

Article

Stored Reference Samples Enable Efficient Non-Target HRMS Screening for Novel Chemical Contamination in Drinking Water

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Abstract: Producers of drinking water (DW) occasionally require chemical identification of new or unexpected contamination, e.g., caused by an incident. The state-of-the-art technique for the identification of organic compounds is High-Resolution Mass Spectrometry (HRMS). The ability to discover unexpected compounds at low concentrations in any sample by HRMS is facilitated by comparison to non-contaminated reference samples. Samples of raw and drinking water were collected regularly over one whole year from six Swedish drinking water treatment plants (DWTPs). The samples were analyzed by LC-HRMS together with spiked samples mimicking an incident. This setup enabled evaluation of the significance of having access to a collection of matrix-matched reference samples. The main variation in the organic compound profile in the data set was explained by the uniqueness of the raw water and purification steps in the individual DWTPs. Seasonal variations were also significant but subordinate. This subject was further explored by the analysis of drinking water sampled once from 90 of the 1750 Swedish DWTPs, where a similarity between DW originating from rock aquifers was observed. No other significant correlation between samples was observed—e.g., other types of raw water, which types of purification steps were involved, or which additives or process chemicals were added—which could aid in the selection of relevant reference samples. The conclusion from the study is that it would be imperative for the DWTPs to have access to their individual reference samples for use in the investigation of an incident. A library of such reference samples, e.g., collected monthly and covering the last 12 months, could be stored and used together with a fresh “suspected sample” for non-target HRMS investigations as described.

Keywords: non-target analysis; drinking water; contaminants; differential analysis; LC-HRMS; GC-HRMS; Orbitrap



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1. Introduction

Sweden has a decentralized drinking water (DW) supply system for its 10 million inhabitants, comprehending approximately 1750 drinking water treatment plants (DWTPs), covering an area of 450,000 km² and the >1500 km from the north to the south [1]. At these DWTPs, the raw water (RW) supply originates from different types of sources, such as lakes, watercourses, or rock and soil aquifers in the ground. The type of source used, together with differences in geology or land use as well as seasonal variations in, e.g., flow and temperature, can all influence the diversity of the profile of organic compounds in RW. A producer of DW is responsible to construct a process that results in compliance with technical parameters listed in the Swedish DW regulation [2], and to assure the water is safe to drink. There are no exact rules regarding which techniques that have to be used, or specific settings for any such step. This has led to a variety of purification steps, and setups consisting of a diversity of filters and process chemicals for DW production. Altogether, it is therefore anticipated that the profile of organic compounds in DW is unique for each

DWTP, and might vary over time. A recent study focusing on chlorinated compounds supports the hypothesis [3].

Swedish DWTPs occasionally require assistance with investigations in order to find new or unexpected chemical contamination. The reasons may vary from any unexpected deviation in standard quality parameters (e.g., Total Organic Carbon), or of smell, taste or appearance, to knowledge of an incident or deviation in the production, mistake, accident or even suspicion of a deliberate attack. The state-of-the-art technique for the identification of organic compounds is High-Resolution Mass Spectrometry (HRMS) by Time-of-Flight or Orbitrap instruments. This technique enables non-target analysis and suspect screening (sometimes combined with target screening) of water and environmental samples and the applications have grown tremendously during the last decade [4–11]. HRMS combined with different types of chromatography and ionization techniques is able to detect organic compounds with a wide variety of physicochemical properties. Powerful multimethods can be developed if the work-up methods and chromatography are adapted for a broad scope of compounds [12,13]. The use of databases and inclusion lists enable target analysis or suspect screening of thousands of compounds, e.g., for broad screening of pesticides and pharmaceuticals. However, an unexpected contaminant might be any of the over 100 million compounds described in chemical databases [14], or a chemical not yet described. Therefore, the use of true non-target approaches for data mining will be required to reach that goal. Such analytical approaches have earlier proven powerful at the Swedish Food Agency for detecting unexpected organic contaminants in food [15,16]. Moreover, similar methods—making use of reference samples in order to detect deviating signals in the sample of interest—is already in use to identify contaminants that are new or show an increase in concentration in, e.g., the river Rhein [17–20]. Our hypothesis was that Swedish DWTPs would benefit from keeping their own set of reference samples in order to facilitate such future investigations. The aim of the present study was to collect and analyze samples from Swedish DWTPs in order to evaluate the necessary characteristics of reference samples and their importance for the screening of new and unexpected DW contaminants. Much of the work makes use of Volcano plots where significance testing and fold change (FC) results are illustrated by displaying $\log_2\text{FC}$ and the negative \log_{10} -transformed p -values of features [21,22].

The starting point of the study was an exercise with 11 external laboratories: A DW sample was spiked with well-known organic compounds and dispatched to the laboratories without any information regarding which, or how many, compounds were added. The participants were instructed to use the analytical methods of their choice to identify them. The preferred analytical approaches of the laboratories were “target analysis” and “suspect screening” [4]. Most of the spiked compounds were not found by several laboratories simply because the compounds were not searched for (Table S3). An example is the false negative detection of acrylamide—spiked at a level of 1 mg/L (10,000 times higher than the European maximum threshold [23])—despite the use of the suitable technique RP-UHPLC-ESI-MS. The results clearly indicated the need for a non-target approach. Such methods, based on the prioritization of unexpected or new signals in HRMS-data, would depend on the access to relevant reference samples. Therefore, a study was initiated to evaluate the necessary characteristics of available reference samples and their importance for finding new or unexpected contamination in DW.

First, a non-target method was set up and a survey was performed for revealing geographical and seasonal variations in RW (Section 3.1 RW profile). Next, the method was tested on spiked DW in order to examine which sets of DW reference samples were most useful (Section 3.2 DW references). Thereafter, DW sampled once from 90 DWTPs were compared to explore if any similarities could be found based on the type of RW or any process-related parameter (Section 3.3 90 DWTPs). Furthermore, the methodology was applied to a real case (Section 3.4 Real case). Finally, the approach was applied to the samples from the initial exercise, also including a preliminary experiment using GC-HRMS (Section 3.5 Exercise follow-up).

2. Materials and Methods

2.1. Chemicals and Consumables

LC-MS grade acetonitrile (ACN) was purchased from Fisher Scientific (Loughborough, UK) and ammonium acetate from Merck (Darmstadt, Germany). The Milli-Q purification system with additional EDS and LC-Pak filters was from Millipore (Billerica, MA, USA). PVDF 0.20 μm filters were from Whatman (Maidstone, UK). The 50 mL polypropylene tubes were Falcon tubes from Corning Science (Reynosa, Mexico). The compounds for spiking in Section 3.2 were D₈-benzoylecgonine, ¹³C₆-carbamazepine, D₆-isoproturon, D₄-nicotine, ¹³C-D₃-tramadol and D₆-venlafaxin from Sigma-Aldrich (St. Louis, MO, USA) and D₇-atenolol from Witega (Berlin, Germany).

