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Characterization of water-soluble synthetic polymeric substances in wastewater using LC-HRMS/MS

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ABSTRACT

Synthetic water-soluble polymeric materials are widely employed in e.g. cleaning detergents, personal care products, paints or textiles. Accordingly, these compounds reach sewage treatment plants and may enter receiving waters and the aquatic environment. Characteristically, these molecules show a polydisperse molecular weight distribution, comprising multiple repeating units, i.e. a homologous series (HS). Their analysis in environmentally relevant samples has received some attention over the last two decades, however, the majority of previous studies focused on surfactants and a molecular weight range <1000 Da. To capture a wider range on the mass versus polarity plane and extend towards less polar contaminants, a workflow was established using three different ionization strategies, namely conventional electrospray ionization, atmospheric pressure photoionization and atmospheric pressure chemical ionization. The data evaluation consisted of suspect screening of ca. 1200 suspect entries and a non-target screening of HS with pre-defined accurate mass differences using ca. 400 molecular formulas of repeating units of HS as input and repeating retention time shifts as HS indicator.

To study the fate of these water-soluble polymeric substances in the wastewater treatment process, the different stages, *i.e.* after primary and secondary clarifier, and after ozonation followed by sand filtration, were sampled at a Swiss wastewater treatment plant. Remaining with two different ionization interfaces, ESI and APPI, in both polarities, a non-targeted screening approach led to a total number of 146 HS (each with a minimum number of 4 members), with a molecular mass of up to 1200 detected in the final effluent. Of the 146 HS, ca 15% could be associated with suspect hits and approximately 25% with transformation products of suspects. Tentative characterization or probable chemical structure could be assigned to almost half of the findings. In positive ionization mode various sugar derivatives with differing side chains, for negative mode structures with sulfonic acids, could be characterized. The number of detected HS decreased significantly over the three treatment stages. For HS detectable also in the biological and oxidative treatment stages, a change in HS distribution towards to lower mass range was often observed.

1. Introduction

Recently, the analysis of polymers has been in the limelight of environmental research, however the focus is often on their solid state namely in terms of micro- and nanoplastics, often neglecting thereby the pressing topic of water-soluble polymeric substances (Arp and Knutsen, 2020). The use of synthetic water-soluble polymeric substances in textile industry, paints, coatings as well as consumer products, such as cleaning detergents, personal care products as well as pharmaceuticals is widespread. The functionality of these diverse substances ranges from e.g. lowering the surface tension, as it is the case for surfactants, to serving as filling substance in pharmaceuticals, or to be applied as dispersing agent in pesticides. Consequently, their prevalent application is also re-

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flected in the production volumes of educts employed in polymer syntheses, reaching up to millions of tons per year (notably, information on synthetic polymers is hardly available, since only their educts are registered under REACH (European Chemicals Agency., 2017). Accordingly, these substances reach municipal wastewater and depending on the elimination during wastewater treatment, may enter receiving waters and the aquatic environment.

By definition, polymeric substances comprise multiple repetition of units typically derived from molecules of low relative molecular mass (McNaught and Wilkinson, 1997). Due to the way of synthesis, polymerization reactions are rarely complete and hence these molecules characteristically show a polydisperse molecular weight distribution, comprising a multiplicity of repeating units (RU), *i.e.* a homologous series (HS). The term "polymeric" essentially describes either part or the whole molecule that consists of these multiple repetitions of these low molecular mass parts (McNaught and Wilkinson, 1997). As a matter of fact, synthetic polymeric substances show high complexity in their chemical structure (Crotty et al., 2016): one polymeric substance can comprise different numbers and types of chains of RU. Hence, there are potentially many constitutional isomers present for one molecular formula of a HS' member. Here, fragmentation spectra, acquired by MS/MS instrumentation, can aid in identifying the structure, however, the degree of freedom, especially in branched chained polymeric substance, is particularly high and therefore the exact chemical structure is often hardly accessible (Lara-Martin et al., 2011; Schymanski et al., 2014b).

