Supporting Information for

Bacterial Opportunistic Pathogen Gene Markers in Municipal Drinking Water are Associated with Distribution System and Household Plumbing Characteristics

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below the level needed for reliable quantification. The median concentrations for aerator biofilm samples are provided in the last column, whereas the concentrations for the water samples are given in Figure 1. PP 1st, PP 2nd, and PP 3rd pertain to the first, second, and third liter of cold building water collected after stagnation

Table S4. Summary of the physical and chemical characteristics of the homes and water samples collected in this study.

Table S5. Details of each OP's linear mixed-effect model, illustrating both the overall model and ANOVA output.

Procedure S1. Details relating to participant enrollment and instruction provided to homes for the day of sampling.

Procedure S2. Details of the analytical method developed to measure and quantify N-Nitrosodimethylamine (NDMA).

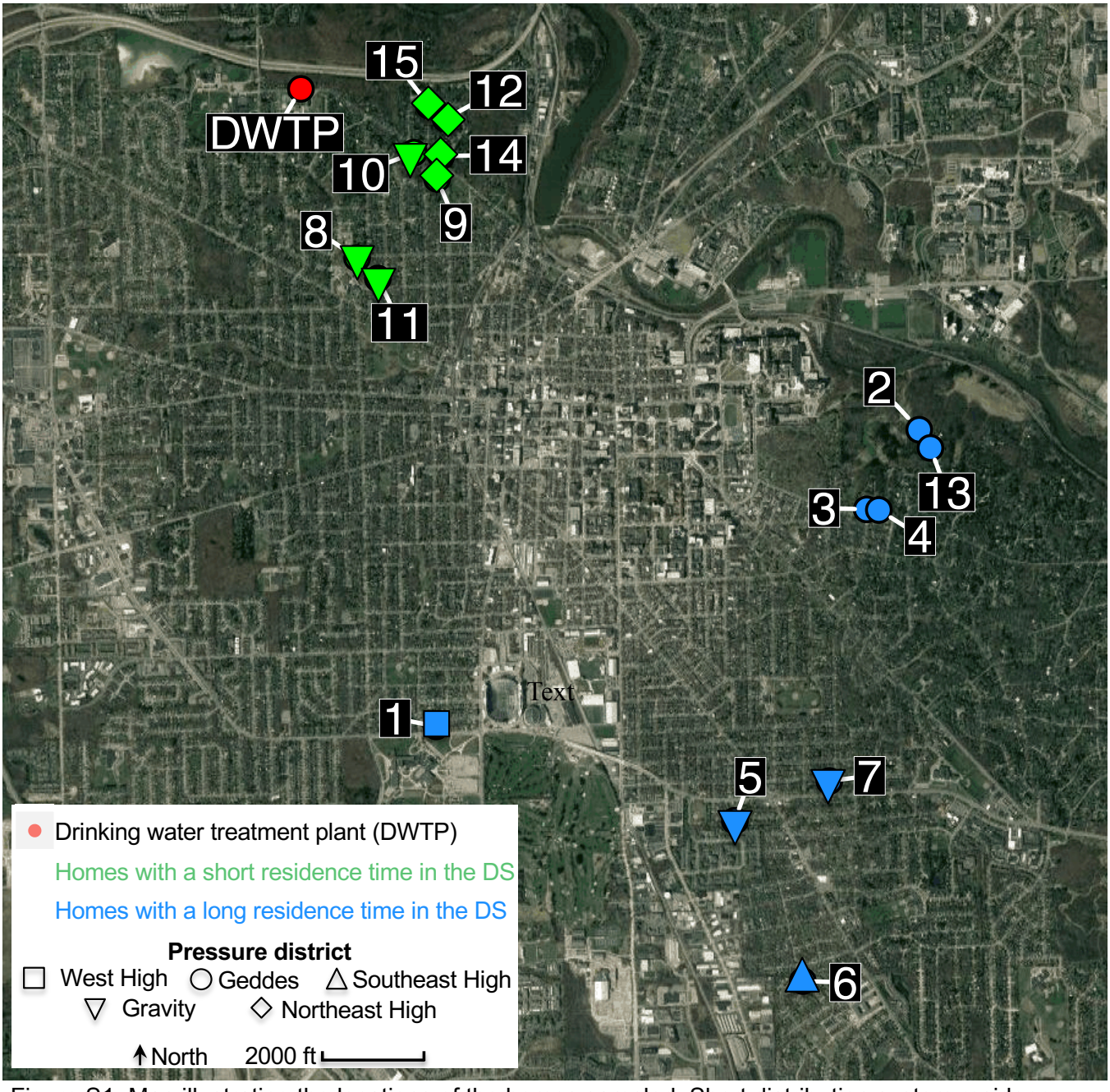

Figure S1. Map illustrating the locations of the homes sampled. Short distribution system residence times are designated as homes receiving water <24 h old and long distribution system residence times are defined as homes receiving water >27 h old. Map data ©2018 Google.