2.2. Samples and Work-Up

For the experiment carried out on samples from 90 DWTPs (described in Section 3.3): Samples—50 mL of DW in polypropylene tubes (sent out from the Swedish Food Agency)—were collected once, during the winter of 2018 (January–February), and sent back at room temperature where they were subjected to work-up either at arrival, or after storage at $-20\text{ }^{\circ}\text{C}$. Work-up consisted of volume reduction by evaporation at a relatively low temperature and filtration as described in detail by Karki and co-workers [24]. Recovery data showed that the method was suitable for the mentioned study of selected common DW contaminants. In short, the samples (45 mL) were reduced to approximately 1.5 mL, i.e., concentrated a factor of approximately 30, by evaporation in SpeedVac equipment (Savant SPD2010 SpeedVac, Thermo Fisher Scientific, Waltham, MA, USA). By means of this equipment, up to 12 samples of 45 mL or 60 samples of 15 mL can simultaneously be processed automatically, saving time and labor. The air pressure was maintained at 5.1 Torr, the temperature was set at $65\text{ }^{\circ}\text{C}$, and the process took approximately 12 h. Due to the evaporation, the samples never reached a temperature above room temperature, i.e., the volume reduction—leading to lower LODs (Limit of Detection)—was carried out at relatively mild conditions. (In future investigations the method might be improved by completing the final volume to 2 mL or possibly by adding internal standards before evaporation allowing the adjustment of intensities by that of the IS.) The samples were thereafter filtered through 0.2 μm PVDF filters before transfer to LC-vials for analysis. The 90 samples were analyzed by LC-Orbitrap on one occasion.

For the experiments carried out on samples collected regularly over one year from six DWTPs (described in Sections 3.1 and 3.2): DWTPs in the Swedish cities of Gothenburg, Karlskrona, Karlstad, Luleå, Gävle and Jönköping were asked to monthly sample duplicates of RW as well as DW in 50 mL polypropylene tubes (sent out from the Swedish Food Agency) and store them at $-20\text{ }^{\circ}\text{C}$. After a sampling period of one year (May 2017 to April 2018) they were asked to send the frozen samples back where they were stored at $-20\text{ }^{\circ}\text{C}$ until thawed prior to work-up and analysis. The type of RW, and main characteristics—although not from exactly the same occasions as for sampling—are presented in Table S1. Due to security reasons, a detailed description of the DWTP's purification processes (and main characteristics of the DW quality) cannot be published. However, a simplified summary of the purification process is presented in Table S2. Work-up was carried out as explained above except for a shorter time of evaporation leading to a volume of 10 mL for RW and 5 mL for DW. A successful sampling would have led to four samples per month per DWTP, i.e., 288 samples in total for the complete study. However, some samples were missing, leading to incomplete sample series. The experiments were for this reason performed on selected samples as explained in the text. Pharmaceuticals commonly occurring as contaminants at low concentrations in RW and DW in Sweden [24,25] were used for spiking of the DW in Section 3.2. In order to avoid any occurrence of these contaminants disturbing the experiment, isotopically labeled versions were used (see Section 2.1). All 30 samples in Section 3.2 were worked-up and analyzed using single injections except for the spiked sample that was injected in triplicate.

For the exercise with external laboratories that was followed up in Section 3.5: Each participant received 50 mL of DW from Uppsala spiked with warfarin (2 mg/L), acrylamide (1 mg/L), triethylamine (7 mg/L), diflufenican (0.05 mg/L) and acetonitrile (80 mg/L). For the LC-Orbitrap analysis, the sample and the reference sample were analyzed in triplicates as such, i.e., no pre-concentration or work-up was employed. For the work-up for GC-Orbitrap analysis see section Section 2.4.

For the application of the approach on a real case (Section 3.4): Work-up was carried out as explained above, reducing samples (45 mL) to a volume of approximately 1.5 mL. A quality control (QC) sample was spiked with the same 19 pesticides as used in a previous study [15], at a concentration of 0.2 µg/L.

2.3. UHPLC-HRMS Analysis

Samples were analyzed with reversed phase UHPLC and an Orbitrap (Q Exactive Focus, Thermo Scientific, Waltham, MA, USA) either by using fullscan mode only (Sections 3.1–3.3), or a combination of fullscan and data-dependent MS/MS (Sections 3.4 and 3.5). Chromatography was performed with an ACQUITY BEH C18 UPLC column, 2.1 × 100 mm fitted with a VanGuard ACQUITY BEH C18 UPLC pre-column, 2.1 × 5 mm, both having a particle size of 1.7 µm (Waters, Manchester, UK). The columns were kept at 40 °C during analysis, and the injection volume was 25 µL. Mobile phase A was 0.1% ammonium acetate in Milli-Q and mobile phase B was 0.1% ammonium acetate in 95% ACN. The gradient elution was performed at 0.4 mL/min as follows: 0–1.0 min, 2% B; 1.0–7.0 min, 2–100% B; 7.0–10.0 min, 100% B; 10.0–10.1 min, 100–2% B; 10.1–12.0 min, 2% B. The mass spectrometer was used with a HESI probe in positive electrospray mode (ES+), with a spray voltage of 3500 kV. The probe heater temperature was 412 °C; sheath gas 47; capillary temperature 256 °C. All gas used was nitrogen generated from pressurized air. The resolution was set at 70,000 and scan range was m/z 100 to m/z 1000. The injection order of samples was always randomized in order to compensate for any drift in, e.g., retention time or instrument response.

2.4. GC-HRMS Analysis

At the follow-up of the inter-laboratory exercise (Section 3.5), spiked DW was analyzed together with three different reference samples: DW from the same building as the spiked sample, DW from a different building and DW from a different city. Before analysis, the DW (50 mL) was extracted twice by 5 mL dichloromethane (Merck KGaA, Darmstadt, Germany) and the combined 10 mL extract was concentrated at a volume of 50 µL under a stream of nitrogen at 40 °C. The sample (1 µL) was injected splitless at 200 °C with helium as the carrier gas at a constant flow of 1.2 mL/min on a Trace 1310 gas chromatograph coupled to an Exactive GC Orbitrap mass spectrometer (Thermo Scientific, Waltham, MA, USA). A DB-5MS column (25 m, ID 0.25 mm, 0.25 µm, Agilent, Santa Clara, CA, USA) was used to separate the compounds. The GC program started at 40 °C for 1 min, followed by a 10 °C/min increase to 80 °C, a 7 °C/min increase to 320 °C and a hold at 320 °C for 5 min. Mass spectrometric scans were performed in the range of 50–550 m/z with a resolution of 30,000. The temperature was set at 250 °C for the transfer line, and 230 °C for the ion source.

