No reference is made to underlying toxicity data. For three compounds, acceptable daily intakes based on non-genotoxic effects have been reported (see Table 2). When we assume a body weight of 70 kg, consumption of 2 L of drinking water per day, and a standard allocation of 20 % of the total exposure to drinking water, provisional drinking water guidelines would approximate 5.8 mg/L for 2-hydroxy-5-nitrobenzoic acid (based on a derived no effect level (DNEL) of 0.83 mg/kg bw/day), 0.01 mg/L for 2,4-dinitrophenol (based on a RfD of 0.002 mg/kg bw/day), and 18 mg/L for 5-nitrovanillin (based on DNEL of 2.6 mg/kg bw/day).

Effect directed analysis

Fractionation

The results of the Ames fluctuation test in the unfractionated and diluted extracts in strains TA98 ± S9 and T100 ± S9 are shown in Electronic Supplementary Material Fig. SI. The methanol blank does not show an increase in mutagenicity compared to the negative control, indicating that the additional methanol used during preparation of the extracts does not affect the test results. The MP UV-treated samples show, as expected, higher responses than the untreated samples, both in the TA98 and TA100 strain. All samples show responses within the detection range of the test. The most diluted (50×) extract of the MP UV-treated water still causes a statistically significant increase in mutagenicity in TA100. These results suggest that Ames test responses above the detection limit will

Table 2 Summary of toxicological properties of the identified N-DBPs

N-DBP	CMR[b]	Measured genotoxicity data	Read across prediction of genotoxicity	Other toxicity data	Conclusion
4-Nitrophenol[a] [100-02-7]	–	Negative in Ames tests ± metabolic activation, positive results in other microorganisms, equivocal results in chromosome aberration tests in vitro, no carcinogenicity in mice after dermal exposure (NLM 2011; OECD 2013; TERA 2010).	Structural alert for DNA binding, not for Ames, micronucleus, and Comet assay or protein binding (Kolkman et al. 2015).	Insufficient data to establish acceptable daily intake; moderate to high toxicity in the aquatic compartment (TERA 2010).	Overall evidence points to absence of mutagenicity in Ames test; insufficient data to assess other genotoxicity and carcinogenic potential.
4-Nitrocatechol[a] [3316-09-4]	–	–	Structural alert for DNA binding, not for Ames, micronucleus, and Comet assay or protein binding (Kolkman et al. 2015).	–	Probably not mutagenic in Ames test; insufficient data to assess other genotoxicity and carcinogenic potential.
4-Nitro-1,3-benzenediol [3163-07-3]	–	–	Potential for DNA binding through production of ROS/nitrenium ions. Structural alert for genotoxic carcinogenicity, not for protein binding (OECD 2013).	–	**Structure suggests genotoxic potential.**
2-Nitrohydroquinone [16090-33-8]	–	–	Potential for DNA binding through production of ROS/nitrenium ions. Structural alert for genotoxic carcinogenicity, not for protein binding (OECD 2013).	–	**Structure suggests genotoxic potential.**
2-Hydroxy-5-nitrobenzoic acid [96-97-9]	–	–	Potential for DNA binding through production of ROS/nitrenium ions. Structural alert for genotoxic carcinogenicity, not for protein binding (OECD 2013). ECHA reports predicted negative result in Ames test and in vitro mammalian chromosome aberration test (ECHA 2015).	DNEL[c] 0.83 mg/kg bw/day based on most sensitive endpoint: effect on fertility (ECHA 2015).	Structure suggests genotoxic potential but no mutagenicity.
4-Hydroxy-3-nitrobenzoic acid [616-82-0]	–	–	Potential for DNA binding through production of ROS/nitrenium ions. Structural alert for genotoxic carcinogenicity, not for protein binding (OECD 2013).	–	**Structure suggests genotoxic potential.**
2-Hydroxy-3-nitrobenzoic acid [85-38-1]	–	–	Potential for DNA binding through production of ROS/nitrenium ions. Structural alert for genotoxic carcinogenicity, not for protein binding (OECD 2013).	–	**Structure suggests genotoxic potential.**
2,4-Dinitrophenol [51-28-5]	–	Negative in Ames test ± metabolic activation, negative for genotoxicity in most in vivo and in vitro studies in prokaryotic and mammalian cells, some positive results in chromosome aberration tests; metabolites (2-amino-4-nitrophenol, 4-amino-2-nitrophenol, and 2,4-diaminophenol) appear to be mutagenic; no carcinogenicity	Potential for DNA binding through production of ROS/nitrenium ions. Structural alert for genotoxic carcinogenicity, not for protein binding (OECD 2013).	RfD[d] 0.002 mg/kg bw/day based on human LOAEL[e] (EPA 2016); DNEL[c] 0.008 mg/kg bw/day based on rat NOAEL[f] (ECHA 2015).	Weight of evidence indicates no mutagenicity and genotoxicity, but clastogenicity and carcinogenicity cannot be excluded.

