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BTO report

Integration of non-target screening, statistical analyses and bioassays to globally assess chemical water quality

KWR Watercycle Research Institute

BTO

Integration of non-target screening, statistical analyses and bioassays to globally asses chemical water quality

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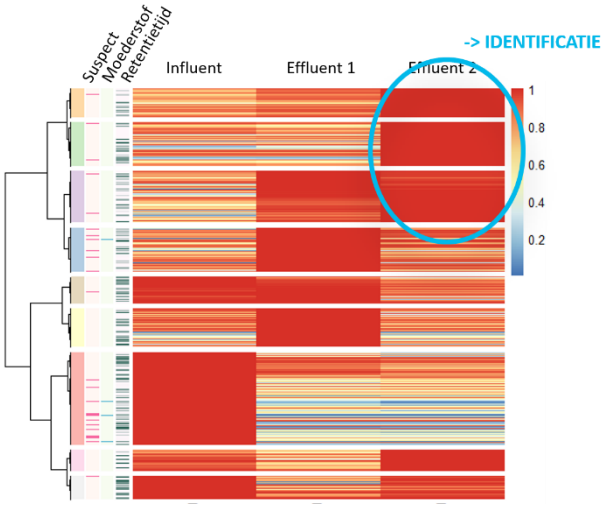
BTO *Managementsamenvatting*

Combineer data uit non-target screening en bioassays via nieuwe statistische tools voor meer inzicht in waterkwaliteitsveranderingen

Auteur(s) Dr. Andrea Brunner, Dr. Thomas ter Laak

Er zijn verschillende statistische tools en workflows ontwikkeld waardoor het nu mogelijk is conclusies te trekken over de effecten van waterbehandeling(stappen) op basis van de grote hoeveelheden data die worden gegenereerd met non-target screening (hoge resolutie massaspectrometrie gecombineerd met vloeistofchromatografie) en bioassays. Deze statistische of data science tools en methoden zijn getest in twee casestudies: een pilot-scale data set uit de drinkwaterbehandeling met gedoseerde organische-microverontreinigingen (*DPWE robuustheid zuiveringen*) en een real-scale data set uit de innovatieve afvalwaterzuivering (*H2020 AquaNES*). Non-target-resultaten zijn met bioassay metingen geïntegreerd om een uitgebreider beeld van de chemische waterkwaliteit te krijgen. Daardoor wordt informatie gegenereerd, die bij alleen target screening ontbreekt: verschillen tussen monsters en behandelingstappen worden op een efficiënte manier aangetoond. De visualisatie helpt hierbij om een duidelijk beeld van complexe data te geven en vereenvoudigt de prioritering.



Data science methoden ondersteunen de prioritering in non-target screening analyses

Belang: data effectief inzetten voor beoordeling van de waterkwaliteit, o.a. tijdens waterbehandeling

Dankzij ontwikkelingen in op hoge-resolutie-massaspectrometrie (HRMS) gebaseerde screeningsmethoden zijn bij de detectie van chemische stoffen in water nu niet alleen specifieke stoffen op te sporen (doelstofanalyse), maar is het ook mogelijk breder te screenen (non-target screening). Non-target screening is een veelbelovend hulpmiddel geworden bij de evaluatie van veranderingen in de chemische waterkwaliteit tijdens waterbehandeling, maar omdat bij deze methode erg veel data worden gegenereerd, wordt structurele identificatie van alle gevonden ‘pieken’ vrijwel onmogelijk. Transformatieproducten ontbreken bovendien vaak in de beschikbare databanken, maar kunnen wel worden voorspeld aan de hand van bekende transformatieregels. Als alternatief kunnen data science methoden worden gebruikt om verschillen bloot te leggen tussen

waterbehandelingsstappen. Daarom is het belangrijk te onderzoeken hoe en hoe zinvol verschillende niveaus van met non-target screening gegenereerde data kunnen worden ingezet om bijvoorbeeld de effectiviteit van waterbehandeling te evalueren. Bij non-target screening worden drie verschillende niveaus van gegenereerde informatie onderscheiden: van "onbekende pieken" tot "suspects" (pieken die voorkomen op een bekende “verdachtenlijst”) en "trendprofielen" (datapatronen die voortkomen uit de combinatie van de voorgaande niveaus). Daarnaast komt ook informatie uit bioassays op basis van effecten.

Aanpak: data science tools verkennen en toepassen in workflows; inzet in twee case-studies

Verschillende niveaus data uit eerdere projecten zijn in dit onderzoek aan statistische hulpmiddelen onderworpen. De suspect screening werd verbeterd en versneld door het online ophalen van chemische kenmerken en uitgebreide *suspect lists*, inclusief semi-automatisch gegenereerde *suspect lists* van transformatieproducten. Piekverschuivingen als gevolg van een enkele behandelingsstap werden gevisualiseerd in *Volcano-plots*. Piekveranderingen over meerdere behandelingsstappen en tussen verschillende behandelingstreinen werden beoordeeld met behulp van multivariate statistische analyses, zoals *principal component analysis* en hiërarchische clustering. Door de trendprofielen in *heat maps* te visualiseren op basis van de clusteringuitkomst en de bioassay readouts ermee te integreren, kon efficiënt worden geprioriteerd welke pieken moeten worden geïdentificeerd.
De ontwikkelde nieuwe data science tools en workflows zijn toegepast in twee casestudies om de waterkwaliteit en veranderingen in de waterkwaliteit tijdens de waterbehandeling te monitoren: H2020-project *AquaNES* (*real-scale* innovatieve zuiveringsinstallaties voor afvalwaterzuivering) en *DPWE Robuustheid Zuiveringen (pilot scale-installaties* met gedoseerde organische-microverontreinigingen bij PWN en Dunea). Op LC-HRMS-gebaseerde non-target screening en bioassays werden op deze monsters toegepast. Door data uit de chemische non-target screening te integreren met de biologische effect data, is ook de toxiciteit in de prioritering meegenomen en kon een uitgebreider beeld van de chemische waterkwaliteit worden verkregen.

Resultaten: ontwikkelde workflows als monitoringtools van de chemische waterkwaliteit

Het onderzoek heeft diverse tools en workflows opgeleverd, die in de casestudies succesvol zijn toegepast:

- Semi-automatisch aanmaken van transformatieproductensuspect lijsten
- Statistische tool set voor interpretatie van data uit non-target screening
- Set non-target trend profielen geassocieerd met drinkwaterbehandelingsstappen
- Nieuwe workflows voor non-target screening en bioassay data interpretatie in R scripts
- Lijsten van gedetecteerde suspects, inclusief transformatieproducten, voor latere structurele opheldering

Non-target-resultaten zijn met bioassay metingen geïntegreerd om een uitgebreider beeld te krijgen van de chemische waterkwaliteit. Dit levert informatie op die ontbreekt bij alleen maar target screening: verschillen tussen monsters en behandelingstappen worden op een efficiënte manier aangetoond. De visualisatie helpt hierbij om een duidelijk beeld van complexe data te geven en vereenvoudigt de prioritering.

Implementatie: laat data science tools helpen de waterkwaliteit te evalueren

Non-target screening en bioassays in combinatie met de nieuwe tools en workflows hebben een uitgebreide beoordeling van de waterkwaliteit tijdens de waterbehandeling mogelijk gemaakt. De toepassing van de ontwikkelde statistische of data science tools illustreert dat ze potentieel hebben voor het beoordelen van de waterkwaliteit en waterbehandeling en laat ook zien dat ze daar technologisch klaar voor zijn. In dit onderzoek lag een sterke focus op inzet bij het beoordelen van de ontwikkeling van de waterkwaliteit bij waterbehandeling, maar de tools zijn ook in te zetten voor andere ontwikkelingen in de waterkwaliteit, bijvoorbeeld seizoensgerelateerde.

Rapport

Dit onderzoek is beschreven in het rapport *Integration of non-target screening, statistical analyses and bioassays to globally assess chemical water quality* (BTO 2019.002).

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Contents

1 Introduction

1.1 Three levels of information from non-target screening data

Advancements in high-resolution mass spectrometry (HRMS)-based screening methods have enabled a shift from target to non-target analyses to detect chemicals in water samples. Non-target screening has therefore become a promising tool to evaluate the changes of chemical water quality during water treatment (Nürenberg et al., 2015). However, the wealth of data resulting from non-target screenings renders structural identification virtually impossible (Hollender et al., 2017). The aim of the exploratory research project presented here was to evaluate the use of information generated by non-target data to study water treatment, without identification of all HRMS peaks. It focused on three different levels of non-target data for water quality assessment, i.e. the "unknown feature" level, the "suspect" level, and the "trend profile" level.

A feature represents a given compound and consists of a unique combination of an accurate mass and a retention time. Without identifying the feature, information on its response – measured in instrument counts or response relative to an internal standard, presence in a homologous series, mass defect, isotopic pattern and predicted hydrophobicity presented as log octanol water partition coefficient (K_{ow}) can be automatically extracted (Heberger, 2007; Zhang et al., 2009; Carlson et al., 2012; Sleno, 2012; Jobst et al., 2013; Nagao et al., 2014; Bade et al., 2015; Aalizadeh et al., 2016; Parry and Young, 2016; Sjerps et al., 2016; Loos and Singer, 2017). The unknown feature level refers to all this intrinsic information. The suspect level refers to potential candidates that match a feature through automated suspect screening against an in-house curated suspect list consisting of environmentally relevant compounds and predicted transformation products. Finally, the trend profile level combines the two, and reveals patterns in the data through statistical methods, with the goal to cluster both features and the effects of water treatments on water quality (Muller et al., 2011; Schollee, 2015; Schollée et al., 2016). Distinction is made between persistence, elimination and formation during treatments. The trend profile level can then be connected to responses of bioassays.

1.2 Evaluate and develop data science tools for non-target screening data interpretation

Here, we present the data science tools we explored to make use of all three levels of non-target data and effect-based bioassay information for water quality assessment. On the feature level, we utilized both feature intensity and isotopic pattern recognition. Suspect screening was improved and accelerated by the online retrieval of chemical characteristics, and extended suspect lists including semi-automatically generated lists of transformation products. Changes in features induced by a single treatment step were assessed based on the fold change between the treatment effluent and influent, and visualized in Volcano plots. Changes in features across multiple treatment steps and between different treatment trains were assessed using multivariate statistical analyses, such as principal component analysis and hierarchical clustering. By visualizing the trend profiles in heat maps based on the clustering outcome and integrating the bioassay read-outs, features could efficiently be prioritized for their later (structural) identification. Together, these novel tools allowed for comprehensive water quality assessment during water treatment.

The programming language R in R Studio was used for data analysis and visualization. An ever increasing wealth of R packages exist that facilitate and accelerate statistical analyses of non-

target HRMS data, multivariate analyses, and data visualization. In line with the demand for reproducible research, scripts were written with R Markdown. R Markdown allows for html output which can easily be read. The workflows can thus readily be reused and adapted for the next dataset at hand. Scripts written as part of the project are available upon request.

1.3 Two case studies to show the readiness level of the developed workflows

This report presents the developed novel data science tools and workflows by means of two studies in which we successfully applied them to monitor water quality and changes in water quality during water treatment. In these studies, we extended the scope of our research from previously described lab scale experiments with spiked-in compounds (Brunner et al., 2019b) to pilot scale installations at two drinking water utilities and real/full scale treatment plants for waste water treatment. Therefore, we selected relevant water samples generated in the DPWE project *Research on robustness of treatment (trains)* and the EU's Horizon 2020 project *Demonstrating Synergies in Combined Natural and Engineered processes for water treatment systems* (AquaNES), respectively. LC-HRMS based non-target screening and bioassays were performed on these samples. By integrating the chemical non-target screening data with the biological effect-based results potential toxicity during prioritization was accounted for. Together, the developed data science workflows allowed for comprehensive water quality assessment.

1.4 Applicability and implementation for the water sector

Ultimately, we anticipate the application of data science-based tools for data interpretation of non-target screening and effect-based bioassay data as described here, also outside the academic community in monitoring tools or as treatment efficiency indicators. The European Drinking Water Directive encourages the development of risk based monitoring programs (Commision, 2015; European Commission, 2015). Customized monitoring allows irrelevant parameters to be abandoned and alternative parameters and tools to be considered. However, the user and regulator acceptance depends on successful demonstration and evaluation of novel tools (Guillén et al., 2012). The application of the data science tools we developed within the two demonstration studies illustrated their potential for water quality and water treatment assessment, as well as their technology readiness level. They enabled evaluation of treatment efficiency of innovative treatment schemes. Overall, the presented work is a valuable step towards the implementation of such tools in water quality assessment.

2 Integration of non-target screening and effect-based monitoring to assess water quality changes in drinking water treatment

2.1 Introduction

2.1.1 Non-target screening for comprehensive monitoring

With the production and use of chemicals exponentially on the rise, the occurrence of organic micro-pollutants in drinking water sources, and if not removed during drinking water treatment in drinking water, is increasing. To ensure drinking water quality, drinking water utilities are evaluating novel water treatment steps aimed at their removal. However, these steps can lead to transformation product (TP) formation and these products can pose environmental and health risks similar to their parent compounds. Consequently, water quality assessment targeting a defined number of regulated priority substances alone does not suffice. Instead, comprehensive non-target screening (NTS) methods are required to detect a multitude of chemicals simultaneously. Such methods can support the water sector in realistically assessing the human and environmental health risks of (emerging) contaminants.

NTS analyses based on high-resolution mass spectrometry combined with liquid chromatography enable the monitoring of organic micro-pollutants in water in the ng / L range. However, the structural identification of unknown compounds from NTS data remains challenging, and relies on improved databases and novel data analysis approaches. The presence of a compound in a database is often the decisive factor in the identification of a detected feature from NTS data, as a database entry turns an “unknown unknown” into a “known unknown” (McEachran et al., 2017; Schymanski and Williams, 2017). This enables an accurate mass (MS1) based suspect screening, and consecutively a fragmentation (MS2) based similarity search against a spectral library or *in silico* predicted spectra of the compound. However, TPs are only beginning to be listed in chemical databases. For instance, the category “transformation product” was added to the STOFF-IDENT database recently, allowing retrieval of TPs based on the name of parent compound to retrieve its transformation products. with the added advantage that CAS numbers are provided for some TPs which can be used to retrieve MS ready SMILES in the US EPA’s Chemistry Dashboard (McEachran et al., 2018). If transformation products are lacking in the available databases they can be predicted on the basis of "metabolic logic" (Schollee, 2015), i.e. the mass shifts indicative of transformation processes are used to link parent compounds and TPs, and well-known (bio) transformation rules (Fenner et al., 2008; Lee et al., 2017).

2.1.2 Data science methods help focus on what really matters

Alternatively, data science methods can be used to interpret NTS data. Thereby, water quality and in particular water quality changes across water treatment steps, seasons and locations can be assessed without identification of the detected features (Schollée et al., 2016; Schollee et al., 2018). For instance, these strategies can reveal shifts in polarity and mass of compounds, as well as newly formed compounds, i.e. transformation products as a result of a specific process. Thereby, they expose differences between the treatment steps of the drinking water

treatment, including aspects that remain elusive when only target compounds are monitored. Furthermore, the NTS in combination with hazard based prioritization can facilitate defining risk based monitoring strategies as demanded by the European Drinking Water Directive (Commision, 2015), and ultimately safeguarding of drinking water quality (van Wezel et al., under review).

2.1.3 Pilot installations of drinking water treatment trains at Dunea and PWN

Here, we describe the application of novel data science tools for the water quality assessment of drinking water treatment trains at three pilot installations. Thereby, we extended the scope of our research from previously described lab scale experiments (Brunner et al., 2019b) to pilot scale installations at two drinking water utilities. These installations included the advanced oxidation processes ozonation and UV treatment in combination with hydrogen peroxide (H2O2), ultrafiltration (UF) in combination with reverse osmosis (RO), and sequential UV / H_2O_2 treatment and granular active coal filtration. A selection of organic micro-pollutants relevant for the drinking water sector was spiked in these installations. LC-HRMS based non-target screening and bioassays were performed on samples from all three installations. The NTS data was screened for predicted and known transformation products of the spike-in compounds in in-house generated suspect lists. Patterns and trends in the NTS data were evaluated using the multivariate analysis methods principal component analysis and hierarchical clustering. This allowed for efficient visualization of complex data. By integrating the chemical non-target screening data with the biological effect-based results potential toxicity was accounted for during prioritization. Together, the developed data science workflows allowed to monitor water quality and changes in water quality during water treatment.

2.2 Material and methods

2.2.1 Selection of organic micro-pollutants for spike-in

Priority compounds were selected based on their presence in Association of River Water Works (RIWA) databases, research reports, the Water Framework Directive (WFD) guideline, and substances proposed by the water utilities. In addition, selection was based on exceedance of the drinking water standard or target value (0.01 µg / L for genotoxic compounds, 0.1 µg / L for other biologically active compounds and 1.00 µg / L for other anthropogenic compounds without known specific biological activity) in several years between 2011 and 2015 more than twice a year, or frequent detection in concentrations above 50% of the standard or target value. Selected compounds were further filtered for toxicity, removal in drinking water treatment, chemical properties, reference compounds and practical issues such as detectability and availability.

Spike-in concentrations of the selected compounds were based on the detection limit of the target method, the flow rate of the installation, dosing time and the maximum removal efficiency. For most compounds spiked concentrations allowed at least a factor of 100 between the spiked concentration and the limit of quantification. Exceptions were barbital (factor 10), fenobarbital (factor 20), HFPO-DA (Gen-X, factor 50). Compounds used in the pilot installations and their respective spike-in concentrations are shown in Table 1. Based on their solubility, compounds were divided into a soluble (1) and poorly soluble(2) group and stock solutions were made accordingly. Due to the late delivery of the substance HFPO-DA, a separate stock solution (3) was made, to which the volatile compounds (*) were added on the day of dosage.

TABLE 1. OVERVIEW OF SPIKE-IN COMPOUNDS, FINAL CONCENTRATION

Compound	Final concentration (µg/L)	Limit of Quantification (LOQ) (µg/L)	Stock
Acesulfame-K	10	0,10	1
AMPA	2	0,02	1
Aniline	1	0,01	1
Barbital	10	1,0	1
1H-Benzotriazole	1	0,01	1
Carbamazepine	1	0,01	2
Carbendazim	1	0,01	2
Diatrizoic acid	3	0,03	1
Diclofenac	1	0,01	2
Dimethenamid	1	0,01	1
1,3-dimethylbenzene	5	0,05	3*
1,4-dimethylbenzene	5		3*
Dimethomorph	1	0,01	2
EDTA	5	0,05	1
Phenobarbital	10	0,50	1
Furosemide	3	0,03	2
Gabapentin	1	0,01	1
HFPO-DA (Gen-X)	10	0,20	3
Glyphosate	5	0,05	1
HMMM	3	0,03	1
Hydrochlorothiazide	5	0,05	1
Melamine	5	0,05	1
Metformin	5	0,05	1
4-Methyl-1H-benzotriazole*	1	0,01	1
5-Methyl-1H-benzotriazole*	1	0,01	1
Propranolol	1	0,01	1
Pyrazole	10	0,50	1
Sucralose	10	1,0	1
Terbuthylazine	1	0,01	2
Tetraglyme	3	0,03	1
TFA	5	0,05	1
Tiamulin	1	0,01	1
TPPO	1	0,01	2
Tramadol	1	0,01	
Urotropin	5	0,05	
		0,20	

* Mixture of 35% 4-methyl-1H-benzotriazool and 65% 5-methyl-1H-benzotriazole.