2.5. Data Analysis

All data analysis (e.g., peak detection, retention time alignment, assignment of isotopes and common adducts, principal component analysis (PCA) and differential analysis (volcano plot)) was performed using the software Compound Discoverer (Thermo Scientific, Waltham, MA, USA) PCA and volcano plots were based on area counts after peak detection, retention time alignment, assignment of isotopes and common adducts. PCA was performed on scaled but not normalized data. Visualization by volcano plots, which is one way of doing differential analyses, can be used for prioritization of compounds based on fold change and significance [21,22]: For each feature, i.e., combination of m/z

and RT (isotopes and common adducts removed), detected in a data set consisting of samples for investigation and reference samples, the fold change (i.e., the ratio between the median peak area of the samples for investigation and the median peak area of the reference samples) is calculated (x -axis: Log2 fold change). The area counts from both groups are then used in order to calculate the level of significance of the fold change (y -axis: $-\text{Log}_{10} p$ -value). Compound signals with the highest fold change (x -axis) and highest significance (y -axis) would be prioritized for further evaluation. The practical approach is to evaluate these signals one by one, judge if the collected data make sense and attempt to identify the compounds behind them based on the collected data (accurate mass, isotopes, any fragments, etc.). For the fold-change calculations, a “gap-filling” function was used when needed to obtain a baseline value, thereby avoiding division by zero for compounds not detected in all samples (e.g., the isotopically labeled compounds in unspiked samples). For the evaluation of data behind Table 1, the number of reference samples used in each comparison was kept at four, always from four different months, and never from the same month as the analyzed sample.

Table 1. The number of “false positives”—defined as compounds exceeding the set criteria of relative concentration and significance—detected in the 30 individual DW samples when using four different sets of reference samples (Jönköping, Karlskrona, Karlstad, or ultrapure water). A total of 120 volcano plots were generated; each evaluated using two different sets of criteria. Cases when it was most beneficial to use reference samples from the same DWTP (although sampled at other months) are labeled green. Other cases are labeled yellow. MQ is ultrapure water (Milli-Q) mimicking an analytical setup without having access to any DW reference sample at all.

Sample		Number of “False Positives” When Using Moderate Criteria (Fold Change > 8 and p -Value < 0.001) and 4 Months of Reference Water from:				Number of “False Positives” When Using Strict Criteria (Fold Change > 64 and p -Value < 1×10^{-5}) and 4 Months of Reference Water from:			
		Jönköping	Karlskrona	Karlstad	MQ	Jönköping	Karlskrona	Karlstad	MQ
Jönköping	Jan	6	107	44	994	3	5	6	592
	Feb	9	70	44	1004	2	2	5	571
	Mar	0	112	24	1004	0	4	6	586
	Apr	3	78	47	976	0	1	2	528
	May	11	127	47	1041	3	6	10	614
	June	13	86	60	927	0	5	5	451
	July	18	106	54	1029	9	5	9	580
	Aug	4	49	28	981	0	2	3	568
	Sep	12	118	52	1040	2	7	9	630
	Oct	1	51	30	1000	0	4	6	596
	Nov	3	120	27	1010	0	5	4	597
	Dec	2	45	35	977	0	3	4	550
	Median	5	96	44	1002	0	5	6	583
Karlskrona	Jan	157	38	164	1035	25	23	32	619
	Mar	125	7	120	1015	1	0	2	621
	Apr	134	0	86	983	3	0	1	611
	May	98	8	93	1015	0	0	1	648
	June	158	3	95	990	4	0	4	576
	July	126	5	121	1010	0	0	6	611
	Sep	62	10	45	999	0	0	0	616
	Oct	174	11	111	933	12	0	7	469
	Nov	186	28	169	1060	21	13	22	627
	Dec	127	0	70	957	5	0	0	580
	Median	131	8	103	1005	4	0	3	614

Table 1. Cont.

Sample	Number of “False Positives” When Using Moderate Criteria (Fold Change > 8 and p -Value < 0.001) and 4 Months of Reference Water from:				Number of “False Positives” When Using Strict Criteria (Fold Change > 64 and p -Value < 1×10^{-5}) and 4 Months of Reference Water from:				
Karlstad	May	38	15	9	1010	0	1	1	570
	June	10	26	14	948	0	0	0	472
	July	45	12	10	977	0	1	0	536
	Aug	23	4	0	906	0	1	0	509
	Sep	35	16	1	982	0	1	0	554
	Oct	13	18	12	969	4	5	4	479
	Nov	22	28	25	980	6	1	4	508
	Dec	17	9	3	955	0	0	0	497
	Median	23	16	10	973	0	1	0	509

3. Results and Discussion

3.1. RW Profile: Seasonal and Geographical Variation in the Profile of Organic Compounds

In order to investigate the dynamic characteristics of reference samples, raw water was sampled every other month for one year from six Swedish DWTPs (see Section 2.2 Samples and work-up) and thereafter analyzed by UHPLC-HRMS. In short, the analytical method comprises sample volume reduction followed by analysis using RP-UHPLC-Orbitrap-HRMS. Sample preparation using solid-phase extraction was avoided due to the risk of losing compounds that are not retained on, or not eluted from, the SPE column [6]. In addition, low reproducibility in the recovery of some (matrix) compounds with a weak affinity to the solid phase may lead to erroneous conclusions when using statistical tools such as PCA or volcano plots (see below). By instead using a mild evaporation process—to concentrate the samples in order to reach lower LODs—the aforementioned problems can be avoided. The obtained data set—from the HRMS analyses of the samples from the six DWTPs—was evaluated by PCA in order to find trends or similarities based on the specific raw water supply, month or season. No systematic variation due to month or season was demonstrated when all samples were included in the PCA (Figure 1A). When labeling the sample according to specific raw water supply, as shown in Figure 1B, it becomes apparent (although the contribution ratios for PC1 and PC2 are quite low) that samples from Karlskrona and Gothenburg (both from rivers) form one cluster each, separated from the samples from the other four raw water supplies (river, lakes and groundwater). Moreover, both these raw water supplies showed seasonal variation: Karlskrona winter water and Gothenburg summer water were the most deviating samples in the two PCA score plots in Figure 1. The RW samples from Karlskrona were the only ones in the study that appeared brownish prior to work-up. In the corresponding PCA loadings plot (not shown), high mass and doubly charged compounds dominated the region corresponding to Karlskrona winter water, possibly due to a high occurrence of humic substances [26]. Relatively high concentrations, and a large variation thereof, of humic substances have been reported for the DWTP in Karlskrona [27]. In addition, several standard water quality parameters (Total Organic Carbon, color, Fe content, and chemical oxygen demand) show significantly higher values for the RW from Karlskrona as compared to the other samples (Table S1), especially in November 2017 and January 2018—the two most deviating samples from Karlskrona in the PCA. For Gothenburg, the explanation for deviating samples might be the degree of environmental contamination. Although low as compared to the European river Rhine, the contamination of the RW in Gothenburg is relatively high compared to other Swedish RWs [25]. According to information from the DWTP, seawater occasionally intrudes into the river, possibly leading to another organic profile or even (other) contaminants from the harbor situated nearby. Signs of seawater intrusion (increased values of conductivity, sodium and chloride, see Table S1) were observed especially in July 2017—the most deviating sample from Gothenburg in the PCA.