Table 2 (continued)

N-DBP	CMR[b]	Measured genotoxicity data	Read across prediction of genotoxicity	Other toxicity data	Conclusion
		studies available (NLM 2011; OECD 2013; TERA 2010).			
5-Nitrovanillin [6635-20-7]	–	–	Potential for DNA binding through production of ROS/nitrenium ions. Structural alert for genotoxic carcinogenicity, not for protein binding (OECD 2013). ECHA reports predicted negative result in Ames test and in vitro mammalian chromosome aberration test (ECHA 2015).	DNEL[c] 2,6 mg/kg bw/day based on inhalation LOEL[g] (ECHA 2015).	Structure suggests genotoxic potential but no mutagenicity.
4-Nitrobenzene-sulfonic acid [138-42-1]	–	Negative in Ames test ± metabolic activation (one study) (NLM 2011).	No structural alerts for DNA or protein binding, in vitro mutagenicity or carcinogenicity (OECD 2013).	–	Mutagenicity and genotoxicity are not expected.
4-Nitrophthalic acid [610-27-5]	–	–	Potential for DNA binding through production of ROS/nitrenium ions. Structural alert for (non-) genotoxic carcinogenicity, not for protein binding (OECD 2013).	–	**Structure suggests genotoxic potential.**
2-Methoxy-4,6-dinitrophenol[a] [4097-63-6]	–	–	Structural alert for DNA binding, not for Ames, micronucleus, and Comet assay or protein binding. Read across predicts a positive response in the Ames test with and without metabolic activation (Kolkman et al. 2015).	–	**Potentially mutagenic in Ames test; insufficient data to assess other genotoxicity and carcinogenic potential.**
3,5-Dinitrosalicylic acid [609-99-4]	–	–	Potential for DNA binding through production of nitrenium ions. Structural alert for genotoxic carcinogenicity (OECD 2013).	–	**Structure suggests genotoxic potential.**
Dinoterb [1420-07-1]	Developmental toxicity (ECHA 2015; SZW 2016)	–	Potential for DNA binding through production of nitrenium ions. Structural alert for genotoxic carcinogenicity (OECD 2013).	Substance of high concern (RIVM 2015); acute toxicity and very toxic to aquatic life with long lasting effects (ECHA 2015); EC authorization for plant protection products withdrawn in 1998 due to concerns for human health and data gaps.	**Structure suggests genotoxic potential.**

Substances in bold: potential mutagenicity in the Ames test based on read across analysis cannot be excluded in absence of additional data

– No data present in databases consulted

[a] Already evaluated in previous study

[b] Classified as carcinogenic, mutagenic, or toxic for reproduction according to IARC, SZW, or HSDB

[c] Derived no effect level

[d] Reference dose

[e] Lowest observed adverse effect level

[f] No observed adverse effect level

[g] Lowest observed effect level

still be obtained when fractions are prepared from 1 mL of extract.

