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BTO report

Aromatic amino acids as a source for nitrogen containing by-products formed by advanced oxidation water treatment

KWR Watercycle Research Institute

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Samenvatting

Geavanceerde oxidatie processen zoals UV/H_2O_2 en ozon worden steeds vaker ingezet voor de productie van drinkwater vanwege desinfectie, maar ook doordat het een belangrijke barrière vormt tegen organische microverontreinigingen in bronnen van drinkwater. Uit voorgaand onderzoek is gebleken dat middendruk (MP) UV/H_2O_2 behandeling leidt tot een positieve respons in de Ames mutageniteitstests, die geheel verwijderd wordt doormiddel van GAC en duininfiltratie. Deze positieve respons wordt vermoedelijk veroorzaakt door de vorming van stikstofhoudende bijproducten (N-DBPs) die gevormd worden door de reactie van fotolyseproducten van nitraat met (fotolyseproducten van) natuurlijk organisch materiaal (NOM). In voorgaand onderzoek is met behulp van stabiel isotoop gelabeld nitraat ($^{15}NO_3^-$) aangetoond dat na MP UV behandeling van kunstmatig water dat NOM en nitraat bevat, verschillende stikstofhoudende bijproducten worden gevormd. Met deze aanpak zijn er in totaal 84 N-NBPs gedetecteerd, waarvan 22 ook zijn aangetoond in monsters van een full-scale drinkwaterzuiveringsinstallatie op basis van MP UV/H_2O_2 behandeling. Tot op heden zijn 14 van de 84 bijproducten geïdentificeerd. Echter kan de positieve response in de Ames test niet verklaard worden door het genotoxisch potentieel van deze geïdentificeerde bijproducten, wat dus vraagt om verder onderzoek.

Uit de literatuur blijkt dat aromatische aminozuren (tyrosine, fenylalanine en tryptofaan) een mogelijke bron kunnen zijn voor de vorming van stikstofhoudende bijproducten. Wanneer water dat aromatische aminozuren en nitraat bevat, wordt behandeld met UV, laat dit een verhoogde mutageniteit zien in de Ames test. In de huidige studie wordt de rol van aminozuren in de vorming van stikstofhoudende producten verder onderzocht. Hiervoor zijn stabiele isotoop labeling experimenten uitgevoerd met nitraat, aromatische aminozuren, MP UV behandeling en hoge resolute massaspectrometrie. Met deze aanpak kon worden aangetoond dat er veel bijproducten gevormd worden, waarvan slechts een aantal gelinkt kon worden aan de N-DBPs die gedetecteerd zijn in het kunstmatig water monster van de vorige studie. Voor tryptofaan kon één N-DBP welke geïdentificeerd is als 3-nitroindole, gelinkt worden aan een N-DBP gedetecteerd in kunstmatig water. De resultaten laten ook zien dat het niet waarschijnlijk is dat 3-nitroindole gevormd wordt uit tryptofaan, maar wordt gevormd uit een andere bron. Voor fenylalanine zijn vier (nog) ongeïdentificeerde bijproducten gelinkt aan bijproducten gedetecteerd in kunstmatig water. De resultaten laten zien dat deze vier bijproducten werkelijk van fenylalanine afkomstig zijn in kunstmatig water, waarmee aangetoond is dat aromatische aminozuren een bron kunnen zijn voor N-DBPs gevormd door MP UV behandeling van artificieel water.

Van vijf N-DBPs is de identiteit opgehelderd tijdens deze studie, waardoor het totaal van geïdentificeerde bijproducten op 19 komt. De toxiciteitsbeoordeling van deze vijf N-DBPS is uitgevoerd op basis van structuur eigenschappen en de uitkomst duidt op mogelijke potentiele mutageniteit.

In het tweede deel van deze studie is een LC-QToF doelstofmethode ontwikkeld voor de bepaling van de 19 geïdentificeerde N-DBPs in drink- en oppervlaktewater. De prestatiekenmerken van de methode, zoals aantoonbaarheids- en detectiegrenzen zijn bevredigend. De methode is vervolgens toegepast voor een monitoringstudie van zeven maanden van een full-scale drinkwaterzuiveringsinstallatie op basis van MP UV/H_2O_2 behandeling. Tijdens de monitoring studie zijn 15 van de 19 N-DBPs gedetecteerd in

concentraties tussen de 1,0 en 44 ng/L. De totale hoeveelheid aan gedetecteerde verbindingen in een full-scale drinkwaterzuiveringsinstallatie laat de relevantie zien van de geïdentificeerde N-DBPs en de ontwikkelde N-DBPs doelstofmethode.

Contents

1 Introduction

1.1 Disinfection by-products

For the production of drinking water, surface water is gaining importance due to increasing populations and limited availability of groundwater. However, surface waters may contain a large variety of organic micropollutants, such as pharmaceuticals, pesticides and industrial compounds, for which the traditional water treatment systems (e.g. rapid sand filtration, coagulation, granular activated carbon filtration) do not constitute a robust barrier [1]. Advanced oxidation processes (AOP), such as UV/H_2O_2 and ozone are becoming increasingly important for the preparation of drinking water, for effectively removing these micropollutants [2-4]. However, it is known that advanced oxidation processes can produce potentially harmful disinfection by-products (DBPs) [5-8]. Many studies were performed to investigate the formation, identities and occurrence of these DBPs [9]. Such studies are challenging, due to the vast amount of DBPs that can be formed. Many factors can contribute to the formation of DBPs such as, disinfection method, type of source water and process conditions. Information on the potential human health risk of these DBPs is often unknown because of their unknown identity and/or the lack of toxicity data. A relatively new subgroup of DBPs are the nitrogenous DBPs (N-DBPs) [10-12], which have a higher human toxicological potential than the well-known chlorinated DBPs [9, 10, 12].

Earlier research has shown that medium pressure (MP) UV/H_2O_2 treatment in drinking water production may lead to the formation of N-DBPs [7, 13, 14]. These N-DBPs are formed through a complex mechanism of nitrate photolysis by UV in which nitrate is converted into the stable nitrite [15, 16]. During this nitrate to nitrite reduction, various nitrate intermediate radicals are formed [16], which have the ability to react with natural organic matter (NOM) that is present in source water. This ultimately results in the incorporation of the nitrogen-atom of nitrate into aquatic NOM [14].

In previous research, an innovative approach was developed in order to trace N-DBPs, combining stable isotope labeled nitrate with high-resolution mass spectrometry (HRMS) [13]. It was shown that multiple N-DBPs were formed after MP UV treatment of artificial water containing nitrate and NOM. Using this approach a total of 84 N-DBPs were detected in artificial water. A suspect screening for these 84 N-DBPs in water samples from a full-scale drinking water facility using MP UV/H_2O_2, resulted in the detection of 22 N-DBPs. The Ames mutagenicity test, a way to determine genotoxicity of (treated) water [5, 6, 17], was also performed. It was shown that chemical response detected by the suspect screening was comparable with the response obtained with Ames fluctuation assay using Salmonella strains TA98 and TA100. This implies that some of the 22 N-DBPs are possibly responsible for the positive response in the Ames fluctuation test. The genotoxic effect was shown to be effectively removed from treated drinking water with granular activated carbon (GAC) filtration and/or dune infiltration [5, 7].

Without the identity and any information about the toxic potency of N-DBPs, it is not possible to perform substance-specific health risk assessment. Therefore, it is important to identify these N-DBPs and to investigate what their mutagenic response is in the Ames test. In our follow-up study this was investigated by applying a fractionation method to MP UV treated water containing nitrate and NOM. Next, the different fractions were analysed by mutagenicity testing and chemical suspect screening [18]. This showed that the presence of

N-DBPs and mutagenicity in the Ames fluctuation test were correlated. Five potentially genotoxic by-products, with relatively high concentrations, were linked to fractions in which mutagenicity was observed. Of the 84 known N-DBPs formed by MP UV treatment, 14 by-products were unambiguously identified [13, 18]. However, the genotoxic potential of the identified by-products does not explain the observed Ames response, and the subject for the present research project is to further identify products that may explain the observed genotoxicity.

1.2 Objective BTO study

A different strategy for detecting by-products and assessment of their mutagenic response, is to use model compounds and apply MP UV treatment. Literature has shown that a possible source for the by-products, besides NOM, could be aromatic amino acids, which are present in NOM and surface water [19, 20]. Suzuki et al. [21] showed that aromatic amino acids (i.e. tryptophan, phenylalanine and tyrosine) become mutagenic after UV irradiation in water containing nitrate and nitrite. For the three aromatic amino acids the highest Ames response was observed for tryptophan, using the salmonella strain TA98 with and without S9 mix. Furthermore Aljammaz [22] showed that in water containing aromatic amino acids the concentration of inorganic nitrogen (i.e. nitrate, nitrite and ammonia) is substantially decreased after MP UV treatment, which indicates that at least a part of inorganic nitrogen is converted to organic nitrogen by nitration of the aromatic amino acids. Once again water containing tryptophan showed the largest nitrogen gap, indicating that tryptophan is most susceptible for nitration and thus by-product formation. Because aromatic amino acids are expected to be present in source water (i.e. surface water) [19, 20] for MP UV drinking water treatment, the role of aromatic amino acids in by-products formation needs to be further investigated.

In order to explain the observed genotoxic response in MP UV treated water and to perform substance-specific health risk assessment, the identities of these N-DBPs needs to be known. Based on evidence by Suzuki and Aljammaz, the following hypothesis was made: aromatic amino acids are a source for the formation of some of the genotoxic N-DBPs formed by MP UV water treatment.

The first part of the present study therefore aims to: (i) investigate the role of aromatic amino acids in N-DBPs formation, by irradiation of aromatic amino acids under MP UV conditions, in combination with stable isotope labeling and high resolution mass spectrometry; and (ii) identification of N-DBPs formed from aromatic amino acids and further identification of relevant N-DBPs formed during full-scale MP UV water treatment. To address the first goal, the labeling strategy developed by Kolkman et al. [13] using ^{14}N and ^{15}N nitrate was used, and was expanded by also using labeled and unlabeled aromatic amino acids, for the detection of N-DBPs and for obtaining structural information.

The second part of the study aims to: (i) perform a toxicological evaluation of newly identified N-DBPs in the present study; and (ii) conduct and evaluate a seven month monitoring study for identified by-products in order to determine the relevance of N-DBPs in a full-scale drinking water treatment facility using MP UV treatment.

1.3 Structure of this report

In the first part of the present study, the role of aromatic amino acids in N-DBPs formation is investigated. Therefore the presence of aromatic amino acids in in source water (Lake IJssel) and artificial water (i.e. NOM and nitrate dissolved in ultrapure water) has to be demonstrated first. Therefore an analytical method is developed in chapter 2, using liquid chromatography (LC) coupled to a high resolution quadrupole time of flight mass spectrometer (QToF), for the quantitative determination of aromatic amino acids in source and artificial water. In chapter 3 the developed LC-QToF method is optimised for stable isotope labeling experiments involving aromatic amino acids. Experiments are conducted using tyrosine, phenylalanine, tryptophan, labeled and unlabeled nitrate, labeled tryptophan and MP UV treatment. Subsequently, the artificial water and full-scale water treatment facility sample of the prior study [13] in which 84 and 22 N-DBPs were detected, is screened for aromatic amino acids N-DBPs (formed with labeling experiments) in order to determine if some of unidentified N-DBPs found in the prior study could originate from aromatic amino acids and be identified. In chapter 4, more of the 84 previously detected N-DBPs are identified and a toxicological evaluation is performed on the identified N-DBPs. Finally, an analytical target method (LC-QToF) is developed and validated for all identified N-DBPs in the current and prior study, in order to perform and evaluate a seven month monitoring study for N-DBPs in a full-scale drinking water treatment facility using MP UV treatment. Finally, in chapter 5 the conclusions of this study are presented and recommendations are discussed.

2 Development and validation of a LC-QToF method for the determination of aromatic amino acids in water

Only if aromatic amino acids are truly present in source water (Lake IJssel) and artificial water (i.e. NOM and nitrate dissolved in ultrapure water), N-DBPs originating from aromatic amino acids can be formed and labeling experiments are meaningful. Therefore, first an analytical method has to be developed for the determination of free dissolved aromatic amino acids (i.e. tyrosine, phenylalanine and tryptophan) in water.

This chapter describes the analytical method, method development, validation and sample analysis of free dissolved aromatic amino acids in water.

2.1 Amino acids analysis

Amino acids present in surface water play an important role in the biogeochemistry of nitrogen and carbon [23], and are therefore studied extensively. They can be analysed with a large variety of analytical techniques (e.g. HPLC, GC-MS, CE, IC) in many different types of matrices. Due to their hydrophilicity and zwitterionic nature, analysis of amino acids can be challenging. Derivatization techniques are therefore widely used to improve detection and chromatographic separation in biological and environmental matrices. But derivatization techniques have some major drawback such as, instable derivatives, low derivatives yield and being labour intensive. Therefore direct analysis techniques without derivatization are becoming more popular using analytical methodologies such as, CE-MS [24], HPAEC-PAD [25], ion-pair chromatography (LC) coupled to MS [26], and HILIC-MS [27]. For the analysis of the total amount of amino acids in a sample (i.e. bound species and biopolymer), often an acid or alkaline hydrolysis is employed before chromatographic separation. Since it was shown that free dissolved amino acids are a potential source for N-DBP formation [11, 21, 22], hydrolysis is not needed.

Because this study focuses on the moderately polar aromatic amino acids and their by-products, sufficient retention and detection is expected using a regular reversed phase C18 method. As a starting point for the analytical method development of free dissolved tyrosine (Tyr), phenylalanine (Phe) and tryptophan (Trp) (see Figure 1 for structures) in water, the non-target high resolution screening method employed in the previous studies was used [13, 18]. A high resolution mass spectrometer was used because of its capabilities for detecting unknown aromatic amino acids by-products, which is needed for the labeling experiments in chapter 3.

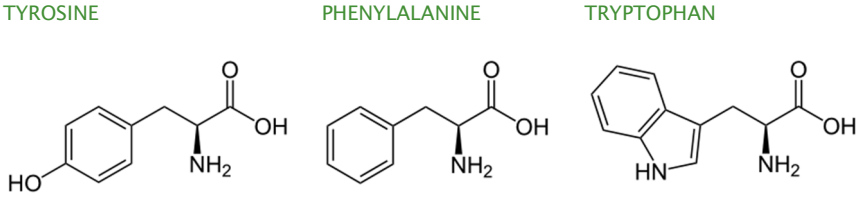

Figure 1: Structures of tyrosine, phenylalanine and tryptophan

2.2 Material & methods

In this section, the final optimized analytical method and sample pre-treatment is described for the analysis of tyrosine, phenylalanine and tryptophan in water.

2.2.1 Chemicals

All solvents used were of analytical grade quality. Acetonitrile and methanol (ultra gradient HPLC grade) were obtained from Avantor Performance Materials B.V. (Deventer, the Netherlands). Formic acid (HPLC quality) was purchased from Sigma-Aldrich (Steinheim, Germany). The aromatic amino acids reference standards; L-tyrosine, L-phenylalanine and L-tryptophan were acquired from Sigma-Aldrich. The isotopically labeled internal standards; L-tyrosine-d4, L-phenylalanine-d5 and L-tryptophan-d5 were purchased from Toronto Research Chemicals (Toronto, Canada). In table 1, the CAS number, formula, accurate mass of the protonated molecule ($[M+H]^+$) and Log D are shown for the aromatic amino acids and their corresponding internal standards. Ultrapure water was obtained by purifying demineralized water in an Elga Purelab Chorus ultrapure water system. (High Wycombe, United Kingdom). Stock solutions of the reference and internal standards were prepared in methanol and ultrapure water (20/80% v/v) at a concentration of 100 and 50 mg/L, respectively. Stock solutions were stored at -25° C. Standards were prepared from the stock solutions by dilution in ultrapure water, and were prepared shortly before analysis.

TABLE 1: CAS NUMBER, FORMULA, ACCURATE MASS PROTONATED MOLECULE AND LOG D OF AROMATIC AMINO ACIDS AND INTERNAL STANDARDS

Name	CAS number	Formula	Accurate mass $[M+H]^+$	Log D* (pH 4)
L-tyrosine	60-18-4	$C_9H_{11}NO_3$	182.0812	-1.49
L-phenylalanine	63-91-2	$C_9H_{11}NO_2$	166.0863	-1.20
L-tryptophan	73-22-3	$C_{11}H_{12}N_2O_2$	205.0972	-1.10
Internal standards				
L-tyrosine-d4	62595-14-6	$C_9H_7D_4NO_3$	186.1063	n.d.
L-phenylalanine-d5	56253-90-8	$C_9H_6D_5NO_2$	171.1176	n.d.
L-tryptophan-d5	62595-11-3	$C_{11}H_7D_5N_2O_2$	210.1285	n.d.

n.d. not determined

** calculated using ChemAxon*

2.2.2 Sample pre-treatment

Fifty mL of water sample was transferred into a 50 mL flask, to which the internal standards were added (2.0 µg/L). After homogenization the samples were filtered using a 0.2 µm Phenomenex Phenex regenerated cellulose filter (Utrecht, Netherlands) and were transferred to an autosampler vial for LC-QToF analysis.

2.2.3 LC-QToF analysis

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 achieved using a Xbridge BEH C18 XP (2.1 x 100 mm, 2.5 µm, Waters, Milford, MA, USA) preceded by a Phenomenex SecurityGuard Ultra column (Phenomenex, Torrance, USA) at a temperature of 25° C. The mobile phase consisted out of solvent A; ultrapure water with 0.05% formic acid (v/v) and solvent B; acetonitrile with 0.05% formic acid (v/v). The gradient elution started with 4% B and was held constant for 1 minute, and was then followed by a linear gradient to 100% B in 7 min, and was held constant at 100% B for 4 min. Then the mobile phase was returned to initial gradient conditions in 0.5 min and

was held for 4.5 min. The mobile phase flow rate was 0.3 mL/min and the injection volume was set to 50 µL.

Detection was performed using an AB Sciex TripleTOF 5600+ high resolution QToF mass spectrometer (AB Sciex, Concord, Canada) operated in positive electrospray (ESI) mode with a DuoSpray ion source. External mass calibration was automatically performed after thirty consecutive samples by a calibration delivery system (AB Sciex) using the APCI probe of the DuoSpray ion source. The source conditions were as follows: ion spray voltage, 5.0 kV; ion source gas 1 and 2 at 40 and 50 psi, respectively; curtain gas, 25 psi; temperature, 500 °C and declustering potential, 70 V. Full scan accurate MS and MS/MS mass spectra were acquired from 100 to 800 Da with a resolving power of 30,000 FWHM (at m/z 400). In order to unambiguously confirm the identities of tyrosine, phenylalanine and tryptophan, MS/MS spectra were recorded with a collision energy of 35 eV and collision energy spread (CES) of 15 eV. The recording of MS/MS spectra of the analytes, specified in the mass list, was continuously acquired (no threshold) from 40 to 300 Da. Data acquisition and processing were performed using Analyst TF 1.6 and Multiquant 3.0 software (AB Sciex).

2.3 Results method development and optimisation

For the method development of free dissolved aromatic amino acids in water, the LC-QToF non-target screening method employed in the previous study [18] was used as starting point . Due to the hydrophilicity of the aromatic amino acids (see table 1 for Log D values), sample pre-treatment using the solid phase extraction method described in the previous study is not possible. The recovery for these aromatic amino acids would just be too low. Therefore the decision was made, to use a direct injection approach in which water samples are directly injected onto the column, in order to minimize the loss of aromatic amino acids. The same approach is used for the labeling experiments described in chapter 3, also to minimize the loss of N-DBPs during sample pre-treatment.

A reversed phase Xbridge BEH C18 XP analytical column was used for the method development. And for the mobile phase a combination of ultrapure water (A) and acetonitrile (B) with formic acid as modifier (0.05% v/v) was used. The initial gradient of the non-target screening method started with 5% B. First the injection volume was optimised. In order to obtain the most sensitivity, a large injection volume (for a 2.1 mm column) of 100 µL was tested first. This resulted in a broad peak for tyrosine (most polar) and also a moderately broad peak for phenylalanine. Satisfactory peak shapes were obtained for all aromatic amino acids using a 50 µL injection. The gradient was then further optimised by lowering the initial gradient to 4% B, and by holding the gradient for 1 min, improving retention for tyrosine. Furthermore the linear gradient was shortened from 40 min (100% B) to 7 min (100% B), improving total analysis time from 52 min to 17 min.

Since mass spectrometric analysis of amino acids can be performed in the positive or negative mode using electrospray ionisation, first a comparison was made between both ionisation modes. It was determined that the sensitivity was improved substantially (> 2x) in the positive mode. Therefore the mass spectrometric detection was performed by the detection of the protonated molecular ion ($[M+H]^+$) using an extracted ion chromatogram window of 10 ppm. In order to improve selectivity and sensitivity, continuous MS2 spectra recording of the aromatic amino acids was added to the QToF acquisition method. The most intense fragments per compound were selected for quantification purposes. This resulted in improved selectivity and thus lower detection limits for all aromatic amino acids. See figure 2 for a comparison between the extracted ion chromatogram (EIC) of the protonated molecular ion ($[M+H]^+$) and EIC of the most intense fragment per compound.

Fragment ions were acquired with a collision energy of 20, 35 and 50 eV, which was automatically averaged (CES) to obtain MS2 spectra with many fragment ions (see attachment I for MS2 spectra of tyrosine, phenylalanine and tryptophan).