Stock solutions 1 and 2 were prepared in 20 L stainless steel tanks filled with ~10 L of demineralized water. The weighed compounds were added sequentially, after which demineralized water was added to ~15 L. Stock solutions were kept at 35 ° C under constant stirring for 6 days, however, complete dissolving couldn't be achieved. Stock solution 3 was prepared in a 20 L stainless steel tank filled with ~15 L of demineralized water. After addition of HFPO-DA (GenX), the solution was left at room temperature overnight with constant stirring. Subsequently, all stock solutions were filtered through a 0.20 μm filter and demineralized water was added to 20 L by weight in jerry cans. Stock solutions were stored at 3 ± 2° C until

the day of dosage. Then, the volatile compounds 1,3-dimethylbenzene and 1,4-dimethylbenzene were added to stock solution 3. Chemicals were purchased from Sigma, Acros, TCI, Merck, TRC and Duchefa Farma.

2.2.2 Set-up pilot installations

The Dutch water utility Dunea produces drinking water from surface water. Their pilot installation included the advanced oxidation processes ozonation and UV treatment in combination with hydrogen peroxide (H_2O_2). Experiments were performed at Dunea on October 5th and 7th, 2017. Organic micro-pollutants were dosed in the pilot installation of the O_3 / H_2O_2 - UV / H_2O_2 process that is fed with filtrate from the rapid sand filters in Bergambacht, The Netherlands. The schematics of the pilot installation and process conditions are shown in Figure 1and Table 2, respectively.

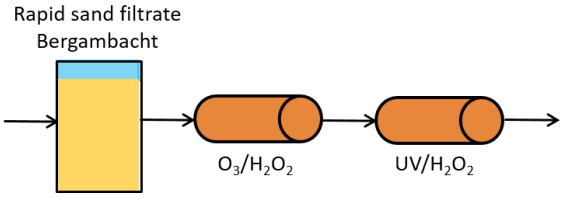


FIGURE 1 SCHEMATICS OF PILOT INSTALLATION AT DUNEA

TABLE 2. PROCESS CONDITIONS PILOT INSTALLATION DUNEA

Parameters	Value	Unit
Feed rate	5	m^3/h
H_2O_2	6	mg/L
O_3	45	g O_3/Nm^3
UV-dose	600 – 700	mJ/cm^2

The Dutch water utility PWN also produces drinking water from surface water. PWN tested two pilot installations; experiments with the first installation combining ultrafiltration (UF) with reverse osmosis (RO) were performed at a feed rate of 9.7 m^3/h from September 18th to 22nd 2017 (Figure 2). Experiments with the second installation of sequential UV / H_2O_2 treatment and granular active coal filtration were performed September 19th and October 3rd to 5th (Figure 3 and Table 3).

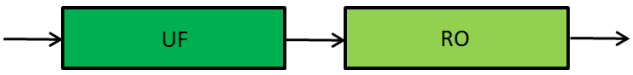


FIGURE 2. SCHEMATICS OF PILOT INSTALLATION UF-RO AT PWN

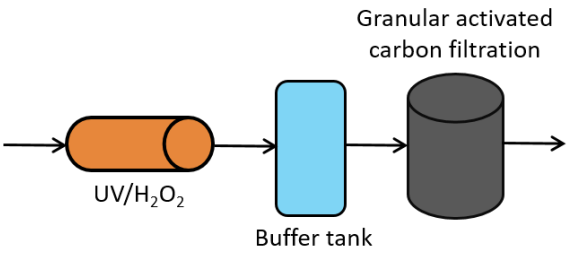


FIGURE 3. SCHEMATICS OF PILOT INSTALLATION UV/H_2O_2 – GAC AT PWN

TABLE 3. PROCESS CONDITIONS PILOT INSTALLATION UV/H_2O_2 – GAC PWN

Parameters	Value	Unit
Feed rate	18,3	m^3/h
H_2O_2	14,5	mg/L
UV-dose	500 - 600	mJ/cm^2
Empty bed contact time (EBCT, GAC)	25 - 30	min

2.2.3 Non-target screening

2.2.3.1 Selected samples and sample preparation

The list of samples, description and sample codes can be found in Table 4.

TABLE 4. OVERVIEW OF NON-TARGET SCREENING SAMPLES

Sample codes Dunea/PWN	Sample description	Sample codes KWR
DUN-INF-GD-1	Influent no spike	LMC-41312-OW-B
DUN-INF-DOS-1	Influent spike-in	LMC-41313-OW-B
DUN-EFFL-OZ-1	Effluent O3/H2O2	LMC-41314-OW-B
DUN-EFFL-UV-1	Effluent UV/H2O2	LMC-41315-OW-B
PWN-UV-INF-GD-2	Influent no spike	LMC-39883-OW-B
PWN-RO-INF-DOS-1	Influent spike-in	LMC-39888-OW-B
PWN-RO-EFFL-1	Effluent UF-RO	LMC-39890-OW-B
PWN-UV-INF-GD-3	Influent no spike	LMC-41336-OW-B
PWN-UV-INF-DOS-2	Influent spike-in	LMC-41338-OW-B
PWN-UV-EFFL-2	Effluent UV/H2O2	LMC-41342-OW-B
PWN-UV-INF-GAC-2	Influent GAC	LMC-41340-OW-B
PWN-UV-EFFL-GAC-2	Effluent GAC	LMC-41344-OW-B

50 ml measuring flasks were pre-rinsed with acetone, PE and the sample, prior to addition of internal standards to a final concentration of 0.98 μg / L atrazine-d5, 0.85 μg / L bentazone-d6, and 1 μg / L fenuron, chloroxuron and diuron. Next, samples were filtered with a 0.2 μm regenerated cellulose filter, and 100 μL injected into the LC-HRMS.

2.2.3.2 Non-target analyses based on LC - HRMS

A Tribrid Orbitrap Fusion mass spectrometer (ThermoFisher Scientific, Bremen, Germany) with a heated electrospray ionisation source was connected to a Vanquish HPLC system (ThermoFisher Scientific). An XBridge BEH C18 XP column (150 mm × 2.1 mm I.D., particle size 2.5 μm, Waters, Etten-Leur, The Netherlands) was used in combination with a 2.0 mm × 2.1 mm I.D. Phenomenex SecurityGuard Ultra column (Phenomenex, Torrance, USA), at a temperature of 25 ° C. The LC gradient started with 5% acetonitrile, 95% water and 0.05% formic acid (v / v / v), increased to 100% acetonitrile, 0.05% formic acid in 25 min, and then remained constant for 4 min. The flow rate was 0.25 mL / min. Mass calibration was performed with ESI positive and negative ion calibration solution (Pierce). The evaporator and capillary temperature was set at 300 ° C. Sheath, auxiliary and sweep gas were set to arbitrary units of 40, 10 and 5. The source voltage was 3.0 kV in positive mode, and -2.5 kV in negative mode.

The RF lens was set to 50%. Full scan high resolution mass spectra were recorded from m / z 80-1300 with a resolution of 120,000 FWHM. Quadrupole isolation was used for acquisition with a 5 ppm mass window. Data dependent acquisition was performed with High Collision Dissociation (HCD) energy of 35% and FT resolution of 15,000 FWHM. Each sample was measured in triplicate.

2.2.3.3 Data processing, analysis and interpretation

The non-target data were processed with Compound Discoverer 3.0 (Beta version, Thermo Fisher) for peak picking, componentization, and suspect screening. An overview of the Compound Discoverer workflow and parameters can be found in SI. The output of this is a feature list, i.e. a table with accurate mass / retention time pairs (features) and their intensity. The feature intensity is reported as peak area. Depending on the statistical analysis, the "Area" (response of each technical triplicate is reported individually) or the "Group Area" (median response of the triplicate) output was used. Only features that were 5 times the intensity of the blank were clustered in the treatment train specific heat maps.

For the spike-in compounds that are included in the chemical database of the US EPA called Chemistry Dashboard (McEachran et al., 2017), a suspect screening was carried out with an in-house curated suspect list that also included potential transformation products (TP) of the spike-in compounds. The suspect list consisted of both known and predicted TPs. Known TPs were retrieved from the water-relevant database STOFF-IDENT and from data from the Bayerisches Landesamt für Umwelt, kindly supplied by Dr. Manfred Sengl. TP prediction was based on "metabolic logic" (Schollee, 2015), biotransformation rules from the EAWAG BDD database (Fenner et al., 2008) and the ozonation prediction tool from Lee et al. (Lee et al., 2017). The suspect list was generated in R based on the packages *RMassScreening* and *rcdk*. R script and curated suspect list are available upon request.

TABLE 5. TP REACTIONS IN RMASSSCREENING

Transformation reaction	Formula change
hydroxylation	**+O**
demethylation	$-CH_2$
deethylation	**$-C_2H_4$**
dehydrogenation	$-H_2$
hydrogenation	**$+H_2$**
dehydration	$-H_2O$
chlorine reduction	**-Cl/+H**
acetylation	$+C_2H_2O$
deacetylation	**$-C_2H_2O$**
glucuronidation	$+C_6H_8O_6$
deglucuronidation	**$-C_8H_8O_6$**
sulfonation	$+SO_3$
desulfonation	**$-SO_3$**

The Compound Discoverer output was imported into R Studio for further data analysis and visualisation (R Core Team, 2017). Data preprocessing in R included the application of a retention time cut-off of 2 min, and for the separate analyses of the three different treatment trains the removal of background features. Principal Component Analysis (PCA) provided an overview of the differences between the samples and treatment groups (Masia et al., 2014).

After normalization of the data through division of feature areas across samples by the maximum area of the respective feature, both samples and features were clustered together based on Euclidean distances using the complete method (Everitt, 1974; Schollée et al., 2016). In a second hierarchical clustering (HC) with Pearson correlations as distance matrices and the Ward's minimum variance method (Ward, 1963), treatment induced trends in the features were revealed, i.e. clusters of features that decrease in intensity, increase or remain the same. The first two could represent parent substances and transformation products. To investigate this further, a theoretical number of clusters X per treatment train was calculated where

$$X = 3^{(n-1)}$$

n ... number of samples (group)

Based on the hierarchical clustering with Pearson correlations as distance matrices, X can be used to cut the dendrogram generated by the clustering, resulting in a table of features per cluster. Clustering results were visualized in heat maps using the *pheatmap* package in R. R scripts are available upon request.

2.2.4 Bioassays

Effect-based measurements were performed with the Ames tests for mutagenicity listed in Table 6 as described previously (Heringa et al., 2011), and the CALUX tests for anti-androgenic activity, estrogenic activity, polycyclic aromatic hydrocarbons (PAHs) and oxidative stress response listed in Table 7 according to the supplier's protocols (Murk et al., 1996b; Sonneveld et al., 2005a; Pieterse et al., 2013a). Bioassay responses were categorized as active and inactive based on whether they exceeded the limit of quantification without further differentiation on the intensity of the response, and the binary output integrated with the non-target screening data through visualization in the HC heat maps.

TABLE 6. AMES TESTS. STRAINS, S9 CONDITIONS AND POSITIVE CONTROLS.

Strain + S9 condition	Positive controls
TA98 no metabolism	20 µg/mL 4-NQO, 500 µg/mL 4-NOPD
TA98 + S9 metabolism	5 µg/mL 2-AA
TA100 no metabolism	12.5 µg/mL NF
TA100 + S9 metabolism	20 µg/mL 2-AA

TABLE 7. CALUX TESTS APPLIED, AND ASSOCIATED MECHANISMS.

CALUX	Mechanism
anti-AR	anti-androgenic activity
ER	estrogenic activity
PAHs	PAHs activity
nrf2	oxidative stress response

2.3 Results and discussion

2.3.1 26 parent compounds and 130 potential TPs thereof detected

Non-target screening analyses based on high-resolution mass spectrometry combined with liquid chromatography of the water samples enabled the monitoring of organic micro-pollutants. A total of 2821 and 1180 features were detected across all samples using positive and negative ionization, respectively (Table 8). Application of a retention time cut-off of 2

minutes and removal of features that were also present in the background, resulted in 927 and 310 features in positive and negative ionization mode. By means of suspect screening based on accurate mass and retention time, the non-target data was searched for accurate masses of spike-in compounds, i.e. parent compounds. The 26 parent compounds (20 pos, 6 neg) that were detected are listed in Table 9. To increase the level of confidence of identification (Schymanski et al., 2014), MS2 fragmentation spectra of the parent compound matches were searched against mzCloud. Compounds with mzCloud scores >90 showed good spectral matching between experimental and theoretical spectra, and were categorized as level 2/3 identification. Next, the non-target data was screened against the in-house generated suspect list of known and predicted TPs of the spike-in parent compounds. Thereby, 130 suspects (81 pos, 49 neg) could tentatively be identified based on their accurate mass. The list of TP matches can be found in suspectsMatched.xlsx.

TABLE 8. OVERVIEW OF FEATURE NUMBERS, DETECTED PARENT COMPOUNDS AND TRANSFORMATION PRODUCTS

	Positive ionization mode	Negative ionization mode
Number features Compound Discoverer	2821	1180
Retention time cut-off >2min	1002	366
Without background compounds	927	310
Parent compound matches	20	6
Transformation product suspect matches	81	49
Unmatched features	826	255

TABLE 9. LIST OF PARENT COMPOUNDS DETECTED

Parent compound	Molecular weight	RT [min]	mzCloud Score	Parent compound screening	MS2	ionization mode
Hexamethylenetetramine	140.10591	2.114	91.8	Single match	yes	+
*Melamine**	126.06512	2.118	79.2	Single match	yes	+
Metformin	129.10115	2.19	84.8	Single match	yes	+
Barbital	184.0845	2.287	*mzCloud Best Sim. Match:71	Single match	yes	+
*Melamine**	*126.06513*	*2.292*	*No result*	Single match	*yes*	*+*
Phenylamine	93.05769	2.335	*No result*	Single match	no	+
Carbendazim	191.06903	6.318	97.5	Single match	yes	+
Gabapentin	171.12554	6.365	91.7	Single match	yes	+
Tetraglyme	222.14633	7.799	97.1	Single match	yes	+
Benzotriazole	119.04804	7.974	88.3	Single match	yes	+
Tramadol	263.18801	9.36	99	Single match	yes	+
4-Methylbenzotriazole	133.06365	10.009	77.7	Multiple matches	yes	+
4-Methylbenzotriazole	133.06365	10.124	73.1	Multiple matches	yes	+
Propranolol	259.15676	11.831	96.9	Single match	yes	+
Carbamazepine	236.09449	13.289	99.8	Single match	yes	+
Tiamulin	493.32176	13.784	97	Single match	yes	+
Triphenylphosphine oxide	278.0855	15.389	94.3	Single match	yes	+
Dimethomorph	387.12312	16.212	90.4	Single match	yes	+

Parent compound	Molecular weight	RT [min]	mzCloud Score	Parent compound screening	MS2	ionization mode
Dimethomorph	387.12306	16.597	95.7	Single match	yes	+
Terbuthylazine	229.10905	16.906	92.3	Single match	yes	+
Acesulfame	162.99389	3.312	95.1	Single match	yes	-
Hydrochlorothiazide	296.96442	7.181	86.8	Single match	yes	-
Barbital	184.08468	8.023	*No result*	Single match	yes	-
Sucralose	396.01462	8.158	*No result*	Single match	yes	-
Phenobarbital	232.08474	11.386	*No result*	Single match	yes	-
Furosemide	330.00768	13.411	91.9	Single match	yes	-

*In the case of melamine, RP LC does not allow for good peak shape: melamine is split in two peaks of the same accurate mass, but two different retention times, potentially due to tautomerism (Klotz and Askounis, 1947).

Despite tailored suspect lists, over 1000 features remained unmatched in the non-target data. These features included compounds that had not been spiked-in, but were present in the source water, and TPs thereof, as well as TPs of the parent compounds that had not been included in the suspect list used for screening. Figure 4 illustrates the contribution of features representing parent compounds, TP matches and unknown compounds, to the overall feature intensities across samples. Suspect screening matches, both parent compound and TP matches together, account for roughly 85% of the signal intensity. However, regarding the feature numbers, the unknown features account for 90% of the total feature number. This confirms, that a prioritization strategy based on tailored suspect lists can effectively reduce feature numbers. Nevertheless, as the feature intensity does not necessarily reflect the concentration of a compound in a sample (Sjerps et al., 2016), it can still be relevant to consider low intensity features when comprehensively assessing water quality.

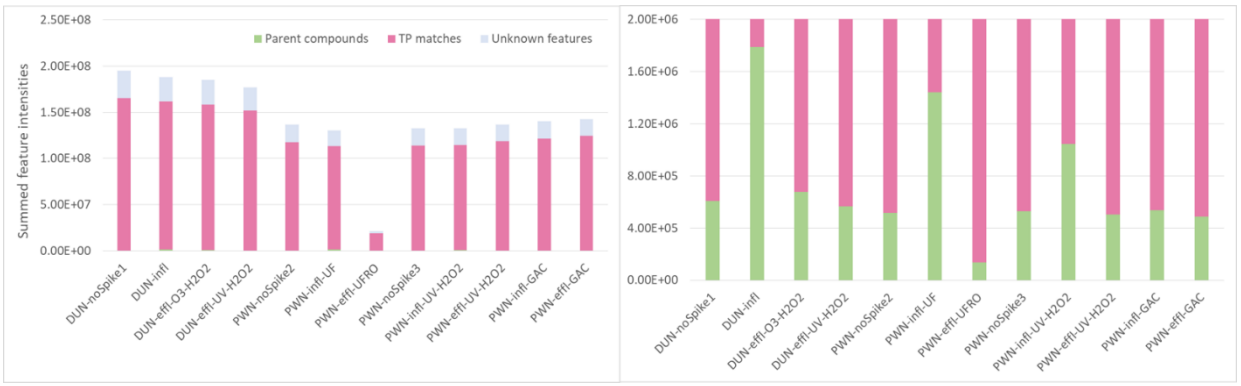


FIGURE 4 SUMMED INTENSITIES OF FEATURES REPRESENTING PARENT COMPOUNDS, TP MATCHES, AND UNKNOWN COMPOUNDS (LEFT). ZOOM-IN ON LOWER INTENSITIES (RIGHT).