Eight fractions were produced from the untreated and MP UV-treated water extracts. These fractions showed visible differences in coloration, probably caused by the unequal distribution of NOM components across the fractions (data not shown). A comparison was made between the summed concentration of N-DBPs detected in the eight fractions, with the summed concentration of N-DPBs detected in the untreated and MP UV-treated extracts before fractionation. This was done to ensure that no losses had occurred during fractionation and corresponding sample treatment (i.e., distillation) and thus to determine recovery of the by-products. The results are shown in Fig. 2. A summed concentration of 607 ng/L (ISTD) of N-DBPs was detected in the fractions of the MP UV-treated sample, and in the undiluted, unfractionated sample, a concentration of 672 ng/L (ISTD) of N-DBPS was detected. The summed concentration of by-products detected in the fractions is thus in agreement with the total concentration detected in the unfractionated samples. The small difference shows that there is only a minimal loss of by-products during fractionation and additional sample treatment and that the by-products are stable under these conditions.

Ames fluctuation test

The Ames fluctuation test results for the untreated and MP UV-treated samples after fractionation for strain TA98 and TA100 are shown in Fig. 3a, b, respectively. From fraction 3 onward, the Ames test responses are higher in the MP UV samples than in the untreated samples in both bacterial strains and statistically significantly increased compared to the negative control. In the fractions of the untreated sample, some significantly positive responses are detected as well, in particular in fractions 5 (TA98) and 6 (TA100), albeit less pronounced than the responses in the fractions of the treated water. The MP UV-treated fractions 4 to 8 roughly cause a similar response. Strain TA98 shows higher responses in these fractions than in the extract before fractionation (see Electronic Supplementary Material Fig. SI).

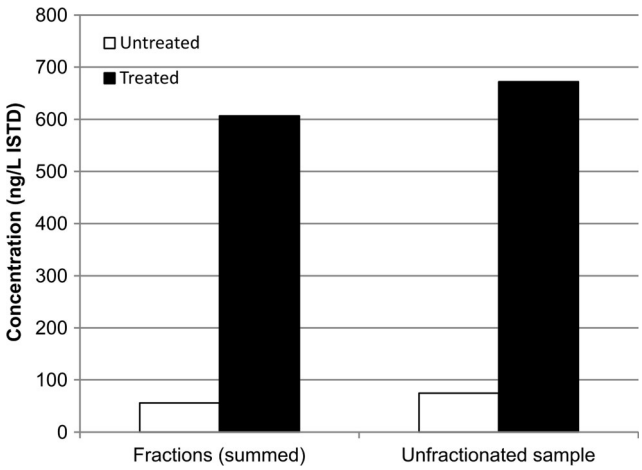

Fig. 2 Sum of N-DBPs detected in fractionated extracts compared with the concentration of by-products found in the unfractionated extracts

LC-Orbitrap MS analysis

The results of the Orbitrap MS analysis of the 84 N-DBPs in untreated and MP UV-treated samples after fractionation are shown in Fig. 3c. Of the 84 previously detected N-DBPs, 81 were detected again in the MP UV-treated sample before and after fractionation. As expected, the summed concentration (in ng/L internal standard equivalents, semi quantification) of N-DBPs in the MP UV-treated fractions is higher than or equal to the concentration in the corresponding untreated fraction. In the MP UV-treated water, the majority of the by-products are detected in fractions 4 to 8. In the first two fractions, no by-products are detected, and in fraction 3, only a low summed concentration of by-products is found.

In order to obtain an overview of the by-products detected per fraction, a top five was composed for each fraction based on the N-DBPs with the highest concentration. The results (Table 3) indicate that overall, the identities of the N-DBPs with the highest concentrations in treated water are known, with the exception of by-product m/z 316.1413 (1) which is detected in fraction 5 at a concentration of 34.9 ng/L internal standard equivalents. All other N-DBPs with a concentration greater than 15 ng/L internal standard equivalents in any fraction were identified. 4-Nitrophenol and 2-hydroxy-5-nitrobenzoic acid are not expected to cause health effects at the detected concentrations based on provisional drinking water guidelines related to endpoints other than carcinogenicity. For the other substances, no health-based drinking water guidelines could be derived. Based on the (predicted) genotoxic potential, 4-nitrophthalic acid, 4-hydroxy-3-nitrobenzoic acid, 2-methoxy-4,6-dinitrophenol, dinoterb, and 3,5-dinitrosalicylic acid may have contributed to the observed mutagenicity. Most of detected by-products at lower concentrations (<15 ng/L internal standard equivalents) remain unidentified.