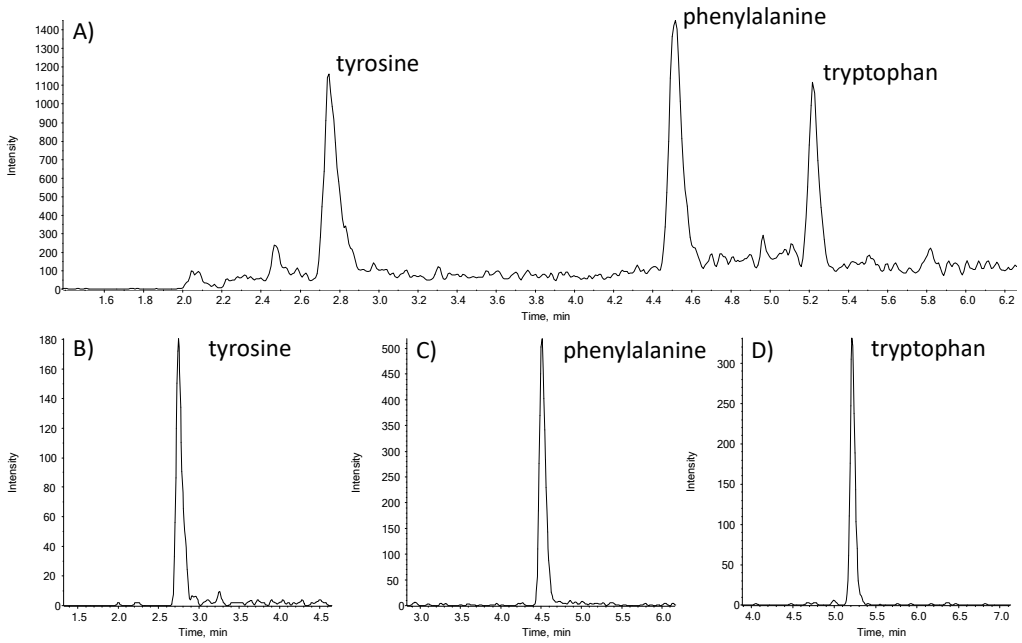

Figure 2: Chromatograms of drinking water spiked with 0.25 µg/L aromatic amino acids. A) Summed EIC $[M+H]^+$ trace (10 ppm) of aromatic amino acids. B) Tyrosine MS2 EIC of m/z 91.0542. C) Phenylalanine MS2 EIC 120.0808. D) Tryptophan MS2 EIC of m/z 118.0651.

Sample pre-treatment consisted of adding isotopically labeled internal standards of the aromatic amino acids to the sample followed by filtration using a 0.20 µm filter prior to LC-QToF analysis. No loss of aromatic amino acids was observed during the filtration step.

2.4 Method validation

The developed analytical method was validated for drinking and surface water. The limit of detection (LOD), limit of quantification (LOQ), repeatability and recovery were determined for all aromatic amino acids in both matrices. The validation results are shown in table 2 for drinking water and table 3 for surface water.

TABLE 2: VALIDATION RESULTS AROMATIC AMINO ACIDS IN DRINKING WATER (N=8)

Compounds	LOD (µg/L)	LOQ (µg/L)	Repeatability 1 µg/L (%)	Recovery 1 µg/L (%)
L-tyrosine	0.033	0.10	3.5	98.5
L-phenylalanine	0.008	0.10	2.6	98.5
L-tryptophan	0.016	0.10	2.2	101.9

TABLE 3: VALIDATION RESULTS AROMATIC AMINO ACIDS IN SURFACE WATER (N=8)

Compounds	LOD (µg/L)	LOQ (µg/L)	Repeatability 1 µg/L (%)	Recovery 1 µg/L (%)
L-tyrosine	*	0.10	2.5	93.1
L-phenylalanine	*	0.10	0.8	94.3
L-tryptophan	*	0.10	2.4	101.6

** Due to the presence of significant amounts of aromatic amino acids in surface water, it was not possible to determine all validation characteristics. The validation results of drinking water can be used as reference.*

Satisfactory LOD and LOQ results were obtained for the developed analytical method in drinking water. For surface water the LOD could not be determined due to the presence of significant amounts of aromatic amino acids and therefore the LOD of drinking water was used as a reference. The LOQ (i.e. ≥ 3x LOD) was determined for all aromatic amino acids at 0.10 µg/L. Recoveries in drinking- and surface water are between 90.0 and 105 % and are satisfactory. The reproducibility for all compounds is lower than 4 % (at 1 µg/L). The validation results for aromatic amino acids in drinking- and surface water show that the analytical method developed can successfully be applied for the determination of aromatic amino acids in water.

2.5 Source and artificial water analysis

After validation, the method developed was applied to the analysis of aromatic amino acids in source and artificial water. A sample was taken from Lake IJssel which is used as source water for Heemskerk drinking water treatment facility, which uses UV/H_2O_2 for disinfection. 22 N-DBPs were detected after MP UV treatment in this drinking water treatment facility. Two artificial water samples were also prepared containing Pony Lake or Suwannee river NOM. Artificial water containing Pony Lake NOM was treated with MP UV in the previous study [13], resulting in the detection of 84 N-DBPS. Suwannee River NOM is probably the best characterized NOM [28] and was therefore used as a reference. Results of aromatic amino acid analysis in source and artificial water are shown in table 4.

TABLE 4: RESULTS AROMATIC AMINO ANALYSIS IN SOURCE AND ARTIFICAL WATER

Compounds	Lake IJssel water intake (µg/L)	Pony Lake NOM 5.4 mg/L (µg/L)	Suwannee river NOM 5.5 mg/L (µg/L)
L-tyrosine	0.15	2.7	0.80
L-phenylalanine	0.16	1.5	0.44
L-tryptophan	< 0.1 (0.08)*	0.61	0.14

** Detected concentration was lower than LOQ but higher than LOD.*

Tyrosine, phenylalanine and tryptophan were detected in all samples with concentrations ranging from 0.08 to 2.7µg/L. Tryptophan was detected in Lake IJssel below the LOQ but higher than the LOD, therefore the reported concentration is semi-quantitative. It was shown that aromatic amino acids are present in moderate concentrations in Lake IJssel, meaning, meaning that aromatic amino acids can be a potential source for N-DBPs.

2.6 Summary/conclusion

A LC-QToF method was developed for the determination of aromatic amino acids in drinking- and surface water. Satisfactory LOD and LOQ results were obtained for both drinking- and surface water. Aromatic amino acids were detected in source and artificial water, showing that they can be a potential source for the formation of N-DBPS.

The developed analytical method demonstrated that it is well applicable for analysis of aromatic amino acids, and will therefore be used for the labeling experiments in chapter 3. However some adjustment will be made to the method in order to perform non-target screening for unknown aromatic N-DBPs (e.g. longer gradient and information dependent MS2 acquisition).

3 Labeling experiments

In chapter 2 it was shown that aromatic amino acids are present in source and artificial water, meaning that the possibility exist that some of the N-DBPs could originate from aromatic amino acids after MP UV treatment. In this chapter stable isotope labeling experiments will be conducted to really find out whether this is the case.

3.1 Stable isotope labeling strategy

In the previous study, an innovative stable isotope labeling strategy was developed for tracing N-DBPs in artificial water [13], based on incorporation of the nitrate atom originating from nitrate into an newly formed N-DBP after MP UV treatment. This strategy will also be used for labeling experiments with aromatic amino acids.

The labeling strategy works as follows: when stable isotope nitrate ($^{15}NO_3^-$) is added to artificial water and normal nitrate ($^{14}NO_3^-$) is added to another artificial water sample from the same source and both are MP UV treated, ^{15}N will be incorporated into a newly formed N-DBP in the first artificial water sample, and ^{14}N will be incorporated into the same N-DBP in the second artificial water. This will result in a mass difference of 0.99704 Da between the N-DBP formed with normal nitrate ($^{14}NO_3^-$) and labeled nitrate ($^{15}NO_3^-$). This mass difference can be detected by high resolution mass spectrometry in combination with a non-target screening approach (see figure 3). Only N-DBPs will have this characteristic mass difference, and can therefore be distinguished from regular DBPs and background ions.

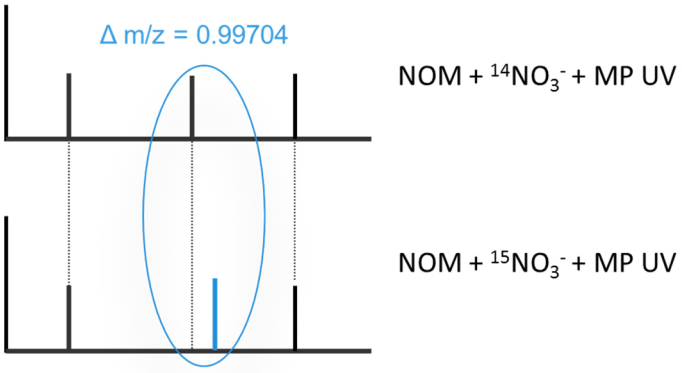

Figure 3: Stable isotope labeling strategy for the detection of N-DBPs. A mass spectrum is shown with a Δm/z of 0.99704 between the stable isotope labeled N-DBP compared to the normal N-DBP.

3.2 Tryptophan labeling experiments

Since tryptophan showed the highest Ames response and nitrogen gap in other studies [21, 22] after MP UV treatment, tryptophan is the obvious choice for starting the labeling experiments. Two stable isotope-labeled substances were used for labeling experiments, i.e. nitrate ($^{15}NO_3^-$) and tryptophan-$^{13}C_{11}$ ($^{13}C_{11}H_{12}N_2O_2$).The stable isotope-labeled tryptophan was used to provide additional certainty that the formed by-product really originates from tryptophan itself, and not from an unwelcome contamination. Another advantage of using labeled tryptophan is that the number of carbon atoms present in the formed by-products can easily be determined. A sample scheme was made for the tryptophan labeling experiments, in which different sample compositions are tested (table 5). All the samples consisted out of ultrapure water to which a combination of; unlabeled tryptophan, labeled tryptophan, unlabeled nitrate, labeled nitrate and Pony Lake NOM was added. For the

labeling experiments a relatively high concentration (5 mg/L) of labeled and unlabeled tryptophan was used, in order to ensure that high concentration of by-products were formed in, which easily should be detected using mass spectrometry. Nitrate and NOM concentration were at the same level as in the previous studies [13, 18], in order to obtain comparable results and to ensure by-product formation. NOM was added to some samples to find out if NOM or NOM intermediate products can react with tryptophan to form other by-products.

All samples were prepared in fourfold (with exception of the untreated reference) which then were subdivided into two duplicate sample sets, of which one duplicate sample set was MP UV treated while the other remained untreated. Of each sample 100 mL was prepared and was transferred to a glass sample bottle and was stored at 1-5 °C until MP UV treatment.

TABLE 5: OVERVIEW TRYPTOPHAN LABELING SAMPLE SCHEME

Samples	MP UV treatment	Unlabeled Tryptophan 5 mg/L	Labeled Tryptophan 5 mg/L	Unlabeled nitrate 10 mg/L	Labeled nitrate 10 mg/L	Pony Lake NOM 5 mg/L
Untreated reference (ultrapure water)						
Untreated + Trp, without nitrate		x				
UV treated + Trp, without nitrate	x	x				
Untreated + Trp + $^{14}NO_3^-$		x		x		
UV treated + Trp + $^{14}NO_3^-$	x	x		x		
Untreated + Trp + $^{15}NO_3^-$		x			x	
UV treated + Trp + $^{15}NO_3^-$	x	x			x	
Untreated + Trp + $^{14}NO_3^-$ + NOM		x		x		x
UV treated + Trp + $^{14}NO_3^-$ + NOM	x	x		x		x
Untreated + Trp + $^{15}NO_3^-$ + NOM		x			x	x
UV treated + Trp + $^{15}NO_3^-$ + NOM	x	x			x	x
Untreated + Trp-^{13}C, without nitrate			x			
UV treated + Trp-^{13}C, without nitrate	x		x			
Untreated + Trp-^{13}C + $^{14}NO_3^-$			x	x		
UV treated + Trp-^{13}C + $^{14}NO_3^-$	x		x	x		
Untreated + Trp-^{13}C + $^{15}NO_3^-$			x		x	
UV treated + Trp-^{13}C + $^{15}NO_3^-$	x		x		x	
Untreated + Trp-^{13}C + $^{14}NO_3^-$ + NOM			x	x		x
UV treated + Trp-^{13}C + $^{14}NO_3^-$ + NOM	x		x	x		x
Untreated + Trp-^{13}C + $^{15}NO_3^-$ + NOM			x		x	x
UV treated + Trp-^{13}C + $^{15}NO_3^-$ + NOM	x		x		x	x
UV treated + Trp-^{13}C + $^{14}NO_3^-$ + $^{15}NO_3^-$	x	x		x*	x*	
UV treated + Trp-^{13}C $^{14}NO_3^-$ + $^{15}NO_3^-$ + NOM	x	x		x*	x*	x

All samples were prepared in duplicate
** 5 mg/L*

3.2.1 MP UV treatment

The samples were sent to PWN technologies for MP UV treatment using a collimated beam set-up. Fifty-five mL of sample was transferred into a 60 × 35 mm crystallizing dish and was MP UV treated in open air at room temperature. The MP UV dose was delivered by a Trojan collimated beam apparatus using a 3 kW medium pressure Hg lamp. UV dose calculations were performed according to Bolton and Linden [29]. UV intensity was measured using a radiometer (International Light IL1700). A MP UV dose of 600 mJ/cm^2 was applied to each

sample. After irradiation the samples were returned and stored at 1-5 °C awaiting sample pre-treatment and LC-QToF analysis.

3.2.2 Sample pre-treatment

Twenty-five mL of water sample was transferred into a 25 mL flask, to which the internal standard tryptophan-d5 was added (100 µg/L). After homogenization, the samples were filtered using a 0.20 µm filter and were transferred to an autosampler vial for LC-QToF analysis.

3.2.3 LC-QToF analysis

For the analysis of tryptophan labeling samples, the aromatic amino acid method used in chapter 2 was partially adjusted. A longer linear gradient was applied, increased from 7 minutes to 100 %B, into 40 minutes to 100% B, in order to achieve a better separation for by-products and also to detect less-polar by-products. Data acquisition was performed in positive and negative ionisation mode. And instead of using a mass list for triggering MS/MS spectra, information dependent acquisition (IDA) was used for triggering MS/MS spectra. Eight IDA MS/MS spectra were triggered per full scan cycle, only for signals higher than 100 counts in combination with background subtraction and dynamic exclusion. The remainder of the LC-QToF settings can be found in attachment II (materials & methods labeling experiments).

3.2.4 Mass spectrometric data analysis

After LC-QToF analysis, the raw mass spectrometric data was processed using MasterView (Sciex) and differential analysis was performed in order to detect differences between $^{14}NO_3^-$, $^{15}NO_3^-$, Trp$^{12}C_{11}$, Trp-$^{13}C_{11}$, TRP-$^{12}C_{11}D_5$ and NOM MP UV treated samples, and the control samples. The intensity threshold for MasterView was set at 2000 counts for the positive and negative mode. The chromatographic data was compared from 1.5 to 35 min, with a mass range of 65-800 Da and an EIC width of 0.02 Da and retention window of 1min.

The nitrate labeling strategy was used for the detection of all N-DBPs formed by the MP UV treatment of nitrate. For this the UV treated Trp + $^{14}NO_3^-$ sample was compared with UV treated Trp + $^{15}NO_3^-$ sample, wherein all detected compounds with a mass difference of 0.99704 Da between the $^{14}NO_3^-$ and $^{15}NO_3^-$ sample, which were not present in the control samples, were detected as N-DBPs. Also the UV treated Trp + $^{14}NO_3^-$ sample was compared with UV treated Trp-$^{13}C_{11}$ + $^{14}NO_3^-$ sample, in order to detect all by-products originating from tryptophan, including by-products that were formed only by UV photolysis without interactions of nitro radicals. An overview of the amount of detected by-products is shown in table 6.

TABLE 6: OVERVIEW OF THE NUMBER OF DETECTED TRYPTOPHAN BY-PRODUCTS

Type of compounds	Number of accurate masses		Summed concentration Trp-d5 equivalents (µg/L)	
	Positive	Negative	Positive	Negative
DBPs (all)	957	1127	4049	3292
N-DBPs	157	278	680	741

Many by-products were formed by MP UV treatment of water containing tryptophan. In total 957 and 1127 accurate masses were detected in positive and negative mode, respectively. Of these detected accurate masses only a relative small number were N-DBPs, 157 for positive mode and 278 for negative mode. The number of accurate masses of DBPs and N-DBPs stated in the table are not all unique. Some of the detected accurate masses are from

fragments or adducts of by-products, therefore the actual number of by-products will be lower.

In order to obtain a good overview and to find out if most of the by-products are detected with the applied approach, a mass balance was made up. For this the concentration of tryptophan before UV treatment and after UV treatment was calculated using Trp-d5. All the detected by-products were also semi-quantified as Trp-d5 internal standard equivalent. The mass balance is shown in table 7.

TABLE 7: MASS BALANCE OF TRYPTOPHAN AND BY-PRODUCTS AFTER MP UV (TRP-D5 EQUIVALENTS)

Type of compounds	Positive mode		Negative mode	
	Concentration (µg/L Trp-d5 eq.)	(%)	Concentration (µg/L Trp-d5 eq.)	(%)
Tryptophan before UV	5188	-	5188	-
Tryptophan after UV	1117	21.5	1560	30.1
DBPs after UV	3369	64.9	2551	49.2
N-DBPs after UV	680	13.1	741	14.3
Sum Trp + DBPs	5166	99.6	4852	93.6

The concentration of tryptophan after MP UV is substantially decreased to 21.5% and 30.1% of its initial amount, for positive and negative mode respectively. This means that a considerable amount of tryptophan is converted by UV photolysis and nitro radicals into DBPs and N-DBPs, which is confirmed by the detected amounts DBPs and N-DBPs. In the end, 99.6% and 93.6% (positive and negative mode) of the mass balance is accounted for, demonstrating that most of by-products are detected using this approach. However, there are some remarks for calculating the mass balance like this. First, the concentration of the by-products cannot be determined exactly, because the ionisation efficiency is different for each by-product. Therefore the calculated concentration is an indication. Second, there is no correction made for the amount of nitrate/nitro groups reacting with tryptophan to form N-DBPs. Nevertheless, the mass balance still gives a good overview of the performed experiment and also shows that most of the by-products are probably detected.

3.2.5 Tryptophan labeling results

For the detection of N-DBPs formed by MP UV treatment of tryptophan, the UV treated Trp + $^{14}NO_3^-$ sample was compared with UV treated Trp + $^{15}NO_3^-$ sample as described in 3.2.4. An example of an N-DBP detected using the labeling strategy is shown figure 4. In this figure an extracted ion chromatogram is shown of an N-DBP with the elemental composition $C_8H_6N_2O_2$. In the $^{14}NO_3^-$ MP UV treated sample, a chromatographic peak is detected for m/z 161.0360, but is not present in the $^{14}NO_3^-$ sample without MP UV treatment. In the $^{15}NO_3^-$ MP UV treated sample, a chromatographic peak is detected for m/z 162.0331 and no peak is visible for m/z 161.0360. In the sample with an equal amount (1:1) of $^{14}NO_3^-$ and $^{15}NO_3^-$, both peaks are detected in the same ratio and at the same retention time. This confirms that detected compound at m/z 161.0360 is really a N-DBP. The 1:1 mixture sample can also be used to search for peak pairs with a 1:1 ratio and mass difference of m/z 0.99704 (for a single N incorporation), making data analysis easier.

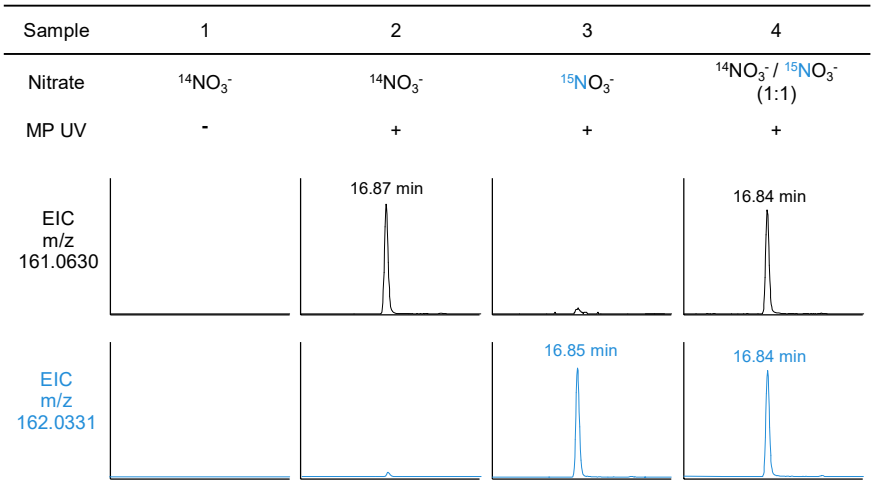

Figure 4: Example of a detected N-DBP ($C_8H_6N_2O_2$) in negative mode that is formed after MP UV treatment. The EICs of m/z 161.0360 and m/z 162.0331 are shown.