2.3.2 PCA and HC to compare drinking water treatment trains

To utilize all the information from non-target data for water quality assessment during the different treatment steps, also without identification of unknown features, data science methods were applied to the data set.

First, data complexity was reduced through Principal Component Analysis (PCA). This allowed visualization of which samples were similar in terms of feature intensities (Figure 5). The first dimension (Dim1) explained 31% (pos) and 25% (neg) of the variation in the data and seemed to represent the total feature intensity, with an increase in intensity going from left to right. Blank and UF-RO samples showing lowest intensities, clustered together on the left, PWN samples at the x-axis, and Dunea samples that had the highest overall response on the right. The PCA thereby showed that UF-RO removed most compounds. The second dimension (Dim2), which explained 12% (pos) and 14% (neg) of the variation clearly separated Dunea samples (red tint) from PWN samples (blue-green tint), both in positive and in negative ionization mode. The source water affected the clustering more than the spike-in compounds, which can be explained by the fact that the unknown features account for 90% of the total feature number observed described above (see 2.3.1).

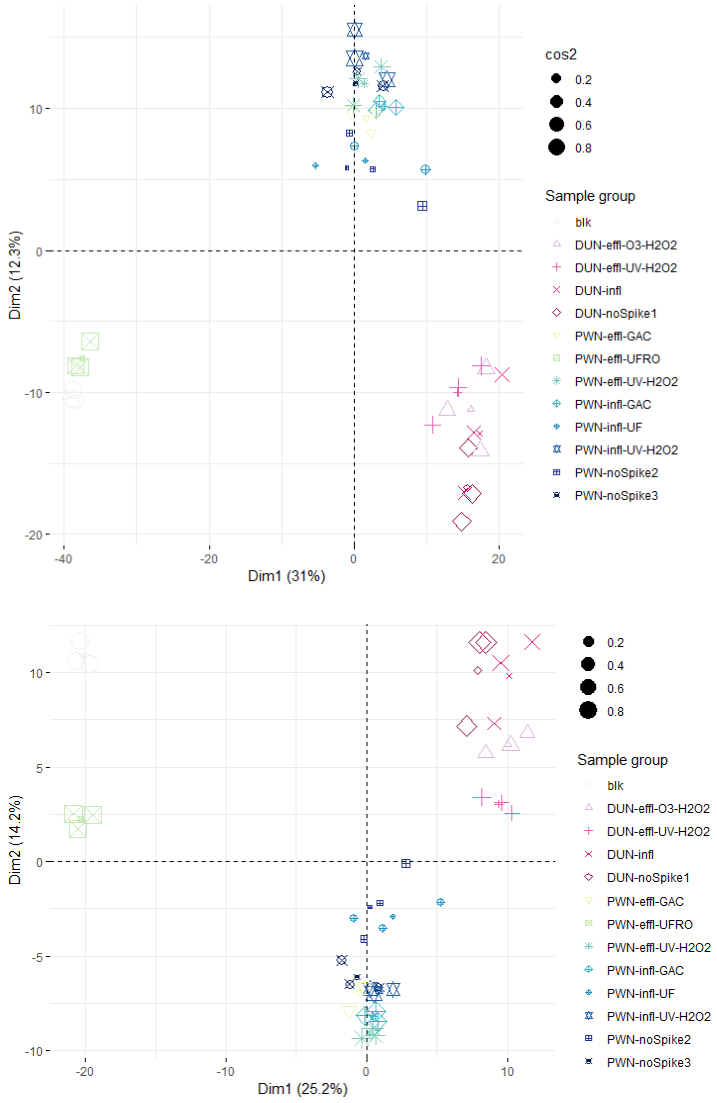
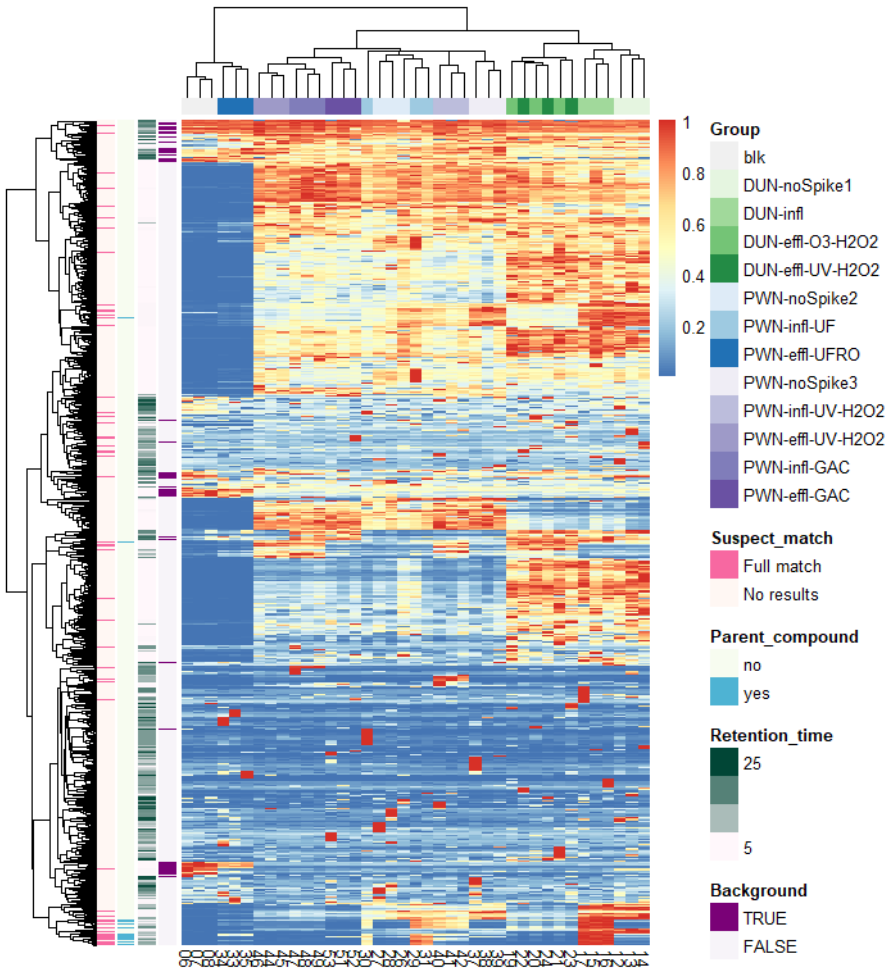


FIGURE 5 PCA PLOT OF FEATURES DETECTED IN POSITIVE (UPPER PANEL) AND NEGATIVE (LOWER PANEL) IONIZATION MODE. SQUARED COSINE OF THE OBSERVATION (COS2) SHOWS THE IMPORTANCE OF A COMPONENT FOR A GIVEN OBSERVATION.

To evaluate whether changes in feature intensities could be related to treatment steps, HC was performed based on Euclidean distances and the complete method and visualized in the heat map shown in Figure 6. Here, samples are clustered horizontally and features vertically. Similarly to the PCA, this visualization reveals which samples are more alike. In the heat map visualization, normalized feature areas are represented in color ranging from blue to red (the most intense feature). Additional feature information is displayed in the columns on the left. From left to right the following is indicated: suspect matches in pink, parent compound matches in turquoise, retention time (early in light grey to late in dark grey) and presence in the background in purple. From this visualization it becomes clear that features that represent parent compounds cluster together. Because the parent compounds are also included in the suspect list, these are shown in both columns.



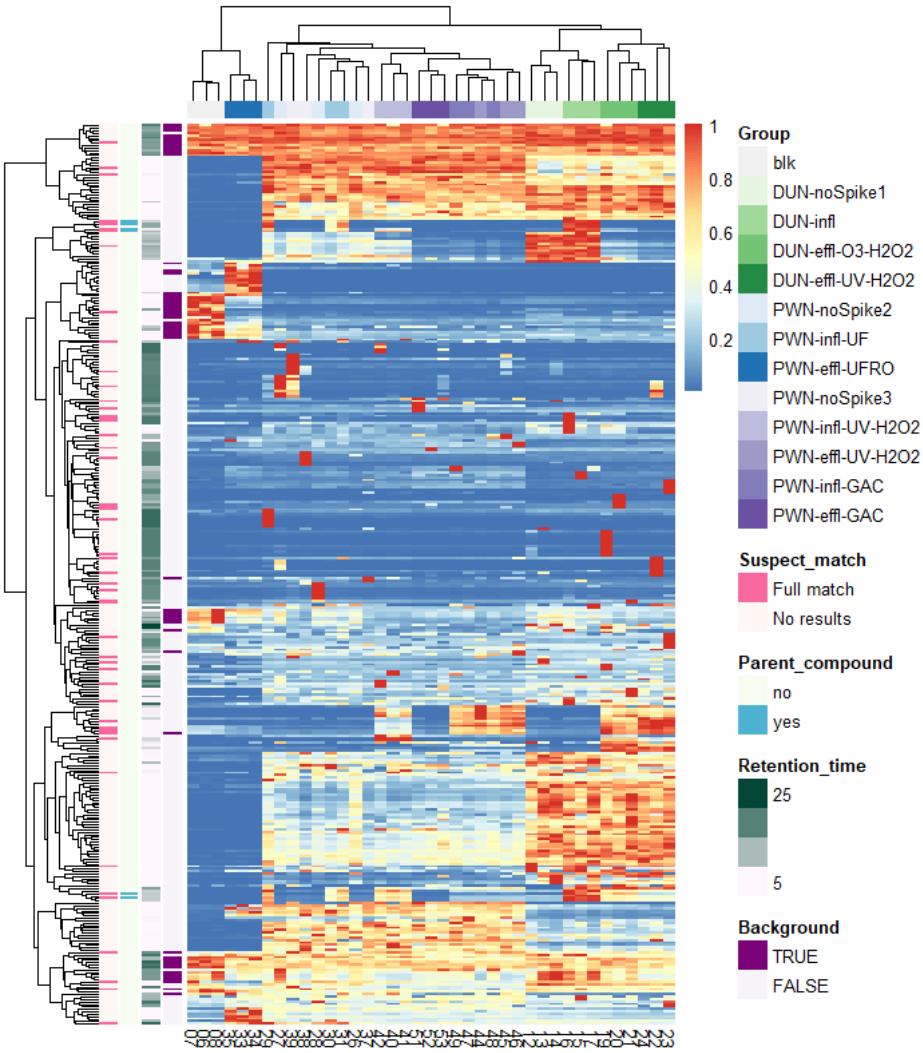


FIGURE 6 HIERARCHICAL CLUSTERING OF FEATURE AND SAMPLES IN POSITIVE (UPPER PANEL) AND NEGATIVE (LOWER PANEL) IONIZATION MODE. EUCLIDEAN DISTANCE, COMPLETE METHOD, MAX NORMALIZED

2.3.3 Treatment train specific analyses per pilot installation

To assess water quality changes due to a specific drinking water treatment train in more detail, the non-target data of the 3 different pilot installations, i.e. (1) sequential O_3 / H_2O_2 - UV / H_2O_2 of rapid sand filtrate at Dunea, (2) UF-RO and (3) sequential UV / H_2O_2 GAC filtration from surface water at PWN, were analysed individually. In these analyses, background subtraction was performed again; Only features that exceeded 5 times the intensity of the blank in a given sample were considered in all subsequent analyses, with exception of the HC where features needed to exceed 5 times the blank intensity in at least one of the samples of the treatment train. The resulting numbers of features detected in the samples, summed feature intensities and parent compound and TP matches are listed in Table 10 for positive ionization mode and Table 11 for negative ionization mode. Lists of detected parent compounds and suspect screening matches can be found in the respective .xlsx files.

TABLE 10. FEATURE NUMBERS, INTENSITIES, PARENT COMPOUND AND TP MATCHES PER TREATMENT TRAIN. POSITIVE IONIZATION MODE DATA. SUMMED FEATURE INTENSITIES ARE ADDED GROUP AREAS.

Dunea	Influent no spike	Influent spike-in	Effluent O3/H2O2	Effluent UV/H2O2	Blank	
Feature number	548	561	553	554		
Summed feature intensities	3.38E+07	3.56E+07	3.59E+07	3.59E+07	2.08E+05	
Parent compound matches	16	20	15	15		
Suspect matches	43	48	43	40		

PWN UF-RO	Influent no spike	Influent spike-in UF-RO	Effluent UF-RO	Blank		
Feature number	533	538	73			
Summed feature intensities	2.68E+07	2.57E+07	5.55E+05	1.63E+05		
Parent compound matches	14	20	11			
Suspect matches	39	48	13			

PWN UV/H2O2 - GAC	Influent no spike	Influent spike-in UV/H2O2	Effluent O3/H2O2	Influent GAC	Effluent GAC	Blank
Feature number	532	554	546	557	522	
Summed feature intensities	2.50E+07	2.62E+07	2.71E+07	2.92E+07	2.74E+07	2.18E+05
Parent compound matches	16	19	15	17	6	
Suspect matches	41	50	41	44	29	

TABLE 11. FEATURE NUMBERS, INTENSITIES, PARENT COMPOUND AND TP MATCHES PER TREATMENT TRAIN. NEGATIVE IONIZATION MODE DATA. SUMMED FEATURE INTENSITIES ARE ADDED GROUP AREAS.

Dunea O3/H2O2 – UV/H2O2	Influent no spike	Influent spike-in	Effluent O3/H2O2	Effluent UV/H2O2	Blank	
Feature number	138	147	149	142		
Summed feature intensities	1.51E+07	1.89E+07	1.90E+07	1.50E+07	7.88E+05	
Parent compound matches	2	6	5	5		
Suspect matches	8	13	13	13		

PWN UF-RO	Influent no spike	Influent spike-in UF-RO	Effluent UF-RO	Blank		
Feature number	134	150	34			
Summed feature intensities	1.22E+07	1.30E+07	2.52E+06	8.32E+05		
Parent compound matches	2	6	4			
Suspect matches	7	12	5			

PWN UV/H2O2 - GAC	Influent no spike	Influent spike-in UV/H2O2	Effluent UV/H2O2	Influent GAC	Effluent GAC	Blank
Feature number	146	155	155	153	141	
Summed feature intensities	1.02E+07	1.35E+07	1.21E+07	1.17E+07	1.08E+07	7.34E+05
Parent compound matches	2	5	4	4	1	
Suspect matches	7	12	12	11	7	

Next, HC based on Pearson correlations between the features using the Ward.D2 method was performed separately on the three datasets to reveal trend profiles of features related to treatment steps, i.e. clusters of features that decrease and increase in intensity. These clusters could represent parent compounds and their transformation products and facilitate prioritization for identification.

2.3.4 Sequential advanced oxidation processes at the DUNEA pilot installation

The sequential O_3/H_2O_2 - UV/H_2O_2 pilot installation at Dunea provided three samples for clustering analyses, the spike-in influent (DUN-infl), the effluent from the O_3/H_2O_2 treatment (DUN-effl-O3-H2O2) and the effluent from the $UV\text{-}H_2O_2$ treatment (DUN-effl-UV-H2O2). Consequently, a maximum of 9 clusters could describe the generated trend profiles:

$$Max.number\ of\ clusters\ Dunea = 3^{(3-1)} = 9$$

The resulting heat map with 9 defined clusters for data recorded in positive ionization mode is shown in Figure 7. As expected, the parent compounds clustered together, predominantly in cluster 1. This cluster contained parent compounds and other compounds that were removed or transformed by the ozone treatment. Interestingly, this cluster also exhibited many suspect matches, i.e. potential TPs. This could mean that the parent compounds were already transformed in the influent or present in the source water. Cluster 1 thus contained compounds that were removed or transformed by ozone, in contrast to clusters 7, 3, 2 and to a lesser extent 6 which included substances that appeared to be generated by ozone treatment. (Note: cluster numbers are merely an aid for communication and do not have any significance.) Cluster 2 included the spike-in compound melamine.

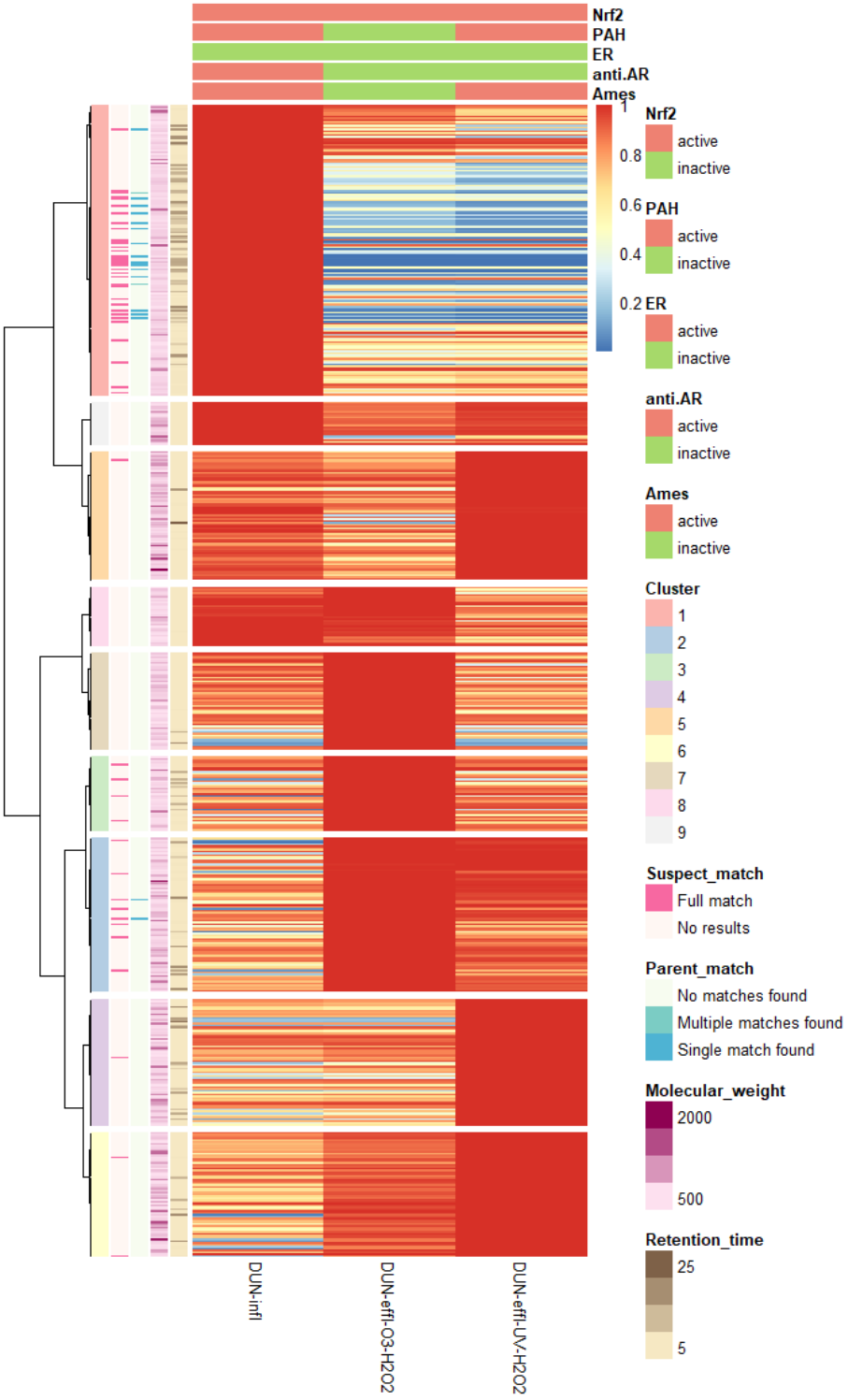


FIGURE 7 HIERARCHICAL CLUSTERING OF DUNEA FEATURES DETECTED IN POSITIVE IONIZATION MODE BASED ON PEARSON CORRELATION USING THE WARD.D2 METHOD AND MAX NORMALIZED FEATURE INTENSITIES.