Typically, in order to understand the fate of anthropogenic contaminants in aquatic environments, high performance liquid chromatography (HPLC) coupled to high resolution mass spectrometry (HRMS) is employed. This front-end separation is essential when dealing with low concentration in complex matrices (Zwiener and Frimmel, 2004). As a pre-requisite of MS-detection, compounds of interest need to be present in their ionized form. The most commonly applied ionization interface is electrospray ionization (ESI). Here, medium-polar to polar molecules in a vast molecular weight range can be ionized. However, if the analytes of interest are potentially extending to a low polarity range, different ionization interfaces, such as atmospheric pressure chemical ionization (APCI) or atmospheric pressure photoionization (APPI), have been used as alternatives (Reemtsma, 2001; Chiaia-Hernandez et al., 2013). The latter two ionization sources are typically limited in terms of molecular weight to roughly 2000 – 3000 Da, since above either in-source fragmentation is drastically increasing or ion formation is not effective (Gruendling et al., 2010) (Terrier et al., 2011). Employing these ionization interfaces for the analysis of polymeric substance was discussed in detail by *e.g.* (Terrier et al., 2011; Andrea and Alessandro, 2003; Reemtsma, 2001). Notably, analytical methods focusing on pure polymer standards (Wesdemiotis, 2017) are not fully transferable to the analysis of these substances in environmental samples, that are characterized by low concentrations and very complex matrices.

As for data evaluation, unknown HS can be detected using different approaches, e.g. most prominently Kendrick mass defect plots (Kendrick, 1963; Fouquet, 2019) or Van Krevelen diagrams (Kim et al., 2003). Notably, here, either generating unique molecular formula for the compounds of interest is required or they are restricted to a small pre-defined set of RUs. More importantly, front-end separation is not considered in the approaches mentioned above. The recently published envihomolog algorithm (Loos and Singer, 2017), however does include this orthogonal information on chromatographic behavior. Moreover, envihomolog does allow both, to screen for any unknown repetitive m/z differences between picked peaks, as well as using pre-defined molecular formulas as RU-input. However, the envihomolog algorithm has so far not been combined with other non-targeted componentization steps in LC-MS signal processing. Namely, any homologous analyte may form several HS over its different ion species and isotopologues, which would remain redundant for later data interpretation, but are also highly indicative for any grouping procedure. Similarly, envihomolog does so far neither account for gapped series nor the combinatorial problems associated with close-eluting and isobaric structural isomers of homologous analytes (*notably the term "isobaric" refers typically to ions of the same nominal masses, in this very context the authors extended this term also to masses of the same accurate mass*).

Over the last decades numerous studies have investigated surfactants in the aquatic environment and have shown that modern surface active compounds are extensively removed but also transformed during the wastewater treatment stages (Brunner et al., 1988; Schröder et al., 1999; Lara-Martin et al., 2011; Faria et al., 2019): Here, prominent examples of surfactants are alcohol ethoxylates (AEO), linear alkylbenzene sulfonates (LAS) and nonylphenol ethoxylates (NPEO). In aqueous as well as solid environmental matrices these surfactants readily degrade and lead to the following well-known degradation products: sulfophenyl carboxylic acids (SPC), nonylphenol ethoxy carboxylates (NPEC) and polyethylene glycols (PEG) (Lara-Martin et al., 2011). Cowan-Ellsberry compiled comprehensively most relevant environmental data regarding surfactants over the last decades, including risk assessment (Cowan-Ellsberry et al., 2014). Recently, risk assessment of surfactants was debated by (Freeling et al., 2019; Dyer et al., 2020).

In a recent review of Hupperstberg *et al.,* the authors argued that if environmental concentrations of water-soluble polymeric substances reach a sufficiently high enough level, these contaminants can act the same way as in their initial field of application, e.g. enhancing solubility of non-polar substances (Huppertsberg et al., 2020). Additonally, as lately pointed out by Arp and Knutsen, there is a lack in understanding the environmental behavior of water-soluble polymers, also in terms of mobility as well as persistence (Arp and Knutsen, 2020).

Qualitative and quantitative information on water-soluble polymeric synthetic substances is still rather scarce and the vast majority of previous studies focused on surfactants and a molecular weight range $<$ 1000 Da (Huppertsberg et al., 2020). The aim of the present work was to investigate what lays outside of the known range of surfactants and potential transformation products. To unravel HS in complex wastewater samples, different ionization strategies were tested to expand the mass polarity plane towards the medium polar compounds. In addition to comprehensive suspect screening, a non-targeted screening workflow with a homologue-based componentization was established.

2. Materials and methods

The complete data acquisition, processing and mining optimized for the characterization of homologous series in wastewater is shown in Fig. 1.