Identification of mutagenic N-DBPs

Figure 3 shows that the presence of the by-products in the individual fractions as detected by the chemical screening corresponds with the induction of significant responses in the Ames fluctuation test. However, the summed concentration of by-products decreases after fraction 4, whereas the magnitude of the Ames test response remains equal until fraction 8. A relatively high Ames test response was observed in fractions 3, 7, and 8 in comparison with the relatively low concentrations of N-DBPs that were detected by the chemical

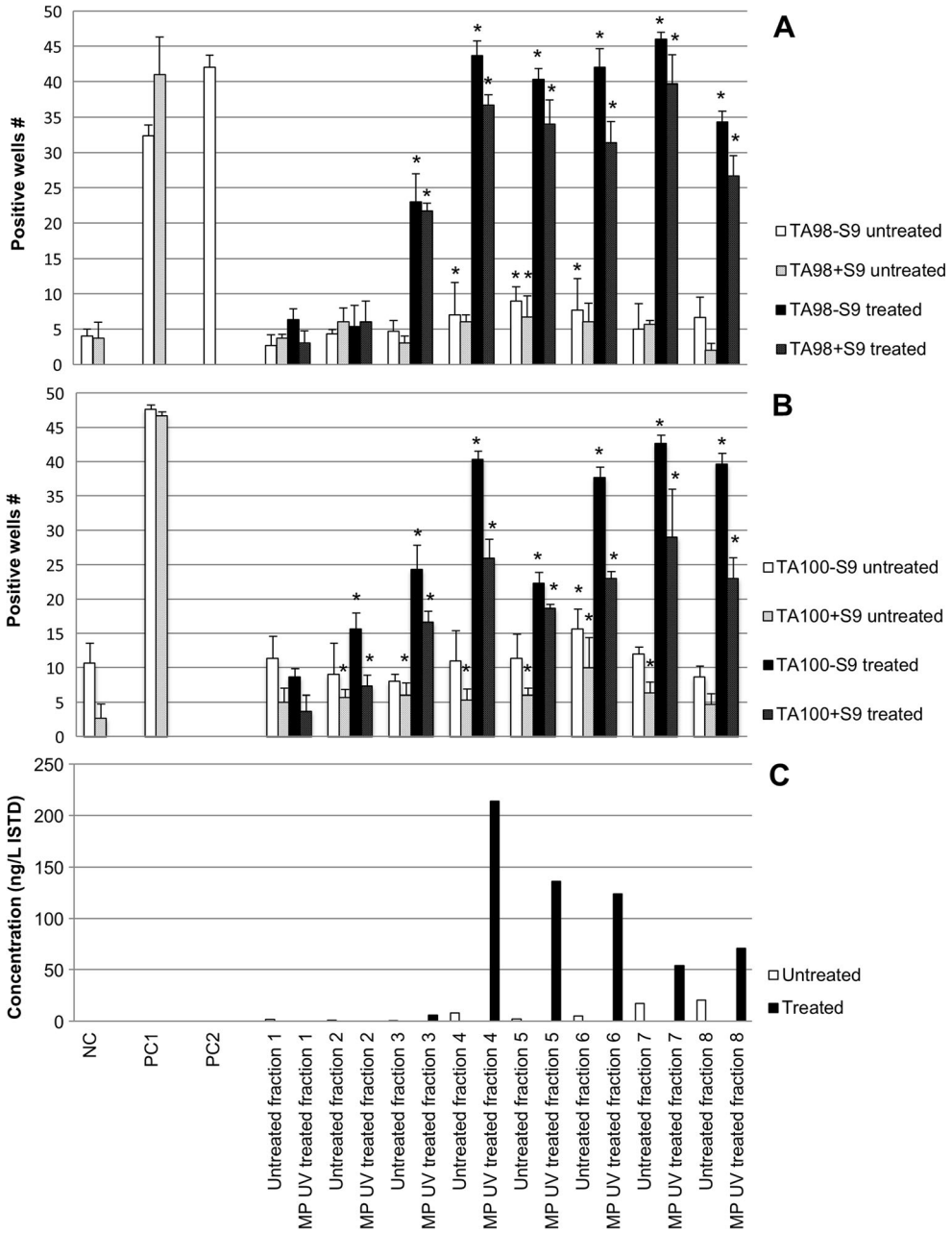

Fig. 3 Ames fluctuation test response in **a** strain TA98 ± S9 and **b** TA100 ± S9 for the fractionated extracts (untreated and MP UV-treated). The *bars* denote average values ($n = 3$). *NC* negative control; *PC* positive control; *PC1* 20 μg/mL 4-NQO (TA98 − S9), 5 μg/mL 2-AA (TA98 + S9), 12.5 μg/mL NF (TA100 − S9), or 20 μg/mL 2-AA (TA100 + S9); *PC2* 500 μg/mL 4-NOPD (TA98 − S9). * = positive response (statistically significantly increased response relative to negative control). **c** Summed concentration of by-products per fraction

screening in these fractions. This could indicate that the by-products present in these fractions may have a relatively high mutagenic potency. Therefore, the by-products detected in these fractions were inspected more closely. By-products that are uniquely present in these three fractions and not in the other fractions (i.e., 4, 5, and 6) are shown in Table 4.

There are no N-DBPs that were commonly detected in fractions 3, 7, and 8. Neither are there any N-DBPs that are uniquely present in fraction 3. In fractions 7 and 8, a total of five by-products are detected that are only present in these fractions and not detected in the corresponding MP UV-untreated fractions. Three of the five by-products mentioned in Table 4 have the same accurate mass, but different retention times (i.e., the isomers at m/z 340.1388) were found in the positive ionization mode in low concentrations. The elemental composition of these three N-DBPs