Figure 8 shows the trend profiles of the parent compounds detected in the positive ionization data. It is apparent that melamine showed an increase in signal intensity through ozone treatment in contrast to all other spike-in compounds showing a decrease.

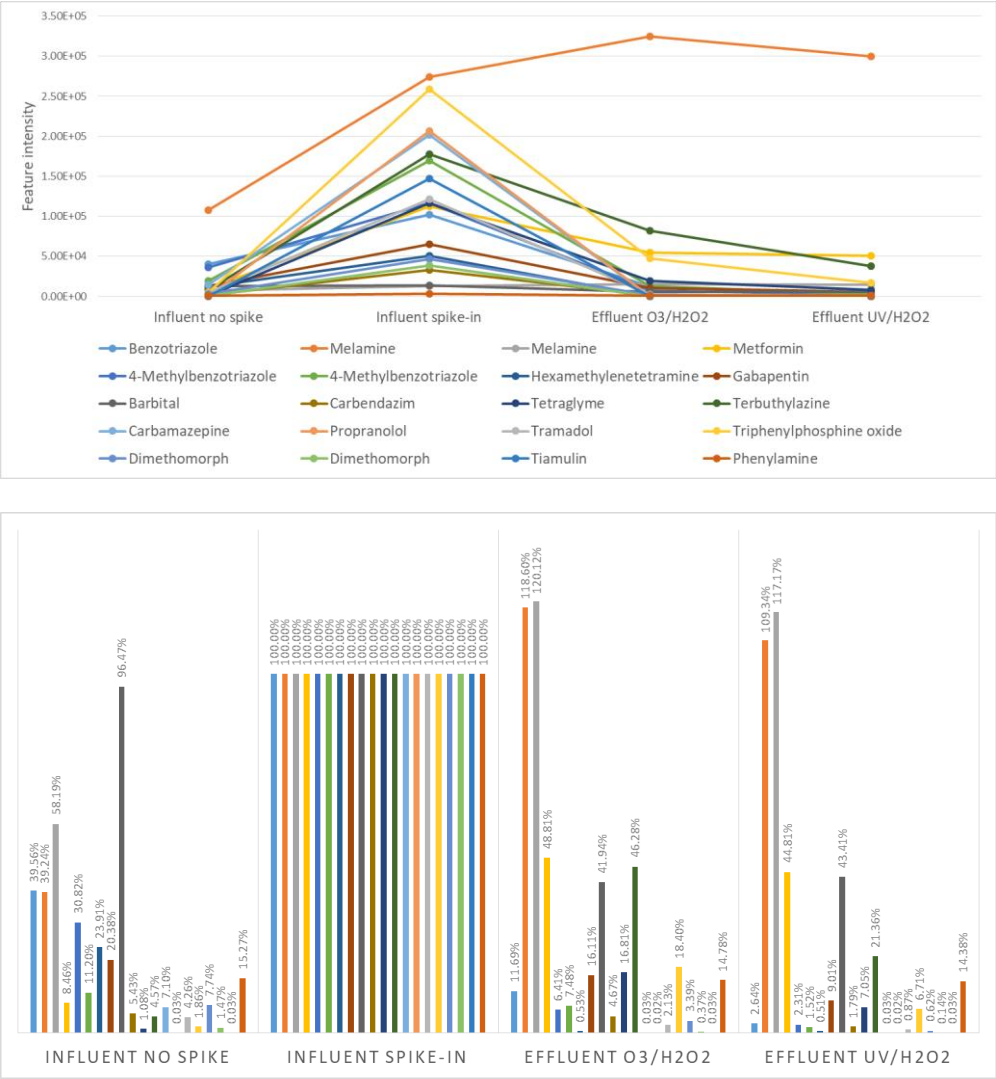


FIGURE 8. TREND PROFILES OF SPIKE-IN PARENT COMPOUNDS IN ABSOLUTE VALUES (UPPER PANEL) AND RELATIVE TO THE SPIKE-IN SAMPLES (LOWER PANEL). POSITIVE IONIZATION DATA.

Compounds in clusters 3, 7 and 8 were removed or transformed by UV/H_2O_2 in the second treatment step. Cluster 8 grouped compounds that were present in the influent already, and persistent against ozonation, but removed or transformed with UV treatment, while compounds in cluster 3 and 7 were generated by ozonation. Cluster 4 contained compounds that were formed by UV/H_2O_2 treatment. Cluster 2 and 6 contained compounds that were generated by ozone treatment and persistent against UV/H_2O_2 treatment. Clusters 5 and 9 included compounds that decreased by ozone treatment, but increased again after UV/H_2O_2 treatment. Furthermore, the addition of the bioassay responses to the non-target screening data showed that anti-AR CALUX response seemed to correlate with the feature intensity profiles of cluster 1, and PAH CALUX and Ames test responses with clusters 5 and 9. PAHs activity and mutagenicity were no longer observed after the O_3/H_2O_2 treatment step, but reappear after UV/H_2O_2 treatment. Oxidative stress was observed across samples from all treatment steps. This could be due to organic micro-pollutants, but also residual oxidants from the UV/H_2O_2 and or O_3/H_2O_2 treatment. However, the binary bioassay read-out used here might lead to an oversimplified picture.

In the negative ionization data, parent compounds clustered together similarly to the positive ionization data, illustrated in Figure 9. They were found mainly in cluster 3 which contained

parent compounds and other compounds that were removed through the ozone treatment. Cluster 1 contained compounds that were not affected significantly by ozone treatment, but removed or transformed by UV/H2O2. Clusters 2, 8 and 9 included ozonation transformation products, those in clusters 8 and 9 were removed by UV, while those in cluster 2 were persistent. Clusters 5 and 7 contained UV transformation products.

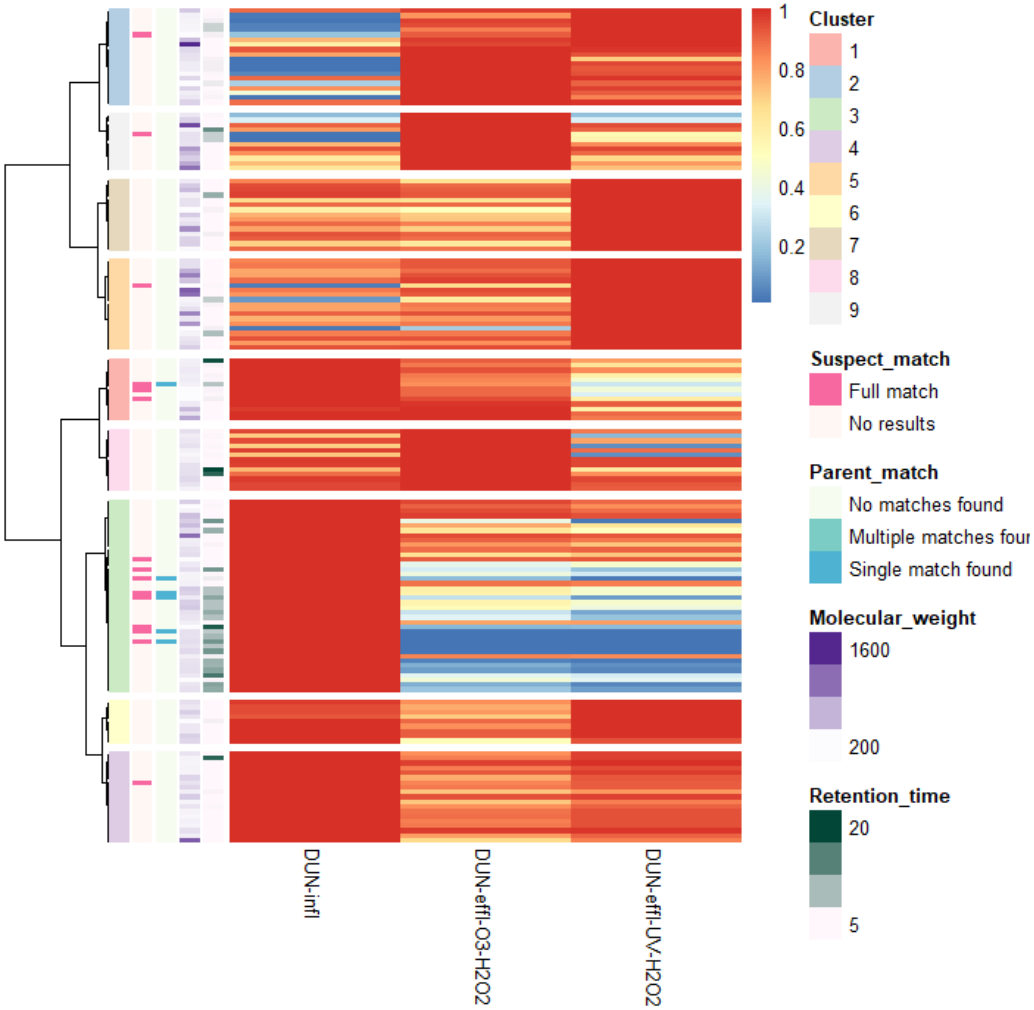


FIGURE 9 HIERARCHICAL CLUSTERING OF DUNEA FEATURES DETECTED IN NEGATIVE IONIZATION MODE BASED ON PEARSON CORRELATION USING THE WARD.D2 METHOD AND MAX NORMALIZED FEATURE INTENSITIES.

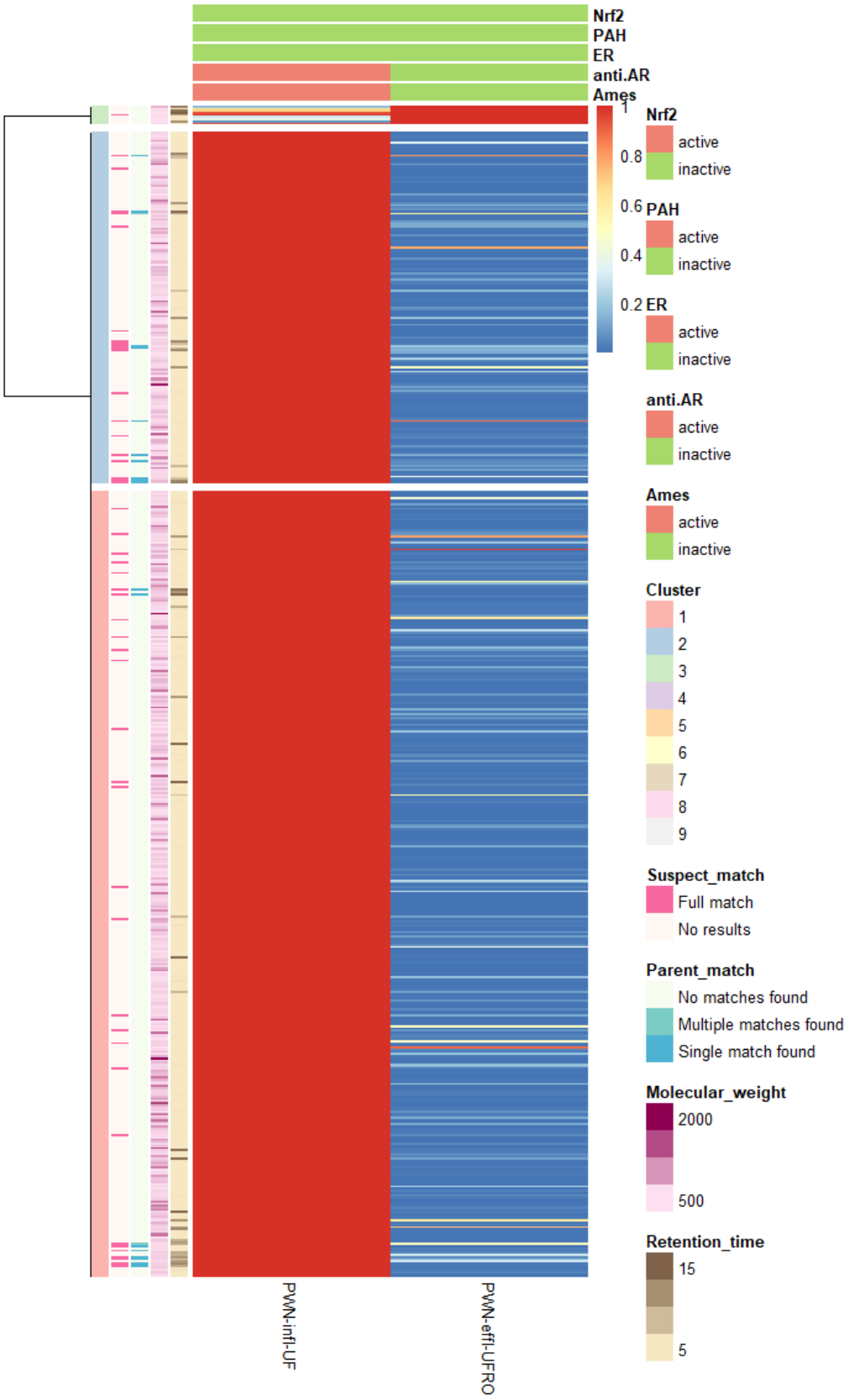
Ultimately, the HC heat maps of the sequential O_3/H_2O_2 - UV/H_2O_2 pilot installation at Dunea could be used for the prioritization of features for identification. For instance, first identification efforts could focus on clusters 2, 4 and 6 and 5 and 9 (pos), and 2, 4 and 6, and 5 and 7 (neg), and thereby on compounds that were present after the last treatment step. As the intensities in the heat maps were normalized, they do not represent the intensity of a feature in the sample. For better prioritization feature intensities could also be considered.

2.3.5 Combination of UF and RO removes most compounds

For the PWN pilot installation data, two separate HC analyses were performed, namely for the PWN-UFRO and PWN-UV-H2O2-GAC treatment trains. PWN-UFRO included the two sample

groups PWN-infl-UF and PWN-effl-UFRO, which are the influent and effluent of the UF-RO pilot installation. This leads to a maximum number of clusters of 3:

$$Max.number\ clusters\ PWN-UFRO = 3^{(2-1)} = 3$$


Nrf2
PAH
ER
anti.AR
Ames
1
0.8
0.6
0.4
0.2
Nrf2
active
inactive
PAH
active
inactive
ER
active
inactive
anti.AR
active
inactive
Ames
active
inactive
Cluster
1
2
3
4
5
6
7
8
9
Suspect_match
Full match
No results
Parent_match
No matches found
Multiple matches found
Single match found
Molecular_weight
2000
500
Retention_time
15
5
PWN-infl-UF
PWN-effl-UFRO