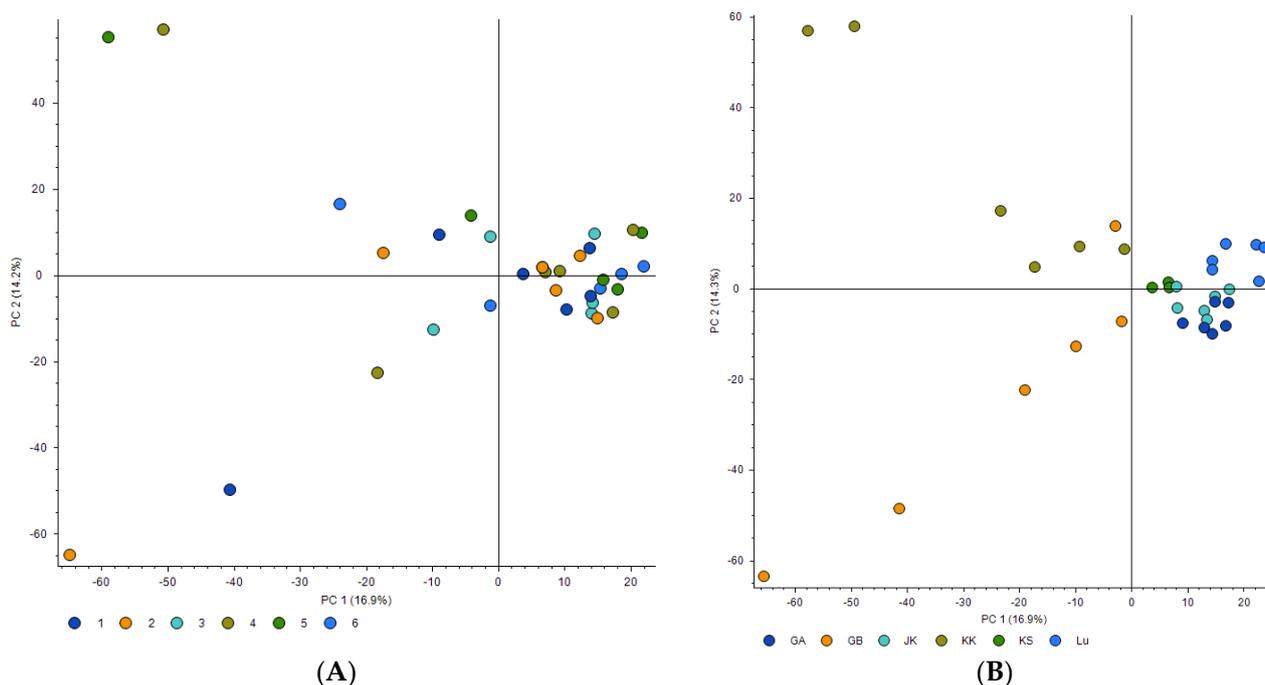


Figure 1. PCA score plots of HRMS-data of RW sampled from six DWTPs: **(A)** (Left) Samples are labeled according to occasion of sampling. 1: May 2017, 2: July 2017, 3: September 2017, 4: November 2017, 5: January 2018, 6: March 2018. **(B)** (Right) Same score plot as in figure A but samples are now labeled according to the city of the DWTP. GA: Gävle, GB: Gothenburg, JK: Jönköping, KK: Karlskrona, KS: Karlstad, Lu: Luleå. Percentages bracketed in axis legends are contribution ratios.

If the Karlskrona and Gothenburg samples were excluded from the PCA, the other DWTPs separated into individual clusters (again despite relatively low values for PC1 and PC2), i.e., the profile of organic compounds of the RW was unique for each raw water supply studied (Figure 2). A score plot of a PCA established for the raw water supply in Luleå revealed a systematic variation dependent on the season (Figure S1), in line with information from the DWTP that the main variation in the RW quality is induced by the snowmelt runoff or autumn rain. However, the main conclusion remains to be that seasonal variations can be significant but are subordinate to the variation between different raw water supplies.

3.2. DW References: Comparison of Different Sets of Reference Samples for Drinking Water Analysis

Drinking water samples were collected regularly (in general monthly) during one year at three Swedish DWTPs, resulting in 30 samples (see materials and methods). Prior to work-up, one of the samples (Karlskrona, November) was spiked with pharmaceuticals at 0.1 µg/L (see Materials and Methods), corresponding to a threshold value often used to indicate the absence of health risks [8]. Differential analysis was used to compare the spiked sample to different sets of reference samples as shown in Figures 3–5. The purpose was to investigate the ability of the method to detect and prioritize the HRMS-signals from the spiked compounds when using different combinations of the remaining 29 samples as reference samples.