2.1. Sampling and sample preparation

Polymeric substances were studied in flow-proportional composite samples (24 h), that were collected at different stages of a wastewater treatment plant (WWTP) in Switzerland, namely after the primary clarifier (grit, sand and oil trap), the secondary clarifier (conventional activated sludge system with nitrification/denitrification and a biological phosphorus elimination step) and after ozonation followed by a sand filter as post treatment step. One sample of each stage was collected by the plant operator in April 2019, filled into 0.5 L annealed glass bottles, and immediately subjected to sample preparation. To prevent sam-

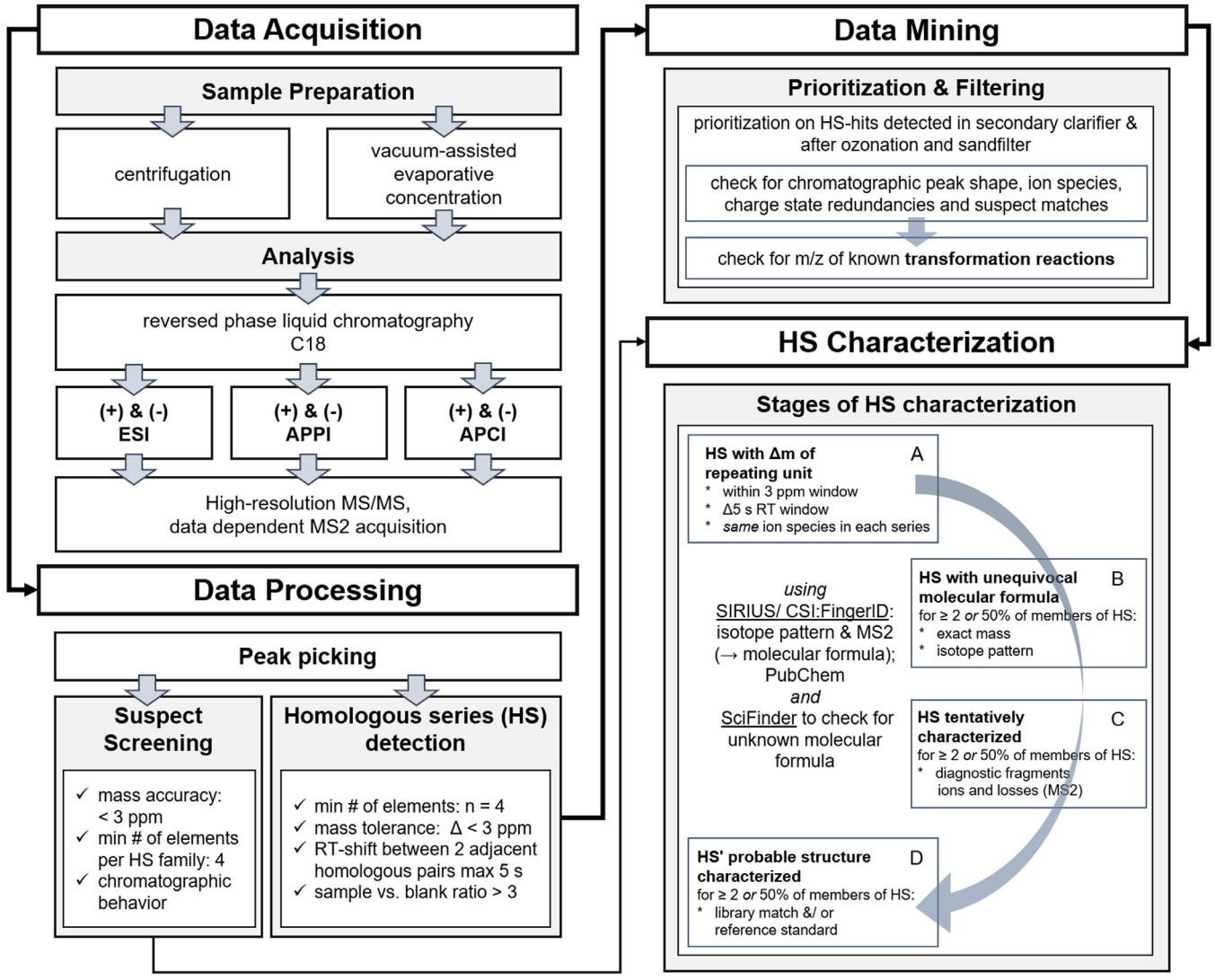


Fig. 1. Analytical workflow, including the data evaluation strategy for the analysis of small water-soluble polymeric substances in waste water using RPLC-HRMS/MS.