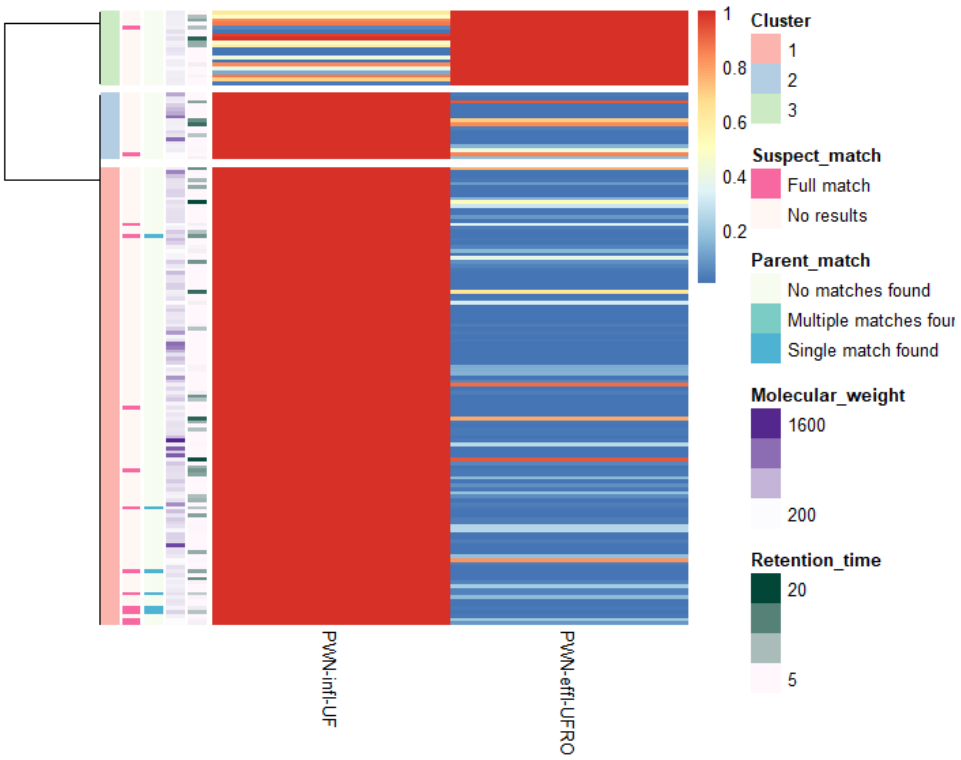


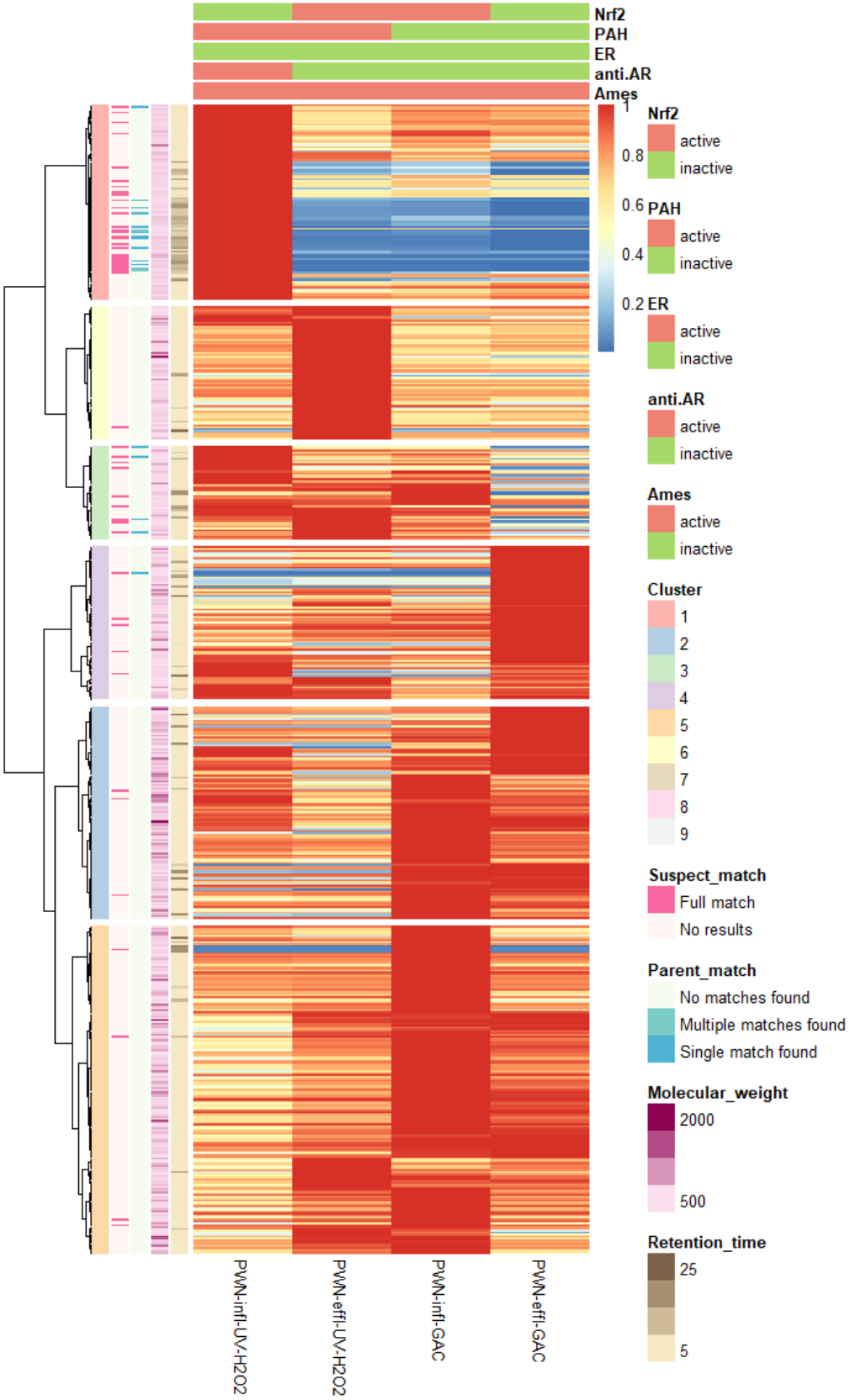
FIGURE 10 HIERARCHICAL CLUSTERING OF PWN-UFRO FEATURES DETECTED IN POSITIVE (UPPER PANEL) AND NEGATIVE (LOWER PANEL) IONIZATION MODE BASED ON PEARSON CORRELATION USING THE WARD.D2 METHOD AND MAX NORMALIZED FEATURE INTENSITIES.

Figure 10 shows the HC derived heat maps. As expected, the parent compounds clustered together, in clusters 1 and 2. These clusters contained parent compounds and other compounds that were removed or transformed by the UF-RO treatment. In line with the observation in the Dunea pilot installation, these clusters also included many features matched to suspects from the TP list. This could mean that the parent compounds were already present in the source water, or transformed in the influent. In contrast to clusters 1 and 2, cluster 3 contained compounds that seemed to be formed by UF-RO treatment. However, these compounds did not result in an active response in any of the bioassays tested. A further identification of the formed compounds might thus not be critical, presuming that the selected bioassays cover the most relevant toxicological endpoints.

2.3.6 PWN-UV-GAC

The second PWN pilot installation combined UV/H2O2 and GAC. The HC contained the sample groups PWN-infl-UV-H2O2, PWN-effl-UV-H2O2, PWN-infl-GAC, and PWN-effl-GAC, i.e. influent and effluent from the UV- H2O2 treatment, and influent and effluent from the GAC treatment step. This lead to a maximum number of clusters of 27:

$$Max.number\ clusters\ PWN-UV-H2O2-GAC = 3^{(4-1)} = 27$$


Nrf2
PAH
ER
anti.AR
Ames
1
0.8
0.6
0.4
0.2
Nrf2
active
inactive
PAH
active
inactive
ER
active
inactive
anti.AR
active
inactive
Ames
active
inactive
Cluster
1
2
3
4
5
6
7
8
9
Suspect_match
Full match
No results
Parent_match
No matches found
Multiple matches found
Single match found
Molecular_weight
2000
500
Retention_time
25
5
PWN-infl-UV-H2O2
PWN-effl-UV-H2O2
PWN-infl-GAC
PWN-effl-GAC

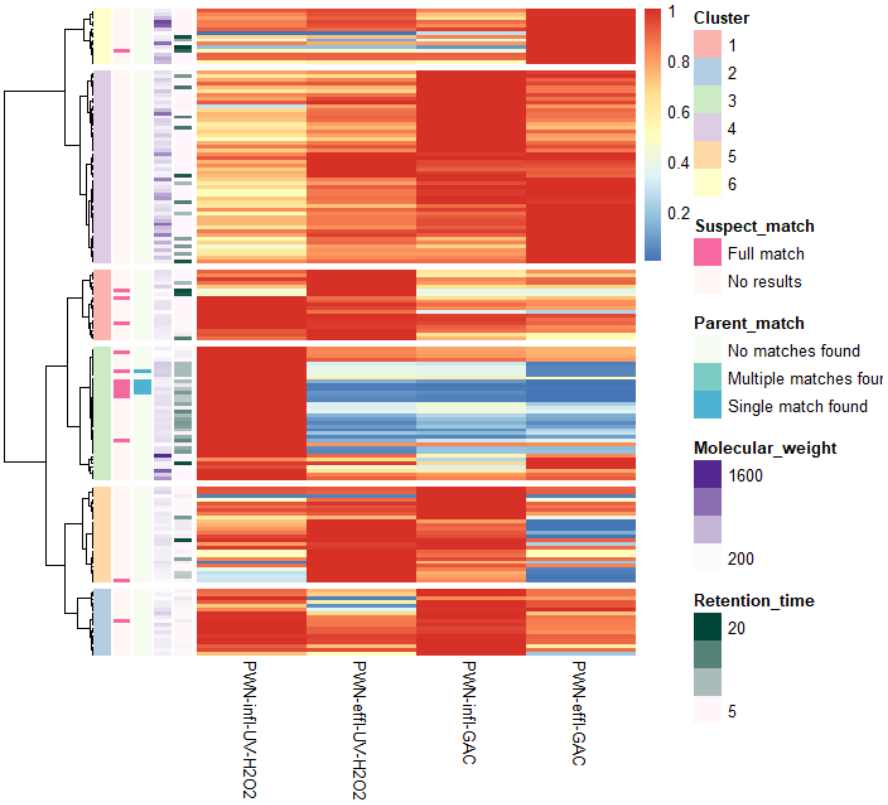


FIGURE 11 HIERARCHICAL CLUSTERING OF PWN-UV-GAC FEATURES DETECTED IN POSITIVE (UPPER PANEL) AND NEGATIVE (LOWER PANEL) IONIZATION MODE BASED ON PEARSON CORRELATION USING THE WARD.D2 METHOD AND MAX NORMALIZED FEATURE INTENSITIES. 6 CLUSTERS.

However, the 27 clusters (see SI HC PWN-UV-GAC5.2) could be grouped into 6 larger clusters for a clearer view, shown in Figure 11. Regarding the positive ionization mode, cluster 1 contained parent compounds and other compounds that decreased due to the treatment, cluster 6 UV/H2O2 transformation products, and clusters 2 and 4 transformation products that originated in the storage vessel before the GAC and during the GAC. Cluster 3 contained compounds that were persistent in the UV / H2O2 treatment, but removed by GAC. Cluster 5 contained substances that increased continuously during the treatment steps, but were removed by GAC. Similar clusters could be distinguished in the negative ionization data, with parent compounds detected in cluster 3. Interestingly, the features that were removed most, i.e. cluster 1 (pos) and cluster 3 (neg) exhibited later retention times than the other features, including those of the respective clusters, indicating that the polarity of a compound affects the UV removal rates.

PAH activity was persistent through UV treatment, but seemed to be removed by the storage step prior to GAC filtration. NRf2 activity was induced by UV treatment and removed by GAC. Mutagenicity was observed across all samples and treatment steps.

2.4 Conclusions and outlook

Non-target screening provides information on water quality and changes thereof due to water treatment, exceeding the information gained by the current monitoring of target compounds alone. The data science methods used here, PCA and hierarchical clustering, demonstrated differences between samples and treatment steps in an efficient and unbiased manner. The visualization through heat maps helped to create a clear picture of highly complex data, in

particular when HC of non-target screening features was related to bioassay readouts. Based on the combined heat maps, features could then be prioritized for identification. Here, the matched suspects could be a good starting point. Moreover, the comparative assessment of treatments with bioassay readouts can indicate a risk to environmental or human health, and/or water treatment problems. In the absence of an active bioassay readout, further identification of unknowns can be renounced, thereby circumventing the challenges in identifying unknown unknowns.

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3 Integration of chemical screening and effect-based bioanalysis to globally asses chemical water quality for water reuse

3.1 Introduction

3.1.1 Water quality assessment

Water quality assessment requires assessment of potential contamination of sources of the water, assessment of treatment efficiency for this (potential) contamination, and assessment of treated water, also in relation to the intended end use. Depending on the end use, water quality must comply with the predefined quality standards for treated wastewater, reuse for irrigation or other purposes and drinking water production demanded by legislation and listed in the European Wastewater Directive, the European Drinking Water Directive, the Groundwater Directive, etc. (European Commission, 1991, 1998, 2000, 2003b, a, 2006b, a; Gawlik and Bidoglio, 2006; European Commission, 2008, 2010, 2015). However, more parameters than those required by legislation might be relevant for a comprehensive water quality - and ensuing, risk assessment. Finally, the time needed to determine the regulated parameters can prevent timely action. To address these shortcomings, innovative, sensitive and fast detection methods have been developed over the past decades to improve monitoring for water quality assessment.

3.1.2 Innovative, fast and integrated approaches

These methods need to be fast as residence times of water in the treatment steps can be short, and up-to-date information is required for timely halt or adaption of treatment. They also need to be sensitive as water quality criteria can be set at low concentrations. Furthermore, the chemical water quality is determined by a plethora of chemicals. Monitoring of individual chemicals does not comprehensively reflect water quality. Therefore, integrative approaches need to enable a more complete water quality assessment.

Effect-based biological and non-target screening based chemical water quality assessment are two complementary methods that together can provide a better and more problem oriented monitoring, in line with the Hazard Analysis and Critical Control Points (HACCP) principle (Dewettinck et al., 2001), the Revision of Annex II of the Drinking water Directive (European Commission, 2015) and upcoming revisions of the regulation on wastewater and reuse.

3.1.3 Effect- based water quality assessment using CALUX bioassays

The Chemical Activated LUciferase eXpression (CALUX) bioassays enable the monitoring of certain biological effects of complex mixtures of chemicals in a water sample. These assays comprise cells that incorporate the firefly luciferase gene coupled to Responsive Elements (REs) as a reporter gene for the presence of compounds activating these REs (Murk et al., 1996a; Sonneveld et al., 2005b; Van der Linden et al., 2008; Pieterse et al., 2013b; Van der Burg et al., 2013; Van der Linden et al., 2014). Cells that are exposed to compounds of interest not only express proteins that are under normal circumstances associated to RE, but also

luciferase. By addition of the appropriate substrate for luciferase, light is emitted. The amount of light is proportional to the amount of ligand-specific receptor binding.

Through a suite of bioassays, specific to generic effect endpoints can be assessed, and an array of chemicals and outputs can be linked to biological effects (Oulton et al., 2010). However, the selection of (various) bioassays is crucial to cover relevant endpoints. Moreover, effect based trigger values of such bioassays, i.e. thresholds that differentiate between acceptable and poor water quality with respect to the organic micro-pollutants are often lacking (Brack et al., 2016; Di Paolo et al., 2016; Wieczerzak et al., 2016).

3.1.4 Comprehensive chemical water quality assessment using LC-HRMS non-target screening

The combined output of CALUX assays provides an integrated assessment of the chemical water quality. However, it does not reveal the individual chemicals responsible for induced or reduced assay responses. These can be due to multiple chemicals. To relate responses to (mixtures of) chemicals present in the samples, as well as to assess chemical water quality and its changes through treatment steps with a complementary method, non-target screening based on liquid chromatography coupled to high resolution mass spectrometry (LC-HRMS) can be performed to detect chemicals in water samples (Hollender et al., 2017). However, the wealth of data resulting from non-target screening renders structural identification of all compounds virtually impossible. Consequently, a prioritisation step needs to be performed to define which of the unknown compounds need to be identified first. This can happen on different levels: the abundance of an unknown feature in the sample, the matching of a feature with a suspect list entry, the trend profile of a feature's intensity across treatment steps and/or its correlation with a bioassay response. A feature represents a given compound and consists of a unique combination of an accurate mass and a retention time. Without identifying the feature, information on its response –measured in instrument counts (Sjerps et al., 2016) or response relative to an internal standard (Parry and Young, 2016) can be automatically extracted. Through suspect screening against a suspect list potential candidates that match a feature based on their accurate mass can be found and ranked according to their occurrence or toxicity (Brunner et al., 2019a). As *in vivo* toxicity data is limited, *in vitro* bioassay data can be used as a proxy, such as the ToxCast database that includes high throughput *in vitro* toxicity information of > 8000 environmentally relevant compounds and >1500 bioassays (Schroeder et al., 2016). To more comprehensively assess changes in water quality, the trend profiles of feature intensities across treatment steps can be considered through application of data science methods that reveal patterns in the data. These profiles allow distinction between persistence, elimination and formation of a feature during treatments and prioritisation based thereupon (Schollée et al., 2016). Ultimately, trend profiles can be integrated with the bioassay read out profiles resulting in a fit for purpose method to monitor water quality in samples and across treatment steps.

3.1.5 Effect-based and non-target screening at Berlin Schoenerlinde

Here, we performed non-target screening analyses on the extracted and not extracted water samples from Berlin Schoenerlinde (AquaNES site 12) from April and July 2018 in technical triplicates, using an Orbitrap Fusion mass spectrometer (Thermo Scientific). Detected features were matched against the Water Framework Directive priority list and the SusDat database of the European Network of reference laboratories, research centres and related organisations for monitoring of emerging environmental substances (NORMAN) consisting of more than 40000 chemicals relevant for environmental monitoring. A novel data analysis workflow was applied to efficiently interpret the wealth of data generated that combined the points mentioned above, including integration of the biological and chemical monitoring data and relate/compare results to applied bioassays on the same samples. The primary sedimentation

effluent was excluded from the data analysis as the amount of features detected exceeded the processing power of the available IT infrastructure.

Overall, the application of these tools within the demonstration site Schoenerlinde illustrated the potential for application in water treatment and its technology readiness level. Additionally it enabled to evaluate treatment efficiency of the innovative treatment schemes. It is thereby a step towards the application of such tools in water quality assessment in a regulatory setting.

3.2 Material and Methods

3.2.1 Sampling points Berlin Schoenerlinde

An overview of the Berlin Schoenerlinde innovative waste water treatment plant including sampling points is depicted in Figure 12. Samples from 7 sampling points were used for the bioassay and NTS analyses with the following sample names: Primary sedimentation effluent, ozonation influent (secondary effluent, S1 in Figure 12), ozonation effluent (S2), constructed wetlands (S3), sand BAC filter (S6), sand anthracite filter (S7) and post GAC (S8). Sampling points S4 and S5 were not included in this study. As can be seen from the overview schematics, the first three sampling points are consecutive, constructed wetland treatment, sand BAC and sand anthracite filtration are performed in parallel, and GAC filtration follows sand anthracite filtration.

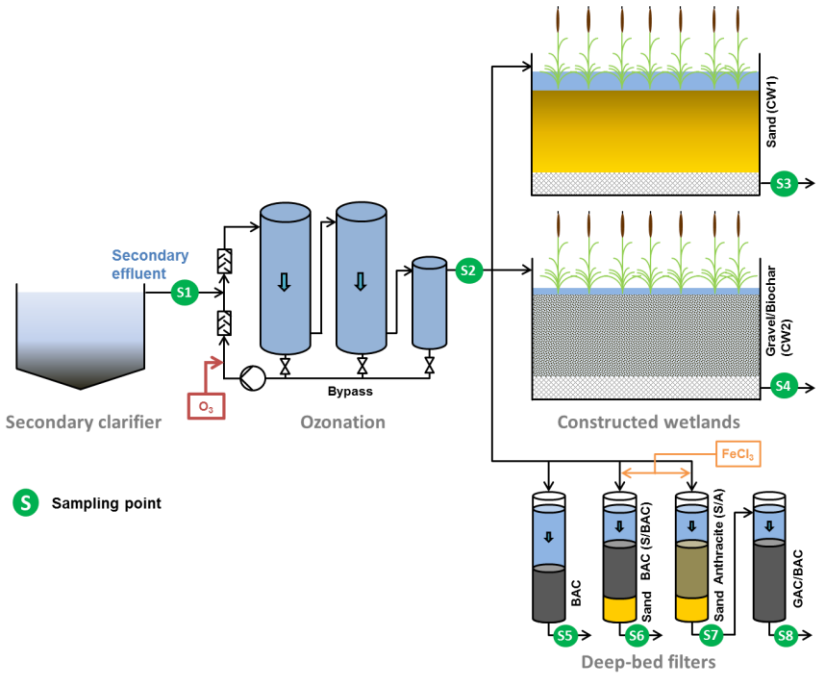

FIGURE 12. SCHEMATICS OF TREATMENT SITE BERLIN SCHOENERLINDE.

3.2.2 Chemicals

Acetonitrile (ACN, HPLC grade) was purchased from Avantor Performance Materials B.V. (Deventer, NL), formic acid (FA) from Fluka Analytical (Sigma-Aldrich, Steinheim, D), the internal standards atrazine-d5 and bentazon-d6 from CDN isotopes (Pointe-Claire, Canada) and LGC Standards (Wesen, Germany), respectively. The ultrapure water used as a blank reference was produced with an Elga Purelab Chorus ultrapure water system through purification of demineralized water in (High Wycombe, UK).