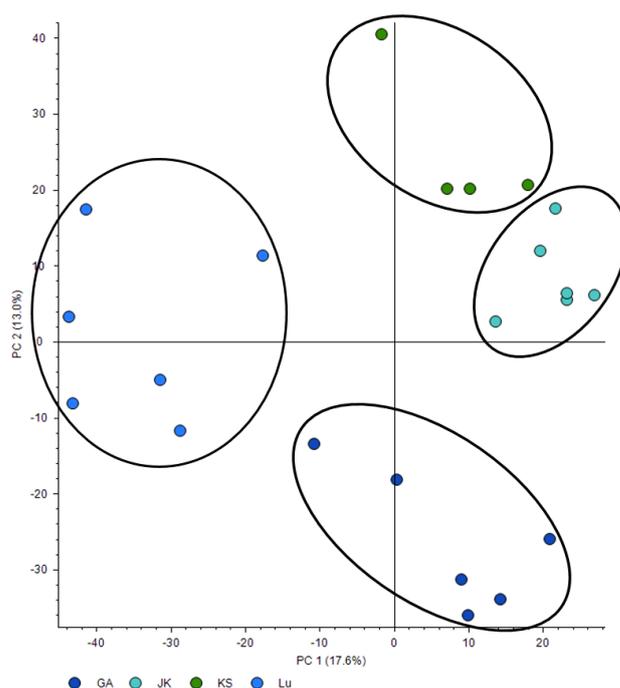


Figure 2. PCA score plot of HRMS-data of RW sampled from four DWTPs. Samples were collected bimonthly, and are in the figure labeled according to the city of DWTP. GA: Gävle, JK: Jönköping, KS: Karlstad, LU: Luleå. The results indicate that the profile of the organic compounds of the RW is unique to each supply studied. Percentages bracketed in axis legends are contribution ratios.

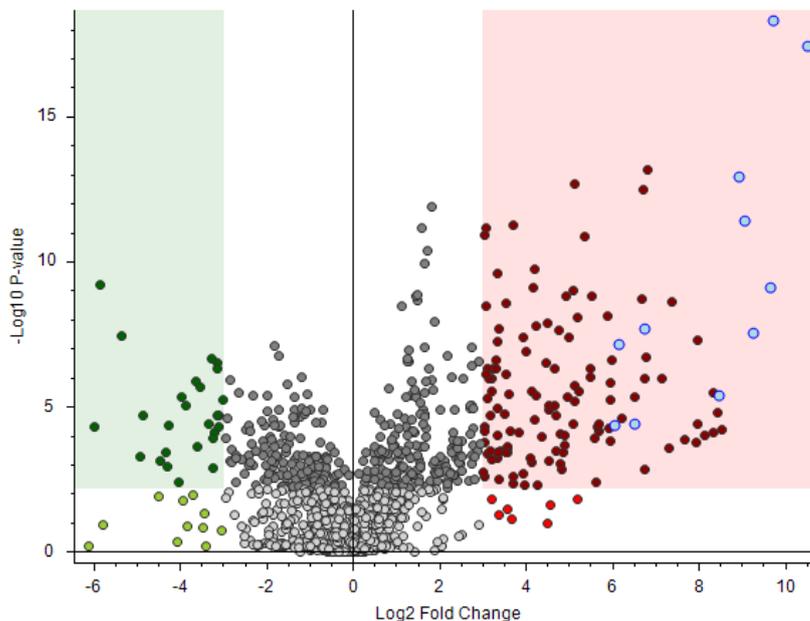


Figure 3. Differential analysis in order to reveal the spiked compounds (labeled in blue), attempting to separate them from background compounds (labeled in red) in the experiment described in Section 3.2. Eight individual samples from another DWTP were used as reference samples. For each feature, i.e., combination of m/z and RT (isotopes and common adducts removed), detected in the data set, the fold change (i.e., the ratio between the median peak area of the spiked sample and the median peak area of the reference samples) was calculated (x-axis: Log2 fold change). Data from all injections (triple injections for the spiked sample and single injections of the eight references) were used in order to calculate the level of significance of the fold change (y-axis: $-\text{Log}_{10} p\text{-value}$). The red shaded area is for comparisons between Figures 3–5 (see text).

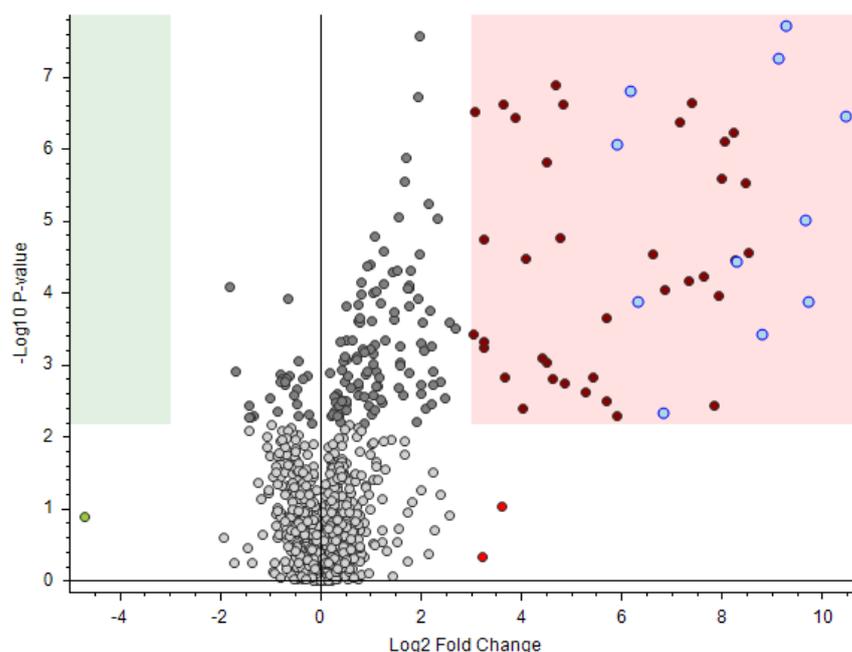


Figure 4. Differential analysis in order to reveal the spiked compound (labeled in blue) in the experiment described in Section 3.2. One reference sample from the same DWTP as the spiked sample (but collected in a different month) was used. For further details regarding volcano plots, see Figure 3 and text.