In total 157 and 278 accurate masses of N-DBPs were detected in the positive and negative mode, respectively The N-DBPs were considered to be "real" when they were present in the same ratio in the $^{14}NO_3^-$ and $^{15}NO_3^-$ sample after MP UV treatment and were present in the 1:1 mixture sample in the same ratio and were also detected in the ^{13}C-Trp sample after MP UV treatment. The ^{13}C-Trp sample was used for confirmation and determination of the amount of ^{13}C atoms in the formed by-products, which give extra information about the elemental compositions of the by-products. The top 10 and top 15 of the highest detected N-DBPs in the positive and negative mode are shown respectively in tables 8 and 9. The elemental composition and mass error were determined for the listed N-DBPs, and for some of the detected N-DBPs the identity was also determined. For the identification, a diagram containing common tryptophan modifications by nitrating agents [30] was used, or were determined using the elemental composition and MS/MS spectrum. All identifications are tentative.(i.e. level 2/3 according to Schymanski [31])

TABLE 8: MOST ABUNDANT TRYPTOPHAN N-DBPS IN POSITIVE MODE

Accurate mass $^{14}NO_3^-$	Accurate mass $^{15}NO_3^-$	Accurate mass ^{13}C-Trp	RT (min)	Intensity	Concn. Trp-d5 equiv (µg/L)	13C atoms	Formula	Δppm	Identity
282.0729	283.0702	293.1097	6.36	344064	61	11	$C_{11}H_{11}N_3O_6$	3.7	dihydroxy-nitrotryptophan
220.0723	221.0691	230.1051	6.97	329039	58	10	$C_{10}H_9N_3O_3$	2.4	
220.0721	221.0691	230.1051	5.59	300943	53	10	$C_{10}H_9N_3O_3$	1.5	
266.0780	267.0749	277.1146	3.90	174436	31	11	$C_{11}H_{11}N_3O_5$	3.2	hydroxy-nitrotryptophan
248.0673	249.0642	259.1038	3.90	139152	25	11	$C_{11}H_9N_3O_4$	2.9	
266.0779	267.0749	277.1145	5.22	137670	24	11	$C_{11}H_{11}N_3O_5$	3.2	hydroxy-nitrotryptophan
264.0625	265.0593	275.0989	4.02	118528	21	11	$C_{11}H_9N_3O_5$	2.7	
250.0828	251.0801	261.1196	8.91	108081	19	11	$C_{11}H_{11}N_3O_4$	2.3	
254.0779	255.0748	264.1112	7.03	104660	19	10	$C_{10}H_{11}N_3O_5$	2.6	
203.0453	204.0423	213.0784	6.96	97688	17	10	$C_{10}H_6N_2O_3$	0.9	

TABLE 9: MOST ABUNDANT TRYPTOPHAN N-DBPS IN NEGATIVE MODE

Accurate mass $^{14}NO_3^-$	Accurate mass $^{15}NO_3^-$	Accurate mass ^{13}C-Trp	RT (min)	Intensity	Concn. Trp-d5 equiv (µg/L)	13C atoms	Formula	Δppm	Identity
189.0311	190.0281	198.0611	14.53	253492	42	9	$C_9H_6N_2O_3$	2.3	nitroindole-carbaldehyde
280.0579	281.0550	291.0944	6.36	240716	40	11	$C_{11}H_{11}N_3O_6$	1.4	dihydroxy-nitrotryptophan
217.0260	218.0229	227.0595	10.85	237069	40	10	$C_{10}H_8N_2O_5$	1.5	
236.0681	237.0649	246.1014	5.59	230689	39	10	$C_{10}H_{11}N_3O_4$	1.4	
264.0632	265.0600	275.0998	3.90	214684	36	11	$C_{11}H_{11}N_3O_5$	1.9	hydroxy-nitrotryptophan
264.0631	265.0600	275.0996	5.21	162315	27	11	$C_{11}H_{11}N_3O_5$	1.5	hydroxy-nitrotryptophan
262.0476	263.0443	273.0839	4.03	136529	23	11	$C_{11}H_9N_3O_5$	1.4	
248.0677	249.0647	259.1052	8.58	104598	18	11	$C_{11}H_{11}N_3O_4$	-0.3	nitrotryptophan
233.0205	234.0174	243.0544	8.89	99350	17	10	$C_{10}H_6N_2O_5$	0.0	
190.0260	191.023	198.0527	10.66	88417	15	8	$C_8H_5N_3O_3$	0.4	
280.0574	281.0543	291.0942	11.9	83280	14	11	$C_{10}H_9N_3O_4$	-0.4	
161.0360	162.0331	169.0629	16.87	70977	12	8	$C_8H_6N_2O_2$	2.2	
189.0308	190.0278	198.0609	15.48	64659	11	9	$C_9H_6N_2O_3$	0.2	
235.0359	236.0330	245.0692	13.67	56744	9	10	$C_{10}H_8N_2O_5$	-1.0	
280.0573	281.0542	291.0939	10.16	50687	8	11	$C_{11}H_{11}N_3O_6$	-1.1	

Dihydroxy-nitrotryptophan is detected as highest in the positive mode and as second highest in the negative mode. The identity of the most intense N-DBP with m/z 189.0311 in the negative mode is uncertain. A possible candidate is nitroindole-carbaldehyde, but many structural isomers are possible. Another frequently detected N-DBP (multiple isomers) is nitrotryptophan, which is detected using both ionisation modes. As expected there is a substantial overlap between the N-DBPs detected in the positive and negative mode, due to the presence of functional groups that are ionisable in the positive (e.g. nitrogen) and negative (e.g. carboxyl and hydoxy) ionisation mode.

3.2.6 NOM samples

For the tryptophan labeling experiments also some samples were prepared containing NOM (see 3.2) in order to find out if NOM or NOM intermediate products can react with tryptophan to form other by-products, or have any effect on by-product formation. For this the UV treated Trp + $^{14}NO_3^-$ + NOM sample was compared with UV treated Trp + $^{15}NO_3^-$ + NOM sample. The top 25 highest detected by-products were then compared with the top 25 by-products formed without NOM to check if there was any difference. See table 10 for an overview of the total amount of N-DBPs detected in the NOM sample, compared with the amount of N-DBPs detected without NOM.

TABLE 10: COMPARSION OF THE AMOUNT OF N-DBPS DETECTED WITH AND WITHOUT NOM

Sample	Detected accurate masses		Summed concentration Trp-d5 equivalents (µg/L)	
	Positive	Negative	Positive	Negative
Trp + $^{14}NO_3^-$ without NOM	157	278	680	741
Trp + $^{14}NO_3^-$ with NOM	165	189	595	459

The same N-DBPs were found with and without NOM, although the concentrations detected in the NOM samples are on average lower. The total amount and concentration of by-products detected in the NOM samples is lower. This is probably due to the available amount of nitrate present in the sample, resulting in competition between the formation of

tryptophan N-DBPs and NOM N-DBPs. So in the end more by-products are probably formed in the presence of NOM, but remain undetected because they fall below the threshold of detection. This experiment shows that presence of NOM has a relatively small effect on tryptophan N-DBP formation, and will therefore not be used for the stable isotope labeling experiments with tyrosine and phenylalanine.

3.2.7 Suspect screening of 84 N-DBPs

The goal of the tryptophan labeling experiments was to find out if some of unidentified N-DBPs detected in artificial water and/or full-scale water treatment facility (both MP UV treated) in the prior study could originate from tryptophan. So a suspect screening was performed for the 84 N-DBPs detected in previous study (see attachment III for the list). For the suspect screening the MP UV treated Trp + $^{14}NO_3^-$ sample was used. In order to confirm a possible match, the artificial water samples and samples of the full-scale water treatment facility (both SPE extracts) of the prior study were analysed again using the analytical method for the tryptophan labeling experiments. The sample extracts were pre-treated in the prior study using the AMES SPE protocol, in order to achieve sufficient sensitivity for the Ames fluctuation assay and non-target HR-MS screening. Because the aromatic amino labeling experiments were conducted at relatively high concentrations, SPE treatment was not needed, and sufficient sensitivity was achieved using direct injection.

With the suspect screening, only one N-DBPs at m/z 161.0360 (in negative mode) was detected in the original $^{14}NO_3^-$ artificial water sample of the prior study, was also detected in the MP UV treated Trp + $^{14}NO_3^-$ sample of the current study. See table 11 for the results. The N-DBP with m/z 161.0360 was not detected in the full-scale water treatment sample.

TABLE 11: RESULTS SUSPECT SCREENING OF THE MOST ABUNDANT TRYPTOPHAN N-DBPS IN THE ARTIFICAL WATER SAMPLE OF THE PRIOR STUDY

Accurate mass $^{14}NO_3^-$	RT (min)	Intensity	Concn. Trp-d5 equiv (µg/L)	Formula	RT Trp N-DBPs Trp + $^{14}NO_3^-$ sample (min)	RT Original $^{14}NO_3^-$ sample (min)	RT, MS1 and MS2 Confirmed
161.0360	16.87	70977	12	$C_8H_6N_2O_2$	16.87	16.88	yes

With the suspect screening there were no N-DBPs detected in the positive mode in the original $^{14}NO_3^-$ artificial water sample. In the negative mode one by-product with m/z 161.0360 was detected and confirmed in the original $^{14}NO_3^-$ artificial water sample by matching retention time and MS/MS spectrum (see figure 5 and table 11). This demonstrates that experimental design of this study has worked, and that one by-product detected in the prior study could potentially originate of tryptophan.

In the prior study only 16 N-DBPs (of which 6 uniquely) of the 84 N-DBPs were detected in the positive mode, therefore it was taken into account that N-DBPs originating from tryptophan most likely would be detected in the negative mode. Of the top 15 by-products formed by the MP UV tryptophan labeling experiments, only number 12 (m/z 161.0360), a relatively low intensity N-DBPs was detected in artificial water. This could mean that the other by-products are not formed during the MP UV irradiation of artificial water, or that these other formed by-products are not sufficiently extracted from the water using AMES pre-treatment protocol, which was used as sample pre-treatment in the prior study. So in order to investigate if the detected N-DBP, really originate of tryptophan, SPE experiment will be conducted to determine the extraction recovery of the top 15 N-DBPs in negative mode. See 3.4 for the SPE experiments.

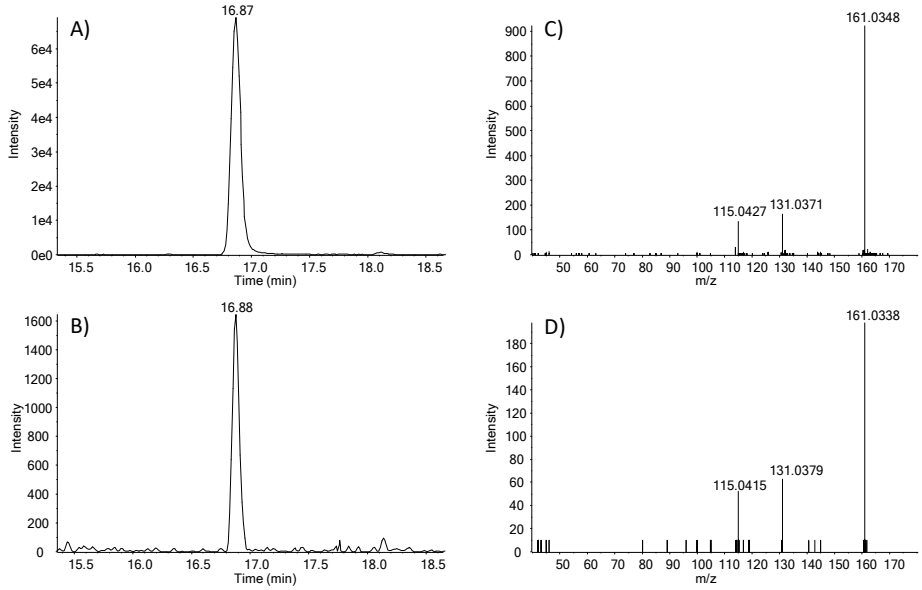

Figure 5: confirmation of N-DBP m/z 161.0360 in artificial water. A) EIC of m/z 161.0360 in Trp + $^{14}NO_3^-$ after MP UV sample. B) EIC of m/z 161.0360 in artificial water after MP UV. C) MS2 spectrum of m/z 161.0360 in Trp + $^{14}NO_3^-$ after MP UV sample. D) MS2 spectrum of m/z 161.0360 in artificial water after MP UV.

3.2.8 Identification of m/z 161.0360

Before a toxicological evaluation can be performed for the N-DBPs with m/z 161.0360, the identity must be known. The identification process for m/z 161.0360 ($C_8H_6N_2O_2$) started with investigating the loss of elements during N-DBP formation. The molecular formula of the by-product ($C_8H_6N_2O_2$) was subtracted from that of tryptophan ($C_{11}H_{12}N_2O_2$). So during N-DBP formation C_3H_6 was lost, and the by-product was very likely nitrated, and gained also a nitro (NO_2) group. This means that of the original tryptophan structure $C_3H_6NO_2$ was lost. This loss shows that the acid functional group and amine functional group of the basic amino acid structure were probably lost during N-DBP formation, whereby the nitrated indole structure remains. See figure 6 for the structure of indole.

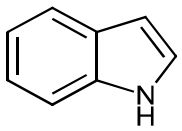

Figure 6: structure of indole (CAS nr. 120-72-9)

The theory that the formed by-product is nitroindole, is supported by the observed retention for this by-product (16.87 min) compared with that of tryptophan (6.86). Nitroindole is less polar due to the missing amine and acid functional group, and should therefore have a substantially higher retention, which is the case. Next a reference spectrum was sought and found of 6-nitroindole in the NIST EI-GC-MS library. This EI-GC-MS spectrum (figure 7) containing nominal masses, was compared with that of the MS2 spectrum of the by-product (figure 5C) and showed similarities. There is a mass difference of one Da between the EI-GC-MS and LC-MS MS2 $[M-H]^-$ spectrum, which can be explained by the loss of a proton (negative mode ionisation).

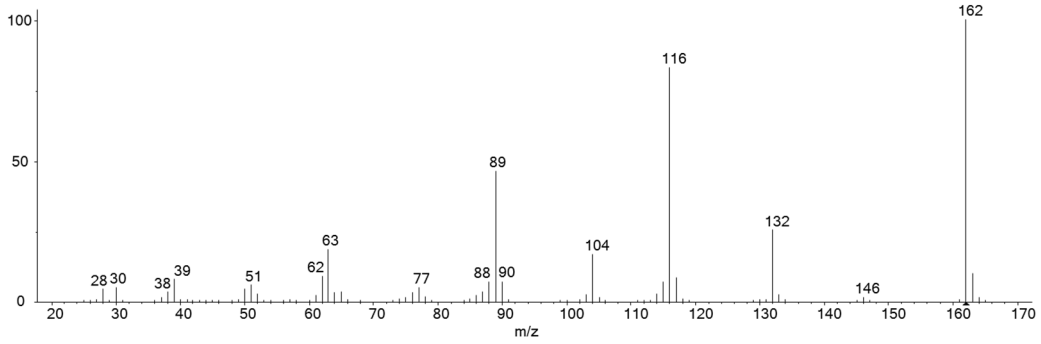

Figure 7: EI-GC-MS reference spectrum of 6-nitroindole

Because nitroindole has many structural isomers, the most common structural isomers were ordered as reference standard for the confirmation of the identity of the by-product. The results of the reference standards and the Trp + $^{14}NO_3^-$ MP UV sample analysis is shown in table 12.

TABLE 12: RESULTS REFERENCE STANDARD ANALYSIS OF NITROINDOLE ISOMERS

Compound	CAS nr	RT sample (min)	RT reference standard (min)	RT, MS1 and MS2 Confirmed
7-Nitroindole	6960-42-5	16.19	18.70	no
6-Nitroindole	4769-96-4	16.19	18.28	no
5-Nitroindole	6146-52-7	16.19	17.49	no
4-Nitroindole	4769-97-5	16.19	17.06	no
3-Nitroindole	4770-03-0	16.19	16.18	yes

The identity of 3-nitroindole was unambiguously confirmed as one of the N-DBPs being formed after tryptophan MP UV irradiation. The retention time and MS2 spectrum of the Trp + $^{14}NO_3^-$ MP UV sample matches exactly with that of the 3-nitroindole reference standard (see figure 8).

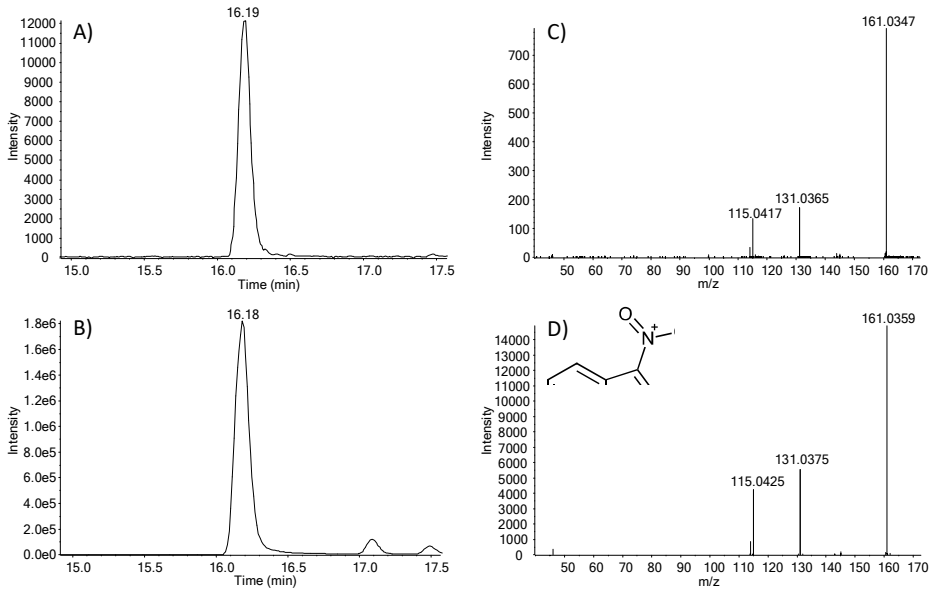

Figure 8: Confirmation of 3-nitroindole. A) EIC of 3-nitroindole (m/z 161.0360) in Trp + $^{14}NO_3^-$ MP UV sample. B) EIC of 3-nitroindole reference standard (m/z 161.0360). C) MS2 spectrum of 3-nitroindole in Trp + $^{14}NO_3^-$ MP UV sample. D) MS2 spectrum of 3-nitroindole reference standard.

3.3 Tyrosine and phenylalanine labeling experiments

After completing the tryptophan labeling experiments, the labeling experiments were continued with the two remaining aromatic amino acids, tyrosine and phenylalanine. The same approach was used as for the tryptophan labeling experiments, with some adjustments. For the tyrosine and phenylalanine labeling experiments no isotope labeled ^{13}C carbon reference standard was used, because the tryptophan experiments showed that the added value of such a standard is limited and therefore too expensive. Also no experiments were conducted with added NOM to the samples, because it had a limited effect on N-DBP formation during the tryptophan labeling experiments. An overview of final sample scheme for the tyrosine and phenylalanine labeling experiments is shown in table 13.

TABLE 13: OVERVIEW OF THE TYROSINE AND PHENYLALANINE LABELING SAMPLE SCHEME

Samples	MP UV treatment	Tyrosine 5 mg/L	Phenylalanine 5 mg/L	Unlabeled nitrate ($^{14}NO_3^-$) 10 mg/L	Labeled nitrate ($^{15}NO_3^-$) 10 mg/L
Untreated reference (ultrapure water)					
Untreated + Tyr, without nitrate		x			
UV treated + Tyr, without nitrate	x	x			
Untreated + Tyr + $^{14}NO_3^-$		x		x	
UV treated + Tyr + $^{14}NO_3^-$	x	x		x	
Untreated + Tyr + $^{15}NO_3^-$		x			x
UV treated + Tyr + $^{15}NO_3^-$	x	x			x
Untreated + Phe, without nitrate			x		
UV treated + Phe, without nitrate	x		x		
Untreated + Phe + $^{14}NO_3^-$			x	x	
UV treated + Phe + $^{14}NO_3^-$	x		x	x	
Untreated + Phe + $^{15}NO_3^-$			x		x
UV treated + Phe + $^{15}NO_3^-$	x		x		x
UV treated + Tyr + $^{14}NO_3^-$ + $^{15}NO_3^-$	x	x		x*	x*
UV treated + Phe + $^{14}NO_3^-$ + $^{15}NO_3^-$	x		x	x*	x*

All samples were prepared in duplicate
** 5 mg/L*

The samples were MP UV treated, pre-treated, and analysed according to the same conditions as described in paragraphs 3.2.1- 3.2.3.

3.3.1 Mass spectrometric data analysis

The mass spectrometric data analysis was performed under same conditions as described in paragraph 3.2.4. The nitrate labeling strategy was used again for the detection of N-DBPs formed by MP UV treatment of nitrate. The MP UV treated Tyr or Phe + $^{14}NO_3^-$ sample was compared with MP UV treated Tyr or Phe + $^{15}NO_3^-$ sample for the detection of N-DBPs. In order to detect all by-products, including by-products that were formed by UV photolysis and without interactions of nitro radicals, the MP UV untreated Tyr or Phe + $^{14}NO_3^-$ sample was compared with the MP UV treated Tyr or Phe + $^{14}NO_3^-$ sample. An overview of the number of detected by-products for tyrosine and phenylalanine is shown in table 14.

TABLE 14: OVERVIEW DETECTED TYROSINE AND PHENYLALANINE BY-PRODUCTS

Type of compounds	Detected accurate masses		Summed concentration (µg/L)	
	Positive	Negative	Positive	Negative
Tyrosine			**(Tyr-d4 equiv.)**	**(Tyr-d4 equiv.)**
DBPs (all)	142	128	1270	2274
N-DBPs	17	24	643	1037
Phenylalanine			**(Phe-d5 equiv.)**	**(Phe-d5 equiv.)**
DBPs (all)	89	126	488	1642
N-DBPs	30	70	246	1138

There are fewer accurate masses detected with the tyrosine (142 and 128; pos/neg) and phenylalanine (89 and 126; pos/neg) labeling experiments in comparison with tryptophan (957 and 1127; pos/neg). An even lower number of N-DBPs was detected for tyrosine (17 and 24; pos/neg) and phenylalanine (28 and 70; pos/neg). Although relatively high concentrations of N-DBPS were detected for tyrosine (1037 µg/L) and phenylalanine (1138 µg/L) compared with tryptophan (741 µg/L). This shows that the average concentration of the detected tyrosine and phenylalanine accurate masses is higher than that of tryptophan and that tyrosine and phenylalanine are also susceptible for nitration.

A mass balance was made up for tyrosine and phenylalanine, in order to find out if most of the by-products are really detected. The mass balance for tyrosine and phenylalanine is shown in table 15.

TABLE 15: MASS BALANCE OF TYROSINE AND PHEYNYLALANINE BY-PRODUCTS AFTER MP UV (TYR-D4 OR PHE-D5 EQUIVALENTS)

Type of compounds	Positive mode		Negative mode	
	Concentration µg/L	(%)	Concentration µg/L	(%)
Tyrosine				
Tyrosine before UV	5178	-	5178	-
Tyrosine after UV	3169	61.2	2936	56.7
DBPs after UV	627	12.1	1237	23.9
N-DBPs after UV	643	12.4	1037	20.0
Sum Tyr + DBPs	4439	85.7	5210	100.6
Phenylalanine				
Phenylalanine before UV	5188	-	5188	-
Phenylalanine after UV	3874	74.7	3771	72.7
DBPs after UV	242	4.7	504	9.7
N-DBPs after UV	246	4.7	1138	21.9
Sum Tyr + DBPs	4362	84.1	5413	104.3

After MP UV treatment a relatively large amount of tyrosine (61.2 and 56.7%) and phenylalanine (74.7 and 72.7%) remains intact in comparison with that of tryptophan (21.5 and 30.1%) for the positive and negative mode. This shows that only a small amount of tyrosine and phenylalanine is converted by UV photolysis and nitro radicals into DBPs and N-DBPs at MP UV conditions. The overall result of the mass balance shows that for tyrosine 85.7% and 100.6% (pos and neg) of the mass balance is accounted for, and for 84.1 and

104.3% (pos and neg) for phenylalanine. This means that most of the by-products are detected. The mass balance results in the negative mode are greater than 100%, which can be explained by that some fragments and adducts are also included in the detection of the by-products. And because the ionisation efficiency is different for each by-product, resulting in less reliable concentrations.

3.3.2 Tyrosine labeling results

For the detection of tyrosine N-DBPs formed by MP UV treatment, the UV treated Tyr + $^{14}NO_3^-$ sample was compared with UV treated Tyr +$^{15}NO_3^-$ sample as described in 3.2.4. Only 17 and 24 accurate masses of N-DBPs were detected in the positive and negative mode. The top 2 and top 5 of the highest detected tyrosine N-DBPs in the positive and negative mode are shown in table 16 and 17.