3.2.3 Sample processing

Sample processing and CALUX bioassays were performed by BioDetectionSystems (BDS, , Amsterdam, The Netherlands. Water samples were extracted by Solid Phase Extraction (SPE) according to BDS protocol p-bds-096. In short, SPE columns (OASIS HLB SPE cartridges, 500 mg, 6 cc, Waters 186000115) were loaded with approximately 500 mL of water and eluted with 10 ml of methanol followed by 10 ml of acetonitrile. Eluates were pooled and evaporated under a gentle stream of nitrogen. The final extracts were re-dissolved in 150 µl of DMSO after which serial dilution in DMSO were prepared.

3.2.4 CALUX bioassays

Effect-based measurements were performed with the CALUX tests for cytotoxicity, androgenic (AR) and anti-androgenic (anti-AR) activity, estrogenic activity (ERa), glucocorticoid receptor-mediated signalling (GR), anti-progesterone receptor-mediated signalling (anti-PR), PPARα-mediated signalling(PPARa2), PPARγ-mediated signalling (PPARg2), PXR, activation of the Nrf2 pathway / oxidative stress response (Nrf2) and p53-dependent pathway activation / genotoxicity response (+/- S9) (P53) listed in Table 12 according to the supplier's protocols (Murk et al., 1996b; Sonneveld et al., 2005a; Pieterse et al., 2013a).

For CALUX activity determination, CALUX cells were seeded in 96 wells plates in assay medium. Following exposure of the CALUX cells to serial dilutions of the sample extracts in triplicate, luciferase production was induced through addition of the substrate luciferin and quantified by luminescence measurements. Per 96-well plate, calibration curves for each bioassay were analysed with the respective reference compounds.

Analysis results of the test samples were interpolated in the calibration curve for quantitative determination of (ant)agonistic potential of the test samples. Only not cytotoxic dilutions (relative induction in the cytotox CALUX bioassay > 80%) were used for the final evaluation of CALUX results. Final results were expressed as µg, ng or pg reference compound equivalents per L of processed water.

The bioassays were performed according to standard BDS protocols p-bds-083 (Culturing U2OS CALUX cells), p-bds-04 (Analysis of Ah-receptor mediated luciferase activity in DRCALUX cells), p-bds-066 (Analysis of luciferase activity in the PAH CALUX bioassay), p-bds-085 (Analysing samples with U2-OS CALUX bioassays using sigmoidal dose response curves (with 0.1% or 1% DMSO)), p-bds-070 (Harvesting the cells and measurement), and p-bds-084 (Calculating U2OS CALUX results using sigmoidal dose response curves).

TABLE 12. CALUX TESTS CONDITIONS AND CELL CULTURE INFORMATION.

Assay	(anti)ERα, (anti)AR, (anti)GR, (anti)PR, PPARα, PPARδ, PPARγ, PXR	Cytotox, Nrf2, P53 (+/-S9)	PAH, DR
Cell type	U2OS	U2OS	H4IIE
Species	Human	Human	Rat
Confluence	10000 cells per well	10000 cells per well	>95% confluence
Medium used	DMEM/F12	DMEM/F12	αMEM
Additions to assay	-Stripped FCS	-Stripped FCS	-FCS
medium	-Non essential amino acids	-Non essential amino acids	
%DMSO	0.1%	1%	0.8%

Exposure time	24 hrs	24 hrs	4 hrs (PAH), 24 hrs (DR)

FCS…Fetal Calf Serum

Based on effect –based trigger (EBT) values, the action rules listed in Table 13 were defined for the bioassay readouts. This color coding was also used for the integration of bioassay and non-target screening data (see below).

TABLE 13. ACTION RULES FOR BIOASSAY READ-OUTS

	If below EBT or LOQ of bioassay	no further action required
	If 1-times <EBT< 3-times	quality check data, continue to monitor every three months, until 1 year and the EBT < 1
	If 3-times <EBT< 10-times	data check, immediate re-sampling and analysis to confirm EBT. It is also required to quantify specific target compounds which are known to cause the effects observed in the respective bioassay. Continue to monitor every three months, until 1 year and the EBT< 1
	If 10-times <EBT< 100-times	all of the above plus enhance source identification program. Also monitoring in the distribution system closer to the point of exposure to confirm attenuation of CEC is occurring and to confirm the magnitude of assumed safety factors associated with removal efficiency, dilution and post-treatment.
	If EBT>100-times	all of the above plus immediately confer with the local environmental authority's to determine the required response action. Confirm plant corrective actions through additional monitoring that indicates the CEC levels are below at least an EBT of 100.

3.2.5 LC-HRMS experiments

LC-HRMS/MS experiments were performed using a Vanquish HPLC system (ThermoFisher Scientific) coupled to a Tribrid Orbitrap Fusion mass spectrometer (ThermoFisher Scientific, Bremen, Germany) with an electrospray ionization source. Chromatographic separation was performed using an XBridge BEH C18 XP column (150 mm × 2.1 mm I.D., particle size 2.5 µm) (Waters, Etten-Leur, The Netherlands) preceded by a 2.0 mm × 2.1 mm I.D. Phenomenex SecurityGuard Ultra column (Phenomenex, Torrance, USA) maintained at a temperature of 25 ˚C. The LC gradient went from 5% acetonitrile, 95% water and 0.05% formic acid (v/v/v) to 100% acetonitrile with 0.05% formic acid in 25 min, after which it was held constant for 4 min at a flow rate of 0.25 mL/min.

Prior to LC-HRMS analysis, the SPE extracted water samples (6667x concentrated compared to the non-extracted original water samples) were diluted 100x, resulting in 66.7x concentrated samples. The internal standards bentazone-d6, atrazine-d5 and benzotriazole-d4 were added to the water samples to a final concentration of 1ug/L. Subsequently, samples were filtered using Phenex™-RC 15mm Syringe Filters 0.2u (Phenomenex, Torrance, USA). 100 µL of each filtered sample was analysed in triplicate. Mass calibration was performed using Pierce ESI calibration solution. The vaporizer and capillary temperature were set to 300 ˚C, sheath,

auxiliary and sweep gas to arbitrary units of 40, 10 and 5, respectively. The source voltage was 3.0 kV in the positive mode. The RF lens was set to 50 %. Full scan high accuracy mass spectra were acquired in the range of 50-1000 m/z with 120,000 FWHM resolution. Quadruple isolation was used for acquisition. Data dependent MS/MS acquisition was performed for the eight most intense ions detected in the full scan, using a High Collision Dissociation (HCD) energy at 35% and 15,000 FWHM resolution.

3.2.6 Data analysis

LC-HRMS raw data files were processed using Compound Discoverer 3.0 (Thermo Scientific, San Jose, USA) for peak picking and suspect screening. Suspect screening was performed using the SusDat database of the European Network of reference laboratories, research centres and related organisations for monitoring of emerging environmental substances (NORMAN, https://www.norman-network.com/?q=node/236) consists of more than 40000 chemicals relevant for environmental monitoring, as well as the Water Framework Directive (WFD) list of priority substances (http://ec.europa.eu/environment/water/water-dangersub/pri_substances.htm). Searches were performed with 5 ppm mass tolerance. The processed data was exported and imported into R Studio as a .csv file for further data analysis and visualisation (R Core Team, 2017). To group and characterize samples and features, the two multivariate analysis techniques principal component analysis (PCA) and hierarchical clustering (HC) were applied. PCA was performed using the R package *FactoMineR*, and results visualized in graph of individuals plots using the R package *factoextra*. Prior to HC, data was normalized through division of feature intensities across samples by the maximum intensity of the respective feature. Both samples and features were clustered based on Euclidean distances (Everitt, 1974) and visualized in a heat map using the *pheatmap* package in R. To show differences in features induced by treatment steps, features were clustered based on their Pearson correlation using the Ward.D2 method (Ward, 1963). In addition, changes in features between two corresponding before and after treatment samples were illustrated in so called Volcano plots displaying the change in intensity as the log 2 fold change (log2FC) and its significance, i.e. the negative log 10-transformed p-values of features (Cui and Churchill, 2003).

3.3 Results and discussion

3.3.1 Ozonation results in decrease of feature numbers and intensities

First, overall feature numbers and intensities, as well as suspect matches against the NORMAN SusDat and the WFD priority lists were determined, the results of which are shown in Table 14 and Figure 13. As expected and consistent with the CALUX assay results (data not shown), the ozonation influent samples showed most features in both sample types and post-GAC filter samples the least, respectively. Apart from the GAC filter step, there was no clear reduction in feature numbers observed through technological an natural treatment steps. Note that the post-ozonation steps constructed wetlands, sand BAC filter and sand anthracite filter are performed in parallel. Post-GAC treatment succeeds the sand anthracite filter treatment. Summed feature intensities, however, did show significant decrease after ozonation of roughly two thirds in April and one third in July, respectively. As feature numbers do not reflect the abundance of a given feature in the sample this could either mean that the features persist at lower concentrations in the samples, or that the features initially present are transformed into new features.

TABLE 14. NUMBERS OF DETECTED FEATURES, SUSDAT SUSPECT AND WFD PRIORITY SUBSTANCE MATCHES ACROSS ALL SAMPLES. TREATMENT STEPS 1,2,3 ARE SEQUENTIAL. 3A,B AND C ARE PERFORMED IN PARALLEL. STEP 4C SUCCEEDS 3C. GREEN – LOW NUMBER, RED – HIGH NUMBER.

	Ozonation influent	Ozonation effluent	Constructed Wetlands	Sand BAC Filter	Sand Anthracite Filter	PostGac Filter
Treatment step	1	2	3a	3b	3c	4c
All Features						
April	26235	23389	24187	22370	25567	8561
July	26394	26228	25567	25648	25691	17598
SusDat suspect matches						
April	13151	11748	12221	11073	12748	4146
July	13203	13087	12764	12755	12859	8820
WFD suspect matches						
April	41	41	41	38	41	11
July	41	42	41	41	41	26

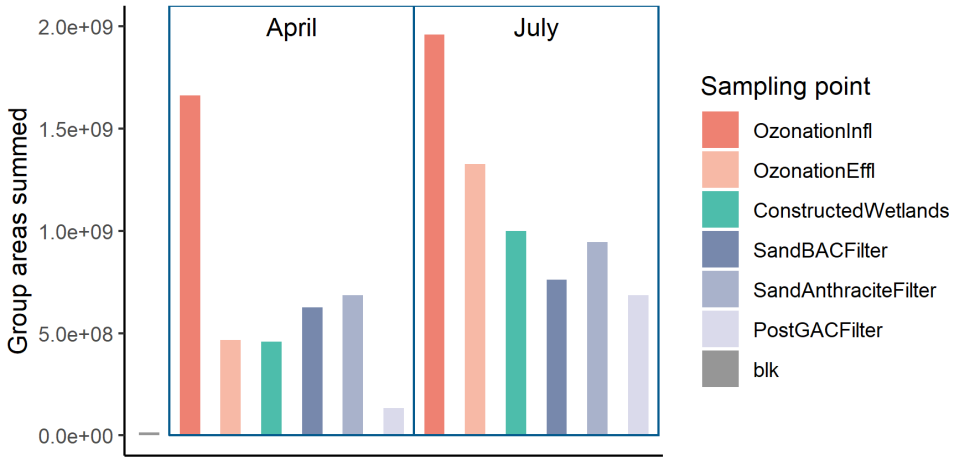

FIGURE 13. SUMMED FEATURE INTENSITIES PER SAMPLE GROUP. BLK = BLANK.

3.3.2 PCA groups samples according to seasonal changes and water treatment steps

Next, principal component analysis (PCA) was applied for a qualitative overview and to group and characterize samples and features. Through reduction of the data complexity PCA can reveal relationships between samples when the principal components are depicted in a so called scores plot (Schollée et al., 2016). Two thirds of the variance in the data could be explained by the first two principal components as shown in the Screeplot in 5.3. Therefore, only the first two components were considered in the following. Figure 14 shows the distribution of the Berlin Schoenerlinde water samples according to the first two components, referred to as dimension 1 and 2. The technical triplicates cluster together indicating good measurement reproducibilty. Dimension 1 is separating the ozonation influent from the other samples. It could thus reflect overall signal intensities. Feature numbers increse along this dimension, with the blank samples on the far left and ozonation influent samples on the far right. Dimension 2 could be representing the seasonal influence, i.e. the variability between April and July samples. In addition, it could be

explaining variability introduced by transformation processes with the parent compounds present in the ozonation influent (negative Dimension 2) and transformation products in the ozonation effluent and other treated samples (positive Dimension 2, decreasing).

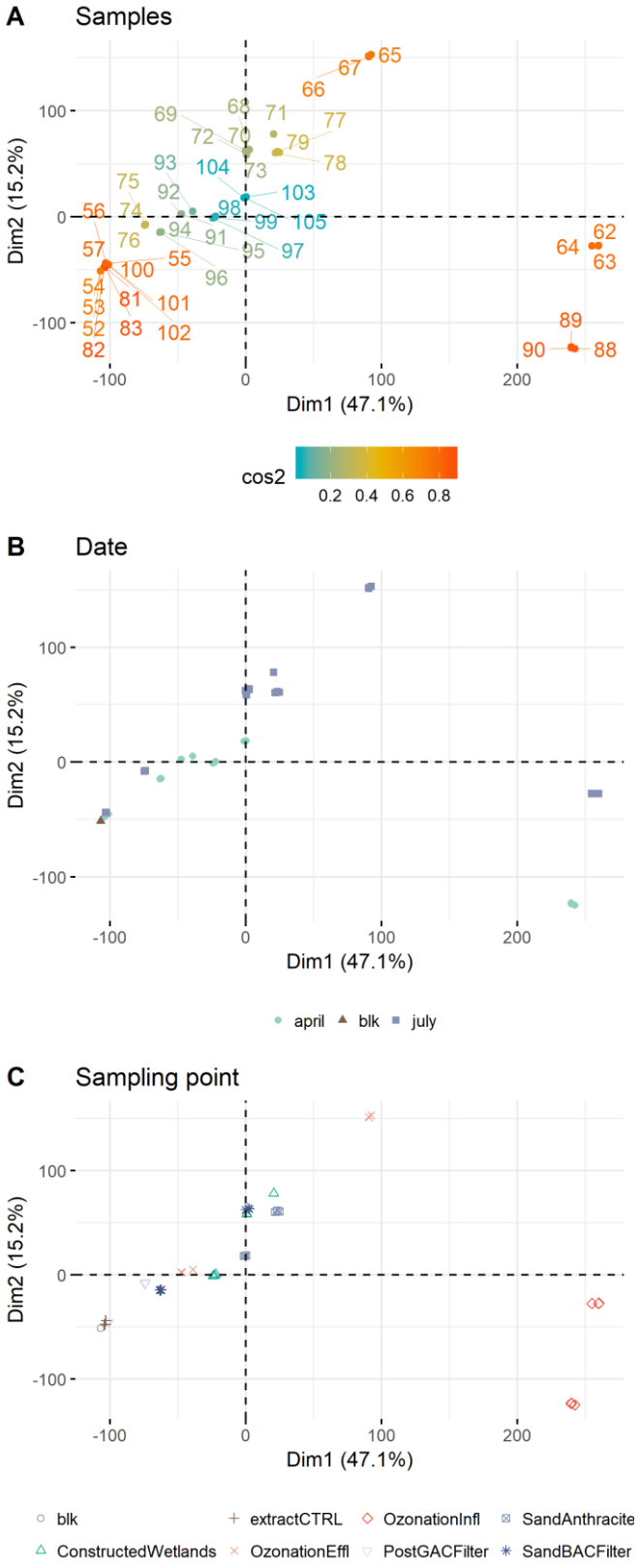

FIGURE 14 PCA GRAPH OF INDIVIDUALS OF WATER SAMPLES FROM SITE 12, APRIL AND JULY. SAMPLES ARE COLOURED ACCORDING TO THEIR SQUARED COSINE OF THE OBSERVATION (COS2, A), DATE (B), AND SAMPLING POINT (C). BLK = BLANK

3.3.3 Volcano plots visualize differences between treatment steps

To investigate changes due to treatment steps, features were consequently plotted according to their changes between two samples and the significance thereof in so called Volcano plots (Cui and Churchill, 2003). Such a Volcano plot is shown in Figure 15 for the changes in features due to ozonation (left panel) and constructed wetland treatment (right panel) samples of the April sampling round. All features above the red line that indicates significance (p-value < 0.05) are significantly different between the treatment steps. The features on the left side of the y-axis represent compounds that are removed through the respective treatment technology. On the contrary, the features on the right side of the y-axis are introduced during these treatment steps and are either formed from parent compounds present in the influent or in the case of the constructed wetlands, through e.g. photo-degradation, biodegradation or hydrolysis within the wetland. As the features are coloured according to their retention time which can serve as a measure for polarity of a compound, the Volcano plot can reveal differences in the chemical space before and after treatment. Visual inspection suggests that the influent sample of the constructed wetlands is more hydrophobic than the wetlands effluent. This is in line with the current understanding that more polar substances are less readily removed in water treatment steps, while the more hydrophobic compounds may sorb to particles and sediment present in the wetland.

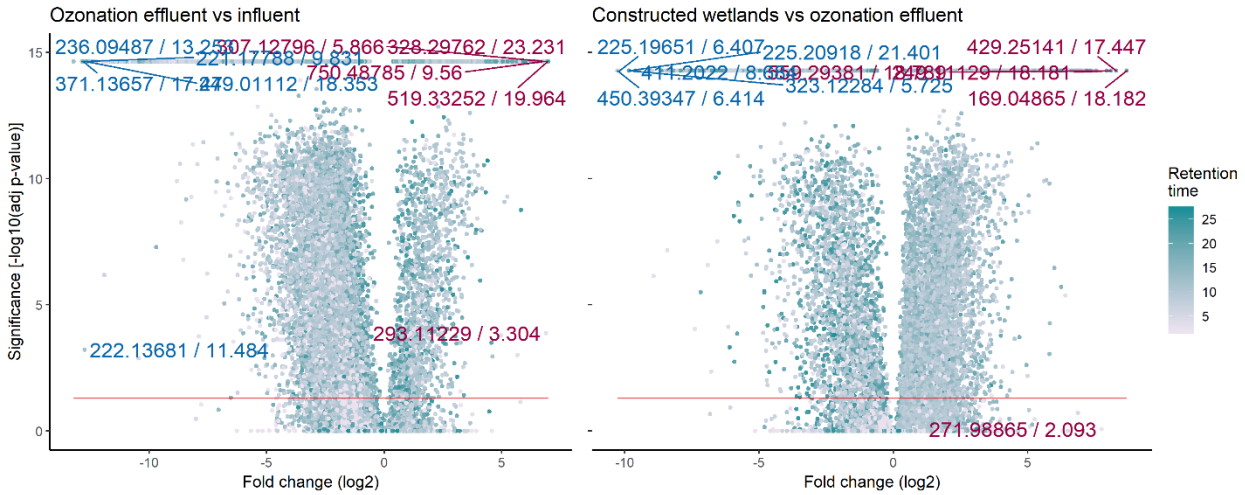

FIGURE 15 COMPARISON OF FEATURE INTENSITIES BETWEEN OZONATION EFFLUENT AND INFLUENT (LEFT PANEL) AND OZONATION EFFLUENT AND CONSTRUCTED WETLANDS (RIGHT) SAMPLES (APRIL SAMPLING ROUND). THE CHANGES BETWEEN FEATURE INTENSITIES (LOG2 FOLD CHANGE, X-AXIS) ARE PLOTTED AGAINST THE SIGNIFICANCE (P-VALUE) IN A VOLCANO PLOT. THE FIVE MOST INTENSE FEATURES OF THE RESPECTIVE BEFORE (BLUE) AND AFTER TREATMENT SAMPLES (RED) ARE LABELLED. THE FEATURES ARE COLOURED ACCORDING TO THEIR RETENTION TIME AS A MEASURE FOR POLARITY.