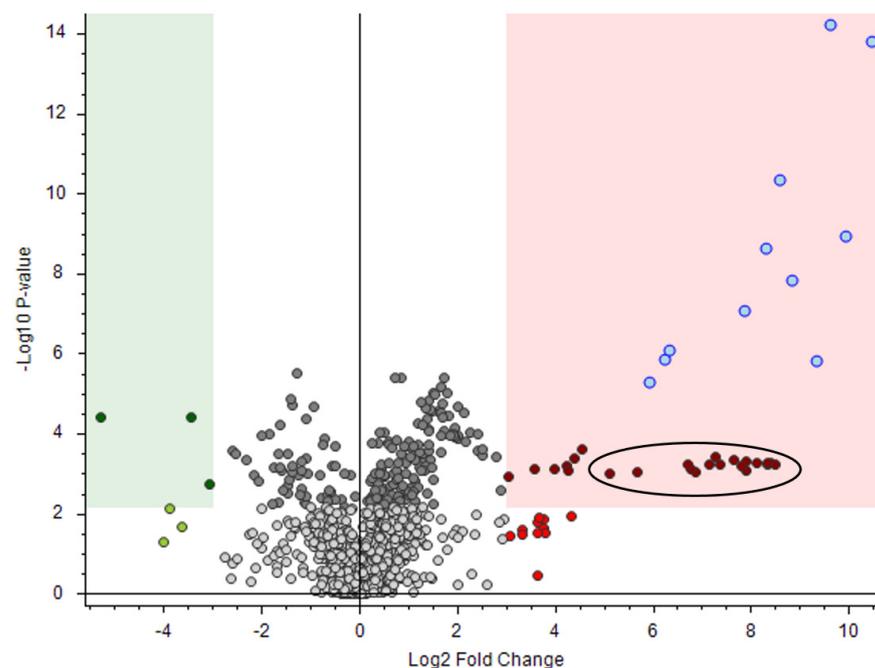


Figure 5. Differential analysis in order to reveal the spiked compound (labeled in blue) in the experiment described in Section 3.2. Seven samples from the same DWTP as the spiked sample (but other months) were used as reference samples. Encircled are the matrix compounds that showed lower significance in this experimental setup as compared to the setup described in Figure 4 (see also text). For further details regarding volcano plots, see Figure 3 and text.

The use of eight individual reference samples from the DWTP Karlstad did not enable effective prioritization of the majority of the compounds spiked into DWTP Karlskrona (Figure 3). The spiked compounds (labeled blue in Figure 3) had high fold change and significance, but were only partially resolved from the DW background compounds (labeled red).

These background compounds were classified as mainly being either humic substances (in line with the findings of such compounds in Karlskrona winter RW in Section 3.1), or disinfection by-products (since the isotopic pattern revealed that the compounds contained one or more chlorine atoms). Hence, the DW from Karlstad did not to a sufficient extent contain the same background of organic compounds as the DW from Karlskrona. A similar result was obtained when using reference samples collected at 12 different months in the city Jönköping (data not shown).

The use of a single reference sample from the same DWTP as for the spiked sample (DWTP Karlskrona) produced a slightly improved result (Figure 4). The reference sample—only one, but injected in triplicate—was sampled in a different month (December) than the spiked sample (which was from November). A lower number of background compounds appearing in the red shaded area indicates the improvement. Essentially, the chlorinated compounds (as explained above) were no longer present in the red-shaded area, indicating that these were specific for Karlskrona as compared to Karlstad and Jönköping. Nevertheless, too many background compounds, mainly humic substances (as explained above), were still present to facilitate an effective prioritization. The conclusion is that the profile of organic compounds in this single reference sample—although it was from the DWTP in question—was not sufficiently similar to the profile of the spiked sample to enable effective prioritization of the spiked compounds.

The most favorable methodological setup comprised the use of several reference samples from the same DWTP as the spiked sample (Figure 5). The reference samples were collected at seven different months (January, March–June, August, and September). Using this set of reference samples, all of the spiked compounds (labeled blue) were separated from the background compounds due to a combination of a high fold change (*X*-axis) and high significance (*Y*-axis), and would all be easy to prioritize. Basically, the humic substances—mentioned when evaluating Figure 4—occurred now in at least one of the reference samples (mainly the January sample), which led to lower significance for those compounds (encircled in Figure 5). To summarize, the experiment showed that the choice of reference samples influenced the efficiency in recognizing the spiked compounds, and the different setups of reference samples that were tested are listed below:

- o Least efficient: Other DWTP(s), several months (Figure 3);
- o Medium efficient: Same DWTP, one month (Figure 4);
- o Most efficient: Same DWTP, several months (Figure 5).

Since the spiking experiment described above focused on one selected sample only (Karlskrona, November) a more systematic investigation was performed. Volcano plots were created for each individual DW sample available in the study using different sets of reference samples. For practical reasons, all of these samples could not be spiked and re-analyzed. Instead, data from the original (non-spiked) samples were used and matrix compounds exceeding certain criteria of fold change and significance were designated as “false positives”. The benefit of using reference samples from the same DWTP as the investigated sample is shown in Table 1. The number of “false positives” was in most cases reduced, sometimes by one order of magnitude, as compared to when using reference samples from another DWTP. The conclusion is that the organic profiles of the cities’ DW deviate substantially from each other. There might be several reasons for these deviations: For Karlskrona, it seems, at least partly, to be due to the RW profile since the data indicated humic substances both in the RW (see Section 3.1) and in the DW (see above). Regarding Karlstad and Jönköping, their RW seem more similar to each other. Both are from big lakes, and they are similar regarding standard quality parameters: TOC, color, COD, turbidity, hardness, conductivity, Fe, Ca, Mg, etc., see Table S1 (although these values could differ between samples and are not from the exact same occasion as the samples that were collected). The reason why the organic profiles of these DWs are different from each other might instead be explained by the fact that the DWTPs used different setups of purification steps: e.g., only the DWTP in Karlstad used artificial infiltration, and only the DWTP in Jönköping used Cl₂ for disinfection (see Table S2).

3.3. 90 DWTPs: Influence of Raw Water and Processes of the DWTPs' on the Profile of the Organic Compounds in the Drinking Water

The previous experiment indicated the benefit of having access to reference samples from the identical DWTP for non-target investigations of DW contamination. However, since there is a significant risk that such samples will not be available at an incident, it would be valuable to investigate further if similar samples from other DWTPs could be used as reference samples. DW from 90 Swedish DWTPs was therefore analyzed with the present LC-Orbitrap method in an attempt to study variations in the profiles of the organic compounds. As a quality assurance measure, the obtained data were compared to data obtained for the same samples using an LC-MS/MS triple quadrupole (QqQ) target method [24]. The retention time and the relative response of the selected common contaminants were similar and stable within the whole batch. In addition, the sensitivity was judged sufficient for the present study (limit of detection for, e.g., carbamazepine was estimated to be 0.4 ng/L).

PCA was applied to the data and the first parameter studied was the influence of the type of RW used for the DW production. The five main types were surface water, natural groundwater from rock or soil aquifers, artificial infiltration, and finally a combination of soil aquifer and artificial infiltration as indicated in Figure 6.