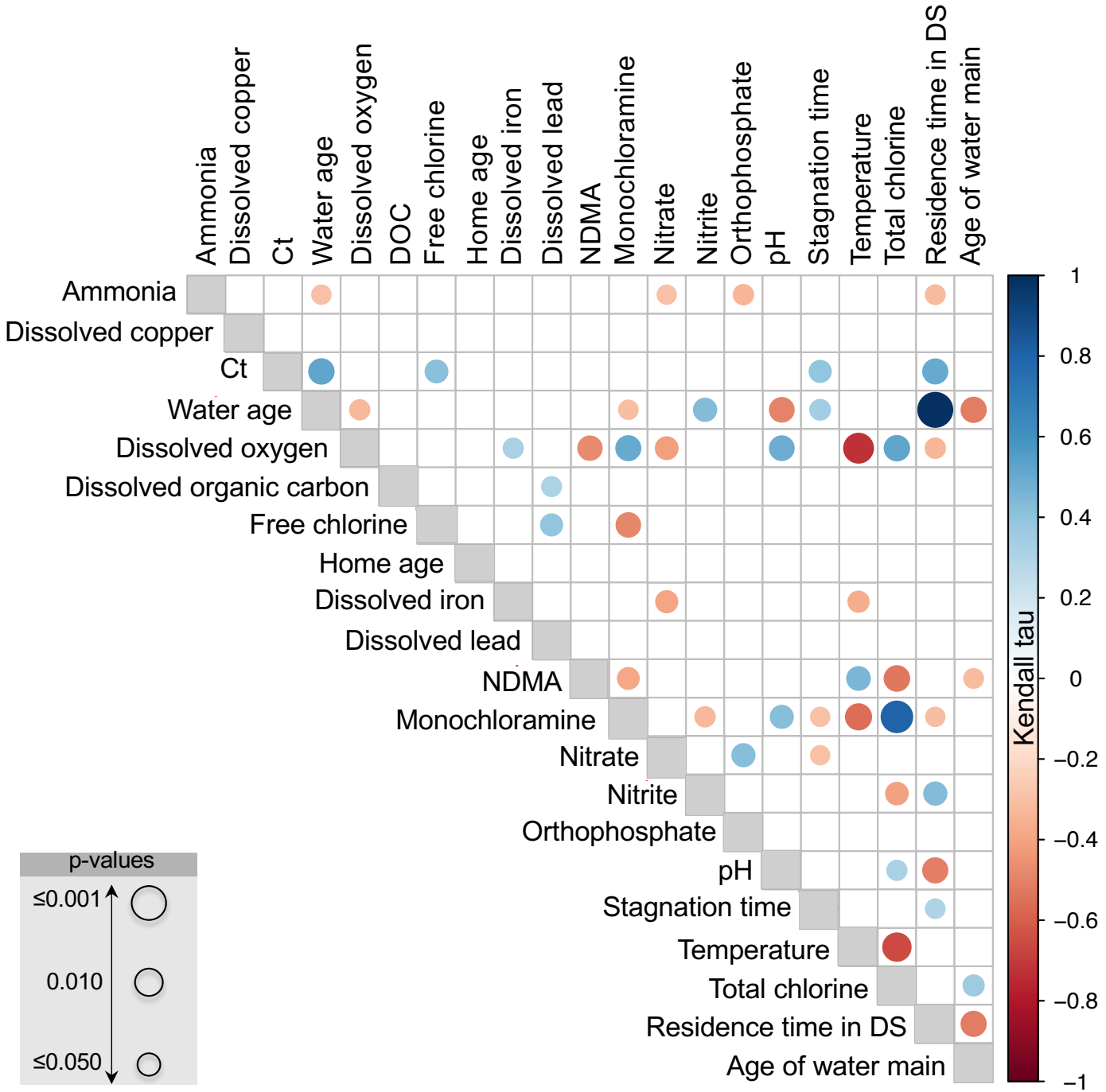

Figure S2: Correlation analysis of statistically significant relationships between the various water quality and abiotic parameters from 15 homes. Correlation results were presented in the form of Kendall correlation. The size and color intensity of the circle indicate the strength and direction of the correlation, with p-values depicted by: *: 0.05, **: 0.01 and ***: 0.001.