with m/z 340.1388 was determined as $C_{16}H_{21}O_7N$, which resulted in 164 hits in Chemspider. Using in silico tools such as MetFrag did not result in a good match for a candidate for the identification of these N-DBPs. In the negative mode, two by-products were detected in fractions 7 and 8. For the N-DBP with m/z 372.1491 with a concentration of 2.1 ng/L ISTD eq., it was not possible to derive the elemental composition. The N-DBP corresponding to m/z 239.0677 was identified as dinoterb and was detected in fraction 7 at a relative high concentration of

Table 3 Top 5 most intensive by-products in fractions 3 to 8

Mass (m/z)	Retention time (min)	Concentration (ng/L ISTD eq.)	Formula	Compound
Fraction 3				
400.1262 (1)	11.77	1.9		
386.1096 (1)	11.44	1.3		
154.0148 (1)	9.89	0.8	$C_6H_5O_4N$	4-Nitrocatechol
210.0048 (1)	7.67	0.7	$C_8H_5O_6N$	4-Nitrophthalic acid
442.1365 (2)	10.56	0.4		
Fraction 4				
182.0098 (2)	10.83	42.2	$C_7H_5O_5N$	4-Hydroxy-3-nitrobenzoic acid
138.0198	12.54	29.2	$C_6H_5O_3N$	4-Nitrophenol
154.0148 (1)	9.89	26.2	$C_6H_5O_4N$	4-Nitrocatechol
400.1262 (2)	12.73	10.6		
408.1308 (2)	13.71	10.0		
Fraction 5				
316.1413 (1)	18.99	34.9	$C_{14}H_{23}O_7N$	
208.0255	12.31	7.9	$C_9H_7O_5N$	
452.1203 (2)	12.70	7.7		
225.9994 (2)	7.80	7.4	$C_8H_5O_7N$	
213.0154	15.24	6.9	$C_7H_6O_6N_2$	2-Methoxy-4,6-dinitrophenol
Fraction 6				
213.0154	15.23	38.5	$C_7H_6O_6N_2$	2-Methoxy-4,6-dinitrophenol
316.1413 (3)	20.45	11.7	$C_{14}H_{23}O_7N$	
238.0726	18.56	9.0	$C_{11}H_{13}O_5N$	
270.0755 (1)	11.89	9.0		
316.1413 (1)	19.08	8.3	$C_{14}H_{23}O_7N$	
Fraction 7				
212.0204	10.93	23.9	$C_8H_7O_6N$	Structural isomer of 5-hydroxy-4-methoxy-2-nitrobenzoic acid
266.1037	23.74	8.4	$C_{13}H_{17}O_5N$	
239.0677	26.78	8.0	$C_{10}H_{12}O_5N_2$	Dinoterb
153.0073	10.93	5.3		
226.9948	13.94	1.8	$C_7H_4O_7N_2$	3,5-Dinitrosalicylic acid
Fraction 8				
182.0098 (3)	13.17	56.2	$C_7H_5O_5N$	2-Hydroxy-5-nitrobenzoic acid
226.9948	13.92	5.5	$C_7H_4O_7N_2$	3,5-Dinitrosalicylic acid
196.0258 (3)	13.30	3.9		
372.1491	24.99	2.1		
239.0677	26.78	0.6	$C_{10}H_{12}O_5N_2$	Dinoterb