The high number of features detected in the non-target screening data calls for prioritisation of relevant features of which the structure should subsequently be identified. Which features are categorized as relevant strongly depends upon context. In the scope of waste water reuse, focus could be on the features that are persistent across treatments as these pose a risk to the final water quality, as well as the features that are different in constructed wetlands treatment compared to other treatments.

The Volcano plot shown in Figure 15 assists in prioritizing features based on their changes and intensities. The five peaks that show the greatest increase in intensity during ozonation and

constructed wetland passage, respectively, are coloured in red, those that show greatest removal are coloured blue. These Top5 features can serve as a starting point for identification.

3.3.4 HC reveals clusters of feature trend profiles

Alternatively, HC can facilitate prioritisation efforts. HC is a strategy that can cluster samples and features based on their similarity and thus reveal trend profiles of features, i.e. clusters of features that are persistent, formed or do not change across treatments. Here, we performed HC on the NTS data set based on Euclidean distances after data normalization, integrated the chemical NTS data with the effect-based data from the CALUX bioassays and visualized the clustering output in the heat map shown in Figure 16.

In this heat map, the relative intensity of each feature (vertical) for each sample (horizontal) is shown ranging from blue (lowest intensity) to red (highest intensity). Based on these intensities the samples are clustered; as expected the technical triplicates cluster together, however, April and July samples do not in all cases, indicating seasonal changes in water quality. Ozonation influent is clearly separated from the treated samples. Based on this heatmap, feature clusters can be selected for identification, for instance those that show high intensities in the ozonation effluent but not influent potentially representing ozonation transformation products, or those that still show high intensities in the Post-GAC filter samples and are thus not removed by the multi-step treatment.

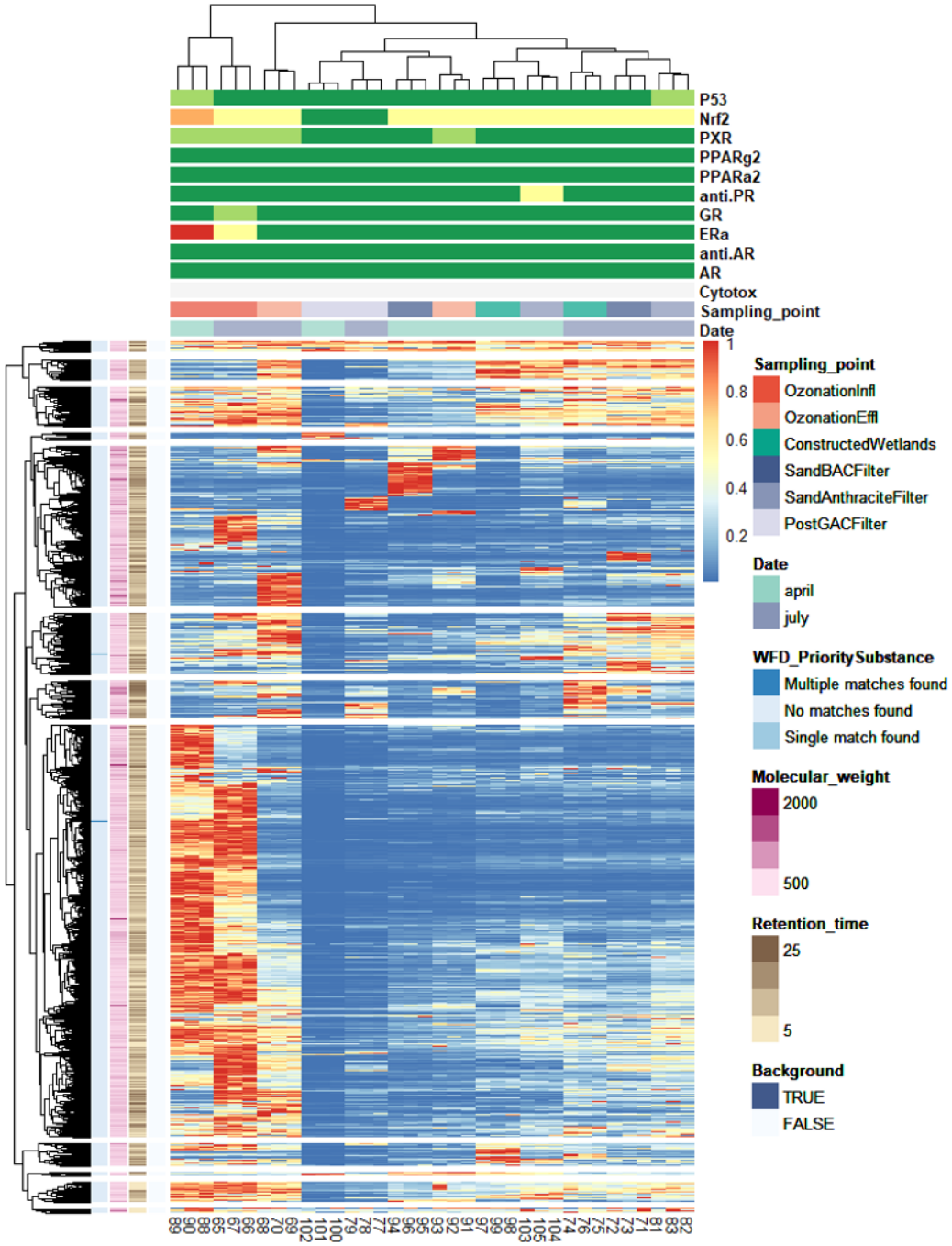

FIGURE 16. CALUX BIOASSAY RESPONSE DATA INTEGRATED WITH NON-TARGET SCREENING DATA. HIERARCHICAL CLUSTERING OF NORMALIZED NON-TARGET SCREENING DATA BASED ON EUCLIDEAN DISTANCE. COLOR CODING OF BIOASSAY READOUTS: < EBT OR LOQ OF BIOASSAY IN DARK GREEN, < 3X EBT IN LIGHT GREEN, < 10X EBT IN YELLOW, < 100X EBT IN ORANGE, > 100X EBT IN RED.

3.4 Conclusions and outlook

Furthermore, through integration of the non-target screening data with the CALUX assay readouts, feature clusters that showed high intensities when a CALUX response is observed can be determined and prioritized for subsequent identification. Moreover, the toxicity of the suspects matched with the WFD list of priority substances indicated with the leftmost column, can be assessed in regards to the CALUX response. Alternatively, bioassay responses can be

used to reduce suspect lists to relevant chemicals. The SusDat suspect list comprises 40000 compounds. The search space resulting from the size of this list can lead to many false positive hits. In earlier research, roughly 90% of tentative suspects based on accurate mass match alone could not be confirmed (van Leerdam et al., 2017). The use of tailored suspect lists comprising relevant suspects expected to be present can significantly increase identification success rate. As samples from all sampling points but the post-GAC filter exceeded EBT values for Nrf2 at least 10 fold, ongoing work is focussing on this CALUX assay. Suspect lists of compounds that lead to an active response in Nrf2 CALUX assays are used to screen for the compounds that are causing the active Nrf2 response in the respective samples. Thereby, the integration of chemical non-target screening data with effect-based bioanalysis can further enhance prioritisation and ultimately assessment of chemical water quality.

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5 Supplementary Info

5.1 Compound Discoverer Setings DPWE

Search name: DPWE_v20180803_supectScreening
Search description: Untargeted environmental research workflow without statistics: Find and identify unknown compounds.
- Performs retention time alignment, unknown compound detection, and compound grouping across all samples. Predicts elemental compositions for all compounds, and hides chemical background (using Blank samples). Identifies compounds using mzCloud (ddMS2), ChemSpider (exact mass or formula) and local database searches against Mass Lists (exact mass and RT) and mzVault spectral libraries. Performs similarity search for all compounds with ddMS2 data using mzCloud. And applies mzLogic to rank order structures from ChemSpider and mass list search results.

Search date: 03/08/2018 09:51:04
Created with Discoverer version: 3.0.0.287

[Input Files (0)]
-->Select Spectra (38)

[Select Spectra (38)]
-->Detect Compounds (24)

[Detect Compounds (24)]
-->Group Compounds (25)
-->Merge Features (14)

[Group Compounds (25)]
-->Search mzCloud (27)
-->Assign Compound Annotations (40)
-->Search ChemSpider (22)
-->Predict Compositions (37)
-->Search Mass Lists (39)
-->Fill Gaps (46)
-->Mark Background Compounds (43)

[Search mzCloud (27)]

[Assign Compound Annotations (40)]

[Search ChemSpider (22)]

[Predict Compositions (37)]

[Search Mass Lists (39)]

[Fill Gaps (46)]

[Mark Background Compounds (43)]

[Merge Features (14)]

[Descriptive Statistics (44)]

[Differential Analysis (45)]

Processing node 0: Input Files

Input Data:

- File Name(s) (Hidden):
D:\Data\2018\DPWE_NTS_20180424\pos\DPWE20180424pos-06.raw
D:\Data\2018\DPWE_NTS_20180424\pos\DPWE20180424pos-07.raw
D:\Data\2018\DPWE_NTS_20180424\pos\DPWE20180424pos-08.raw
D:\Data\2018\DPWE_NTS_20180424\pos\DPWE20180424pos-12.raw
D:\Data\2018\DPWE_NTS_20180424\pos\DPWE20180424pos-13.raw
D:\Data\2018\DPWE_NTS_20180424\pos\DPWE20180424pos-14.raw
D:\Data\2018\DPWE_NTS_20180424\pos\DPWE20180424pos-15.raw
D:\Data\2018\DPWE_NTS_20180424\pos\DPWE20180424pos-16.raw
D:\Data\2018\DPWE_NTS_20180424\pos\DPWE20180424pos-17.raw
D:\Data\2018\DPWE_NTS_20180424\pos\DPWE20180424pos-19.raw
D:\Data\2018\DPWE_NTS_20180424\pos\DPWE20180424pos-20.raw
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D:\Data\2018\DPWE_NTS_20180424\pos\DPWE20180424pos-23.raw
D:\Data\2018\DPWE_NTS_20180424\pos\DPWE20180424pos-24.raw
D:\Data\2018\DPWE_NTS_20180424\pos\DPWE20180424pos-26.raw
D:\Data\2018\DPWE_NTS_20180424\pos\DPWE20180424pos-27.raw
D:\Data\2018\DPWE_NTS_20180424\pos\DPWE20180424pos-28.raw
D:\Data\2018\DPWE_NTS_20180424\pos\DPWE20180424pos-29.raw
D:\Data\2018\DPWE_NTS_20180424\pos\DPWE20180424pos-30.raw
D:\Data\2018\DPWE_NTS_20180424\pos\DPWE20180424pos-31.raw
D:\Data\2018\DPWE_NTS_20180424\pos\DPWE20180424pos-33.raw
D:\Data\2018\DPWE_NTS_20180424\pos\DPWE20180424pos-34.raw
D:\Data\2018\DPWE_NTS_20180424\pos\DPWE20180424pos-35.raw
D:\Data\2018\DPWE_NTS_20180424\pos\DPWE20180424pos-37.raw
D:\Data\2018\DPWE_NTS_20180424\pos\DPWE20180424pos-38.raw
D:\Data\2018\DPWE_NTS_20180424\pos\DPWE20180424pos-39.raw
D:\Data\2018\DPWE_NTS_20180424\pos\DPWE20180424pos-40.raw
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D:\Data\2018\DPWE_NTS_20180424\pos\DPWE20180424pos-42.raw
D:\Data\2018\DPWE_NTS_20180424\pos\DPWE20180424pos-44.raw
D:\Data\2018\DPWE_NTS_20180424\pos\DPWE20180424pos-45.raw
D:\Data\2018\DPWE_NTS_20180424\pos\DPWE20180424pos-46.raw
D:\Data\2018\DPWE_NTS_20180424\pos\DPWE20180424pos-47.raw
D:\Data\2018\DPWE_NTS_20180424\pos\DPWE20180424pos-48.raw
D:\Data\2018\DPWE_NTS_20180424\pos\DPWE20180424pos-49.raw
D:\Data\2018\DPWE_NTS_20180424\pos\DPWE20180424pos-51.raw
D:\Data\2018\DPWE_NTS_20180424\pos\DPWE20180424pos-52.raw
D:\Data\2018\DPWE_NTS_20180424\pos\DPWE20180424pos-53.raw

Processing node 38: Select Spectra
---
1. General Settings:
- Precursor Selection: Use MS(n - 1) Precursor
- Use Isotope Pattern in Precursor Reevaluation: True
- Provide Profile Spectra: Automatic
- Store Chromatograms: False

2. Spectrum Properties Filter:
- Lower RT Limit: 0
- Upper RT Limit: 0
- First Scan: 0
- Last Scan: 0
- Ignore Specified Scans: (not specified)
- Lowest Charge State: 0
- Highest Charge State: 0
- Min. Precursor Mass: 100 Da
- Max. Precursor Mass: 5000 Da
- Total Intensity Threshold: 0
- Minimum Peak Count: 1

3. Scan Event Filters:
- Mass Analyzer: (not specified)
- MS Order: Any
- Activation Type: (not specified)

- Min. Collision Energy: 0
- Max. Collision Energy: 1000
- Scan Type: Any
- Polarity Mode: (not specified)

4. Peak Filters:
- S/N Threshold (FT-only): 1.5

5. Replacements for Unrecognized Properties:
- Unrecognized Charge Replacements: 1
- Unrecognized Mass Analyzer Replacements: ITMS
- Unrecognized MS Order Replacements: MS2
- Unrecognized Activation Type Replacements: CID
- Unrecognized Polarity Replacements: +
- Unrecognized MS Resolution@200 Replacements: 60000
- Unrecognized MSn Resolution@200 Replacements: 30000

Processing node 24: Detect Compounds
---
1. General Settings:
- Mass Tolerance [ppm]: 5 ppm
- Intensity Tolerance [%]: 30
- S/N Threshold: 3
- Min. Peak Intensity: 100000
- Ions:
    [2M+ACN+H]+1
    [2M+ACN+Na]+1
    [2M+FA-H]-1
    [2M+H]+1
    [2M+K]+1
    [2M+Na]+1
    [2M+NH4]+1
    [2M-H]-1
    [2M-H+HAc]-1
    [M+2H]+2
    [M+3H]+3
    [M+ACN+2H]+2
    [M+ACN+H]+1
    [M+ACN+Na]+1
    [M+Cl]-1
    [M+DMSO+H]+1
    [M+FA-H]-1
    [M+H]+1
    [M+H+K]+2
    [M+H+MeOH]+1
    [M+H+Na]+2
    [M+H+NH4]+2
    [M+H-H2O]+1
    [M+H-NH3]+1
    [M+K]+1
    [M+Na]+1
    [M+NH4]+1
    [M-2H]-2
    [M-2H+K]-1
    [M-H]-1
    [M-H+HAc]-1
    [M-H+TFA]-1
    [M-H-H2O]-1
- Base Ions: [M+H]+1; [M-H]-1
- Min. Element Counts: C H
- Max. Element Counts: C90 H190 Br3 Cl4 F6 K2 N10 Na2 O18 P3 S5

2. Peak Detection:
- Filter Peaks: True
- Max. Peak Width [min]: 0.8
- Remove Singlets: False

- Min. # Scans per Peak: 3
- Min. # Isotopes: 1

Processing node 25: Group Compounds
---
1. Compound Consolidation:
- Mass Tolerance: 5 ppm
- RT Tolerance [min]: 0.1

2. Fragment Data Selection:
- Preferred Ions: [M+H]+1; [M-H]-1

Processing node 27: Search mzCloud
---
1. Search Settings:
- Compound Classes: All
- Match Ion Activation Type: True
- Match Ion Activation Energy: Match with Tolerance
- Ion Activation Energy Tolerance: 20
- Apply Intensity Threshold: True
- Precursor Mass Tolerance: 10 ppm
- FT Fragment Mass Tolerance: 10 ppm
- IT Fragment Mass Tolerance: 0.4 Da
- Identity Search: Cosine
- Similarity Search: Similarity Forward
- Library: Reference
- Post Processing: Recalibrated
- Match Factor Threshold: 50
- Max. # Results: 20

Processing node 40: Assign Compound Annotations
---
1. General Settings:
- Mass Tolerance: 5 ppm

2. Data Sources:
- Data Source #1: mzCloud Search
- Data Source #2: MassList Search
- Data Source #3: Predicted Compositions
- Data Source #4: ChemSpider Search
- Data Source #5: (not specified)

Processing node 22: Search ChemSpider
---
1. Search Settings:
- Database(s):
    ACToR: Aggregated Computational Toxicology Resource
    DrugBank
    EAWAG Biocatalysis/Biodegradation Database
    EPA DSSTox
    EPA Toxcast
    FDA UNII - NLM
- Search Mode: By Formula or Mass
- Mass Tolerance: 5 ppm
- Max. # of results per compound: 20
- Max. # of Predicted Compositions to be searched per Compound: 3
- Result Order (for Max. # of results per compound): Order By Reference Count (DESC)

2. Predicted Composition Annotation:
- Check All Predicted Compositions: True

Processing node 37: Predict Compositions

---
1. Prediction Settings:
- Mass Tolerance: 5 ppm
- Min. Element Counts: C H
- Max. Element Counts: C40 H60 Cl5 N10 O18 P3 S5
- Min. RDBE: 0
- Max. RDBE: 40
- Min. H/C: 0.1
- Max. H/C: 3.5
- Max. # Candidates: 10
- Max. # Internal Candidates: 500