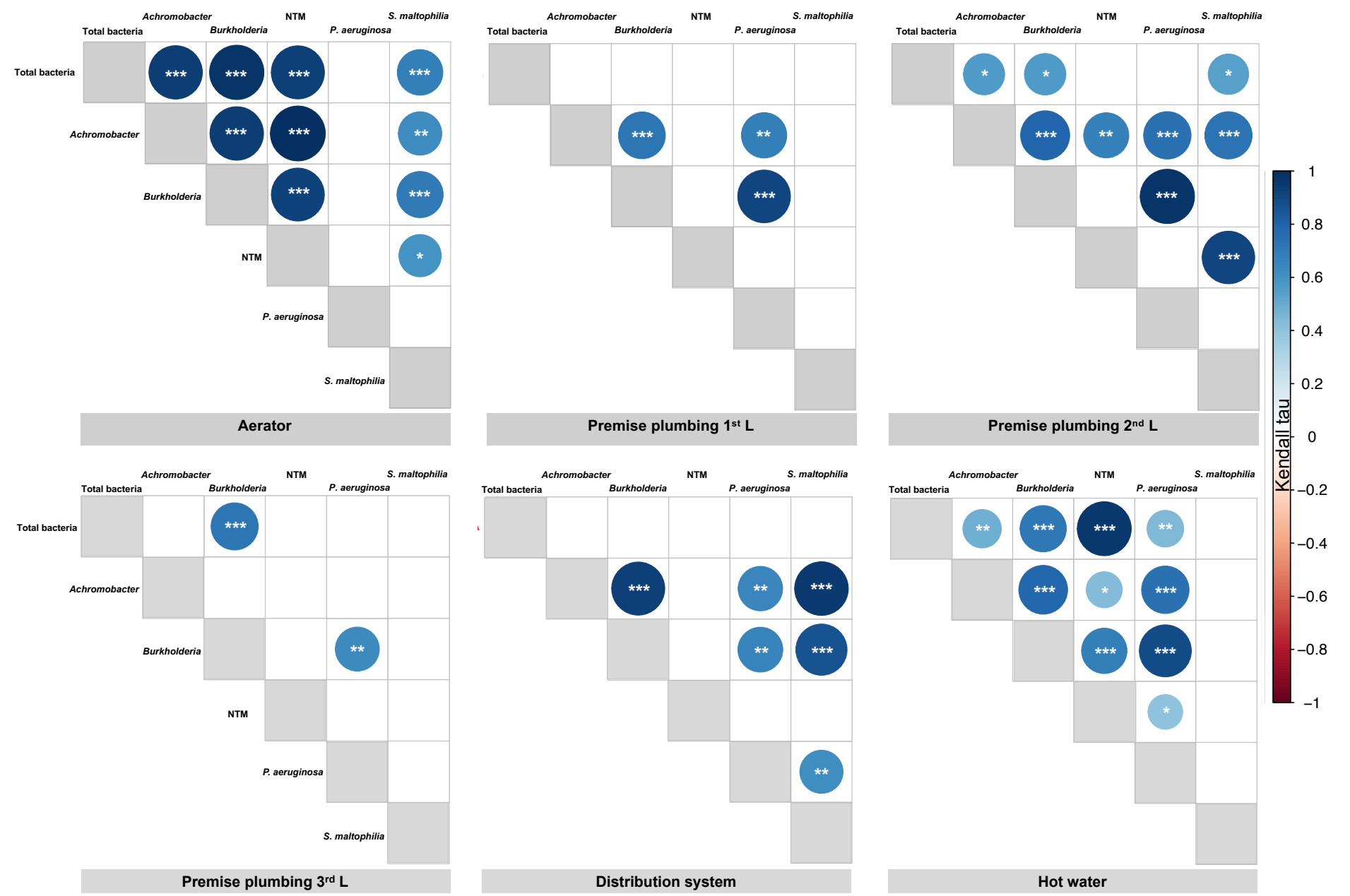

Figure S3: Pairwise Kendall correlation analyses between the different OPs in each type of sample collected. Significance was determined after controlling for multiple comparisons using Benjamini-Hochberg false likelihood correction. The p-values are depicted by: *: 0.05, **: 0.01 and ***: 0.001.