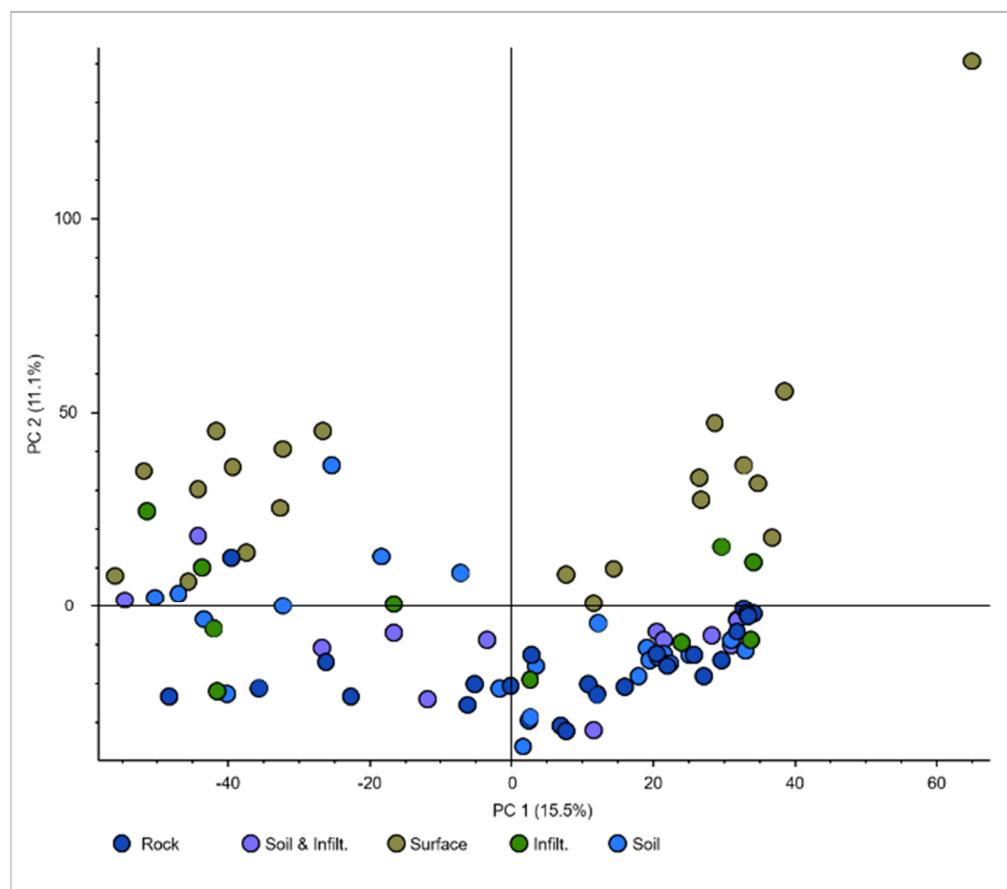


Figure 6. PCA score plot of HRMS-data of DW from 90 Swedish DWTPs sampled once in January–February 2018. The samples are labeled according to the type of RW supply. The five main groups are surface water, rock aquifer, soil aquifer, artificial infiltration, and finally DWTPs that use a combination of soil aquifer and artificial infiltration for the production of the DW. Contribution ratios in brackets (%).

The results indicate a weak similarity between DW originating from rock aquifers since the majority of these appear in the same area of the PCA score plot in Figure 6. Several of the DWs originating from soil aquifers also appear in this area of the PCA,

explicit as for rock aquifers. The corresponding loadings plot (not shown) had relatively few compounds associated with this area of the graph, indicating very clean DW from rock aquifers. Groundwater from rock aquifers can be expected to contain low amounts of natural organic matter (NOM), and has proven to be the least contaminated type of water source in Sweden regarding organic micro-pollutants [24]. Consequently, few purification steps are applied in the DW production from such sources. Hence, the impact on the profile of organic compounds from such DWTPs' treatment process is limited. It could therefore be anticipated that analysis of unexpected or new contaminants using the presented method would be relatively easy for DW produced from a rock aquifer also without reference samples from the DWTP in question. This conclusion does not necessarily apply to soil aquifers in general—as partly indicated in the graph—since they can be expected to contain more NOM, and their contamination seems more similar to surface water [24].

In the PCA plot, shown in Figure 6, all samples of DW produced from surface water appear on the upper half of the graph, opposite the rock aquifers. According to the loadings plot, the DW produced from surface water contained more organic compounds than the DW produced from RW from rock aquifers. The origin of these compounds could be from the RW source (of biological origin or anthropogenic contaminants) or a result of the DWTPs' purification steps. No further conclusion was drawn from the PCA in Figure 6. Other parameters collected from the DWTPs—e.g., type of filters, type of process chemicals, etc.—were evaluated by using the same PCA in further attempts to reveal other trends and correlations. The following parameters were studied when applicable: membrane filter, addition of KMnO_4 , aeration, pre-alkalinization, carbon filter, pH adjustment, UV filter, chlorination, and chemical filter. The only trend observed was that samples from DWTPs that used lime for pre-alkalinization and/or pH adjustment showed a tendency to cluster (Figure S2). However, such samples were too few to make statistically sound conclusions. No other trends or correlations were found. The results suggest that many of the addressed parameters affect the profile of the organic compounds in the DW. However, it was not possible to group the DWTPs based on the type of filters or process chemicals. Conclusively, except for natural groundwater, it will be difficult or impossible to replace reference samples from a DWTP under investigation with reference samples from other DWTPs.

3.4. Real Case: Application of the Suggested Methodology to a Real Case

In October 2020, an intrusion in a DW reservoir connected to a DWTP was committed. The question arose if the water had been intentionally contaminated. As a safety measure, the reservoir (approximately 1000 m^3) was emptied. Prior to this action, a sample (100 mL in duplicate) was collected and sent for chemical analysis. Samples from the effluent from the DWTP as well as DW from another reservoir were collected and used as reference samples since no stored DW reference samples were available. For quality control (QC), a subsample was spiked with 19 pesticides at $0.2 \mu\text{g/L}$. The LC-Orbitrap analytical method was applied to all samples, and the obtained data were processed using differential analysis. The spiked pesticides in the QC sample were detected with a relatively high fold change and significance. When analyzing the original sample using only the DWTP effluent as a reference sample approximately 20 candidate compounds appeared with a high fold change and significance as shown in Figure 7A. Similarly, many candidate compounds appeared with a high fold change and significance when using only the second reservoir as reference (Figure 7B). However, when using a combination of both reference samples, the number of candidate compounds for prioritization was reduced to two compounds (Figure 7C).