TABLE 16: MOST ABUNDANT TYROSINE N-DBPS IN POSITIVE MODE

Accurate mass $^{14}NO_3^-$	Accurate mass $^{15}NO_3^-$	RT (min)	Intensity	Concn. Tyr-d4 equiv (µg/L)	Formula	Δppm	Identity
227.0664	228.0637	5.60	1646000	599	$C_9H_{10}N_2O_5$	0.7	Nitrotyrosine
227.0663	228.0632	2.78	71667	26	$C_9H_{10}N_2O_5$	-0.2	Nitrotyrosine

TABLE 17: MOST ABUNDANT TYROSINE N-DBPS IN NEGATIVE MODE

Accurate mass $^{14}NO_3^-$	Accurate mass $^{15}NO_3^-$	RT (min)	Intensity	Concn. Tyr-d4 equiv (µg/L)	Formula	Δppm	Identity
225.0520	226.0492	5.59	978589	617	$C_9H_{10}N_2O_5$	1.8	Nitrotyrosine
225.0517	226.0487	2.80	194186	122	$C_9H_{10}N_2O_55$	0.5	Nitrotyrosine
146.9667	148.9608	2.19	85259	54	?		2x N15 label
439.0374	440.0343	2.78	17116	11	?		
177.0305	178.0282	17.85	9892	6	$C_8H_6N_2O_3$	1.3	

Two isomers of nitrotyrosine were detected in high concentrations in the positive (625 µg/L) and negative mode (739 µg/L). In the positive mode nitrotyrosine accounted for more than 97% of total amount of the formed N-DBPs and in negative mode for more than 71%. The remaining N-DBPS in the negative mode with exception of m/z 146.9667 were too low in concentration for identification. The N-DBP with m/z 146.9667 showed a double nitrogen label, meaning that it had reacted twice with nitrate, but could not be identified.

3.3.3 Phenylalanine labeling results

Phenylalanine N-DBPs formed by MP UV treatment were detected by comparing the UV treated Phe + $^{14}NO_3^-$ sample with the UV treated Tyr +$^{15}NO_3^-$ sample as described in 3.2.4. In total 30 and 70 accurate masses of N-DBPs were detected in the positive and negative mode. The top 5 and top 10 of the highest detected phenylalanine N-DBPs in the positive and negative mode are shown in table 18 and 19, respectively.

TABLE 18: MOST ABUNDANT PHENYLALANINE N-DBPS IN POSITIVE MODE

Accurate mass $^{14}NO_3^-$	Accurate mass $^{15}NO_3^-$	RT (min)	Intensity	Concn. Phe-d5 equiv (µg/L)	Formula	Δppm	Identity
227.0666	228.0635	5.60	479466	67	$C_9H_{10}N_2O_5$	1.1	Nitrotyrosine
227.0665	228.0635	6.09	438853	61	$C_9H_{10}N_2O_5$	0.8	nitro-hydroxyphenylalanine
227.0664	228.0635	6.39	417768	58	$C_9H_{10}N_2O_5$	0.7	nitro-hydroxyphenylalanine
227.0664	228.0635	5.90	141834	20	$C_9H_{10}N_2O_5$	0.7	nitro-hydroxyphenylalanine
227.0664	228.0633	3.93	53856	8	$C_9H_{10}N_2O_5$	0.7	nitro-hydroxyphenylalanine

TABLE 19: MOST ABUNDANT PHENYLALANINE N-DBPS IN NEGATIVE MODE

Accurate mass $^{14}NO_3^-$	Accurate mass $^{15}NO_3^-$	RT (min)	Intensity	Concn. Phe-d5 equiv (µg/L)	Formula	Δppm	Identity
225.0519	226.0489	6.09	390075	148	$C_9H_{10}N_2O_5$	0.5	nitro-hydroxyphenylalanine
225.0517	226.0490	5.60	353717	134	$C_9H_{10}N_2O_5$	0.0	Nitrotyrosine
222.0156	224.0097	18.45	322402	122	$C_8H_5O_5N_3$	0.7	2x N15 label
225.0519	226.0488	6.39	280288	106	$C_9H_{10}N_2O_5$	0.0	nitro-hydroxyphenylalanine
222.0156	224.0097	17.79	279937	106	$C_8H_5O_5N_3$	-0.2	2x N15 label
267.0005	506.0145	17.31	128372	49	$C_8H_4N_4O_7$	-0.8	3x N15 label
177.0311	178.0279	17.23	99836	38	$C_8H_6N_2O_3$	2.5	nitrooxindole?
504.0208	506.0145	6.08	95040	36	?		2x N15 label
436.0008	437.9951	17.79	89085	34	?		2x N15 label
177.0311	178.0279	14.72	86245	33	$C_8H_6N_2O_3$	3.0	
391.0165	392.0135	14.72	78018	30	?		
243.8992	244.8901	2.29	73738	28	?		
257.0410	258.0384	5.47	71785	27	$C_9H_{10}N_2O_7$	-1.3	
168.0305	169.0274	10.31	52166	20	$C_7H_7NO_4$	1.0	
208.0250	209.0223	5.91	48094	18	$C_9H_7NO_5$	0.5	

Nitrotyrosine and the structural isomer nitro-hydroxyphenylalanine were detected in high concentrations in the positive and negative mode. In the positive mode, nitrotyrosine and nitro-hydroxyphenylalanines together account for more than 86% of the concentration of detected by-products. In the negative mode this accounts only for less than 36% and many other by-products are also detected. For the negative detected phenylalanine by-products, it is striking that four by-products are detected with a double nitrogen label and even one with a triple nitrogen label (see figure 9 for the mass spectrum of m/z 267.0005, containing a triple nitrogen label). This shows that phenylalanine is very susceptible for double or even triple nitration.

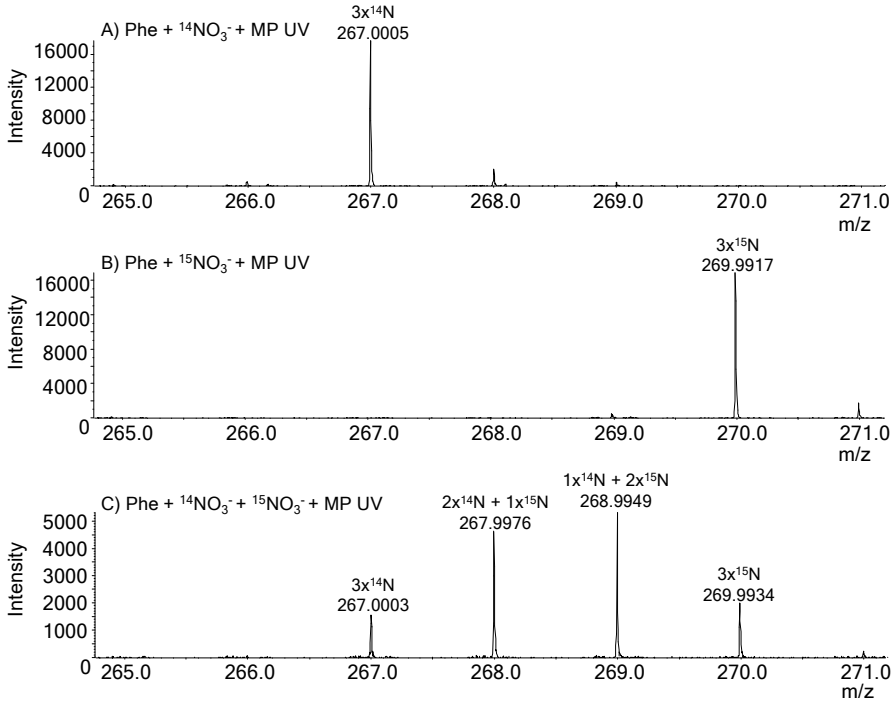

Figure 9: Mass spectra of N-DBP m/z 267.005 in various samples showing different ^{14}N and ^{15}N labels. A) Mass spectrum of Phe + $^{14}NO_3^-$ + MP UV sample, showing a triple ^{14}N label. B) Mass spectrum of Phe + $^{15}NO_3^-$ + MP UV sample, showing a triple ^{15}N label. C) Mass spectrum of Phe +$^{14}NO_3^-$ + $^{15}NO_3^-$ + MP UV sample, showing a triple ^{14}N, double ^{14}N + single ^{15}N, single ^{14}N + double ^{15}N and triple ^{15}N label.

3.3.4 Suspect screening of 84 N-DBPs

After the tyrosine and phenylalanine experiments a suspect screening was performed using the 84 N-DBPs detected in previous study (see attachment III for the list). The suspect screening was performed on the MP UV treated Phe + $^{14}NO_3^-$ and MP UV treated Phe + $^{15}NO_3^-$ sample. The suspect screening was performed as described in 3.2.7. See table 20 for the results of the suspect screening.

TABLE 20: RESULTS SUSPECT SCREENING OF THE MOST ABUNDANT PHENYLALANINE N-DBPS IN THE ARTIFICAL WATER SAMPLE OF THE PRIOR STUDY

Accurate mass $^{14}NO_3^-$	RT (min)	Intensity	Concn. Phe-d5 equiv (µg/L)	Formula	RT Original $^{14}NO_3^-$ sample (min)	RT, MS1 and MS2 Confirmed
222.0156	18.45	322402	122	$C_8H_5O_5N_3$	18.42	yes
222.0156	17.79	279937	106	$C_8H_5O_5N_3$	17.76	yes

With the suspect screening there were no tyrosine N-DBPs detected in the original $^{14}NO_3^-$ artificial water sample in the positive and negative mode and for phenylalanine no N-DBPs were detected in the positive mode. For phenylalanine in the negative mode, two by-products with m/z 222.0156 (isomers) were detected and confirmed in the original $^{14}NO_3^-$ artificial water sample by matching retention time and MS/MS spectrum (see figure 10), but these were not detected in the full-scale water treatment sample. This shows once again that aromatic amino acids could potentially be the source of some of the by-products detected in the prior study.

Of the most abundant 15 by-products formed by the MP UV phenylalanine labeling experiments, only number 3 and 5 (both m/z 222.0156) corresponded with the list of suspects. As before with the tryptophan labeling experiments, this could mean that the other by-products are not formed during the MP UV irradiation of artificial water or that these by-products are not sufficiently extracted from the water. This will be further investigated in 3.4.

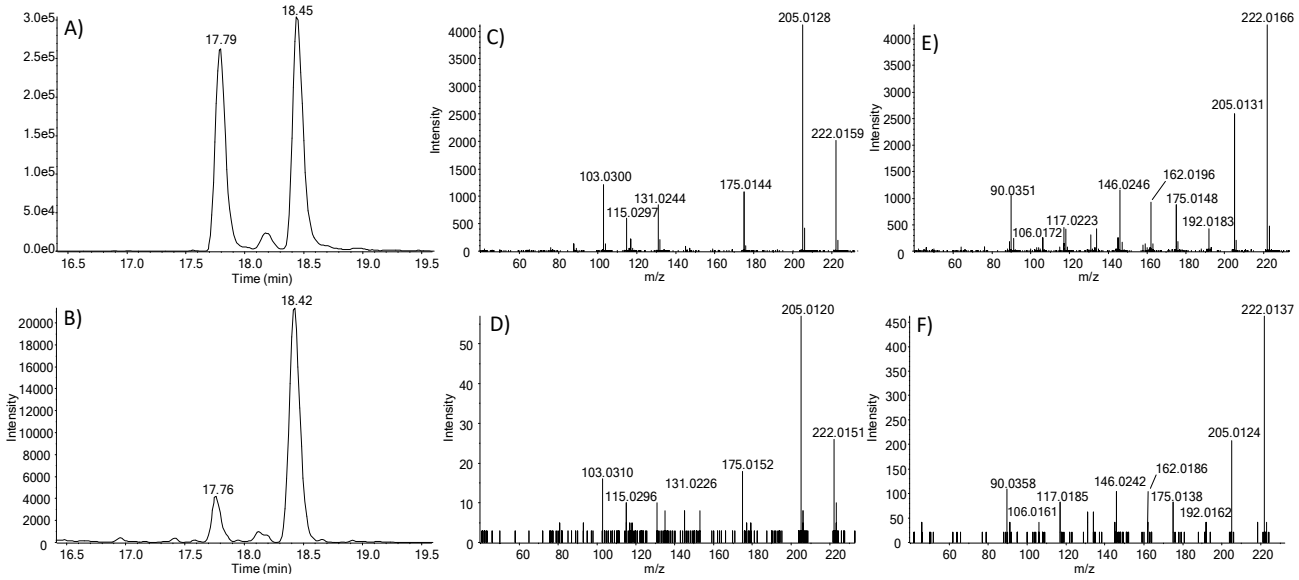

Figure 10: confirmation of N-DBP m/z 222.0156 (2 isomers) in artificial water. A) EIC of m/z 222.0156 (2 isomers) in Phe + $^{14}NO_3^-$ after MP UV sample. B) EIC of m/z 222.0156 in artificial water after MP UV. C) MS2 spectrum at RT 17.79 of m/z 222.0156 in Phe + $^{14}NO_3^-$ after MP UV sample. D) MS2 spectrum at RT 17.76 of m/z 222.0156 in artificial water after MP UV. E) MS2 spectrum at RT 18.45 of m/z 222.0156 in Phe + $^{14}NO_3^-$ after MP UV sample. F) MS2 spectrum at RT 18.42 of m/z 222.0156 in artificial water after MP UV.

3.3.5 Identification of m/z 222.0156

The identification of the two isomers with m/z 222.0156 ($C_8H_5N_3O_5$) containing both a double nitrogen label started by annotating the MS/MS spectrum and searching for known losses (See attachment IV for the annotated MS/MS spectra). This showed mainly losses of NO and OH, meaning that there are probably nitro and hydroxyl functional groups present in the by-products. Next the formula of the by-products ($C_8H_5N_3O_5$) was compared with that of phenylalanine ($C_9H_{11}NO_2$). This showed there was a loss of one carbon and at least one oxygen (assuming that the by-product was double nitrated, and also alcohol oxidation had occurred), indicating that the polar acidic functional group of the amino acid was probably lost during formation of the by-product. This is also supported by the observed retention time (17.79 and 18.45 min) of both by-products in comparison with that of phenylalanine (4.65 min), which means that the by-products are less polar than the parent compound. The by-products are expected to consist of a benzene ring containing two nitro and one hydroxy functional group and also containing the remainder of the amine part of the amino acid (C_2H_xN). Then a ChemSpider and PubChem database search was performed using the formula of the by-products which resulted in 37 and 65 hits. The structures of these possible candidates were thoroughly investigated and finally one good candidate was found: 3,5-Dinitro-4-hydroxy-benzylcyanid (see figure 11). There were no other structural isomers in the ChemSpider and Pubchem database, although there are many more structural isomers possible. The only candidate was checked by computational (in silico) ms/ms fragmentation using MetFrag [32], in which the computational MS/MS spectrum was compared with the measured MS/MS spectrum and showed a decent match (8 of 13 fragments explained) with

the by-product detected at 18.45min. Unfortunately this candidate was not available for purchase, and remains therefore unconfirmed. There are many structural isomers possible for this structure, so synthesis is not option. The only option that remains for identification is using nuclear magnetic resonance (NMR), but that falls beyond the scope of this study.

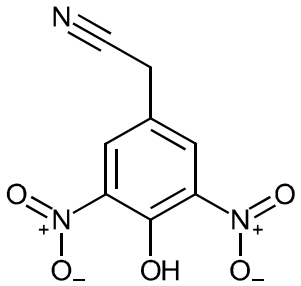

Figure 11: structure of 3,5-dinitro-4-hydroxy-benzylcyanid (CAS nr. 55770-69-9)

The basic structure of this by-product is benzyl cyanide, which is a known toxic compound for which an oral LD50 in mouse was derived at 45.5 mg/kg [33]. Furthermore, Ma et al [34] demonstrated that benzyl cyanide is the major by-product of phenylalanine chlorination in drinking water. This means that it is a possibility that the detected by-product with m/z 222.0156 really could be a derivative from benzyl cyanide and makes this by-product interesting for further research.

3.4 SPE experiments of tryptophan and phenylalanine by-products

A total of three N-DBPs were detected with the tryptophan and phenylalanine MP UV labeling experiments that were also present in the artificial water sample of the prior study, meaning that these by-products in the artificial water sample potentially could originate from aromatic amino acids. However of the top 15 by-products detected with the tryptophan and phenylalanine labeling experiments, only these three by-products (i.e. 3-nitroindole and two isomers with m/z 222.0156) were detected in the artificial water sample, of which 3-nitroindole is a relatively low intensity N-DBP (nr 12). This would imply that other by-products of the top 15 are not formed by MP UV treatment of artificial water, which makes it more likely that the three detected by-products could originate from other sources than from aromatic amino acids. Alternatively, it might be that these other by-products are formed, but are not sufficiently extracted from the water using the AMES pre-treatment protocol, which was used as sample pre-treatment in the prior study for the artificial water sample. In order to investigate if the detected N-DBPs really originate of tryptophan or phenylalanine, SPE experiments were conducted according to the AMES pre-treatment protocol to determine the extraction recovery of the top 15 detected N-DBPs.

For the SPE experiments the same samples were used as for the aromatic amino acids labeling experiments, using the AMES SPE pre-treatment protocol for determining the recovery of the by-products. All samples for the labeling experiments were stored in the freezer at -25°C to reduce the risk of degradation. The following samples were used for the SPE experiment in duplo: UV treated + Trp + $^{14}NO_3^-$ sample, UV treated + Phe + $^{14}NO_3^-$ sample and an ultrapure water sample (blank). In order to compare the by-products in the samples after the AMES SPE pre-treatment with the samples that were not pre-treated, the final SPE extracts were diluted again with ultrapure water so that effectively no sample enrichment had occurred. In this way, a fair comparison could be made between the samples with and without sample pre-treatment in order to determine the recovery of the by-products. See attachment V for the applied conditions for the tryptophan and phenylalanine by-products SPE experiment.

3.4.1 Results of SPE experiments

Because quantification of by-products is not possible without knowing their identity, the recovery was calculated using the intensity of the by-products. The intensity of the by-products in the samples that were not pre-treated was compared with intensity of the by-products after SPE treatment, making it possible to calculate the recovery. The recoveries of the top 15 by-products of tryptophan and phenylalanine are shown in figure 12 and 13.

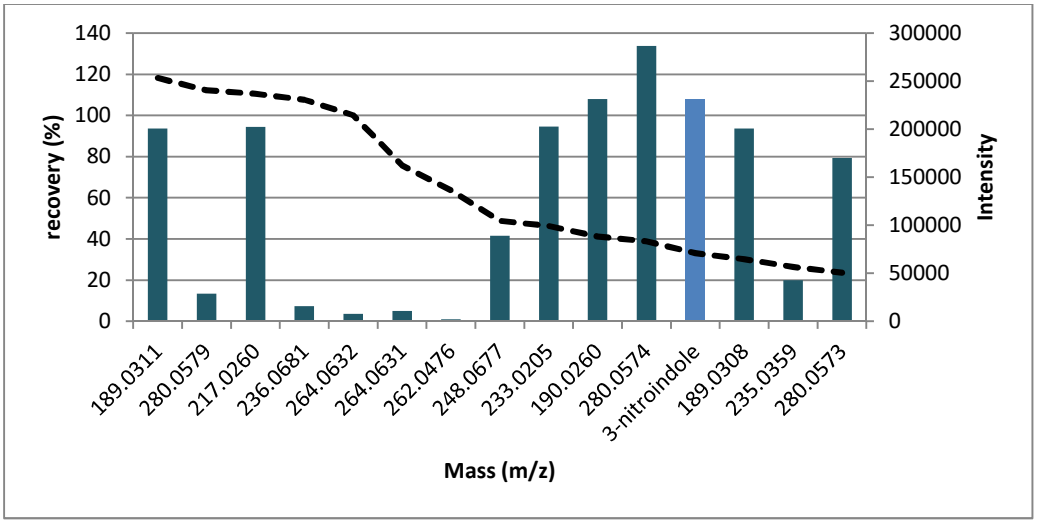

Figure 12: Recoveries of tryptophan by-products in SPE treated samples. Recoveries were calculated as the ratios of intensities of each N-DBP after and before pre-treatment. By-product 3-nitroindole indicated in blue. The dashed line represents the intensity of the by-products when no sample pre-treatment is applied (direct injection)

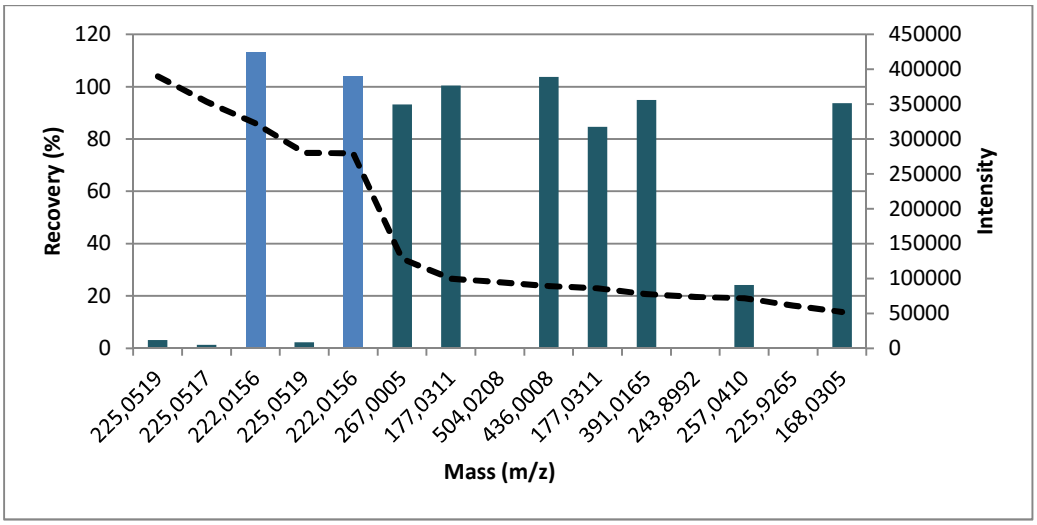

Figure 13: Recoveries of phenylalanine by-products in SPE treated samples. Recoveries were calculated as the ratios of intensities of each N-DBP after and before pre-treatment. The two isomers with m/z 222.0516 are indicated in blue. The dashed line represents the intensity of the by-products when no sample pre-treatment is applied (direct injection).