ple alterations and contamination, sample preparation was as limited as possible, plastic material was avoided whenever possible and glass ware was either annealed or thoroughly cleaned using analytical grade methanol (LC-MS grade, Optima^{TM}, Fisher Scientific, Switzerland) and nanopure water prior use. Two different strategies were pursued using either a simple centrifugation step to eliminate particles or pre-concentration using vacuum-assisted evaporation (pre-concentration factor of 10). For both sample preparation strategies, five replicates were prepared alongside with the same number of replicates for blank samples. To reduce changes of the prepared samples over time, HPLC-HRMS/MS analysis was started as quickly as possible. For quality control in terms of mass accuracy, a composite sample spiked with a mixture of five isotopically labeled substances was prepared and analyzed every fifteenth injection (Table S1e).

Additionally, two PEG standards with an average molecular weight of 600 and 2000 Da (Sigma Aldrich, Switzerland), respectively, were spiked to one aliquot of each sample type (final concentration of 2.5 μg mL^{-1}). Spiking was performed on the centrifugated samples and each spiked sample was injected (and analyzed) three times.

2.2. Analysis via liquid chromatography- high resolution mass spectrometry

For analysis, reversed phase liquid chromatography (RPLC) using a ThermoFisher Scientific Ultimate HPLC, equipped with a PAL autosampler was coupled to a Q-Exactive HRMS (ThermoFisher Scientific, San Jose, CA) in data-dependent acquisition mode (TopN, N = 5) (mass resolution @ m/z 200: MS1: 140K and MS2: 17.5K). Chromatographic separation was performed using a Waters Xbridge C18 column (2.1 × 50 mm, 3.5 μm, 130 Å pore size), equipped with a pre-column (Waters Xbridge BEH C18, 2.1 × 5 mm, 3.5 μm). Ionization was realized by optimizing different interfaces, namely ESI, APCI and APPI in terms of temperature, gas flows and dopant addition. To this end, a sample, collected after the WWTP's primary clarifier, was employed to guarantee the presence of analytes of interest and to mimic an analytical worst-case scenario in terms of matrix complexity. Due to their abundance over a wide molecular mass range and presence in the sample, PEG and polypropylene glycol (PPG) were applied as model HS in an m/z range of up to 3000. Data on optimization is shown in the SI Figure S1- S4. Depending on the ionization source, mobile phases

for gradient elution were adjusted accordingly and are shown together with ion source and MS parameters in Table S1a-c in the SI.

2.3. Data evaluation and data mining

As depicted in Fig. 1 data was evaluated combining suspect screening and non-target analysis workflow in enviMass v4.2beta, further data processing and mining steps were performed with dedicated R packages within RStudio (R Core Team, 2016).

2.3.1. Suspect screening

To generate a suspect screening list, different sources were combined, further extended (NORMAN suspect list (Network et al., 2020), (García et al., 2019) and known PEG derivatives, differing in end groups). The suspect list that was used as an input in enviMass can be found in the SI Table S4. In detail, a primary clarifier sample was taken as basis for characterization. Here, exact masses of the respective HS family were extracted and chromatographic parameters (smooth RT change over the HS family's mass range and chromatographic peak shape) were checked. The term "HS family" is introduced here, since some polymeric substances have different chains/RU structural positions to grow from, *e.g.* alcohol ethoxylates: CnAEOx, and these are therefore summarized to one family. MS2 spectra were checked for library matches and diagnostic fragment ions and losses. Suspect hits would be generally assigned to stage C or even D, if they suffice the requirements shown in Fig. 1 and explained in detail in 2.3.3. To be reported, a minimum number of four members per HS was necessary and suspects needed to be present in all technical replicates. Notably, suspect matches are also indicated in the results of the envihomolog algorithm in enviMass and were checked within this software accordingly.

2.3.2. Non-target HS detection

Employed settings for peak picking, blank peak subtraction and homologous series detection can be found in Table S2 in the SI. Since enviMass allows RUs as input parameter, literature research was conducted on commercially available polymeric substances (Schymanski et al., 2014b; Gago-Ferrero et al., 2015; García et al., 2019) and RUs (Ellis and Smith, 2008). 444 unique RUs were compiled of which 400 had a mass of < 400 Da and were consequently used as input (see Table S3 in the SI). Notably not all of them are to be expected in the wastewater samples. enviMass v4.2beta includes advances on the embedded non-targeted HS extraction tool, envihomolog (Loos and Singer, 2017). A more detailed description on the advances can be found in the supplementary information.