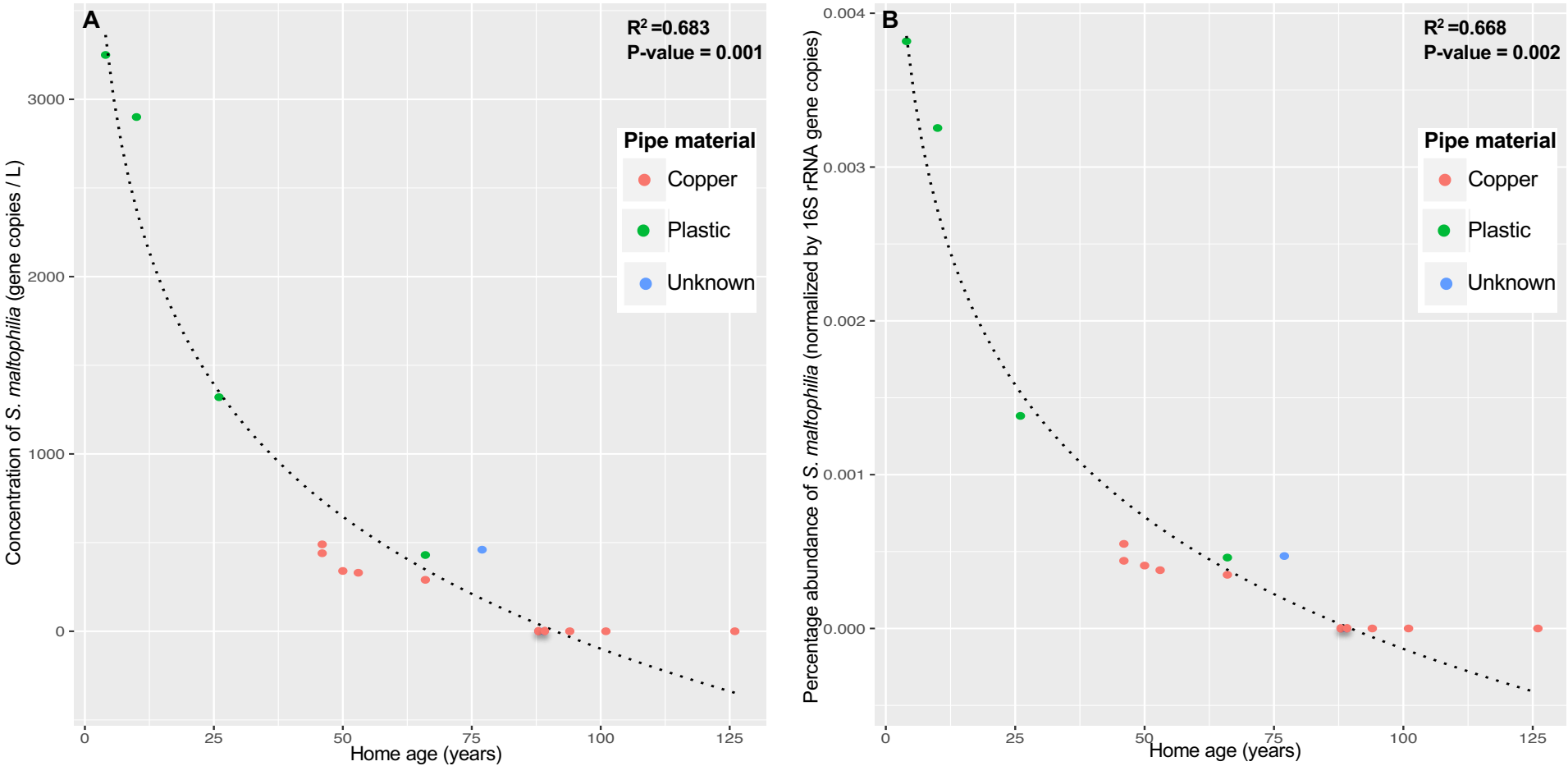

Figure S4. Exponential decay plots of the absolute abundance (A) and relative abundance (B) of S. maltophilia against home age in the first liter of building plumbing water samples. Homes with copper pipes are indicated by red points, with plastic pipes by green points, and with pipes of unknown material by blue points. The black dotted line shows the fitted exponential decay.

Table S1: Different physical and chemical water quality parameters measured in this study and corresponding analytical method used.

Parameter	Units	Analytical Technique
pH		pH electrode
Temperature	°C	Thermometer
Ammonium	mg/L as N	Phenate method
Nitrate	mg/L as N	Cadmium reduction
Nitrite	mg/L as N	Diazotization
Free chlorine	mg/L as Cl_2	DPD method
Total chlorine	mg/L as Cl_2	DPD method
Dissolved copper	µg/L	ICP-MS (0.45 µm filtered)
Dissolved iron	µg/L	ICP-MS (0.45 µm filtered)
Dissolved lead	µg/L	ICP-MS (0.45 µm filtered)
Dissolved organic carbon (DOC)	mg/L	TOC (0.2 µm filtered)
Dissolved oxygen (DO)	mg/L	HRDO method
N-Nitrosodimethylamine (NDMA)	ng/L	High resolution LC-MS
Orthophosphate	mg/L as P	Ascorbic acid method
Total cell counts	cells/L	DAPI staining

Table S2: Details of the qPCR target genes, primers, annealing conditions, and respective LODs and LOQs used in this study.

Target	Forward (5'-3')	Reverse (5'-3')	Approx. Amplicon size (bp)	Annealing temperature (°C)	LOD[I] (copies)	LOQ[II] (copies)	Reference[III]
Achromobacter genus *16S rRNA* gene	AX-F1: GCAGGAAAGAAACGTCGCGGGT	AX-B1: ATTTCACATCTTTCTTTCCG	163	57.4	29.1	30.3	1
Burkholderia genus *RecA* gene	Bur3F: GARAAGCAGTTCGGCAA	Bur4R: GAGTCGATGACGATCAT	385	46	21.8	22.9	2
Nontuberculous mycobacteria *atpE* gene	FatpE: CGGYGCCGGTATCGGYGA	RatpE: CGAAGACGAACARSGCCAT	164	59.6	14.8	15.4	3
Pseudomonas aeruginosa OprL gene	Ps-F: CGAGTACAACATGGCTCTGG	Ps-R: ACCGGACGCTCTTTACCATA	117	57.8	26.4	27.8	4
Stenotrophomonas maltophilia 23S rRNA gene	SM1: CAGCCTGCGAAAAGTA	SM4: TTAAGCTTGCCACGAACAG	531	56.4	20.5	30.9	5
Total bacteria *16S rRNA* gene	Eub338: ACTCCTACGGGAGGCAG	Eub518: ATTACCGCGGCTGCTGG	200	54	80	85	6