The results of the SPE recovery experiment for tryptophan by-products show that there are six by-products with poor recoveries (< 20%) while the other nine by-products have a moderate to good recoveries (41 – 134%), including 3-nitroindole (108%). Of those nine by-

products only the low intensity by-product 3-nitroindole was detected in the artificial water of the prior study. This implies that it is quite unlikely that 3-nitroindole present in the artificial water sample is a MP UV by-product of tryptophan. It is more probable that the 3-nitroindole in the artificial water sample originates from another source. Using a retrospective suspect screening it was determined that unnitrated indole is not the source.

The SPE recovery results for the phenylalanine by-products show that there are seven by-products with poor recoveries (< 25%) and eight others with good recoveries (84 – 114), including the two unidentified isomers with m/z 222.0156. Of those eight by-products, initially only the two isomers with m/z 222.0156 were detected in the artificial water sample of the prior study. However, a suspect screening conducted at a very low intensity also revealed the presence of two isomers with m/z 177.0311(of the top 15) in the artificial water samples, just barely above the detection limit (s/n > 3). This means that four of the eight by-products with a good SPE recovery are present in the artificial water sample. This result makes it quite probable that the two unidentified isomers with m/z 222.0156 present in the artificial water sample are really MP UV by-products of phenylalanine and shows that aromatic amino acids can be responsible for the formation for some of the N-DBPs formed by MP UV treatment of artificial water.

3.5 Conclusion labeling experiments

It was shown that the labeling strategy involving nitrate, aromatic amino acids and MP UV worked. Many by-products were detected using this strategy. For tryptophan, one by-product was detected in the artificial water sample of the prior study and was identified as 3-nitroindole. However, other high intensity tryptophan by-products were not detected in the artificial water, meaning that it is quite likely that the 3-nitroindole in the artificial water sample does not originate from tryptophan, but from another source.

For phenylalanine, two unidentified by-products with m/z 222.0156 (isomers) were initially detected in the artificial water sample of the prior study. Two other phenylalanine by-products with m/z 177.0311 (isomers) were detected at a very low intensity in the artificial water, meaning that four of the eight by-products with a good recovery are detected in the artificial water sample. This makes it quite probable that the two unidentified isomers with m/z 222.0156 present in the artificial water sample are really MP UV by-products of phenylalanine, and shows that aromatic amino acids are probably responsible for some of the N-DBPs formed by MP UV treatment of artificial water.

None of the tryptophan and phenylalanine by-products were detected in the full-scale MP UV water treatment sample of the prior study. This could mean that the concentration of aromatic amino acids in Lake IJssel is just too low in order to detect their by-products. This is supported in chapter 2, in which the detected the concentrations of tryptophan and phenylalanine were 6 and 9 times lower in Lake IJssel in comparison with artificial water. This makes the detected by-products of tryptophan and phenylalanine less relevant for further research.

4 Identification, toxicological evaluation and quantification of N-DBPs

In this chapter, structure elucidation of a number of the 84 previously detected but unidentified N-DBPs was performed and the results of a toxicological evaluation which was performed on the identified N-DBPs are discussed. Furthermore an analytical target method (LC-QToF) was developed and validated for the identified N-DBPs in the current and prior study, in order to conduct and evaluate a seven months monitoring study for N-DBPs in a full-scale drinking water treatment facility using MP UV treatment.

4.1 Identification of N-DBPs

In total 14 N-DBPs of the 84 detected by-products detected in artificial water were identified in the two prior studies [13, 18]. The identification of the remaining unidentified N-DBPs remains important, because the response of the identified N-DBPs does not explain the overall AMES response detected in artificial water and full-scale MP UV treated water [18]. The identification was mainly focussed on N-DBPs that are also formed by full-scale MP UV water treatment. The N-DBPs were identified by using an in silico fragmentation tool (MetFrag), and by expert knowledge. It was assumed that all the N-DBPs consist of a benzene ring to which a nitro group is attached and could contain the following functional groups: hydroxyl, methyl, methoxyl, carboxylic acid, sulfonic acid and amine. Possible candidates were purchased and were analysed using the LC-QToF method as described in 3.2.3 and compared with the LC-QToF data from the N-DBPs in the sample. The results of the QToF analysis for the identification of N-DBPs is shown in table 21. The chromatograms and mass spectra of the identified N-DBPs are shown in attachment VI.

TABLE 21: RESULTS Q-TOF ANALYSIS OF REFERENCE STANDARDS FOR THE UNAMBIGUOUS IDENTIFICATION OF N-DBPS

Compound	CAS nr	Formula	Accurate mass [M-H]	RT sample (min)	RT reference standard (min)	RT*, MS1** and MS2 Confirmed
2-Methyl-4-nitrophenol	99-53-6	$C_7H_7O_3N$	152.0353	16.25	16.25	yes
5-Methyl-2-nitrophenol	700-38-9	$C_7H_7O_3N$	152.0353	16.23	18.88	no
4-Methyl-2-nitrophenol	119-33-5	$C_7H_7O_3N$	152.0353	16.23	18.93	no
3-Methyl-4-nitrophenol	2581-34-2	$C_7H_7O_3N$	152.0353	16.23	15.30	no
2-Methyl-3-nitrophenol	5460-31-1	$C_7H_7O_3N$	152.0353	16.23	16.19	no
4-Methyl-3-nitrophenol	2042-14-0	$C_7H_7O_3N$	152.0353	16.23	15.95	no
3-Methyl-2-nitrophenol	4920-77-8	$C_7H_7O_3N$	152.0353	16.23	16.43	no
2-Methyl-5-nitrophenol	5428-54-6	$C_7H_7O_3N$	152.0353	16.23	16.83	no
2-Methoxy-4-nitrophenol	3251-56-7	$C_7H_7O_4N$	168.0302	13.40	13.43	yes
2-Methoxy-5-nitrophenol	636-93-1	$C_7H_7O_4N$	168.0302	13.40	13.04	no
2-Amino-3-nitrobenzoic acid	606-18-8	$C_7H_6N_2O_4$	181.0255	14.66	14.64	yes
6-Nitroanthranilic acid	50573-74-5	$C_7H_6N_2O_4$	181.0255	14.69	9.17	no
5-Nitroanthranilic acid	616-79-5	$C_7H_6N_2O_4$	181.0255	10.38	12.40	no
4-Nitroanthranilic acid	619-17-0	$C_7H_6N_2O_4$	181.0255	10.38	14.00	no
4-Hydroxy-3-nitrobenzenesulfonic acid	6313-34-4	$C_6H_5O_6NS$	217.9765	4.86	4.87	yes

** Retention time differs less than 0.10 min relative to the reference standard*

*** Measured accurate mass falls within a 5 ppm mass range of the theoretical mass*

Four N-DBPs were unambiguously identified by analysing the reference standards and matching the accurate mass, retention time, and MS/MS fragmentation spectra of the reference standard with the N-DBPs detected in the artificial water sample. The identities of 2-metyl-4-nitrophenol, 2-methoxy-4-nitrophenol, 2-amino-3-nitrobenzoic acid and 4-hydroxy-3-nitrobenzenesulfonic acid were thus confirmed.

4.2 Toxicological evaluation of the identified N-DBPs

Five N-DBPs were newly identified (one in chapter 3 and four in chapter 4) for which it is now possible to conduct a toxicity evaluation. For the toxicity evaluation the following sources were consulted:

- Organizations: European Food Safety Authority (EFSA), European Chemicals Agency (ECHA), US Environmental Protection Agency (EPA), Food and Drug Administration, and Dutch National Institute for Environment and Health (RIVM);
- Toxicological meta-databases: International Toxicity Estimates for Risk (ITER) comprising data from the WHO International Programme on Chemical Safety (IPCS), U.S. EPA, Agency for Toxic Substances and Disease Registry (ATSDR), Health Canada, International Agency for Research on Cancer (IARC), en RIVM; TOXNET; and the OECD eChemPortal linking to e.g. ACToR, ECHA, HSDB, INCHEM, UNEP and IRIS;
- OECD QSAR Toolbox v3.4.0.17 software.

For none of the substances, toxicological study results or human health risk assessments were retrieved from any of the information sources. Additional literature search by name and CAS number of all chemicals using PubMed and Scopus resulted in only two publications, reporting anti-androgenic activity [35] and vasodilating properties [36] of 2-methyl-4-nitrophenol.

Two chemicals, 2-methyl-4-nitrophenol and 2-methoxy-4-nitrophenol (which is in use as a hair dye ingredient), are present in the ECHA database. ECHA labeled these chemicals as eye, skin and respiratory irritants. Both substances have not been REACH registered, but are listed in the REACH Annex III inventory of indications for hazardous toxicological or ecotoxicological properties. The REACH inventory and chemical profiling using the OECD QSAR Toolbox show potential mutagenic and carcinogenic activity for these substances based on their chemical structure (see Appendix VII). The other substances were not included in the OECD QSAR Toolbox. Their InChI identifiers were imported for chemical profiling; structural alerts for mutagenicity and/or carcinogenicity were identified in these chemicals as well (see Appendix VIII). In addition, the OECD QSAR Toolbox reports structural alerts for additional endpoints for all substances (Appendix VII and VIII). Read across was performed to predict mutagenicity, carcinogenicity, and chronic toxicity based on measured data for identified structural analogues included in the OECD QSAR Toolbox. Table 22 summarizes all findings.

TABLE 22: SUMMARY OF TOXICOLOGICAL EVALUATION OF N-DBPS OF WHICH THE IDENTITY WAS CONFIRMED

Substance	Toxicity data	Structural alerts	Read across
2-Amino-3-nitrobenzoic acid	-	mutagenicity carcinogenicity reproductive and developmental toxicity	positive for *in vitro* mutagenicity negative for *in vivo* genotoxicity negative for carcinogenicity
3-Nitroindole	-	mutagenicity skin sensitization	positive for *in vitro* mutagenicity negative for *in vivo* genotoxicity negative for carcinogenicity positive for reproductive toxicity
2-Methyl-4-nitrophenol	anti-androgenic activity vasodilation	mutagenicity carcinogenicity hemolytic anemia & hepatotoxicity *or* energy metabolism dysfunction	positive/negative for *in vitro* mutagenicity positive for carcinogenicity positive for developmental toxicity
2-Methoxy-4-nitrophenol (4-Nitroguaiacol)	-	mutagenicity carcinogenicity energy metabolism dysfunction	negative for *in vitro* mutagenicity positive for carcinogenicity positive for developmental toxicity
4-Hydroxy-3-nitrobenzenesulfonic acid	-	mutagenicity estrogen receptor binding energy metabolism dysfunction	negative for *in vitro* mutagenicity

4.2.1 Conclusion toxicological evaluation

Because limited toxicity data was available for the toxicological evaluation of the by-products identified, the prediction of DNA binding and/or genotoxic potential was based on structural characteristics. For all five N-DBPs the OECD QSAR Toolbox indicated a potential for DNA binding and mutagenicity, and potential carcinogenicity was indicated for 2-amino-3-nitrobenzoic acid, 2-methyl-4-nitrophenol and 2-methoxy-4-nitrophenol. Although these predictions may indicate mutagenic potency, it is not certain that these N-DBPS show a positive response in the Ames test. Therefore it remains uncertain if the identified N-DBPS have contributed to the overall Ames response detected in artificial water and full-scale MP UV treated water in the prior study [13]. In the end, this can be determined by applying the identified N-DBPs for Ames testing.

4.3 Development of a LC-QToF target method for N-DBPS in water

For the analytical method development and validation of the 19 identified N-DBPs in water (table 23), the LC-QTOF method which was applied for the labeling experiments (chapter 3) was used again and was modified for target method analysis. A high resolution LC-QToF method was preferred over a regular triple quadrupole LC-MS system, due to the screening and structure elucidation capabilities of the QToF mass spectrometer for unidentified N-DBPs. The data dependent acquisition of the QToF instrumental method was adapted so that the MS/MS spectra of the 19 N-DBPs were continuously acquired within a specified time window (set in the mass list), while the remaining available scan time (i.e. cycle time) was used for triggering MS/MS spectra of the highest detected peaks (maximum of eight) detected in the full scan MS mode.

The sample pre-treatment consisted of solid phase extraction according to the AMES II protocol and was used for concentrating the samples and thus increasing sensitivity for detecting N-DBPs. The AMES II protocol was also used in the two prior studies and therefore only small changes were made to the pre-treatment procedure, so that it was possible to compare results between different studies. The following modifications were made to the pre-treatment procedure: (i) sample volume was reduced from 1000 mL to 500 mL, due to sample availability and (ii) two additional internal standards were added for quantification purposes, i.e. nitrophenol-d4 and neburon. See attachment IX (materials en methods) for the final sample pre-treatment procedure and LC-QToF method.

TABLE 23: CAS NUMBERS, FORMULAS, AND ACCURATE MASSES OF THE IDENTIFIED N-DBPS

Compound	CAS nr	Formula	Accurate mass [M-H$^-$]
4-Nitrophenol	100-02-7	$C_6H_5NO_3$	138.0197
2-Methyl-4-nitrophenol	99-53-6	$C_7H_7O_3N$	152.0353
4-Nitrocatechol	3316-09-4	$C_6H_5NO_4$	154.0146
4-Nitro-1,3-benzenediol	3163-07-3	$C_6H_5O_4N$	154.0146
2-Nitrohydroquinone	16090-33-8	$C_6H_5O_4N$	154.0146
3-Nitroindole	4770-03-0	$C_8H_6N_2O_2$	161.0357
2-Methoxy-4-nitrophenol	3251-56-7	$C_7H_7O_4N$	168.0302
2-Amino-3-nitrobenzoic acid	606-18-8	$C_7H_6N_2O_4$	181.0255
2-Hydroxy-5-nitrobenzoic acid	96-97-9	$C_7H_5NO_5$	182.0095
4-Hydroxy-3-nitrobenzoic acid	616-82-0	$C_7H_5NO_5$	182.0095
2-Hydroxy-3-nitrobenzoic acid	85-38-1	$C_7H_5NO_5$	182.0095
2,4-Dinitrophenol	51-28-5	$C_6H_4N_2O_5$	183.0047
5-Nitrovanillin	6635-20-7	$C_8H_7NO_5$	196.0252
4-Nitrobenzenesulfonic acid	138-42-1	$C_6H_5NO_5S$	201.9816
4-Nitrophthalic acid	610-27-5	$C_8H_5NO_6$	210.0044
2-Methoxy-4,6-dinitrophenol	4097-63-6	$C_7H_6N_2O_6$	213.0153
4-Hydroxy-3-nitrobenzenesulfonic acid	6313-34-4	$C_6H_5O_6NS$	217.9765
3,5-Dinitrosalicylic acid	609-99-4	$C_7H_4N_2O_7$	226.9946
Dinoterb	1420-07-1	$C_{10}H_{12}O_5N_2$	239.0673

4.4 Method validation in ultrapure water

The developed LC-QToF target method for the determination of 19 N-DBPs in water was validated in ultrapure water first. The validation was performed including SPE sample pre-treatment (n=5). The limit of detection (LOD), limit of quantification (LOQ), repeatability, recovery and instrumental repeatability were determined for all N-DBPs. The instrumental repeatability was determined by analysing a calibration standard in eightfold (no SPE). The validation results are shown in table 24.

TABLE 24: SPE VALIDATION RESULTS (N=5) FOR N-DBPS IN ULTRAPURE WATER AND INSTRUMENTAL REPEATABILITY RESULTS

Compound	LOD (ng/L)	LOQ (ng/L)	Recovery 25 ng/L (%)	Repeatability 25 ng/L (%)	Instrumental repeatability 25 ng/L (%) (n=8)
2-Methoxy-4,6-dinitrophenol	0.26	1.0	105.2	2.4	1.6
4-Nitrophenol	1.7	5.0	91.9	3.0	2.0
4-Nitrocatechol	0.89	2.0	82.6*	6.2*	2.5
2-Hydroxy-5-nitrobenzoic acid	0.65	2.0	92.3	0.9	1.4
5-Nitrovanillin	0.36	1.0	100.9	6.5	2.8
4-Nitrophthalic acid	1.01	10	119.5	7.9	1.7
2,4-Dinitrophenol	0.39	1.0	95.5	3.1	2.5
4-Nitro-1,3-benzenediol	0.61	2.0	94.1	2.7	1.7
2-Nitrohydroquinone	1.95	5.0	71.7*	14.5*	3.5
4-Nitrobenzenesulfonic acid	0.69	3.0	24.2	14.1	1.5
4-Hydroxy-3-nitrobenzoic acid	0.42	2.0	109.5	1.9	1.3
2-Hydroxy-3-nitrobenzoic acid	0.66	2.0	88.8	0.9	1.7
Dinoterb	0.64	2.0	94.1	4.4	2.0
3,5-Dinitrosalicylic acid	0.39	3.0	45.2	13.6	2.9
2-Methyl-4-nitrophenol	0.06	1.0	92.1	1.9	1.6
2-Methoxy-4-nitrophenol	1.33	3.0	95.2	2.7	2.5
4-Hydroxy-3-nitrobenzenesulfonic acid	0.43	2.0	26.2	6.2	1.6
2-Amino-3-nitrobenzoic acid	0.84	2.0	92.1	2.2	2.2
3-Nitroindole	0.33	2.0	90.2	1.7	1.2

** n=4*

Satisfactory LOD and LOQ results were obtained for all N-DBPs in ultrapure water, resulting in a LOQ between 1.0 – 10 ng/L in ultrapure water. The recoveries for most N-DBPs are between 71.1 – 119.5 % and are within acceptable range, except for 4-nitrobenzenesulfonic acid, 4-hydroxy-3-nitrobenzenesulfonic acid and 3,5-dinitrosalicylic acid which have a recovery of 24.2, 26.2 and 45.2%, respectively. The repeatability for all compounds is lower than 15% and is satisfactory. The instrumental repeatability is good and for all N-DBPs lower than 3.6%. The low recovery for 4-nitrobenzenesulfonic acid and 4-hydroxy-3-nitrobenzenesulfonic acid was expected in advance, due to hydrophilic nature (logP = -1.35 and -1.25) of both compounds and therefore limited retention was expected with the applied SPE cartridge. In the end, good validation results are obtained for ultrapure water which can be used to compare to the validation results in drinking- and surface water (4.4.1).

4.4.1 Method validation for drinking- and surface water

For the monitoring study of Heemskerk full-scale drinking water treatment facility the LC-QToF method was also validated for drinking- and surface water. For the preparation of validation samples, drinking water was obtained from the tap at KWR and surface water was obtained from the Lekkanaal. The limit of detection (LOD), limit of quantification (LOQ), repeatability and recovery were determined for all N-DBPs in both drinking- and surface water. The validation results are shown in table 25 and 26.

TABLE 25: SPE VALIDATION RESULTS (N=5) FOR N-DBPS IN DRINKING WATER

Compound	LOD	LOQ	Recovery 25 ng/L	Repeatability 25 ng/L
	(ng/L)	(ng/L)	(%)	(%)
2-Methoxy-4,6-dinitrophenol	0.29	1.0	74.4	1.7
4-Nitrophenol	0.79	5.0	91.1	7.4
4-Nitrocatechol	0.18	2.0	87.2	6.6
2-Hydroxy-5-nitrobenzoic acid	0.50	2.0	93.1	0.7
5-Nitrovanillin	0.38	1.0	112.2	3.8
4-Nitrophthalic acid	5.9	10	275.0	3.0
2,4-Dinitrophenol	0.27	1.0	132.9	6.9
4-Nitro-1,3-benzenediol	0.45	2.0	86.6	5.0
2-Nitrohydroquinone	2.6	5.0	78.0	3.8
4-Nitrobenzenesulfonic acid	1.2	3.0	80.9	19.2
4-Hydroxy-3-nitrobenzoic acid	0.64	2.0	43.4	2.5
2-Hydroxy-3-nitrobenzoic acid	0.19	2.0	80.2	2.3
Dinoterb	0.44	2.0	109.3	2.8
3,5-Dinitrosalicylic acid	1.33	3.0	63.8*	18*
2-Methyl-4-nitrophenol	0.49	1.0	137.7	6.0
2-Methoxy-4-nitrophenol	0.40	3.0	107.0	3.2
4-Hydroxy-3-nitrobenzenesulfonic acid	0.31	2.0	58.5	13.8
2-Amino-3-nitrobenzoic acid	0.47	2.0	48.3	3.1
3-Nitroindole	0.48	2.0	130.0	4.5

** n=4*

TABLE 26: SPE VALIDATION RESULTS (N=5) FOR N-DBPS IN SURFACE WATER

Compound	LOD	LOQ	Recovery 25 ng/L	Repeatability 25 ng/L
	(ng/L)	(ng/L)	(%)	(%)
2-Methoxy-4,6-dinitrophenol	*	1.0	87.4	1.2
4-Nitrophenol	*	5.0	111.4	5.3
4-Nitrocatechol	*	2.0	91.9	2.8
2-Hydroxy-5-nitrobenzoic acid	*	2.0	114.8	2.3
5-Nitrovanillin	*	1.0	116.0	2.0
4-Nitrophthalic acid	*	10	245.1	2.5
2,4-Dinitrophenol	*	1.0	137.6	2.0
4-Nitro-1,3-benzenediol	0.66	2.0	99.2	4.1
2-Nitrohydroquinone	*	5.0	87.9	6.4
4-Nitrobenzenesulfonic acid	*	3.0	80.2	5.4

4-Hydroxy-3-nitrobenzoic acid	*	2.0	57.1	2.4
2-Hydroxy-3-nitrobenzoic acid	*	2.0	95.0	0.7
Dinoterb	0.99	2.0	124.7	3.2
3,5-Dinitrosalicylic acid	*	3.0	71.8**	26.4**
2-Methyl-4-nitrophenol	*	1.0	154.8	3.7
2-Methoxy-4-nitrophenol	*	3.0	118.7	4.0
4-Hydroxy-3-nitrobenzenesulfonic acid	0.69	2.0	43.2	12.4
2-Amino-3-nitrobenzoic acid	0.63	2.0	62.5	2.0
3-Nitroindole	0.49	2.0	134.0	3.7

** Due to the presence of the N-DBPs in surface water, it is not possible to determine all validation characteristics. The validation results of drinking water can then be used as reference.*
*** n=4*

Satisfactory LOD and LOQ results were obtained for the N-DBPs in drinking- and surface water, which results in LOQs between 1.0 – 10 ng/L. The recoveries in drinking and surface water for almost all N-DBPs are between 43.2 – 154.8 % and are within acceptable range, excluding 4-nitrophthalic acid which has a recovery of 275.0 and 245.1 for surface and drinking water. The repeatability for almost all compounds is lower than 20% and is satisfactory. Only the repeatability for 3,5-dinitrosalicylic acid is relatively high, and was determined at 26.4%. The high recovery for 4-nitrophthalic acid is was not observed with the validation in ultrapure water and might therefore be caused by severe ion enhancement in the mass spectrometer. This can possibly solved by using an isotopically labeled internal standard, although it is not commercially available for 4-nitrophthalic acid.