For non-target HS detection, results on components (*i.e.* grouped picked peaks (monoisotopic m/z, isotopologues and respective ion species)), HS as well as peaks within HS were exported from enviMass, and further processed with RStudio (R Core Team, 2016). HS containing components comprising only one ion (single ion events) for each member were excluded from further analysis. Focus was laid on the HS remaining after the secondary clarifier or after the ozonation and sand filtration step. These were visually controlled by plotting extracted ion chromatograms (EIC) using the R package MSnBase (Gatto and Lilley, 2012) and checked manually and case-wise for redundancies due to different adducts or charge states. For that purpose and also to screen for transformation reaction products the R package TPPrioritizR ("TPPrioritizR," n.d.) was employed (Table S5 in the SI shows the list of screened mass differences). MS2 spectra and MS1 isotopologues were consequently extracted using the R package RMassbank (Stravs et al., 2013) and written to *.ms file for characterization using SIRIUS/CSI:FingerID, version 4.0.1 (Dührkop et al., 2019). Here, for molecular formula annotation the following settings were used: if the ion species was known the entry was set accordingly, otherwise, it was set to unknown ($[M+?]^+$ or $[M-?]^-$), though prevalent ion species (*e.g.* $[M+Na]^+$ in case of (+)-APPI) were prioritized in the hit list of molecular formulas; a 5 ppm mass accuracy window was set. For *in silico* structure elucidation, PubChem was used as input database. If molecular formulas could be assigned, but no hit in PubChem was found, molecular formulas were searched within SciFinder using the information on the RU as input.

2.3.3. Characterization of HS

The last step of the workflow comprises the characterization of the HS. Due to the vast structural complexity of polymeric substances an unequivocal structural identification will be hard to achieve with merely RPLC-HRMS/MS (Gruendling et al., 2010; Crotty et al., 2016), this holds especially true for branched chain polymeric substances. Then again, there is the advantage of having multiple members that need to suffice specific requirements and thereby contributing to the confidence of identification. Hence, the concept of communicating the level of confidence of identifications, as suggested by Schymanski et al. (Schymanski et al., 2014a), was adapted to the characterization of HS. Here, the authors define the lowest level (A) as a HS of interest (*analogue level 5*), with a minimum of 4 HS members, with a defined accurate m/z difference within a 3 ppm window and a RT variability between adjacent members of 5 s. It is also to be checked if the HS' members are of the same ion species. In the next step (B), for at least 2 members or $\geq$ 50% of the detected HS a molecular formula is assigned (*analogue level 4*). As a next stage (C), MS2 fragment information is included for tentatively characterizing the HS (analogue to level 3). Here, diagnostic fragment ions are indicative, as described by *e.g.* Thurman et al. for PEGs (Thurman et al., 2017). *In silico* fragmentation prediction is applied here. The final stage (D) includes also library matches (*analogue to level 2*) and/or reference standards *(analogue to level 1)*.

3. Results and discussion

Suspect screening and non-target HS detection were performed in parallel (see Fig. 1). Since centrifugated samples resulted in a significantly lower number of HS detections (e.g. for samples after ozonation: 10 $\pm$ 2 HS compared to 96 $\pm$ 10 HS for pre-concentrated samples in case of (+)-ESI ionization approach), focus was put on the pre-concentrated samples. Table 1 gives a summary on the suspect matches and the number of *non-targeted* HS detected via enviMass and its algorithm envihomolog. Importantly for the latter findings, the number of HS listed in Table 1 correspond to HS findings after the implemented HS-filter advancement and ensuring a minimum number of ions per HS members.

3.1. Method optimization and quality assurance

The variation in detected HS depicted in Table1 as well as in the following figures, is referring to sample preparation replicates (n=5) and can be explained by differences in signal intensity and consequently peak picking. This holds especially true for signals of low intensity. To generally gauge the repeatability of signal intensity, variation in chromatographic peak height of PEG was evaluated for the analytical worst-case scenario, the primary clarifier. Here, an average RSD of the signal of the respective most abundant ion species of 12%, 19% and 12% for pre-concentrated primary clarifier samples using (+)-APCI, (+)-APPI and (+)-ESI, respectively was assessed (Data shown in SI Table "S6_PEG_Int-Repeatability"). Besides, also RT stability was assessed by means of PEG-05EO up

Table 1
Summary of results on suspect screening and non-targeted HS detection using different ionization interfaces and polarities to analyze the different stages of a Swiss WWTP. Samples were pre-concentrated by approximately a factor of 10. *for n=5, ** with at least one non-blank peak, n.d. not determined.