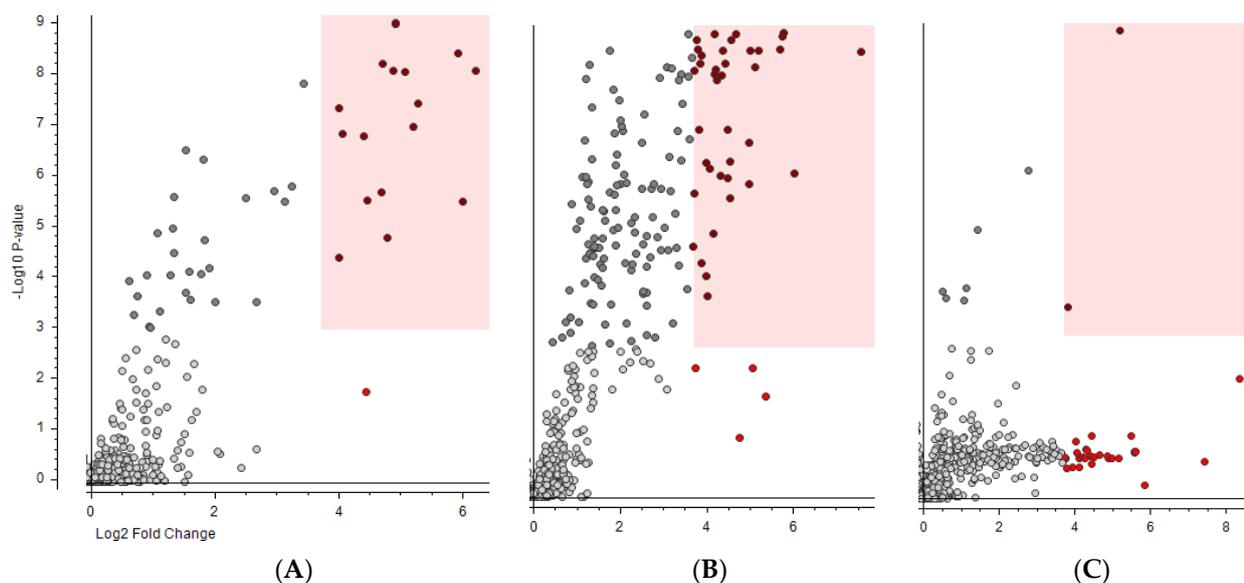


Figure 7. Differential analysis in order to reveal any unexpected compound as described in Section 3.4: (A): The DWTP effluent was used as reference. (B): The second reservoir was used as reference. (C): Both the DWTP effluent and the second reservoir were used as references. The red shaded area corresponds to criteria set only for the purpose of comparison of (A–C) (Log_2 Fold Change > 3.7, p -value < 0.001). (Only compounds with a positive Fold Change are shown, i.e., compounds having a higher concentration in the suspected sample).

These compounds were thereafter tentatively identified as *N,N*-Bis(2-hydroxyethyl) octanamide and the corresponding decanamide by comparison of the simultaneously recorded MS/MS spectra with MS/MS-spectra from PubChem [28]. The compounds were listed by the Norman Network [29], and denoted by PubChem as “environmental hazard” and “irritant”. No link between the findings and the intrusion into the reservoir could be established. Nevertheless, the investigation showed the benefit of having access to more than one relevant reference sample.

3.5. Application of the Suggested Methodology to a Recent Exercise

A follow-up of the recent inter-laboratory exercise that formed the basis for this study was performed (see Section 1 and Table S3). First, the described LC methodology was applied, which easily revealed the compounds suiting LC: warfarin, acrylamide and triethylamine (Figure S3). Since the remaining two spiked chemicals in the test sample—diflufenican and acetonitrile—rather would suit GC, analysis by GC-HRMS was applied. The use of GC-Orbitrap (see Section 2) allowed for the identical data processing software (Compound Discoverer, CD) to be used for both LC- and GC-HRMS data. Acetonitrile was spiked at the highest concentration in the exercise, and by using headspace GC-MS, it was easily discovered without CD software support. However, diflufenican, the compound that was spiked at the lowest concentration in the exercise, required the use of CD and the volcano plot-data display for effective prioritization (Figure S4). With a small set-up of reference samples, it could also be shown for GC-HRMS that the reference sample collected closest to the source of the original sample used for spiking—but months later—was the most relevant (Table S4).

The follow-up of the initial inter-laboratory exercise showed the potential of using a combination of LC- and GC-HRMS—together with non-target analysis based on differential analysis—since all added compounds were now detected. The concentrations of the spiked compounds in this experiment were relatively high (mg/L levels) and it was therefore not expected to be a challenge to detect them using non-target analysis as described. It should be pointed out again that the present non-target method performed successfully

at considerably lower concentration levels as shown, e.g., in the experiment in Section 3.2 (DW references) carried out at 0.1 µg/L.

4. Conclusions

This study shows the significance of having access to relevant reference samples for non-target analysis of unknown contaminants in DW. The observed variability in the organic profile of DW—probably due to a combination of differences in RW type, surrounding land use, seasonal and process-related factors, etc., between the investigated DWTPs—proved to have a significant impact on the results. The overall results indicate that it is invaluable for DWTPs to have a set of reference samples ready for any incident, ideally collected on several occasions during the past year at the DWTP in question. These samples—shown to be stable enough for the purpose when stored at −20 °C up to at least one year—should be sent together with a fresh “suspected sample” to a laboratory capable of performing non-target HRMS investigations as described. The non-target HRMS methodology used in our laboratories proved to be efficient for detecting new contamination while simultaneously excluding compounds normally or often present in an investigated DW sample.

Our study was performed using samples from Swedish DWTPs only, but the conclusions are probably relevant in other countries as well. The methodology is not restricted to being used only when there is an incident. It may also be used whenever any alteration in a DWTP’s production scheme is made, e.g., exchanging the RW source, any filter or supplier of process chemical. It could even be a future standard procedure to be performed at any such modification.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/w14162586/s1>. Table S1: Type of RW, main characteristics, etc., for the RW analysed in Section 3.1; Table S2: Simplified summary of the purification process for DW production at the DWTPs sampled for the experiment in Section 3.2; Table S3: Result of the inter-laboratory exercise described in Section 1; Table S4: Analysis of GC-Orbitrap data in Section 3.5; Figure S1: PCA score plot of HRMS-data of RW sampled from a DWTP in Luleå; Figure S2: Same score plot as in figure 6, but samples are now labelled according to what chemical – if any – was used for any pre-alkalinisation process step; Figure S3: Analysis of LC-Orbitrap data, Differential analysis (volcano plot) used in the follow-up (in Section 3.5) of the inter-laboratory exercise for revealing the signals from the spiked compounds; Figure S4: Analysis of GC-Orbitrap data, Differential analysis (volcano plot) used for revealing the signals from the spiked compounds in the exercise described in Section 3.5.

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