8 ng/L ISTD equivalents. Based on its structure, dinoterb can be expected to cause genotoxic effects.

Discussion

MP UV treatment of nitrate-rich water in the presence of NOM resulted in the formation of 81 by-products. High molecular organic constituents of NOM such as humic and fulvic acids are known to serve as precursors for DBPs (Kleiser and Frimmel 2000). The chemical identity of 14 of the DBPs was confirmed. This leaves a large part of the by-products still unidentified, although the identity of most of the by-products detected in the highest concentrations was confirmed and the identities of 9 of the 22 N-DBPs that were previously detected in water samples from a full-scale drinking water treatment plant based on MP UV/H_2O_2 treatment (Kolkman et al. 2015) are now unraveled. The identification of DBPs is usually complicated, especially when compounds have a molecular weight over 250 g/mol. The number of possible

Table 4 Detected by-products only present in fractions 7 and 8

Mass (m/z)	Retention time (min)	Mode	Fraction	Concentration (ng/L ISTD eq.)	Formula	Compound
340.1388 (1)	27.80	pos	7	0.3	$C_{16}H_{21}O_7N$	
340.1388 (2)	28.16	pos	7	1.3	$C_{16}H_{21}O_7N$	
340.1388 (3)	28.90	pos	8	0.3	$C_{16}H_{21}O_7N$	
239.0677	26.78	neg	7	8.0	$C_{10}H_{12}O_5N_2$	Dinoterb
372.1491	24.99	neg	8	2.1		

chemical structures increases dramatically when molecular weight increases. Due to the relatively high concentration of by-product m/z 316.1413, it is specifically recommended to unravel the identity of this by-product in future research, as well as the identities of the unknown N-DBPs that were only present in the fractions showing the highest mutagenicity.

MP UV treatment caused an increase in the response of the Ames fluctuation test using both the TA98 and TA100 strains, each of which represents a different type of mutagenicity. This result is not surprising, since a mixture of N-DBPs, each with their own mechanism of action, is formed through nitrate photolysis by MP UV treatment. In order to evaluate which of the detected N-DBPs contribute to the mutagenic responses observed, the water samples were split into eight fractions each, based on polarity. Fractionation of the samples resulted in minimal loss of by-products. However, it should be noted that the amount of matrix and chemicals present in the unfractionated sample is much higher than in the individual fractions, which could have effects such as ionization suppression during the mass spectrometric analysis. This may also explain the higher Ames test response in fractions 4 to 8 observed in strain TA98 than the response that was detected in the unfractionated sample, since the concentration of the complete mixture of components present in the extract before fractionation may cause inhibitory effects that mask the mutagenic potency of the sample. Fractionation may thus remove matrix compounds and isolate mutagenic compounds, thereby enhancing the mutagenic response (Brack et al. 2016).