The results of the validation of N-DBPs in drinking- and surface water is satisfactory and show that the LC-QToF target method can be applied for the monitoring study of a full-scale drinking water treatment facility.

4.5 Monitoring study full-scale drinking water treatment facility

For the monitoring of N-DBPs formed by MP UV treatment, the Heemskerk full-scale treatment facility was selected (PWN, Heemskerk, The Netherlands). In the prior study, 22 N-DBPs were detected after MP UV/H_2O_2 treatment in this facility. At this facility surface water from Lake IJssel is treated by applying advanced oxidation and adds H_2O_2 before MP UV treatment. After MP UV/H_2O_2 treatment, granulated active carbon (GAC) filtration is applied for quenching the excess of H_2O_2 and is followed by infiltration into the dunes. Artificial dune recharge water is reclaimed after at least > 21 days, which is first aerated and filtrated (i.e. rapid sand filtration) before drinking water distribution.

For the monitoring study three types of samples were selected:

- Influent MP UV/H_2O_2 treatment
- Effluent MP UV/H_2O_2 treatment (before GAC filtration)
- Aerated reclaimed dune infiltration water

The sampling campaign was conducted from July 2016 until January 2017 with a sampling frequency of once a month. In total seven samples were collected per sampling point. The samples were collected and stored (1-5 °C) in 1L glass sample bottles upon arrival at KWR. Then the samples were transferred to 1L HDPE bottles and stored in a freezer (-25 °C) until LC-QToF analysis.

4.6 Results monitoring study

The results of the monitoring study for the influent MP UV/H_2O_2 and effluent MP UV/H_2O_2 samples are presented in table 27 and 28. No N-DBPs were detected in the aerated reclaimed dune infiltration water sample and the results for this sample can be found in attachment X.

TABLE 27: RESULTS MONITORING STUDY - INFLUENT MP UV/H_2O_2 TREATMENT SAMPLE

Compound	Influent MP UV/H_2O_2 treatment						
	27/07/16 ng/L	05/09/16 ng/L	21/09/16 ng/L	20/10/16 ng/L	21/11/16 ng/L	14/12/16 ng/L	11/01/17 ng/L
2-Methoxy-4,6-dinitrophenol	< 1.0	5.9	6.8	< 1.0	< 1.0	1.0	1.2
4-Nitrophenol	< 5.0	< 5.0	< 5.0	< 5.0	5.8	5.5	8.8
4-Nitrocatechol	< 2.0	< 2.0	< 2.0	< 2.0	< 2.0	< 2.0	< 2.0
2-Hydroxy-5-nitrobenzoic acid	< 2.0	< 2.0	< 2.0	< 2.0	< 2.0	< 2.0	< 2.0
5-Nitrovanillin	< 1.0	7.5	5.6	< 1.0	< 1.0	< 1.0	< 1.0
4-Nitrophthalic acid	< 10	< 10	< 10	< 10	< 10	< 10	< 10
2,4-Dinitrophenol	4.2	5.0	8.8	8.0	7.3	7.7	8.7
4-Nitro-1,3-benzenediol	< 2.0	< 2.0	< 2.0	< 2.0	< 2.0	< 2.0	< 2.0
2-Nitrohydroquinone	< 5.0	< 5.0	< 5.0	< 5.0	< 5.0	< 5.0	< 5.0
4-Nitrobenzenesulfonic acid	< 3.0	< 3.0	< 3.0	< 3.0	< 3.0	< 3.0	3.0
4-Hydroxy-3-nitrobenzoic acid	< 2.0	2.2	< 2.0	< 2.0	< 2.0	< 2.0	2.0
2-Hydroxy-3-nitrobenzoic acid	< 2.0	< 2.0	< 2.0	< 2.0	< 2.0	4.3	2.2
Dinoterb	< 2.0	< 2.0	< 2.0	< 2.0	< 2.0	< 2.0	< 2.0
3,5-Dinitrosalicylic acid	< 3.0	< 3.0	< 3.0	< 3.0	< 3.0	< 3.0	< 3.0
2-Methyl-4-nitrophenol	1.4	1.6	2.4	2.5	6.5	7.1	10.2
2-Methoxy-4-nitrophenol	< 3.0	< 3.0	< 3.0	< 3.0	< 3.0	< 3.0	< 3.0
4-Hydroxy-3-nitrobenzenesulfonic acid	< 2.0	< 2.0	< 2.0	< 2.0	< 2.0	< 2.0	< 2.0
2-Amino-3-nitrobenzoic acid	< 2.0	< 2.0	< 2.0	< 2.0	< 2.0	< 2.0	< 2.0
3-Nitroindole	< 2.0	< 2.0	< 2.0	< 2.0	< 2.0	< 2.0	< 2.0
Summed concentration	5.6	22	24	11	20	26	36

TABLE 28: RESULTS MONITORING STUDY - EFFLUENT MP UV/H_2O_2 TREATMENT SAMPLE

Compound	Effluent MP UV/H_2O_2 treatment						
	27/07/16 ng/L	05/09/16 ng/L	21/09/16 ng/L	20/10/16 ng/L	21/11/16 ng/L	14/12/16 ng/L	11/01/17 ng/L
2-Methoxy-4,6-dinitrophenol	< 1.0	2.6	6.9	1.4	22	21	41
4-Nitrophenol	< 5.0	7.2	9.2	6.6	25	29	31
4-Nitrocatechol	< 2.0	< 2.0	< 2.0	< 2.0	2.1	3.1	3.7
2-Hydroxy-5-nitrobenzoic acid	< 2.0	15	16	11	28	44	44
5-Nitrovanillin	< 1.0	< 1.0	1.4	< 1.0	< 1.0	< 1.0	< 1.0
4-Nitrophthalic acid	< 10	35	28	19	26	42	39
2,4-Dinitrophenol	4.5	1.0	1.2	< 1.0	4.7	3.1	5.6
4-Nitro-1,3-benzenediol	< 2.0	< 2.0	< 2.0	< 2.0	< 2.0	< 2.0	< 2.0
2-Nitrohydroquinone	< 5.0	< 5.0	< 5.0	< 5.0	< 5.0	< 5.0	< 5.0
4-Nitrobenzenesulfonic acid	< 3.0	9.7	8.1	12	28	29	40
4-Hydroxy-3-nitrobenzoic acid	< 2.0	3.5	5.8	3.6	11	16	16
2-Hydroxy-3-nitrobenzoic acid	< 2.0	2.4	< 2.0	2.3	5.6	8.8	8.6
Dinoterb	< 2.0	< 2.0	< 2.0	< 2.0	< 2.0	< 2.0	< 2.0
3,5-Dinitrosalicylic acid	< 3.0	< 3.0	< 3.0	< 3.0	3.0	5.6	4.6
2-Methyl-4-nitrophenol	1.3	< 1.0	1.2	< 1.0	1.3	2.3	2.3

2-Methoxy-4-nitrophenol	< 3.0	< 3.0	< 3.0	< 3.0	< 3.0	< 3.0	< 3.0
4-Hydroxy-3-nitrobenzenesulfonic acid	< 2.0	< 2.0	< 2.0	< 2.0	16	7.3	23
2-Amino-3-nitrobenzoic acid	< 2.0	< 2.0	< 2.0	< 2.0	< 2.0	2.4	2.9
3-Nitroindole	< 2.0	< 2.0	< 2.0	< 2.0	< 2.0	2.0	2.3
Summed concentration	5.8	76	78	55	174	216	263

Fifteen of the 19 N-DBPs were detected during the monitoring study in MP UV/H_2O_2 treated water, with concentrations ranging from 1.0 to 44 ng/L. Five N-DBPs were detected at a relatively high concentrations in MP UV treated water; 2-methoxy-4,6-dinitrophenol (41 ng/L), 4-nitrophenol (31 ng/L), 2-hydroxy-5-nitrobenzoic acid (44 ng/L), 4-nitrophthalic acid (42 ng/L) and 4-nitrobenzenesulfonic acid (40 ng/L). As expected, the highest concentrations of N-DBPs were detected in the MP UV treated water, while much lower concentrations were detected in the influent sample. Three N-DBPs (5-nitrovanillin, 2,4-dintrophenol, and 2-methyl-4-nitrophenol) were detected in higher concentrations in the influent sample than in the MP UV treated sample, which suggest that these N-DBPs are probably not formed by MP UV in the full-scale treatment facility. Furthermore, there were no N-DBPs detected in the sample after dune infiltration, meaning that there are no N-DBPs present in the final drinking water.

3-Nitroindole, a by-product detected in the tryptophan labeling experiments, was also detected in the MP UV/H_2O_2 treated water, although at low concentrations of 2.0 - 2.3 ng/L in two of the seven samples. A suspect screening was performed for detection of other by-products of tryptophan, but none were found. A suspect screening was also performed for by-products of phenylalanine, but yielded no results. These results show that the N-DBPs of aromatic amino acids play a limited role in in the Heemskerk full-scale treatment facility.

In order to obtain a good overview of the detected N-DBPs in the monitoring study, the concentrations were summed per sample (see figure 14).

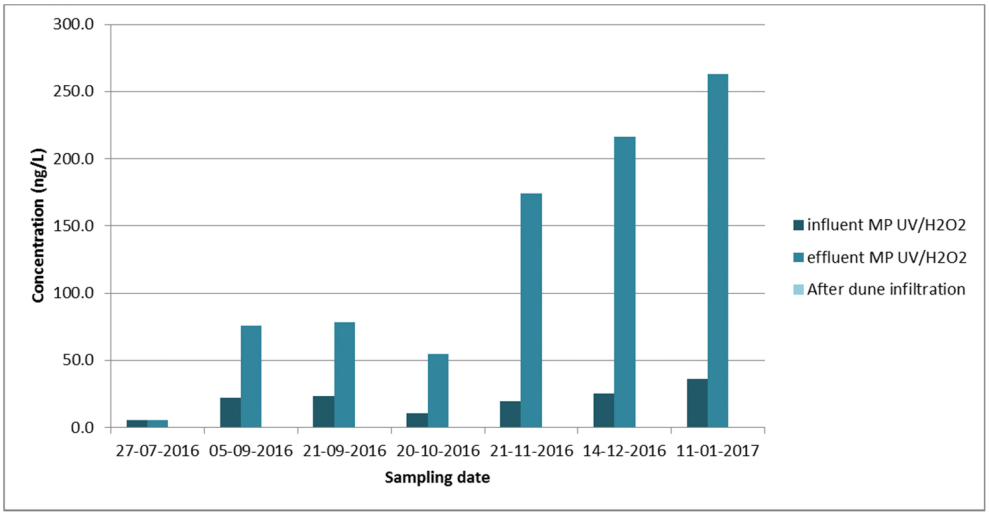

Figure 14: Summed concentrations of the detected by-products in the Heemskerk full-scale water treatment facility in influent MP UV/H_2O_2, effluent MP UV/H_2O_2 and after dune infiltration water, analysed using the QToF N-DBP target method.

A clear trend is visible when looking at the total amount of detected by-products per month. Low summed concentrations of N-DBPs were detected in July (sum is 5.8 ng/L) but increases considerably during the year, with a maximum of 263 ng/L detected in January. This trend cannot be explained by the concentration of dissolved organic carbon (DOC) or nitrate present in the water. The concentration of DOC does not fluctuate much during the year (5-7

mg/L), and the concentration of nitrate was low from August to December (< 2 mg/L) [37]. The increase in N-DBP concentration can currently not be explained and is subject for further research.

Furthermore the results show that the best period for detecting by-products is from November until January, although the period from February until June was not monitored and could possibly yield even higher concentrations.

4.7 N-DBP concentration in extracts of previous study

In the previous study [13] the Heemskerk full-scale drinking water treatment facility was also monitored and in March 2013 the same sampling points were also sampled. In the previous study the concentration of the mainly unidentified N-DBPs was estimated by using bentazon-d6 equivalents. Now a large portion of by-products is identified which are formed in a full-scale MP UV facility, it is possible to determine the actual concentrations of these by-products in these "old" extracts. The extracts were pre-treated in 2013 according to the Ames II protocol and were stored in the freezer at -25°C. The extracts were analysed with the LC-QToF target method as described in 4.3. The results of the target N-DBP analysis is shown in table 29. In the previous study a total amount of 82 ng/L bentazon-d6 equivalents were detected in influent MP UV/H_2O_2 water, whereof 47 ng/L were of identified N-DBPs.

TABLE 29: RESULTS N-DBP ANALYSIS USING THE QTOF TARGET METHOD IN EXTRACTS OF 2013

Compound	**influent MP UV/H_2O_2 15-03-2013 (ng/L)**	**effluent MP UV/H_2O_2 15-03-2013 (ng/L)**	**After dune infiltration 15-03-2013 (ng/L)**
2-Methoxy-4,6-dinitrophenol	< 0.5	1.4	< 0.5
4-Nitrophenol	< 3.0	4.7	< 3.0
4-Nitrocatechol	< 1.0	1.7	< 1.0
2-Hydroxy-5-nitrobenzoic acid	< 1.0	6.1	< 1.0
5-Nitrovanillin	< 0.5	0.9	< 0.5
4-Nitrophthalic acid	< 5.0	6.3	< 5.0
2,4-Dinitrophenol	0.6	< 0.5	< 0.5
4-Nitro-1,3-benzenediol	< 1.0	< 1.0	< 1.0
2-Nitrohydroquinone	< 3.0	< 3.0	< 3.0
4-nitrobenzenesulfonic acid	< 2.0	4.0	< 2.0
4-Hydroxy-3-nitrobenzoic acid	< 1.0	2.4	< 1.0
2-Hydroxy-3-nitrobenzoic acid	< 1.0	< 1.0	< 1.0
Dinoterb	< 1.0	< 1.0	< 1.0
3,5-Dinitrosalicylic acid	< 2.0	< 2.0	< 2.0
2-Methyl-4-nitrophenol	1.5	< 0.5	< 0.5
2-Methoxy-4-nitrophenol	< 2.0	< 2.0	< 2.0
4-Hydroxy-3-nitrobenzenesulfonic acid	< 1.0	< 1.0	< 1.0
2-Amino-3-nitrobenzoic acid	< 1.0	< 1.0	< 1.0
3-Nitroindole	< 1.0	< 1.0	< 1.0
Summed concentration	2.1	28	0.0

The total amount of N-DBPs detected in the MP UV/H_2O_2 treated sample (28 ng/L) is relatively low in comparison with the results of the current monitoring study and is also lower than 47 ng/L bentazon-d6 equivalents of the previous study. A possible explanation for the low concentrations in comparison with previous study could be that the concentration of some of N-DBPs declined due to break down over time, although such effects were previously not

observed. Another explanation is due to the quantification with bentazon-d6, which yield a different ionisation response [38] in comparison with the individual N-DBP.

4.7.1 Conclusion

In the end it is good to finally confirm the presence of several N-DBPs in the samples of the previous study and that it is finally possible to quantify them. Because the identity and concentration is now known for many N-DBPs detected in full-scale drinking water treatment facility using MP UV, it is now possible to test the identified N-DBPs (or mixture of) in the Amest test and to gain more insight in the relation between the observed mutagenicity and the identified N-DBPs.

5 Conclusion & recommendations

In this study a LC-QToF method was developed for the determination of aromatic amino acids for which satisfactory LOD and LOQ results were obtained for both drinking- and surface water. Aromatic amino acids were detected in Lake IJssel source water and artificial water, indicating that they can be a potential source for the formation of N-DBPs.

Labeling experiments were conducted using a non-target screening LC-QToF method and it was demonstrated that the labeling strategy involving nitrate, aromatic amino acids and MP UV treatment worked and many by-products were detected using this strategy. For tryptophan, one by-product was detected in the artificial water sample and was identified as 3-nitroindole. However, other high intensity tryptophan by-products were not detected in artificial water, suggesting that 3-nitroindole in the artificial water sample does not originate from tryptophan, but originates from another source. For phenylalanine, four unidentified phenylalanine N-DBPs were detected in artificial water, for which was shown that these N-DBPs are truly MP UV by-products of phenylalanine. This demonstrates that aromatic amino acids are probably responsible for some of the N-DBPs formed by MP UV treatment of artificial water. However, none of the tryptophan and phenylalanine by-products were detected in the full-scale MP UV water treatment sample of the prior study, making the detected by-products of tryptophan and phenylalanine less relevant for further research.

Of the 84 by-products detected in artificial water (prior study), five N-DBPs were unambiguously identified during this study, bringing the total of identified N-DBPs in artificial water to 19. For all of the newly identified N-DBPs an indication for DNA binding and mutagenicity was obtained and an indication for potential carcinogenicity for 2-amino-3-nitrobenzoic acid, 2-methyl-4-nitrophenol and 2-methoxy-4-nitrophenol.

A LC-QToF target method was developed for determination of the 19 identified N-DBPs in drinking- and surface water and satisfactory LOD and LOQ results were obtained. The method was applied for a seven months monitoring study of a full-scale drinking water facility using MP UV treatment. Fifteen of the 19 known N-DBPs were detected during the monitoring study in MP UV/H_2O_2 treated water, with concentrations ranging from 1.0 to 44 ng/L. Five N-DBPs were detected at relatively high concentrations in MP UV treated water; 2-methoxy-4,6-dinitrophenol (41 ng/L), 4-nitrophenol (31 ng/L), 2-hydroxy-5-nitrobenzoic acid (44 ng/L), 4-nitrophthalic acid (42 ng/L) and 4-nitrobenzenesulfonic acid (40 ng/L). The monitory study showed a large seasonal variation in the summed concentration of detected N-DBPs (5.8 ng/L in July and 263 ng/L in January) and also showed there were no N-DBPs detected in the samples after dune infiltration. The amount of detected N-DBPs in a full-scale treatment facility demonstrates the relevance of the identified N-DBPs and the developed N-DBP target method.

Because the identity and concentration is now known for many N-DBPs detected in a full-scale drinking water treatment facility using MP UV; it is recommended to conduct N-DBP target analysis of MP UV treated water of a drinking water facility, and then to perform Ames testing with and without (mixtures of) identified N-DBPs at their detected concentration, in order to gain more insight between the relation of identified N-DBPs and observed mutagenicity in MP UV treated water.

Other recommendations:

- Conducting a monitoring study of a year for a full-scale drinking water treatment facility using MP UV in order to obtain a good overview of the concentrations of the N-DBPs per month and to find an explanation between the concentration differences per month
- Performing labeling experiments in MP UV influent water, in order to detect other types of N-DBPs, such as N-DBPs formed from organic micro pollutants.

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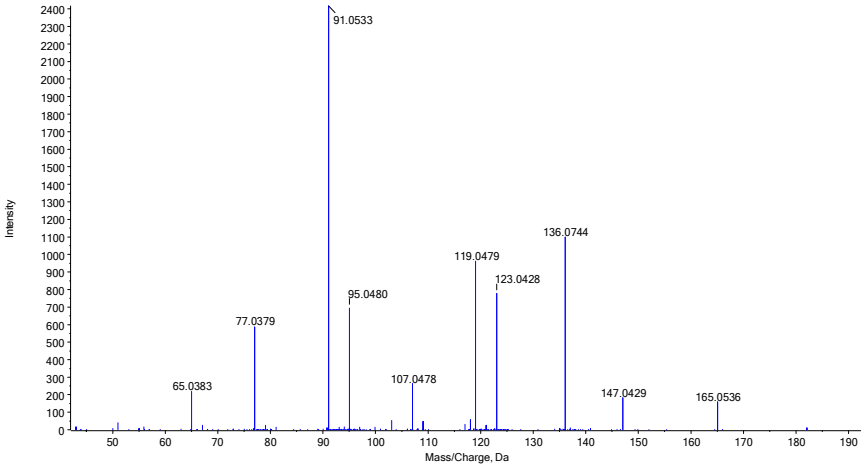
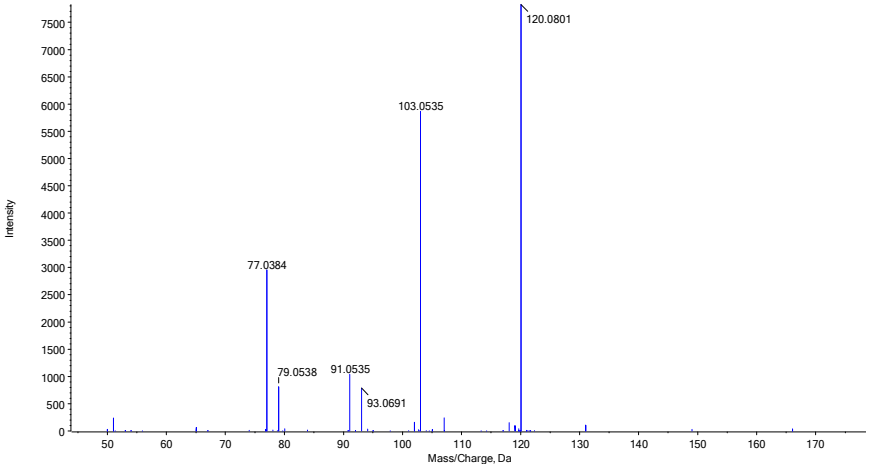
Attachment I MS/MS spectra of aromatic amino acids

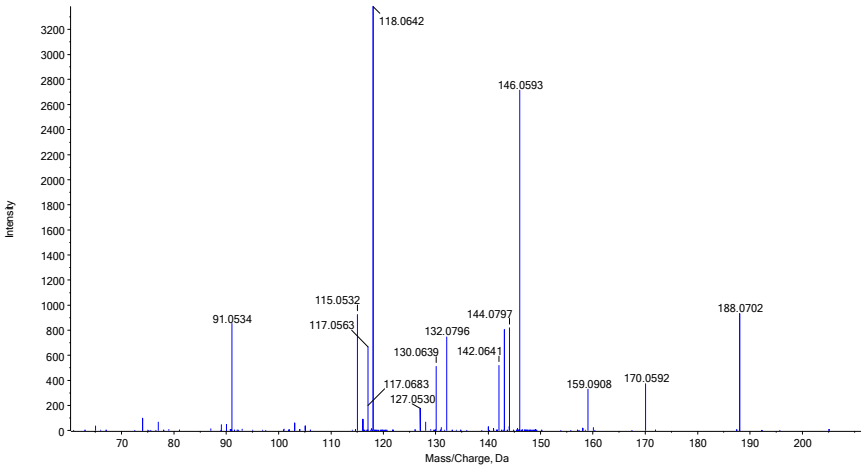
TYROSINE MS/MS SPECTRUM (20, 35 AND 50 EV, AVERAGED)



PHENYLALANINE MS/MS SPECTRUM (20, 35 AND 50 EV, AVERAGED)



TRYPTOPHAN MS/MS SPECTRUM (20, 35 AND 50 EV, AVERAGED)



Attachment II Materials & Method labeling experiments

II.1 Chemicals

All solvents used were of analytical grade quality. Acetonitrile and methanol (ultra gradient HPLC grade) was obtained from Avantor Performance Materials B.V. (Deventer, the Netherlands). Formic acid (HPLC quality), potassium nitrate ($K^{14}NO_3$) and $K^{15}NO_3$ (98% enrichment) were purchased from Sigma-Aldrich (Steinheim, Germany). The aromatic amino acids reference standards; L-tyrosine, L-phenylalanine and L-tryptophan were acquired from Sigma-Aldrich. The isotopically labeled internal standards; L-Tyrosine-d4, L-Phenylalanine-d5 and L-Tryptophan-d5 were purchased from Toronto Research Chemicals (Toronto, Canada). L-tryptophan-$^{13}C_{11}$ was obtained from Cambridge Isotope Laboratories. Ultrapure water was obtained by purifying demineralized water in an Elga Purelab Chorus ultrapure water system. (High Wycombe, United Kingdom). Pony Lake NOM was obtained from International Humic Substances Society. Stock solutions of the reference and internal standards were prepared in methanol and ultrapure water (20/80% v/v) at a concentration of 100 and 50 mg/L, respectively. Stock solutions were stored at -25 °C.