[I]LOD: Limit of detection, [II]LOQ: Limit of quantification

[III]

[1] Liu, L. et al., 2002. J. Clin. Microbiol, 40(4), 1210-1213.
[2] Payne, G. W., et al., 2005. App. Env. Microbiol, 71(7), 3917-3927.
[3] Radomski, N., et al., 2013. BMC Microbiol, 13(1), 277.
[4] Feizabadi MM et al. 2010. Infect Genet Evol 10:1247–1251.
[5] Whitby, P. W., et al., 2000. J. Clin. Microbiol, 38(12), 4305-4309.
[6] Fierer, N., et al., 2005. App. Env. Microbiol, 71(7), 4117-4120.

Table S3. The occurrence rates of quantifiable OPs in water samples and aerator biofilms. The detection rates are indicated in parentheses when the concentration of the OP was below the level needed for reliable quantification. The median concentrations for aerator biofilm samples are provided in the last column, whereas the concentrations for the water samples are given in Figure 1. PP 1st, PP 2nd, and PP 3rd pertain to the first, second, and third liter of cold building water collected after stagnation

Target group	Occurrence rate (%) of quantifiable (detectable) OPs							Aerator biofilm median concentration (gene copies / g wet biomass)	Limit of detection (copies)	Limit of quantification (copies)
	Sites (n = 15)						All home locations (n =90)			
	Aerator	PP 1st	PP 2nd	PP 3rd	DS	Hot water				
Achromobacter	100	100	93 (100)	93 (100)	100	100	98 (100)	3.63×10^6	29.1	30.3
Burkholderia	100	100	100	100	100	100	100	5.37×10^8	21.8	22.9
NTM	100	100	100	93 (100)	100	93 (100)	98 (100)	6.46×10^6	14.8	15.4
P. aeruginosa	100	100	100	93 (100)	100	93 (100)	98 (100)	5.89×10^5	26.4	27.8
S. maltophilia	100	80 (100)	87 (100)	93 (100)	87 (100)	87 (100)	87 (100)	8.51×10^3	20.5	30.9
Total bacteria	100	100	100	100	100	100	100	2.19×10^9	80	85

Procedure S1 Details relating to participant enrollment and instructions provided to homes for the day of sampling.

The document provided below was circulated through fitness groups and neighbourhood watch forums to recruit individuals who would be willing to participate in the study.

Individuals who expressed and interest in participating in the study were contacted following their specified method of communication and details regarding sampling were discussed. A reminder communication was performed 24 h prior to sampling to confirm sampling was still possible and to remind participants that no water could be used for at least 6 h prior to arrival of the sampling team.

A drinking water use survey was performed in each home to obtain further information on individuals water use habits, and home and plumbing characteristics.

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Microbial Water Quality in Household Plumbing in Ann Arbor, MI

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Why we are performing this study:
Approximately 90% of the U.S. population receives drinking water treated in water treatment plants using processes such as filtration and disinfection. This treated drinking water reaches our faucets after passage through household plumbing, which includes water meters, household pipes, water heaters, etc. Microbial biofilms consist of microbes that prefer to grow attached to the inside surfaces of our household water pipes. Stagnation of water in household plumbing for several hours at a time, which happens at night, when residents are at work or at school, etc., favors the formation of microbial biofilms. This can result in increased bacterial numbers in water that passes by these biofilms. Some bacteria in our drinking water are so-called "opportunistic pathogens". While the vast majority of people never become ill when exposed to these bacteria, individuals who are immunocompromised have an increased risk of becoming ill when they come into contact with opportunistic bacterial pathogens. Few studies have explored the role of municipally treated drinking water in exposing immunocompromised individuals to opportunistic bacterial pathogens. In this study, we aim to identify factors in household plumbing (e.g., water stagnation time, pipe material, water temperature) that influence the types of bacteria present in tap water. Determining these factors and identifying the opportunistic bacterial pathogens present in household plumbing is a necessary first step in developing strategies to reduce the risk of infection for immunocompromised individuals.

What you can expect if you agree to participate:
If your home receives water from the Ann Arbor Drinking Water Treatment Plant, we invite you to be part of our study. If you agree to participate, up to three researchers would be present in your kitchen for approximately 60-90 minutes during a time the water in your home has been stagnant (not used) for at least 6 hours (e.g., early morning or late afternoon/early evening). During our sampling, you can expect the following to take place:

1. We will ask you a short list of questions on water use in your home.
2. We will collect water and biofilm samples from your kitchen faucet. Specifically, we will remove the faucet aerator, collect microbial biofilm from the faucet aerator, carefully rinse the faucet aerator and place it back. Note that we have experience removing and re-installing many different types of faucet aerators. We then collect three water samples of 1 liter as well as a larger water sample of 5 liter after running the faucet for approximately 5 minutes. After sampling cold water, we will collect a 5-liter hot water sample.
3. We will bring a portable battery operated filtration system to your home to filter the samples on site.