Matrix	Ionization interface	Total number of picked peaks * avg ± SD		Number of non-target components ** avg ± SD		*Results on suspect screening*				*Results on HS detection using envihomolog algorithm*							
						Components containing suspect compounds avg ± SD		Number of HS families		Number of HS families avg ± SD		Total number of members involved in detected HS avg ± SD		Number of unique members involved in detected HS avg ± SD		Total number of suspect matches avg ± SD		Number of unique suspect matches avg ± SD	
		(+)	(-)	(+)	(-)	(+)	(-)	(+)	(-)	(+)	(-)	(+)	(-)	(+)	(-)	(+)	(-)	(+)	(-)
primary clarifier	ESI	6733± 61	6398± 94	4119± 56	4459± 54	447± 14	334± 17	26	29	790± 28	208± 10	4392± 148	1103± 45	2438± 66	892± 23	1017± 54	326± 30	230± 9	116± 7
	APPI	1542± 60	1720± 79	888± 54	1160± 53	157± 17	101± 8	15	10	82± 7	50± 6	411± 32	343± 23	271± 23	251± 11	238± 29	144± 15	66± 8	49± 4
	APCI	1421± 31	1094± 177	964± 33	820± 26	182± 9	113± 8	13	9	99± 6	55± 2	542± 27	319± 10	334± 8	219± 5	391± 39	109± 21	91± 7	40± 5
secondary clarifier	ESI	3251± 28	2536± 23	1989± 14	1510± 17	106± 6	145± 4	14	4	66± 5	49± 5	326± 22	212± 22	254± 8	165± 18	14± 4	76± 11	11± 2	22± 4
	APPI	2256± 175	3683± 47	846± 261	2319± 15	118± 15	73± 2	11	9	15± 3	8± 1	80± 15	32± 6	56± 7	28± 5	67± 12	6± 6	21± 2	3± 2
	APCI	481± 21	239± 14	311± 12	130± 7	8± 1	6± 1	1	0	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
after ozonation & sand filtration	ESI	2543± 34	1431± 28	1294± 32	563± 17	118± 3	122± 7	15	5	96± 10	26± 5	499± 45	116± 23	361± 20	89± 12	54± 9	71± 15	22± 2	27± 3
	APPI	1506± 34	1624± 37	470± 14	824± 34	104± 6	52± 2	7	3	23± 4	6± 1	136± 28	33± 8	89± 13	23± 2	74± 24	23± 8	21± 3	8± 1
	APCI	383± 7	286± 11	185± 5	103± 6	13± 2	4± 1	1	0	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.

to PEG-24EO. Excellent RT stability throughout the different sample matrices was observed for all ionization sources ($\leq$ 2%). Only for APPI variation up to around 5% was observed for the earlier eluting compounds (i.e. < 1.5 min) most probably due to addition of isopropanol to the mobile phase as APPI-modifier (Data shown in SI Table S7_PEG_RTStability).

Importantly, the different sample types are characterized by their different matrix loads, leading to effects on chromatographic separation and ionization that are compound dependent. For target quantification, considering this matrix effect is essential. However, in non-targeted analysis and suspect screening, where compound-specific information is lacking, such considerations become challenging. Since the aim of this work is focusing on the characterization and not the quantitation of polymeric substances, no matrix factor was added and only inter-sample type changes in intensities of above one orders of magnitude, and a signal to blank factor > 3 were considered as actual changes during the wastewater treatment process.

It is noteworthy that RU are depicted as m/z and hence are dependent on the charge state of the respective HS (*e.g.* C_2H_4O can be found as 44.02 for singly charged and 22.01 for doubly charged HS). Besides, RUs can be also described as multiple of each other, e.g. CH_2 and C_2H_4 units. Another factor contributing to the overall complexity of HS analysis is the redundancy arising from the dependence of molecular weight and ion species: especially in ESI, one HS may change its members' ionization behavior, i.e. charge state, over (increasing) mass. The diminished overlap of co-eluting peaks over these different HS makes their grouping difficult, despite the additional HS filtering steps which were implemented here. In order to reduce this complexity, further filtering steps were implemented into envihomolog approach. Generally, the chromatographic separation and difference in masses aid in indicating on what type of monomeric unit was employed for synthesizing the polymeric substance, however, the head groups are often significantly more difficult to be characterized. Especially, if depletion or transformation reactions have taken place and where it is hardly possible to track the parent polymeric substance.