2. Pattern Matching:
- Intensity Tolerance [%]: 30
- Intensity Threshold [%]: 0.1
- S/N Threshold: 3
- Min. Spectral Fit [%]: 30
- Min. Pattern Cov. [%]: 80
- Use Dynamic Recalibration: True

3. Fragments Matching:
- Use Fragments Matching: True
- Mass Tolerance: 5 ppm
- S/N Threshold: 3

Processing node 39: Search Mass Lists
---
1. Search Settings:
- Mass Lists:
ExpectedCompounds20180731.massList|suspects_allcombined_NO_SMILES.massList
- Mass Tolerance: 5 ppm
- Use Retention Time: False
- RT Tolerance [min]: 0.5

Processing node 46: Fill Gaps
---
1. General Settings:
- Mass Tolerance: 5 ppm
- S/N Threshold: 1.5
- Use Real Peak Detection: True

Processing node 43: Mark Background Compounds
---
1. General Settings:
- Max. Sample/Blank: 5
- Max. Blank/Sample: 0
- Hide Background: True

Processing node 14: Merge Features
---
1. Peak Consolidation:
- Mass Tolerance: 5 ppm
- RT Tolerance [min]: 0.1

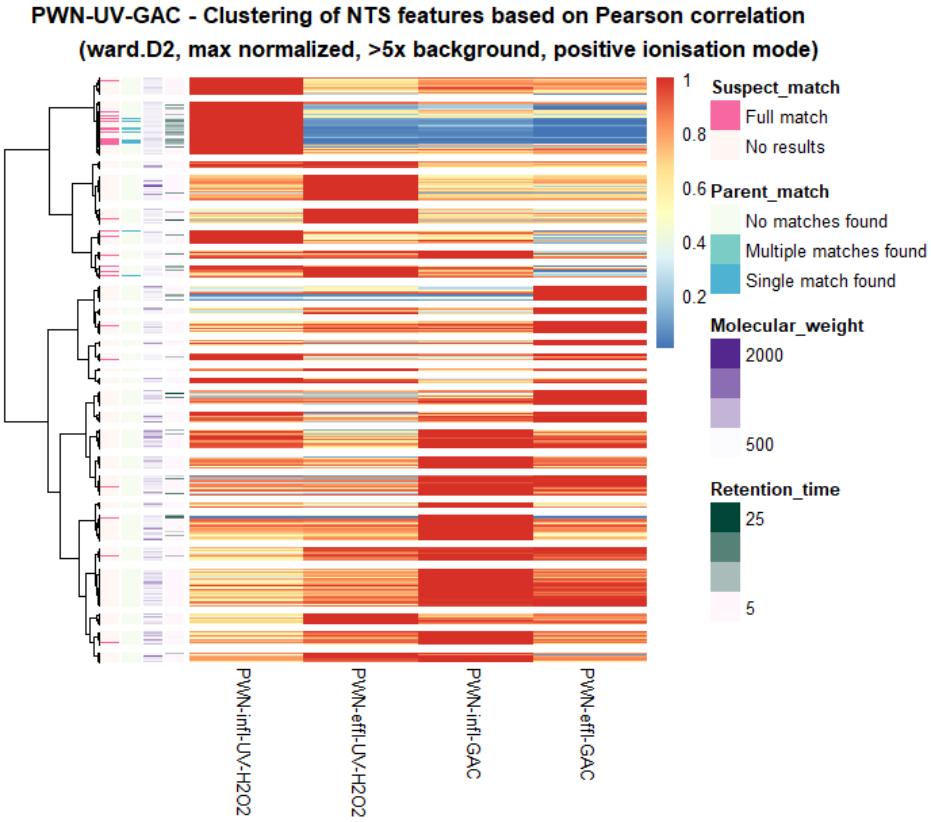
Processing node 44: Descriptive Statistics

No parameters

Processing node 45: Differential Analysis
---
1. General Settings:

- Log10 Transform Values: True

5.2 HC PWN-UV-GAC



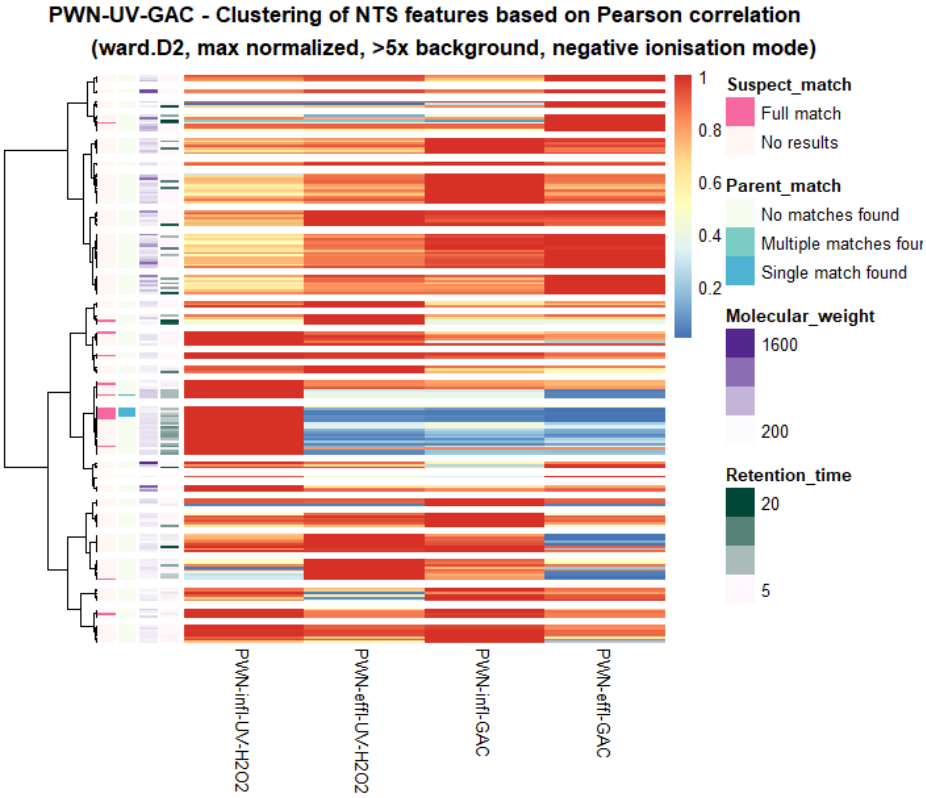


FIGURE 17 HIERARCHICAL CLUSTERING OF PWN-UV-GAC FEATURES DETECTED IN POSITIVE (UPPER PANEL) AND NEGATIVE (LOWER PANEL) IONIZATION MODE BASED ON PEARSON CORRELATION USING THE WARD.D2 METHOD AND MAX NORMALIZED FEATURE INTENSITIES. 27 CLUSTERS.

5.3 Compound Discoverer Settings AquaNES

Search name:
AquaNES_pos_20181207_noPrimSedEfl_log2FC_toxcast_WFD_Btargets_extractsOnly
Search description: Untargeted environmental research workflow without statistics: Find and identify unknown compounds.
- Performs retention time alignment, unknown compound detection, and compound grouping across all samples. Predicts elemental compositions for all compounds, and hides chemical background (using Blank samples). Identifies compounds using mzCloud (ddMS2), ChemSpider (exact mass or formula) and local database searches against Mass Lists (exact mass and RT) and mzVault spectral libraries. Performs similarity search for all compounds with ddMS2 data using mzCloud. And applies mzLogic to rank order structures from ChemSpider and mass list search results.

Search date: 07/12/2018 10:15:20
Created with Discoverer version: 3.0.0.294

[Input Files (0)]
-->Select Spectra (38)

[Select Spectra (38)]
-->Detect Compounds (24)

[Detect Compounds (24)]
-->Group Compounds (25)
-->Merge Features (14)

[Group Compounds (25)]
-->Search mzCloud (27)
-->Assign Compound Annotations (40)
-->Search ChemSpider (22)
-->Predict Compositions (37)
-->Search Mass Lists (39)
-->Fill Gaps (44)

[Fill Gaps (44)]
-->Mark Background Compounds (43)

[Search mzCloud (27)]

[Assign Compound Annotations (40)]

[Search ChemSpider (22)]

[Predict Compositions (37)]

[Search Mass Lists (39)]

[Mark Background Compounds (43)]

[Merge Features (14)]

[Differential Analysis (45)]

[Descriptive Statistics (46)]

Processing node 0: Input Files

Input Data:
- File Name(s) (Hidden):

D:\Users\Brunnan\MSData\Aquanes\20180914pos-105.raw
D:\Users\Brunnan\MSData\Aquanes\20180914pos-104.raw
D:\Users\Brunnan\MSData\Aquanes\20180914pos-103.raw
D:\Users\Brunnan\MSData\Aquanes\20180914pos-102.raw
D:\Users\Brunnan\MSData\Aquanes\20180914pos-101.raw
D:\Users\Brunnan\MSData\Aquanes\20180914pos-100.raw
D:\Users\Brunnan\MSData\Aquanes\20180914pos-99.raw
D:\Users\Brunnan\MSData\Aquanes\20180914pos-98.raw
D:\Users\Brunnan\MSData\Aquanes\20180914pos-97.raw
D:\Users\Brunnan\MSData\Aquanes\20180914pos-96.raw
D:\Users\Brunnan\MSData\Aquanes\20180914pos-95.raw
D:\Users\Brunnan\MSData\Aquanes\20180914pos-94.raw
D:\Users\Brunnan\MSData\Aquanes\20180914pos-93.raw
D:\Users\Brunnan\MSData\Aquanes\20180914pos-92.raw
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D:\Users\Brunnan\MSData\Aquanes\20180914pos-90.raw
D:\Users\Brunnan\MSData\Aquanes\20180914pos-89.raw
D:\Users\Brunnan\MSData\Aquanes\20180914pos-88.raw
D:\Users\Brunnan\MSData\Aquanes\20180914pos-83.raw
D:\Users\Brunnan\MSData\Aquanes\20180914pos-82.raw
D:\Users\Brunnan\MSData\Aquanes\20180914pos-81.raw
D:\Users\Brunnan\MSData\Aquanes\20180914pos-79.raw
D:\Users\Brunnan\MSData\Aquanes\20180914pos-78.raw
D:\Users\Brunnan\MSData\Aquanes\20180914pos-77.raw
D:\Users\Brunnan\MSData\Aquanes\20180914pos-76.raw
D:\Users\Brunnan\MSData\Aquanes\20180914pos-75.raw
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D:\Users\Brunnan\MSData\Aquanes\20180914pos-86.raw
D:\Users\Brunnan\MSData\Aquanes\20180914pos-85.raw
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D:\Users\Brunnan\MSData\Aquanes\20180914pos-60.raw
D:\Users\Brunnan\MSData\Aquanes\20180914pos-59.raw
D:\Users\Brunnan\MSData\Aquanes\20180914pos-58.raw

Processing node 38: Select Spectra
---
1. General Settings:
- Precursor Selection: Use MS(n - 1) Precursor
- Use Isotope Pattern in Precursor Reevaluation: True
- Provide Profile Spectra: Automatic
- Store Chromatograms: False

2. Spectrum Properties Filter:
- Lower RT Limit: 2
- Upper RT Limit: 27
- First Scan: 0
- Last Scan: 0
- Ignore Specified Scans: (not specified)

- Lowest Charge State: 0
- Highest Charge State: 0
- Min. Precursor Mass: 100 Da
- Max. Precursor Mass: 5000 Da
- Total Intensity Threshold: 0
- Minimum Peak Count: 1

3. Scan Event Filters:
- Mass Analyzer: (not specified)
- MS Order: Any
- Activation Type: (not specified)
- Min. Collision Energy: 0
- Max. Collision Energy: 1000
- Scan Type: Any
- Polarity Mode: (not specified)

4. Peak Filters:
- S/N Threshold (FT-only): 1.5

5. Replacements for Unrecognized Properties:
- Unrecognized Charge Replacements: 1
- Unrecognized Mass Analyzer Replacements: ITMS
- Unrecognized MS Order Replacements: MS2
- Unrecognized Activation Type Replacements: CID
- Unrecognized Polarity Replacements: +
- Unrecognized MS Resolution@200 Replacements: 60000
- Unrecognized MSn Resolution@200 Replacements: 30000

Processing node 24: Detect Compounds
---
1. General Settings:
- Mass Tolerance [ppm]: 5 ppm
- Intensity Tolerance [%]: 30
- S/N Threshold: 3
- Min. Peak Intensity: 100000
- Ions:
  [M+2H]+2
  [M+ACN+H]+1
  [M+Cl]-1
  [M+H]+1
  [M+H+MeOH]+1
  [M+H-H2O]+1
  [M+K]+1
  [M+Na]+1
  [M+NH4]+1
  [M-H]-1
- Base Ions: [M+H]+1; [M-H]-1
- Min. Element Counts: C H
- Max. Element Counts: C90 H190 Br3 Cl4 F6 K2 N10 Na2 O18 P3 S5

2. Peak Detection:
- Filter Peaks: True
- Max. Peak Width [min]: 0.8
- Remove Singlets: False
- Min. # Scans per Peak: 3
- Min. # Isotopes: 1

Processing node 25: Group Compounds
---
1. Compound Consolidation:
- Mass Tolerance: 5 ppm
- RT Tolerance [min]: 0.1

2. Fragment Data Selection:
- Preferred Ions: [M+H]+1; [M-H]-1

Processing node 27: Search mzCloud
---
1. Search Settings:
- Compound Classes: All
- Match Ion Activation Type: True
- Match Ion Activation Energy: Match with Tolerance
- Ion Activation Energy Tolerance: 20
- Apply Intensity Threshold: True
- Precursor Mass Tolerance: 10 ppm
- FT Fragment Mass Tolerance: 10 ppm
- IT Fragment Mass Tolerance: 0.4 Da
- Identity Search: Cosine
- Similarity Search: Similarity Forward
- Library: Reference
- Post Processing: Recalibrated
- Match Factor Threshold: 50
- Max. # Results: 20

Processing node 40: Assign Compound Annotations
---
1. General Settings:
- Mass Tolerance: 3 ppm

2. Data Sources:
- Data Source #1: MassList Search
- Data Source #2: mzCloud Search
- Data Source #3: ChemSpider Search
- Data Source #4: Predicted Compositions
- Data Source #5: (not specified)

Processing node 22: Search ChemSpider
---
1. Search Settings:
- Database(s):
    EAWAG Biocatalysis/Biodegradation Database
    EPA DSSTox
    EPA Toxcast
- Search Mode: By Formula or Mass
- Mass Tolerance: 3 ppm
- Max. # of results per compound: 20
- Max. # of Predicted Compositions to be searched per Compound: 3
- Result Order (for Max. # of results per compound): Order By Reference Count (DESC)

2. Predicted Composition Annotation:
- Check All Predicted Compositions: True

Processing node 37: Predict Compositions
---
1. Prediction Settings:
- Mass Tolerance: 3 ppm
- Min. Element Counts: C H
- Max. Element Counts: C90 H190 Br3 Cl8 F18 N10 O18 P3 S5
- Min. RDBE: 0
- Max. RDBE: 40
- Min. H/C: 0.1
- Max. H/C: 3.5
- Max. # Candidates: 10
- Max. # Internal Candidates: 500

2. Pattern Matching:
- Intensity Tolerance [%]: 30
- Intensity Threshold [%]: 0.1

- S/N Threshold: 3
- Min. Spectral Fit [%]: 30
- Min. Pattern Cov. [%]: 80
- Use Dynamic Recalibration: True

3. Fragments Matching:
- Use Fragments Matching: True
- Mass Tolerance: 5 ppm
- S/N Threshold: 3

Processing node 39: Search Mass Lists
---
1. Search Settings:
- Mass Lists:
SusDat4cd30.massList|P53_chemPar.massList|PXR_chemPar.massList|PPARg2r_chemPar.massList|PPARa2_chemPar.massList|Nrf2_chemPar.massList|ERa_chemPar.massList|Cytotox_chemPar.massList|AR_chemPar.massList|GR_chemPar.massList|WFD_prioritySubst_chemPar.massList|anti.AR_chemPar.massList|BerlinTargets.massList
- Mass Tolerance: 3 ppm
- Use Retention Time: False
- RT Tolerance [min]: 0.5

Processing node 44: Fill Gaps
---
1. General Settings:
- Mass Tolerance: 5 ppm
- S/N Threshold: 1.5
- Use Real Peak Detection: True

Processing node 43: Mark Background Compounds
---
1. General Settings:
- Max. Sample/Blank: 5
- Max. Blank/Sample: 0
- Hide Background: False

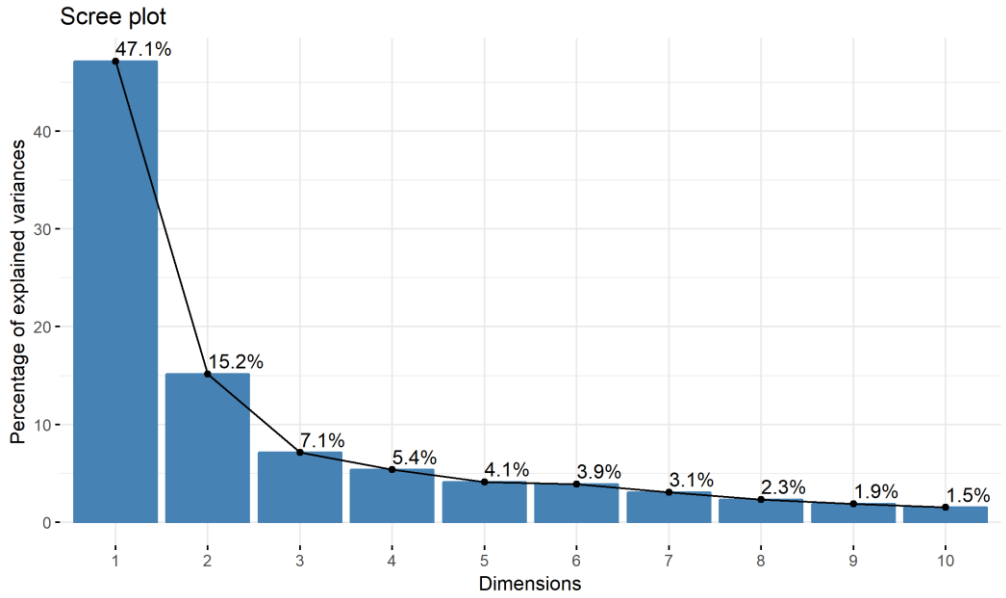
Processing node 14: Merge Features
---
1. Peak Consolidation:
- Mass Tolerance: 3 ppm
- RT Tolerance [min]: 0.1

Processing node 45: Differential Analysis
---
1. General Settings:
- Log10 Transform Values: True

Processing node 46: Descriptive Statistics

No parameters

5.4 AquaNES Screeplot



Two thirds of the variance in the NTS data could be explained by the first two principal components as shown in the Screeplot.