How to let us know you are interested in participating:
If you are interested in helping us with this research study, please email Dr. Sarah Haig (sjhaig@umich.edu) with your name, address, and preferred phone number or email address, so we can organize a convenient time to visit your home for sample collection. Please email Sarah by Wednesday, October 28, 2015 if you want to be included in our study.

Thank you for your help with this project!
We have been granted a determination of exemption by the Institutional Review Board of the University of Michigan because our study does not provide a risk to the participants.
All data will be de-identified if published

ANN ARBOR HOME WATER USE SURVEY

[INPUT STREET ADDRESS]_____ Ann Arbor, MI [INPUT ZIP CODE] _____

[CHOOSE THE HOUSING TYPE THAT BEST DESCRIBES THIS HOME]

- □ House
- □ Condominium
- □ Other [PLEASE SPECIFY] _____

[VERIFY THAT THE PERSON YOU ARE SPEAKING WITH IS OVER 18 YEARS OLD AND LIVES AT THE HOME.]

- □ Yes
- □ No
- □ No answer at the door

[IF R RESPONDS NO] ***Is there another time during the day that would be better?***

- □ Yes [SPECIFY TIME] _____
- □ No

[IF R RESPONDS NO] *Ok, thank you for your time.*

First, we'd like to ask you some general questions about how your household uses tap water.

1. ***At home, do you and other household members usually drink tap water or commercially bottled water?***
   - □ Tap water
   - □ Commercially bottled water
   - □ R volunteers: I don't know
   - □ Other [PLEASE SPECIFY] _____

2. ***Which of the following ways have you and the people in your household used tap water in the last 30 days?*** *I will read different activities aloud. Please answer yes or no to each—yes if you use tap water for that activity, no if you do not. [CHECK YES OR NO FOR EACH OPTION AS R ANSWERS.]*

Activity	Yes	No	R volunteers: I don't know
Drinking			
Making cold drinks			
Making hot drinks			
Making ice			
Rinsing produce			
Cooking			
Hand washing dishes			
Bathing/showering			
Brushing teeth			
Filling vaporizer/humidifier			
Watering plants indoors/outdoors			

   - □ R volunteers: Other [PLEASE SPECIFY _____]

3. [IF R RESPONDS "TAP WATER"] ***Is your tap water treated by boiling, filtering, or a water softener?***
   - ☐ No
   - ☐ Boiling
   - ☐ Filtering
   - ☐ Water Softener
   - ☐ R volunteers: Other [PLEASE SPECIFY] _____

4. (a) [IF R RESPONDS WITH "FILTERING"] *I am going to read off different locations of water filters that can be used in your house.* ***Please answer yes or no to each of the following as I read them aloud, indicating if you use them in your household or not.*** [CHECK ALL THAT R INDICATES]

Type of filter	Yes	No	I don't know
A. Water pitcher with a filter (e.g. Brita)			
B. Refrigerator dispenser with a filter			
C. Filter installed on the faucet			
D. Filter installed under the sink			

   - ☐ No water filter used [VERIFY THAT THE HOUSEHOLD DOES NOT FILTER THEIR TAP WATER]

   (b) [IF R RESPONDS YES TO MORE THAN ONE FILTER ABOVE] ***Which of these filters is used in the household the most for drinking?*** _____

   (c) ***Is this a carbon filter or a reverse osmosis filter or both?***
   - ☐ Carbon filter
   - ☐ Reverse osmosis filter
   - ☐ Both carbon and reverse osmosis

   (d) ***Do you know the brand name of this filter?*** _____

5. (a) ***Have you or other household members replaced your*** *[FILL IN MOST USED FILTER FROM PREVIOUS QUESTION]* ***in the last year?***
   - ☐ Yes
   - ☐ No
   - ☐ R volunteers: I don't know

   (b) [IF R RESPONDS YES] ***When was the last time you or other household members replaced your*** *[FILL IN MOST USED FROM PREVIOUS QUESTION]?*
   - ☐ [WRITE HOW LONG AGO HERE] _____Months _____Years
   - ☐ If **R** volunteers: I don't know

6. ***How many bathrooms <u>with showers</u> are used in your home?*** _____

7. ***Please tell me all the ways that you or household members use the bathroom faucet(s).*** *Please answer yes or no to the following activities as I read them aloud.* [CHECK ALL THAT R INDICATES]

Activity	Yes	No
Brushing teeth		
Washing hands		
Washing face		
Drinking		

- ☐ R volunteers: Other [PLEASE SPECIFY _____]
- ☐ R volunteers: I don't know

8. *(*a) ***During the last seven days, how often did you and household members use the shower in total?*** *Do you estimate that your household took 0, less than 5, between 5 and 10, or more than 10 showers in the last seven days?*
   - ☐ 0
   - ☐ Less than 5
   - ☐ Between 5 and 10
   - ☐ More than 10
   - ☐ R volunteers: I don't know