Evidently, the number of detected HS is depending on the employed ionization source and the polarity (as shown in Table 1). By far the highest number of HS is clearly detected using ESI in positive mode. Here, it has to be pointed out, that during ESI multiple charges can be transferred to the analytes and additionally various ion species are possible. This holds especially true for positive ionization mode. The differences in charge states and ion species for the different ionization interfaces is shown for the model polymeric substance PEG in Figure S5 and Figure S6 in the SI. They clearly show that one HS can be present in different ionization species as well as carrying different number of charges (up to z=8 detected). Notably, the occurrence of multiple ion species is generally matrix-dependent, however, no significant difference was observed for the model polymeric substance for the three different sample types (data not shown). Data processing might represent another contributing factor: many non-targeted analysis algorithms are intended for the analysis of ions with low charge states ($z \leq 2$) and the grouping of isotopologues and adducts to one component might be hampered for ions showing higher charge states. Consequently, (+)-ESI might be more prone to redundancies and false-positive findings in detected HS, compared to the other two ionization interfaces.

In the following the results on suspect hits and the non-target HS detection approach will be discussed in more detail.

3.2. Suspect screening

Results on suspect screening can be found in Table S8, showing the name and abbreviation, RU and molecular structure of the

respective HS family, level of confidence and in which ionization mode it was found. Hits in the suspect list were checked for MS2 availability and MS2 matches, corresponding to a level of confidence of at least 3 (Schymanski et al., 2014a) and a characterization stage of C according to Fig. 1. For certain HS family members, MS2 library spectra were available at MassBank/ MassBank of North America (Horai et al., 2010) and allowed for a level of confidence of 2 /stage D.

As it is shown in Table S8, in total 21 different HS families were found; 6 unique HS families were found for APCI and 9 for APPI. ESI clearly shows the highest number on hits and all detected suspect HS families were amenable to this ionization interface, either in positive or negative mode. However, this finding might be biased due to the sources of the suspect list, since ESI is definitely the most prominently used ionization interface for LC-based separations.

As expected, the primary clarifier clearly represents the sample with the highest number of detectable HS and all suspects listed in Table S8 were found in the primary clarifier (PEG-EOn, PEG monomethyl ether (PEG-MME-EOn), PEG dimethyl ether (PEG-DME-EOn), PEG monobutyl ether (PEG-MB-EOn), PPG-POn, tetramethylbutyl phenol, ethoxylated (TMBP-EOn), Cn- AEOx, octylphenol ethoxylates (OPEOn), coconut diethanolamide (Cn – DEA), nonylphenol polyethoxylates (NPEOn), glycol ether sulfate (GES-n), nonylphenol ethoxylates sulfate (NPEOn- SO4), alkyl sulfate (Cn – AS), secondary alkane sulfonate (Cn- SAS), alkyl ethoxy sulfates (Cn- AExS), dialkyl tetralinsulfonates (Cn – DATS), linear alkylbenzene sulfonate (Cn- LAS), sulfophenyl carboxylic acids (SPA-Cn), sulfophenyl alkyl dicarboxylated (SPA-nDC), sulfotetralin alkyl carboxylated (STA-nC)).

Within the suspect list hits, the following HS families were still detected in the secondary clarifier: Cn-AEOx, NPEOn, PEG-EOn, PEG-DME-EOn, PPG, SPA-Cn, SPA-nDC, STA-nC. Finally, the following suspect hits persisted partially treatment and found in the final effluent, *i.e.* after ozonation and sand filter: GES-n, NPEOn, PEG-EOn, PEG-DME-EOn, PEG-MME-EOn, PPG, SPA-Cn, SPA-nDC, STA-nC.

In fact, the HS length of the PEG present in the wastewater samples was surprising, since typically the mass range studied in environmental samples has an upper limit of 1000 m/z. Here, with APPI up to roughly 3540 Da (corresponding to 80 ethoxy units) in the primary clarifier and down to roughly 1380 Da in the final effluent were observed. This can be seen in Fig. 2, showing the fate of this widely used water soluble polymeric substance during the three steps of the wastewater treatment process. Similar observation for WWTP effluent samples were reported recently by (Freeling et al., 2019), showing PEG with up to 40 ethoxy units.