All fractions were tested in the Ames test and screened for the presence of N-DBPs. From fraction 3 onward, the presence of by-products and an increased Ames test response was demonstrated in the MP UV-treated samples. In MP UV-treated fractions 4 to 8, the highest Ames test response and the majority of the by-products were found. The detection of by-products thus correlates with the presence of mutagenicity as revealed by positive responses in the Ames tests. However, the Ames test response was similarly high in fractions 4 to 8, whereas the concentration of N-DBPs decreased after fraction 4. The results of both analyses are therefore not quantitatively correlated. This may be explained by the fact that the set of by-products differs per fraction. In addition, not all by-products are (equally) mutagenic and the detected Ames fluctuation test response represents the total effect of a mixture of mutagenic and non-mutagenic N-DBPs. The mutagenic potency is not yet established for the majority of these N-DBPs. Some N-DBPs may only show mutagenicity in different *Salmonella* strains, such as YG strains, that are more responsive to nitro compounds (Cerna et al. 1996; Umbuzeiro Gde et al. 2004). Moreover, other mutagenic by-products might be present in fractions 5 to 8 that did not make part of the selection of 84 substances that were screened for. Besides, the intensity of each compound in the MS is dependent on ionization efficiency that, in turn, may be influenced by the (different) matrix present in each fraction. And finally, both the bioassay and chemical measurements yield semiquantitative results.

Since there was not a specific fraction that caused a high response in the Ames test and contained by-products that were uniquely present in this fraction, it cannot be concluded which subset of N-DBPs was responsible for the observed mutagenicity. Of special interest is fraction 3, in which a high response in the Ames test was observed, but only a low summed concentration of by-products was found. Potent mutagens are expected to be present in this fraction, but no N-DBP(s) could be indicated that occurred only in fraction 3. It is therefore advised to chemically analyze this fraction more in depth by using other ionization techniques, like APPI and APCI, or by using other analytical techniques such as GC-MS. Nevertheless, we could indicate five N-DBPs (4-nitrophthalic acid, 4-hydroxy-3-nitrobenzoic acid, 2-methoxy-4,6-dinitrophenol, dinoterb, and 3,5-dinitrosalicylic acid) that are potentially genotoxic and were present in relatively high concentrations in the fractions in which mutagenicity was observed. 4-Hydroxy-3-nitrobenzoic acid and 2-methoxy-4,6-dinitrophenol were previously shown to be formed in a full-scale MP UV/H_2O_2 treatment plant as well (Kolkman et al. 2015).

In the fractions of the untreated water sample, some positive Ames test responses and by-products were detected as well, albeit to a much smaller extent than in the treated water samples. The exact cause of this elevated level of by-products and mutagenicity in these reference fractions is unknown, but it is likely that some mutagenic substances are already present in NOM. Besides, it cannot be completely excluded that minor amounts of compounds are introduced during sample pretreatment, since a procedure blank was not included due to the

large sample volume and wide range of sample treatment steps.

Conclusions

By applying a fractionation method to MP UV-treated nitrate rich and NOM containing water followed by chemical screening and mutagenicity testing, the presence of N-DBPs and mutagenicity in the Ames fluctuation test were shown to be correlated. Five by-products demonstrated to be present in relatively high concentrations in the fractions in which mutagenicity was observed are potentially genotoxic: 4-nitrophthalic acid, 4-hydroxy-3-nitrobenzoic acid, 2-methoxy-4,6-dinitrophenol, dinoterb, and 3,5-dinitrosalicylic acid. Evaluation of these substances by more advanced QSAR and read across analysis and final testing of (mixtures of) the N-DBPs in the Ames fluctuation tests, also including strains that are particularly responsive to nitroso compounds, is necessary to provide further insight into the relationship between these N-DBPs and the observed mutagenicity in MP UV-treated water. Refinement of the EDA methodology applied by using a larger number of fractions to relate the analytical results to responses in a larger arrays of bioassays may also aid in identifying N-DBPs of potential health concern. In addition, further elucidation of the identity of the other N-DBPs that are formed during MP UV drinking water treatment may be accomplished by using mass spectrometry with a higher resolution and advanced data analysis software. Finally, evaluation of the relevance of identified by-products with potential mutagenicity for full-scale drinking water treatment plants and under varying process conditions (such as composition of source water with regard to NOM, nitrate and anthropogenic substances, UV dose, addition of H_2O_2, and LP instead of MP UV) is recommended.

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