   (b) ***During the last seven days, how often did you and household members take a bath in total?*** *Do you estimate it was 0, less than 5, between 5 and 10, or more than 10 baths during the last seven days?*
   - ☐ 0
   - ☐ Less than 5
   - ☐ Between 5 and 10
   - ☐ More than 10
   - ☐ R volunteers: I don't know

9. ***Which faucet in your home is used <u>least</u> often?*** *Is it the bathroom sink faucet, the kitchen sink faucet or another faucet?*
   - ☐ Bathroom sink faucet
   - ☐ Kitchen sink faucet
   - ☐ R volunteers: Other [PLEASE SPECIFY] _____
   - ☐ R volunteers: I don't know

10. ***Which faucet in your home is usually used first in the morning?*** *Is it the bathroom sink faucet, the shower, the bathtub faucet or the kitchen sink faucet?*
    - ☐ Bathroom sink faucet
    - ☐ Shower
    - ☐ Bathtub faucet
    - ☐ Kitchen sink faucet
    - ☐ R volunteers: Other [PLEASE SPECIFY] _____
    - ☐ R volunteers: I don't know

Now I'm going to ask about some household water practices.

First, I'm going to ask you about your [FILL IN FROM THE LAST RESPONSE]. *Some people run their faucet for a few minutes before they use the water in the morning and some people don't.*

11. (a) ***Do you or other members of your household ever run the*** [FILL IN FROM THE LAST RESPONSE] ***before the tap water is used?***
    - ☐ Always
    - ☐ Never
    - ☐ Sometimes

    (b) [IF R VOLUNTEERS ALWAYS OR SOMETIMES FOR LAST RESPONSE] ***When is the* [FILL IN FROM RESPONSE FROM QUESTION 7]** ***run before the water is used? Is it something that you and household members do in the morning only, or a few times throughout the day, or is it every time someone uses the water from this faucet?***
    - ☐ In the morning
    - ☐ A few times throughout the day
    - ☐ Every time I use the water from this faucet

12. (a) *Some people are curious about their drinking water and some people aren't.* ***Have you ever had your water tested for germs by a professional or a company?***
    - ☐ Yes
    - ☐ No
    - ☐ R volunteers: I don't know

    (b). [IF R RESPONDS YES] *When did you have it tested?* [WRITE DATE] _____

13. (a) *Some people clean their showerhead and some people replace it.* ***Have you cleaned or replaced your showerhead in the last year?***
    - ☐ Yes
    - ☐ No

    (b) [IF R RESPONDS YES] ***When was the last time you cleaned your showerhead?***

    - ☐ [WRITE HOW LONG AGO HERE] _____Months _____Years
    - ☐ If **R** volunteers: I don't know

14. ***Have you ever cleaned or replaced the faucet aerator(s) in your home?***
    - ☐ Yes
    - ☐ No

15. [IF R RESPONDS YES] ***When was the last time you cleaned or replaced your faucet aerator(s)?***

    - ☐ [WRITE HOW LONG AGO HERE] _____Months _____Years
    - ☐ If **R** volunteers: I don't know

16. [If R RESPONDS YES] ***What do you do to clean your faucet aerator?***

17. ***For which faucet did you clean/replace the aerator?***
    - ☐ Bathroom faucet
    - ☐ Kitchen faucet
    - ☐ Other faucet [PLEASE SPECIFY] _____

18. ***Do you have a tank or tankless water heater in your home?*** *Tankless water heaters are also known as on demand water heaters. They heat water directly without the use of a storage tank.*
    - Tank
    - Tankless
    - R volunteers: I don't know

19. ***What temperature is your hot water heater set at?***
    - [WRITE TEMPERATURE HERE] _____
    - R volunteers: I don't know

20. ***Have you ever increased the temperature of your hot water heater for a short time to kill germs in your hot water pipes or for other reasons?***
    - Yes
    - No
    - R volunteers: I don't know
    - R volunteers: Other [PLEASE SPECIFY] _____

21. ***Do you reduce the temperature of your hot water heater when you are traveling and no one is home?***
    - Yes
    - No

22. ***What are your drinking water pipes made of?***
    - Copper
    - Plastic (e.g., polyvinyl chloride-PVC, cross-linked polyethylene-PEX)
    - Stainless steel
    - R volunteers: I don't know
    - Other [PLEASE SPECIFY] _____

23. ***Have you replaced any of your water pipes in the last five years?***
    a. Yes[PLEASE SPECIFY] _____
    b. No

In this final section of the survey, I am going to ask you some general questions about your household.