II.2 LC-QTOF analysis

HPLC settings (Shimadzu Nexera; LC-30AD, SIL-30AC, CTO-20AC):

- Column: Waters Xbridge C18 XP, 2.1 x 150 mm, 2.5 µm
- Mobile phase A: ultrapure water + 0.05% formic acid
  Mobile phase B: acetonitrile + 0.05% formic acid
- Gradient: 4% B held for 1 min. Then linear from 4% to 100% B in 40 min. Held at 100% B for 5 min. Then return to initial conditions in 1 min and held for 6 min.
- Flow: 300 µL/min
- Injection volume: 50 µL
- Column oven temperature: 25°C

QToF-MS settings (AB SCIEX TripleTOF 5600+):

- Resolution: > 30.000 @ m/z 400 (MS and MS/MS mode)
- Mass accuracy < 5 ppm
- Mass range Full scan: 65-800 Da
- Mass range MS2 scan: 40-800 Da
- Ionisation: positive and negative mode
- Source: electrospray (ESI)
- TurboIonSpray heater: 500°C
- Ion Spray Voltage: 5000 and 3000 volt for positive and negative mode
- Curtain gas: 25 psi
- Gas 1: 40 psi
- Gas 2: 50 psi
- Divert valve: 3.0 min
- Collision energy: 20, 35, 50 eV (averaged)
- Data dependant MS/MS scans: 8 per cycle (50ms), threshold 100 counts and dynamic background subtraction.

Attachment III Screening list of nitrogenous disinfection by-products

Accurate mass	Retention time* (min)	Positive/ Negative mode	Most probable formula	Detected in fractionated samples	Detected in unfractionated samples	Compound
138.0199	11.79	Negative	$C_6H_5O_3N$	yes	yes	4-nitrophenol
152.0359 (1)	10.10	negative	$C_7H_7O_3N$	yes	yes	
152.0361 (2)	15.18	negative	$C_7H_7O_3N$	yes	yes	
153.0073	10.54	negative		yes	yes	
154.0148 (1)	9.23	negative	$C_6H_5O_4N$	yes	yes	4-nitrocatechol
154.0148 (2)	10.06	negative	$C_6H_5O_4N$	yes	yes	2-nitrohydroquinone
154.0148 (3)	**	negative	$C_6H_5O_4N$	yes?	yes	4-nitro-1,3-benzenediol
161.0364	15.06	negative	$C_8H_6O_2N_2$	yes	no	
168.0306	12.23	negative	$C_7H_7O_4N$	yes	yes	
179.0101	10.62	negative	$C_7H_4O_4N_2$	yes	yes	
181.0263	13.59	negative	$C_7H_7O_4N_2$	yes	yes	
182.0098 (2)	10.21	negative	$C_7H_5O_5N$	yes	yes	4-hydroxy-3-nitrobenzoic acid
182.0098 (3)	12.81	negative	$C_7H_5O_5N$	yes	yes	2-hydroxy-5-nitrobenzoic acid
182.0098 (4)	**	negative	$C_7H_5O_5N$	yes	yes	2-hydroxy-3-nitrobenzoic acid
183.0055	14.03	negative	$C_6H_4O_5N_2$	yes	yes	2,4-dinitrophenol
195.0055	10.97	negative	$C_7H_4O_5N_2$	yes	yes	
196.0254	7.68	negative	$C_8H_7O_5N$	yes	yes	
196.0258 (1)	10.10	negative		yes	yes	
196.0259 (2)	11.51	negative		yes	yes	5-nitrovanillin
196.0260 (3)	12.89	negative		yes	yes	
198.0047	7.88	negative	$C_7H_5O_6N$	yes	yes	
201.9818	5.00	negative	$C_6H_5NO_5S$	yes	yes	4-nitrobenzenesulfonic acid
208.0255	11.77	negative	$C_9H_7O_5N$	yes	yes	
210.0048 (1)	7.48	negative	$C_8H_5O_6N$	yes	yes	4-nitrophthalic acid
210.0048 (2)	8.90	negative	$C_8H_5O_6N$	yes	yes	
211.0004	12.90	negative	$C_7H_4O_6N_2$	yes	yes	
212.0204	10.55	negative	$C_8H_7O_6N$	yes	yes	Structural isomer of 5-Hydroxy-4-methoxy-2-nitrobenzoic acid
213.0154	14.58	negative	$C_7H_6O_6N_2$	yes	yes	2-methoxy-4,6-dinitrophenol
216.0268	11.53	positive		yes	yes	
216.0268	6.81	positive		yes	yes	
217.9767	5.73	negative		yes	yes	
222.0165	16.75	negative	$C_8H_5O_5N_3$	yes	yes	
223.0005	10.63	negative	$C_8H_4O_6N_2$	yes	yes	
223.9957	13.52	negative	$C_7H_3O_6N_3$	yes	yes	
225.9994 (1)	6.37	negative	$C_8H_5O_7N$	yes	yes	
225.9996 (2)	7.39	negative	$C_8H_5O_7N$	yes	yes	
226.9948	13.28	negative	$C_7H_4O_7N_2$	yes	yes	3,5-dinitrosalicylic acid
238.0726	17.84	negative	$C_{11}H_{13}O_5N$	yes	yes	
239.0677	25.87	negative	$C_{10}H_{12}O_5N_2$	yes	yes	dinoterb
240.0151	7.67	negative	$C_9H_7O_7N$	yes	yes	
243.9923	5.20	negative		no	no	
252.0153	8.74	negative	$C_{10}H_7O_7N$	yes	yes	
254.0314	10.64	negative	$C_{10}H_9O_7N$	yes	yes	
266.1037	22.94	negative	$C_{13}H_{17}O_5N$	yes	yes	
270.0755 (1)	11.32	negative		yes	yes	
270.0755 (2)	11.61	negative		yes	yes	
272.0891	11.53	positive		yes	yes	
274.0935	13.89	negative	$C_{11}H_{17}O_7N$	yes	yes	

298.0940	13.10	negative	$C_{13}H_{17}O_7N$	yes	yes	
316.1413 (1)	18.35	negative	$C_{14}H_{23}O_7N$	yes	yes	
316.1417 (2)	19.00	negative	$C_{14}H_{23}O_7N$	yes	yes	
316.1417 (3)	19.74	negative	$C_{14}H_{23}O_7N$	yes	yes	
318.1550 (1)	19.80	positive	$C_{14}H_{23}O_7N$	yes	yes	
318.1550 (2)	18.42	positive	$C_{14}H_{23}O_7N$	yes	yes	
319.0209	16.16	negative		yes	yes	
331.0554 (1)	12.39	negative		yes	yes	
331.0554 (2)	12.57	negative		yes	yes	
331.0554 (3)	13.08	negative		yes	yes	
335.0534	18.95	negative		yes	yes	
340.1388	27.36	positive	$C_{16}H_{21}O_7N$	yes	yes	
340.1388	26.99	positive	$C_{16}H_{21}O_7N$	yes	yes	
340.1388	28.08	positive	$C_{16}H_{21}O_7N$	yes	yes	
372.1491	24.10	negative		yes	yes	
386.1096 (1)	10.94	negative		yes	yes	
386.1096 (2)	11.84	negative		yes	yes	
386.1653	26.10	negative		yes	yes	
387.1091	20.87	negative		yes	no	
400.1262 (1)	11.24	negative		yes	yes	
400.1262 (2)	12.19	negative		yes	yes	
400.1802	28.34	negative		yes	yes	
404.2758 (1)	17.34	positive	$C_{19}H_{37}O_8N_3$	yes	yes	
404.2758 (2)	17.54	positive	$C_{19}H_{37}O_8N_3$	yes	no	
404.2758 (3)	17.72	positive	$C_{19}H_{37}O_8N_3$	yes	no	
404.2758 (4)	18.02	positive	$C_{19}H_{37}O_8N_3$	yes	yes	
404.2758 (5)	18.36	positive	$C_{19}H_{37}O_8N_3$	yes	yes	
404.2758 (6)	18.86	positive	$C_{19}H_{37}O_8N_3$	no	no	
404.2758 (7)	20.40	positive	$C_{19}H_{37}O_8N_3$	yes	no	
408.1308 (1)	11.70	negative		yes	yes	
408.1310 (2)	13.21	negative		yes	yes	
410.1468	14.46	negative		yes	yes	
414.1418 (1)	11.48	negative		yes	yes	
414.1421 (2)	12.57	negative		yes	yes	
428.1213	10.26	negative		yes	yes	
442.1365 (1)	9.49	negative		yes	yes	
442.1365 (2)	10.19	negative		yes	yes	
447.1455	24.00	negative		no	no	
474.1501	11.66	positive		yes	yes	
448.2675 (1)	17.32	negative	$C_{20}H_{39}O_8N_3$	no	no	
448.2675 (2)	17.52	negative	$C_{20}H_{39}O_8N_3$	no	no	
448.2675 (3)	17.70	negative	$C_{20}H_{39}O_8N_3$	no	no	
448.2675 (4)	17.99	negative	$C_{20}H_{39}O_8N_3$	no	no	
448.2675 (5)	18.35	negative	$C_{20}H_{39}O_8N_3$	no	no	
448.2675 (6)	18.86	negative	$C_{20}H_{39}O_8N_3$	no	no	
448.2675 (7)	20.40	negative	$C_{20}H_{39}O_8N_3$	no	no	
452.1203 (1)	11.12	negative		yes	yes	
452.1210 (2)	12.44	negative		yes	yes	

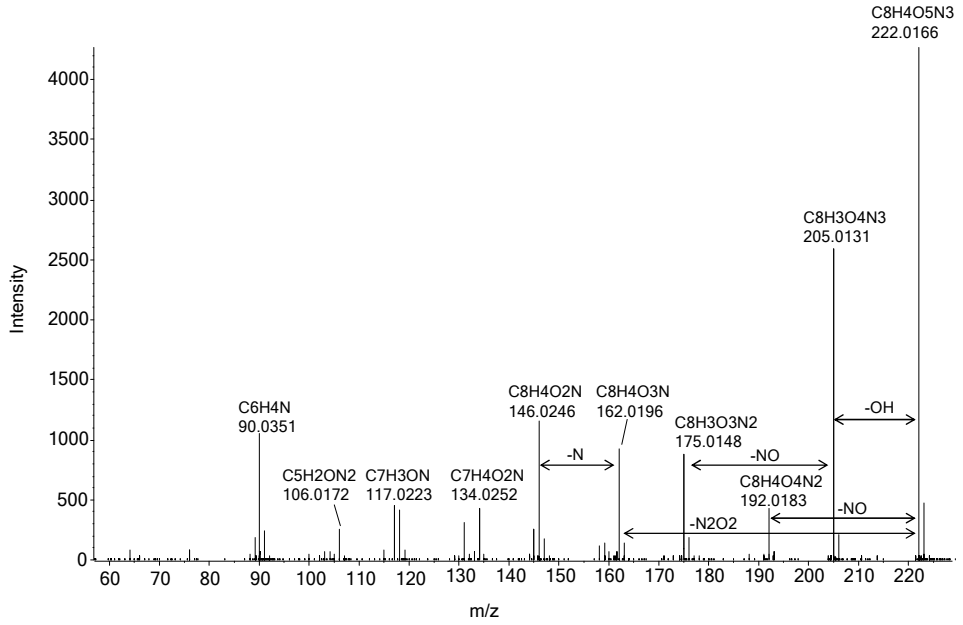
** Retention time of the by-products as determined with stable isotope labeling using the Orbitrap mass spectrometer*

*** These N-DBPs were not detected during the initial isotope labeling experiment, but in the follow-up study by Vughs et al.*

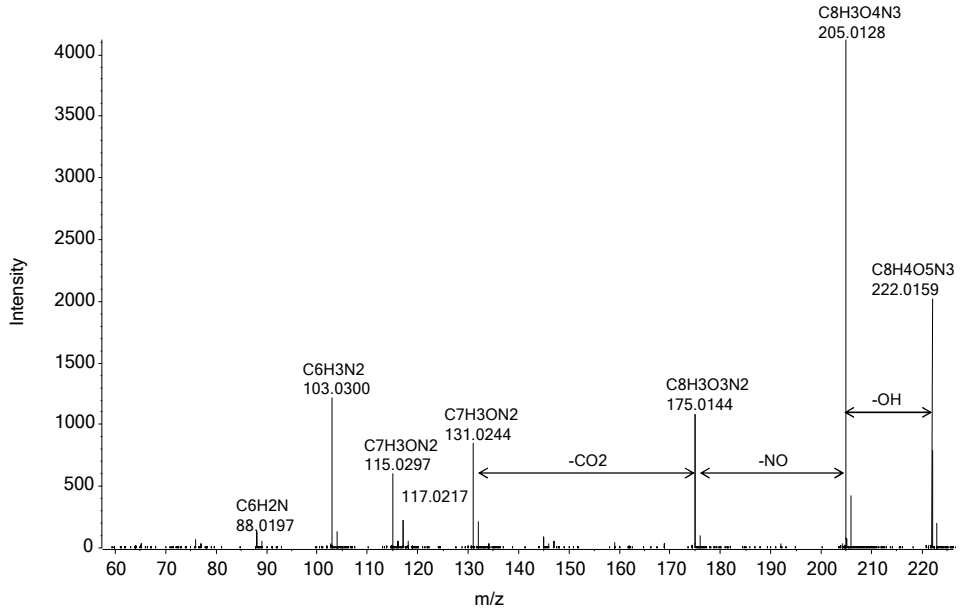
For more information about the detection and identification of the by-products, see publication: Kolkman, A.; Martijn, B. J.; Vughs, D.; Baken, K. A.; van Wezel, A. P., Tracing Nitrogenous Disinfection Byproducts after Medium Pressure UV Water Treatment by Stable Isotope Labeling and High Resolution Mass Spectrometry. Environmental Science & Technology 2015, 49, (7), 4458-4465.

Attachment IV Annotated MS/MS spectra of phenylalanine N-DBPs

ANNOTATED MS/MS SPECTRUM (20, 35 AND 50 EV, AVERAGED) OF BY-PRODUCT MZ 222.0156 (RT 18.45)



ANNOTATED MS/MS SPECTRUM (20, 35 AND 50 EV, AVERAGED) OF BY-PRODUCT MZ 222.0156 (RT 17.79)



Attachment V Tryptophan and phenylalanine N-DBPs SPE experiment conditions

V.1 Samples tryptophan and phenylalanine N-DBPs SPE experiment

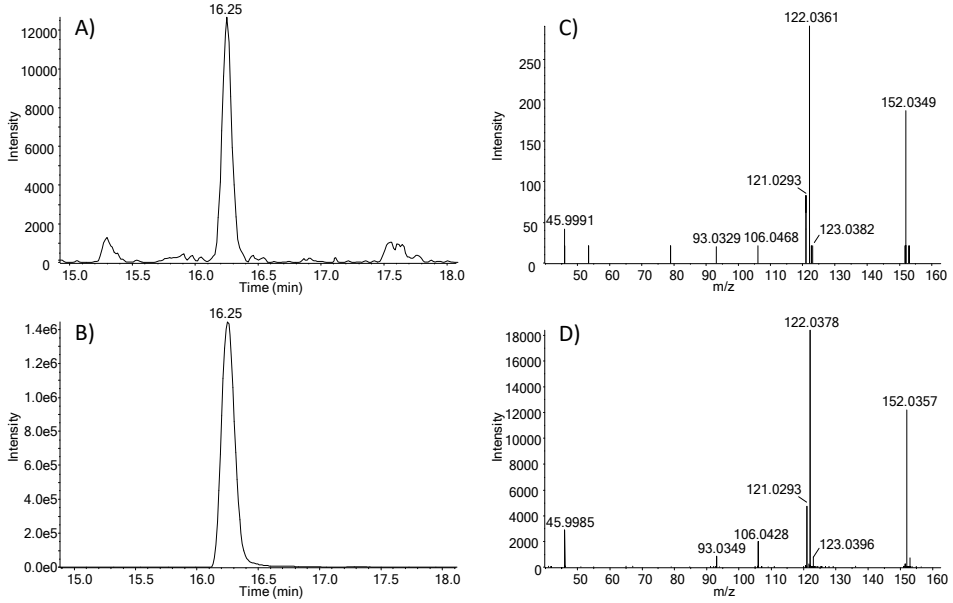
The following samples were used for the SPE experiment in duplo: UV treated + Trp + $^{14}NO_3^-$ sample, UV treated + Phe + $^{14}NO_3^-$ sample and an ultrapure water sample (blank). Ten mL of sample was diluted with 990 ml of ultrapure water prior the SPE sample pre-treatment.

V.2 AMES SPE pre-treatment protocol

For the AMES sample pre-treatment, 1L of sample was acidified to pH 2.3 using hydrochloric acid and loaded on a 200 mg Waters OASIS HLB glass SPE cartridge (Etten-Leur, Netherlands). Then the SPE column was dried for 1 hour by air and elution was performed with 7.5 mL of 8:2 (v/v) acetonitrile/methanol. The eluate was evaporated using a Barkey optocontrol (Leopoldshöhe, Germany) with a gentle nitrogen stream at circa 75 °C (block temperature at 300 °C) until a volume of 250 µL was reached. In order to compare the by-products in the samples after the AMES SPE pre-treatment with the samples that were not pre-treated, 9750 µL of ultrapure water added to the extract, so that effectively no sample enrichment had occurred (10 mL before SPE to 10 mL after SPE). In this way, a fair comparison could be made between the samples with and without sample pre-treatment in order to determine the recovery of the by-products. Finally, the samples were analysed using the LC-QToF as described in paragraph 3.2.3.

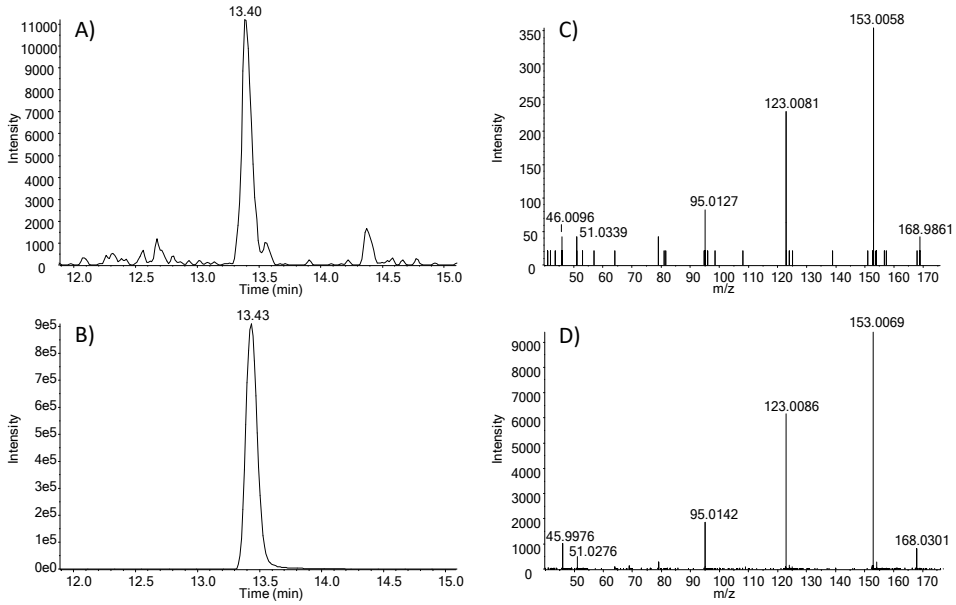
Attachment VI Confirmation of identified N-DBPs

CONFIRMATION OF 2-METHYL-4-NITROPHENOL IN ARTIFICIAL WATER



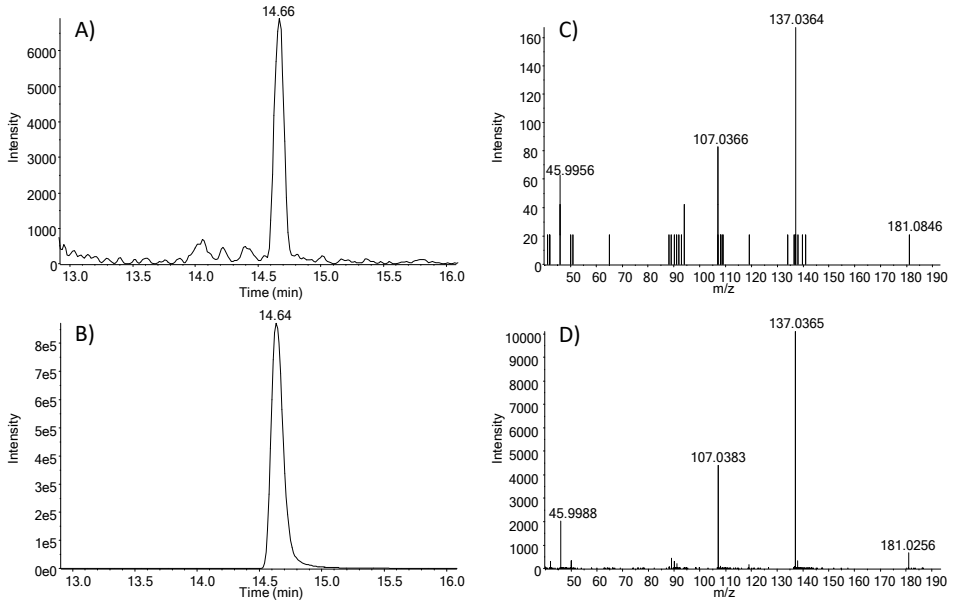
A) EIC of m/z 152.0353 in artificial water after MP UV. B) EIC of 2-methyl-4-nitrophenol reference standard (m/z 152.0353). C) MS2 spectrum of m/z 152.0353 in artificial water after MP UV. D) MS2 spectrum of 2-methyl-4-nitrophenol reference standard.