24. ***When was your house built?*** _____

25. (a) ***How many people, including you, live in your household?*** *(Please do not include short-term visitors.)* _____

    (b) *How many of the people living in your household are children (younger than 18)?* _____
    (c) *How many of them are adults?* _____

26. ***Would you be willing to provide us with your age and gender for demographics purposes?***

AGE: _____ GENDER: M F OTHER[SPECIFY]_____

27. *This is our last question. As part of this study on water use, our research group at the University of Michigan will also be collecting water samples from households in Ann Arbor. We won't do this today but would schedule a time to come back in the next couple of months. We are trying to better*

understand drinking water quality as a first step in a larger project that will determine how drinking water might affect health. ***Would you be willing to allow a researcher on this project to collect some water samples from your house?***

- ☐ Yes [WRITE NAME, ADDRESS, PHONE NUMBER AND/OR EMAIL ADDRESS]
  Name: _____
  Address: _____
  Phone number: _____
  Email address: _____
- ☐ No

Procedure S2 Analytical method for N-Nitrosodimethylamine (NDMA).

The disinfection byproduct N-Nitrosodimethylamine (NDMA) was measured using liquid chromatography–mass spectrometry (LC-MS). A high-resolution LC-MS system (EQuan MAX system with Exactive Plus Orbitrap, Thermo Fisher Scientific, Waltham, MA, USA) with a heated electrospray interface was used according to a procedure adapted from Ngongang *et al.,* 2015.

Reagents: NDMA and reagent-grade formic acid (>95 %) were purchased from Sigma Aldrich (St. Louis, USA), whereas HPLC-grade methanol and water for dilutions were purchased from Fisher Scientific (New Jersey, USA).

Standard solution and calibration curves: A primary stock solution of 2 mg/ml of NDMA in methanol was used for the preparation of intermediate solutions, which were stored in a freezer at -20 °C. Working solutions of NDMA were prepared daily from intermediate solutions to achieve the desired concentrations prior to LC-MS analysis.

Sample collection and storage: Immediately after collection of each water sample, 20 ml was aliquoted into amber vials containing 2 mg of sodium thiosulfate (Fisher Scientific, New Jersey, USA) to dechlorinate the sample and halt NDMA formation (Munch and Bassett, 2004). Samples were then transported on ice and stored at 4 °C. All samples were analyzed within 12 days of collection.

Instrument analysis: A Thermo Scientific EQuan MAX online sample concentration UHPLC-MS system equipped with a Thermo Scientific Hypersil GOLD column (100 x 2.1 mm, 1.9 µm particle size) and a Thermo Scientific Accucore aQ C18 polar endcapped analytical column (30 x 2.1 mm, 2.6 µm particle size) was used. A 1.2 ml sample injection volume was used for each sample.

The Thermo Scientific Exactive Orbitrap mass spectrometer was operated in full-scan mode with resolving power set to 70,000. Conditions for the online sample concentration injection and the operation of the Orbitrap mass spectrometer are summarized in Table 1. All samples and six standards were run in triplicate alongside three negative controls. Final NDMA concentration was determined by comparing the sample's peak area to that of the generated calibration curve.

Linearity, limit of detection (LOD), limit of quantification (LOQ), and extraction recovery: A single run consisting of a calibration curve of seven concentration levels, each ran five times, and three replicates of both a low (10 ng/L) and high (250 ng/L) sample spike were processed to establish linearity, LOD, and LOQ, and extraction recovery. The calibration curve and spike samples were prepared from intermediary solutions by spiking calculated volumes of HPLC grade water or the drinking water sample. The seven points of the calibration curve were from 5 to 500 ng/L. Details of the linearity, LOD, LOQ and extraction recovery are shown in Table 2.

Procedure S2 Analytical method for N-Nitrosodimethylamine (NDMA).

Table 1: Summary of the mass spectrometry optimized parameters.

Parameter	Value
Sheath gas flow rate	25
Auxiliary gas flow rate	10
Ion sweep gas flow rate	1
S-lens RF level	65
Resolution	70,000
AGC Target	2×10^5
Maximum IT	50 ms
Scan type	Full scan
Scan range	50-150 m/z
Detection mode	Positive
Spray Voltage	4 KV

Table 2: Method validation results for linearity (R^2), limit of detection (LOD), quantification (LOQ), and recovery.

Linearity R^2	LOD (ng/L)	LOQ (ng/L)	Recovery (Percentage)	
			10 ng/L spike	250 ng/L spike
0.999	4.26	10.65	73 ± 2	88 ± 3

References

Munch, J. W., and Bassett, M. V. 2004. Method 521 determination of nitrosamines in drinking water by solid phase extraction and capillary column gas chromatography with large volume injection and chemical ionization tandem mass spectrometry (MS/MS). *National Exposure Research Laboratory Office of Research and Development, US Environmental Protection Agency, Cincinnati*.

Ngongang, A. D, Duy, S. V, and Sauvé, S. 2015. Analysis of nine N-nitrosamines using liquid chromatography-accurate mass high resolution-mass spectrometry on a Q-Exactive instrument. *Analytical Methods* **7** (14), pp:5748-5759.