CONFIRMATION OF 2-METHOXY-4-NITROPHENOL IN ARTIFICIAL WATER



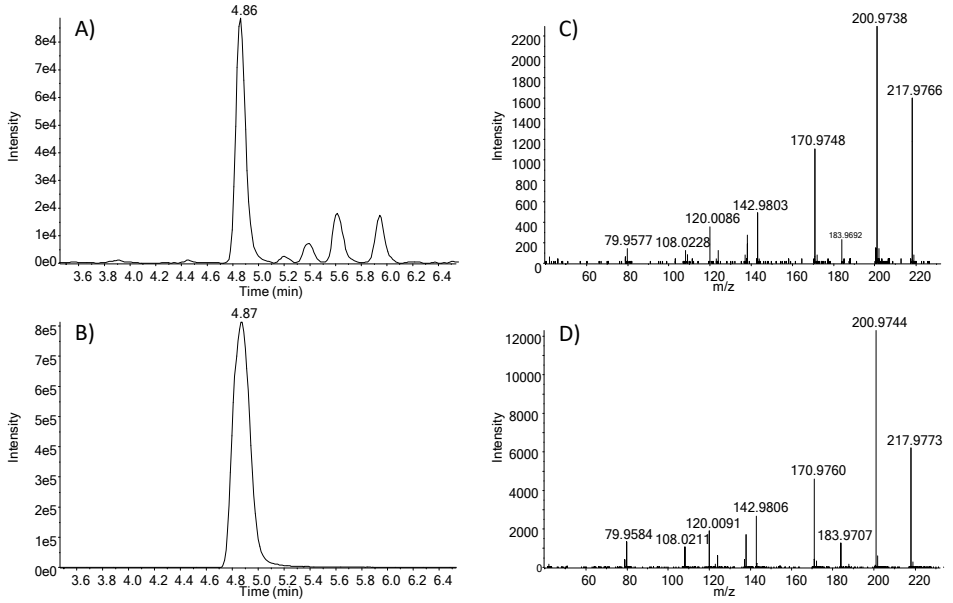
A) EIC of m/z 168.0302 in artificial water after MP UV. B) EIC of 2-methoxy-4-nitrophenol reference standard (m/z 168.0302). C) MS2 spectrum of m/z 168.0302 in artificial water after MP UV. D) MS2 spectrum of 2-methoxy-4-nitrophenol reference standard.

CONFIRMATION OF 2-AMINO-3-NITROBENZOIC ACID IN ARTIFICIAL WATER



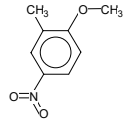
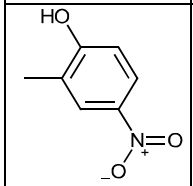
A) EIC of m/z 181.0255 in artificial water after MP UV. B) EIC of 2-Amino-3-nitrobenzoic acid reference standard (m/z 181.0255). C) MS2 spectrum of m/z 181.0255 in artificial water after MP UV. D) MS2 spectrum of 2-Amino-3-nitrobenzoic acid reference standard.

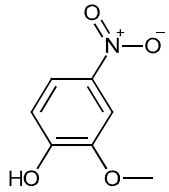
CONFIRMATION OF 4-HYDROXY-3-NITROBENZENESULFONIC ACID IN ARTIFICIAL WATER



A) EIC of m/z 217.9765 in artificial water after MP UV. B) EIC of 4-hydroxy-3-nitrobenzenesulfonic acid reference standard (m/z 217.9765). C) MS2 spectrum of m/z 217.9765 in artificial water after MP UV. D) MS2 spectrum of 4-hydroxy-3-nitrobenzenesulfonic acid reference standard.

Attachment VII Structural alerts reported by ECHA and the OECD QSAR Toolbox

CAS	Substance	ECHA database	OECD QSAR Toolbox
99-53-6[1]	2-Methyl-4-nitrophenol		
		**Suspected mutagen:** The Toolbox profiler 'DNA alerts for AMES, MN and CA by OASIS v.1.3' gives an alert for mutagenicity; The Toolbox profiler 'in vitro mutagenicity (Ames test) alerts by ISS' gives an alert for mutagenicity; ISS Mutagenicity model in VEGA (Q)SAR platform predicts that the chemical is Mutagen (moderate reliability) **Suspected carcinogen:** The Toolbox profiler 'Carcinogenicity (genotox and nongenotox) alerts by ISS' gives an alert for carcinogenicity; ISS Carcinogenicity model in VEGA (Q)SAR platform predicts that the chemical is Carcinogen (moderate reliability) **Suspected persistent in the environment:** Ready biodegradability model (IRFMN) in VEGA (Q)SAR platform predicts that the chemical is Possible NON Readily Biodegradable (good reliability);The Danish QSAR database contains information indicating that the substance is predicted as non readily biodegradable[2]	**DNA Binding & DNA alerts for AMES, CA and MNT by OASIS v.1.4:** Radical >> Radical mechanism via ROS formation (indirect) >> Nitrophenols, Nitrophenyl Ethers and Nitrobenzoic Acids SN1 >> Nucleophilic attack after reduction and nitrenium ion formation >> Nitrophenols, Nitrophenyl Ethers and Nitrobenzoic Acids **DNA Binding by OECD:** SN1 >> Nitrenium Ion formation >> Aromatic nitro **Carcinogenicity (genotox and nongenotox) alerts by ISS:** Nitro-aromatic (Genotox) Structural alert for genotoxic carcinogenicity **in vitro mutagenicity (Ames test) alerts by ISS** Nitro-aromatic **in vitro mutagenicity (Micronucleus) alerts by ISS** Nitro-aromatic **Oncologic Primary Classification** Aromatic Amine Type Compounds **Repeated dose (HESS)** Nitrobenzenes (Hemolytic anemia with methemoglobinemia) Rank A Nitrobenzenes (Hepatotoxicity) Rank C
			**DNA Binding by OASIS v.1.4:** Radical >> Radical mechanism via ROS formation (indirect) >> Nitrophenols, Nitrophenyl Ethers and Nitrobenzoic Acids SN1 >> Nucleophilic attack after reduction and nitrenium ion formation >> Nitrophenols, Nitrophenyl Ethers and Nitrobenzoic Acids **DNA Binding by OECD:** Michael addition >> P450 Mediated Activation to Quinones and Quinone-type Chemicals >> Alkyl phenols SN1 >> Nitrenium Ion formation >> Aromatic nitro **Carcinogenicity (genotox and nongenotox) alerts by ISS:** Nitro-aromatic (Genotox)

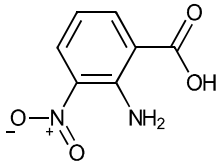
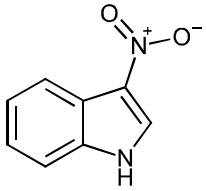
			Structural alert for genotoxic carcinogenicity **in vitro mutagenicity (Ames test) alerts by ISS** Nitro-aromatic **in vitro mutagenicity (Micronucleus) alerts by ISS** Nitro-aromatic **Oncologic Primary Classification** Aromatic Amine Type Compounds Phenol Type Compounds **Repeated dose (HESS)** Nitrophenols/ Halophenols (Energy metabolism dysfunction) Rank B
3251-56-7	2-Methoxy-4-nitrophenol 	**Suspected mutagen:** The Toolbox profiler 'DNA alerts for AMES, MN and CA by OASIS v.1.3' gives an alert for mutagenicity; The Toolbox profiler 'in vitro mutagenicity (Ames test) alerts by ISS' gives an alert for mutagenicity; CAESAR Mutagenicity model in VEGA (Q)SAR platform predicts that the chemical is Suspect Mutagen (moderate reliability); ISS Mutagenicity model in VEGA (Q)SAR platform predicts that the chemical is Mutagen (good reliability); KNN Mutagenicity model in VEGA (Q)SAR platform predicts that the chemical is Mutagen (moderate reliability) **Suspected carcinogen:** The Toolbox profiler 'Carcinogenicity (genotox and nongenotox) alerts by ISS' gives an alert for carcinogenicity; CAESAR Carcinogenicity model in VEGA (Q)SAR platform predicts that the chemical is Carcinogen (good reliability); ISS Carcinogenicity model in VEGA (Q)SAR platform predicts that the chemical is Carcinogen (good reliability) **Suspected persistent in the environment**: Ready biodegradability model (IRFMN) in VEGA (Q)SAR platform predicts that the chemical is Possible NON Readily Biodegradable (good reliability);The Danish QSAR database contains information indicating that the substance is predicted as non-readily biodegradable[3]	**DNA Binding & DNA alerts for AMES, CA and MNT by OASIS v.1.4:** Radical >> Radical mechanism via ROS formation (indirect) >> Nitrophenols, Nitrophenyl Ethers and Nitrobenzoic Acids SN1 >> Nucleophilic attack after reduction and nitrenium ion formation >> Nitrophenols, Nitrophenyl Ethers and Nitrobenzoic Acids **DNA Binding by OECD:** Michael addition >> P450 Mediated Activation to Quinones and Quinone-type Chemicals >> Hydroquinones SN1 >> Nitrenium Ion formation >> Aromatic nitro **in vitro mutagenicity (Ames test) alerts by ISS** Nitro-aromatic **in vitro mutagenicity (Micronucleus) alerts by ISS** H-acceptor-path3-H-acceptor Nitro-aromatic **Carcinogenicity (genotox and nongenotox) alerts by ISS:** Nitro-aromatic (Genotox) Structural alert for genotoxic carcinogenicity **Oncologic Primary Classification** Aromatic Amine Type Compounds Phenol Type Compounds **Repeated dose (HESS)** Nitrophenols/ Halophenols (Energy metabolism dysfunction) Rank B

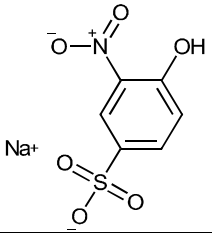
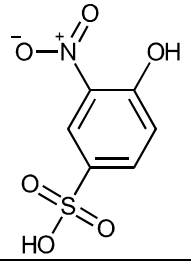
1 OECD QSAR Toolbox reports two structures for the same CAS number

2 https://echa.europa.eu/nl/information-on-chemicals/annex-iii-inventory/-/dislist/details/AIII-100.002.512

3 https://echa.europa.eu/nl/information-on-chemicals/annex-iii-inventory/-/dislist/details/AIII-100.019.854

Attachment VIII Structural alerts reported by the OECD QSAR Toolbox

CAS	InChI	Substance	OECD QSAR Toolbox
606-18-8	InChI=1S/C7H6N2O4/c8-6-4(7(10)11)2-1-3-5(6)9(12)13/h1-3H,8H2,(H,10,11)	2-Amino-3-nitrobenzoic acid 	**DNA Binding by OASIS v.1.4:** Radical >> Radical mechanism via ROS formation (indirect) >> Nitrophenols, Nitrophenyl Ethers and Nitrobenzoic Acids SN1 >> Nucleophilic attack after reduction and nitrenium ion formation >> Nitrophenols, Nitrophenyl Ethers and Nitrobenzoic Acids **DNA Binding by OECD:** SN1 >> Nitrenium Ion formation >> Aromatic nitro **in vitro mutagenicity (Ames test) alerts by ISS** Nitro-aromatic **in vitro mutagenicity (Micronucleus) alerts by ISS** H-acceptor-path3-H-acceptor Nitro-aromatic **Carcinogenicity (genotox and nongenotox) alerts by ISS:** Nitro-aromatic (Genotox) Structural alert for genotoxic carcinogenicity **Oncologic Primary Classification** Aromatic Amine Type Compounds **Protein binding by OASIS v1.4** AN2 >> Michael-type addition to quinoid structures >> Substituted Anilines **DART scheme v.1.0** Known precedent reproductive and developmental toxic potential NO2-alkyl/NO2-benzene derivatives (8b)
4770-03-0	InChI=1S/C8H6N2O2/c11-10(12)8-5-9-7-4-2-1-3-6(7)8/h1-5,9H	3-nitroindole 	**DNA Binding & DNA alerts for AMES, CA and MNT by OASIS v.1.4:** Radical >> Radical mechanism via ROS formation (indirect) >> Conjugated Nitro Compounds SN1 >> Nucleophilic attack after reduction and nitrenium ion formation >> Conjugated Nitro Compounds **DNA Binding by OECD:** SN1 >> Nitrenium Ion formation >> Unsaturated heterocyclic nitro **in vivo mutagenicity (Micronucleus) alerts by ISS** H-acceptor-path3-H-acceptor **Oncologic Primary Classification** Aromatic Amine Type Compounds **Protein binding alerts for skin sensitization by OASIS v1.4** Michael Addition >> Michael addition on conjugated systems with electron withdrawing group >> Nitroalkenes

6313-34-4	InChI=1S/C6H5NO6S.Na/c8-6-2-1-4(14(11,12)13)3-5(6)7(9)10;/h1-3,8H,(H,11,12,13);/q;+1	sodium;4-hydroxy-3-nitrobenzenesulfonic acid 	**DNA Binding by OASIS v.1.4:** Radical >> Radical mechanism via ROS formation (indirect) >> Nitrophenols, Nitrophenyl Ethers and Nitrobenzoic Acids SN1 >> Nucleophilic attack after reduction and nitrenium ion formation >> Nitrophenols, Nitrophenyl Ethers and Nitrobenzoic Acids **in vivo mutagenicity (Micronucleus) alerts by ISS** H-acceptor-path3-H-acceptor **Oncologic Primary Classification** Aromatic Amine Type Compounds Phenol Type Compounds **Estrogen Receptor Binding** Strong binder, OH group **Repeated dose (HESS)** Benzene/ Naphthalene sulfonic acids (Less susceptible) Rank C Nitrophenols/ Halophenols (Energy metabolism dysfuntion) Rank B
616-85-3	*In OECD QSAR Toolbox database*	*4-hydroxy-3-nitrobenzenesulphonic acid* 	

Attachment IX Materials & Methods LC-QToF N-DBPs target method

IX.1 Chemicals

All solvents used were of analytical grade quality. Acetonitrile and methanol (ultra gradient HPLC grade) was obtained from Avantor Performance Materials B.V. (Deventer, the Netherlands). Formic acid (HPLC quality) and hydrochloric acid 30% suprapur were purchased from Sigma-Aldrich (Steinheim, Germany) and Merck (Darmstadt, Germany), respectively. The internal standards 4-nitrophenol and neburon were obtained from Sigma-Aldrich and bentazon-d6 was obtained from LGC Standards GmbH (Wesel, Germany). The following N-DBPs were purchased from Sigma-Aldrich, 4-nitrophenol, 4-nitrocatechol, 2-hydroxy-5-nitrobenzoic acid, 5-nitrovanillin, 4-nitrophthalic acid, 2,4-dinitrophenol, 4-hydroxy-3-nitrobenzoic acid, 2-hydroxy-3-nitrobenzoic acid, dinoterb, 3,5-dinitrosalicylic acid, 2-methyl-4-nitrophenol, 2-methoxy-4-nitrophenol and 2-amino-3-nitrobenzoic acid. 2-methoxy-4,6-dinitrophenol and 4-hydroxy-3-nitrobenzenesulfonic acid were obtained from Vitas-M laboratory (Moscow, Russia). 4-nitro-1,3-benzenediol was purchased from Santa Cruz Biotechnology. 2-nitrohydroquinone and 3-nitroindole were obtained from Chemos GmbH (Regenstauf, Germany) and Oxchem (Wood Dale, IL, USA), respectively. 4-nitrobenzenesulfonic acid was obtained from TCI Europe (Zwijndrecht, Belgium).Ultrapure water was obtained by purifying demineralized water in an Elga Purelab Chorus ultrapure water system. (High Wycombe, United Kingdom). Stock solutions of the reference and internal standards were prepared in methanol and ultrapure water (20/80% v/v) at a concentration of 100 and 50 mg/L, respectively. Stock solutions were stored at -25 °C.

IX.2 Sample pre-treatment

For the sample pre-treatment, 500 mL of sample was acidified to pH 2.3 using hydrochloric acid and was loaded onto a SPE cartridge (OASIS HLB, 200 mg, glass, 6 cc) obtained from Waters (Etten-Leur, Netherlands). Then the SPE column was dried for 1 hour by air and elution was performed with 7.5 mL of 8:2 (v/v) acetonitrile/methanol. The eluate was evaporated using a Barkey optocontrol (Leopoldshöhe, Germany) with a gentle nitrogen stream at circa 75 °C (block temperature at 300 °C) until a volume of 250 µL was reached. Then 750 µL ultrapure water was added to extract, containing nitrophenol-d4, bentazon-d6 and neburon internal standards at a concentration of 13.33 µg/L. Then the extracted was filtered using a 0.2 µm Phenomenex Phenex regenerated cellulose filter (Utrecht, Netherlands) and was transferred to a 1.8 mL autosampler vial for LC-QToF analysis.

IX.3 LC-QTOF analysis

HPLC settings (Shimadzu Nexera; LC-30AD, SIL-30AC, CTO-20AC):

- Column: Waters Xbridge C18 XP, 2.1 x 150 mm, 2.5 µm
- Mobile phase A: ultrapure water + 0.05% formic acid
  Mobile phase B: acetonitrile + 0.05% formic acid
- Gradient: linear from 5% to 100% B in 40 min. Held at 100% B for 5 min. Then return to initial conditions in 1 min and held for 6 min.
- Flow: 300 µL/min
- Injection volume: 10 µL

- Column oven temperature: 25°C

QToF-MS settings (AB SCIEX TripleTOF 5600+):

- Resolution: > 30.000 @ m/z 400 (MS and MS/MS mode)
- Mass accuracy < 5 ppm
- Mass range Full scan: 120-500 Da
- Mass range MS2 scan: 40-460 Da
- Ionisation: negative mode
- Source: electrospray (ESI)
- TurboIonSpray heater: 500°C
- Ion Spray Voltage: 3000 volt
- Curtain gas: 25 psi
- Gas 1: 40 psi
- Gas 2: 50 psi
- Divert valve:0 - 3.0 min to waste
- Collision energy: 20, 35, 50 eV (averaged)
- Data dependant MS/MS scans: 8 per cycle (50ms), threshold 100 counts and dynamic background subtraction.
- MS/MS inclusion list, see table below

MS/MS INCLUSIONLIST (2 MINUTE WINDOW)

Mass (Da)	Retention time (min)
213.0153	15.56
138.0197	12.95
154.0146	10.46
182.0100	13.56
196.0252	12.68
210.0040	8.46
183.0047	15.10
154.0146	13.04
154.0146	11.25
201.9816	6.31
182.0095	11.32
192.0095	10.97
239.0673	26.68
226.9946	14.69
152.0353	16.26
168.0302	13.40
217.9765	4.83
181.0255	14.64
161.0357	16.18

Attachment X Result monitoring study

RESULTS MONITORING STUDY - AERATED RECLAIMED DUNE INFILTRATION WATER SAMPLE

Compound	Aerated reclaimed dune infiltration water						
	27/07/16 ng/L	05/09/16 ng/L	21/09/16 ng/L	20/10/16 ng/L	21/11/16 ng/L	14/12/16 ng/L	11/01/17 ng/L
2-Methoxy-4,6-dinitrophenol	< 1.0	< 1.0	< 1.0	< 1.0	< 1.0	< 1.0	< 1.0
4-Nitrophenol	< 5.0	< 5.0	< 5.0	< 5.0	< 5.0	< 5.0	< 5.0
4-Nitrocatechol	< 2.0	< 2.0	< 2.0	< 2.0	< 2.0	< 2.0	< 2.0
2-Hydroxy-5-nitrobenzoic acid	< 2.0	< 2.0	< 2.0	< 2.0	< 2.0	< 2.0	< 2.0
5-Nitrovanillin	< 1.0	< 1.0	< 1.0	< 1.0	< 1.0	< 1.0	< 1.0
4-Nitrophthalic acid	< 10	< 10	< 10	< 10	< 10	< 10	< 10
2,4-Dinitrophenol	< 1.0	< 1.0	< 1.0	< 1.0	< 1.0	< 1.0	< 1.0
4-Nitro-1,3-benzenediol	< 2.0	< 2.0	< 2.0	< 2.0	< 2.0	< 2.0	< 2.0
2-Nitrohydroquinone	< 5.0	< 5.0	< 5.0	< 5.0	< 5.0	< 5.0	< 5.0
4-Nitrobenzenesulfonic acid	< 3.0	< 3.0	< 3.0	< 3.0	< 3.0	< 3.0	< 3.0
4-Hydroxy-3-nitrobenzoic acid	< 2.0	< 2.0	< 2.0	< 2.0	< 2.0	< 2.0	< 2.0
2-Hydroxy-3-nitrobenzoic acid	< 2.0	< 2.0	< 2.0	< 2.0	< 2.0	< 2.0	< 2.0
Dinoterb	< 2.0	< 2.0	< 2.0	< 2.0	< 2.0	< 2.0	< 2.0
3,5-Dinitrosalicylic acid	< 3.0	< 3.0	< 3.0	< 3.0	< 3.0	< 3.0	< 3.0
2-Methyl-4-nitrophenol	< 1.0	< 1.0	< 1.0	< 1.0	< 1.0	< 1.0	< 1.0
2-Methoxy-4-nitrophenol	< 3.0	< 3.0	< 3.0	< 3.0	< 3.0	< 3.0	< 3.0
4-Hydroxy-3-nitrobenzenesulfonic acid	< 2.0	< 2.0	< 2.0	< 2.0	< 2.0	< 2.0	< 2.0
2-Amino-3-nitrobenzoic acid	< 2.0	< 2.0	< 2.0	< 2.0	< 2.0	< 2.0	< 2.0
3-Nitroindole	< 2.0	< 2.0	< 2.0	< 2.0	< 2.0	< 2.0	< 2.0
Summed concentration	0.0	0.0	0.0	0.0	0.0	0.0	0.0