3.3. Non-target HS detection

Over the different stages of the sampled Swiss WWTP a significant decrease in number of detected HS was observed using the non-target HS detection approach. This observation is in agreement with findings in literature for target surfactants (Brunner et al., 1988; Schröder et al., 1999; Traverso-Soto et al., 2016; Freeling et al., 2019) and is shown in Fig. 3 for HS detectable in positive ionization mode and in Figure S7 for HS detectable in negative ionization mode, respectively.

Inspecting m/z vs. RT distribution of detected HS, as can be seen in Fig. 3 and (Figure S7 for negative mode), the chosen m/z range of 200 – 3000 proved reasonable especially for APCI and APPI, since mainly singly or doubly charged ions were observed. In case of ESI, charge states of the model HS PEG of up to +8 were detected, hence reducing the upper bounds of observed m/z values. It is worth mentioning, that in environmental samples, typically singly charged ions in a mass range of 50 – 1000 m/z are in the focal point of analysis. Hence, depending on the sample matrix, HS of higher masses would be missed.

3.4. Characterization of HS

As described in Fig. 1, after HS detection, suspect and non-target screening findings were checked manually for potential redundancies, ion species mis-classifications, etc. Besides, hits were limited to a charge state z $\leq$2 and MS2 availability of at least 2 HS members or $>$ 50% of the members was set as a pre-requisite for tentative and probable structure elucidation, respectively. EICs of HS of interest were generated for all three stages of the WWTP and were checked visually for changes in distribution over the course of the treatment. Often the HS were present in all sample types, but differing in the distribution of the HS' members. In the blank sample hardly any HS could be observed.

The main focus of the present study was laid on the fate of water-soluble polymeric substances and discharge in receiving water bodies, hence characterization of detected HS was performed in samples taken after the second and third stage of the WWTP. The second stage was included in the fate study, since globally, ozonation does not represent a commonly performed stage in WWTP. Since APCI did not show any significant benefit, neither in the number of detected HS using the non-targeted screening approach, nor the suspect screening (Table 1), this dataset was not further investigated.

Results of the detected and filtered HS, using the two different ionization interfaces, ESI and APPI, and present in the two final WWTP stages, can be found in Table S10- S13 (including information on HSs' presence depending on the sample type and comments on changes in distributions). A summary of the findings (occurring RUs, minimum and maximum detected m/z, length and characterization classification) is shown in the SI Table S9. Due to the described filtering measures, the number of HS to be characterized decreased, namely to 10 HS for (+)-APPI, 5 for (-) APPI as well as 91 for (+)-ESI and 40 in case of (-)-ESI. This results in a total of 774 members being grouped to 146 HS.

Suspect and non-target analysis (envihomolog) are in good agreement as can be seen in Table 1 and in Table S10 – 13. Out of the 146 filtered HS, 20 HS were assigned to suspect-HS and 36 had matches regarding accurate mass of potential transformation products of suspects. Notably, also naturally occurring "HS" were expected to be present, but were not included in the suspect list, e.g. fatty acids being detectable using (-)-ESI. In the following, the findings are discussed and to showcase challenges associated with the characterization of HS, six examples are shown in more detail in Fig. 4.

In case of APPI, the m/z of detected and filtered HS was ranging between roughly 390 up to 1320 m/z and HS were eluting between 2 to 22 minutes. For ESI the lowest observed m/z for the filtered HS was around 210, whereas the highest m/z value was around 820, the RT space was similar to APPI. Generally, positive ionization lead to the detection of HS containing a higher number of members (up to 19 for (+)-APPI and 15 for (+)-ESI), whereas the maximum length in negative mode was only 6 members. Note that sodium adducts, being prevalent in (+)-APPI, give generally MS2 spectra with lower information content compared to other adducts such as protonated species or ammonium adducts. This definitely affects the success of structural characterizations of the (+)-APPI mode.

In APPI the number of HS in the secondary clarifier decreased significantly to 2 HS for positive mode and 2 for negative mode. In case of positive mode, one of the detected polymeric substance comprises 19 members (from roughly 525 to 1320 Da) and was characterized as PEG by comparing MS2 library spectra in MassBank and a PEG 600 and PEG 2000 spiked sample. For the

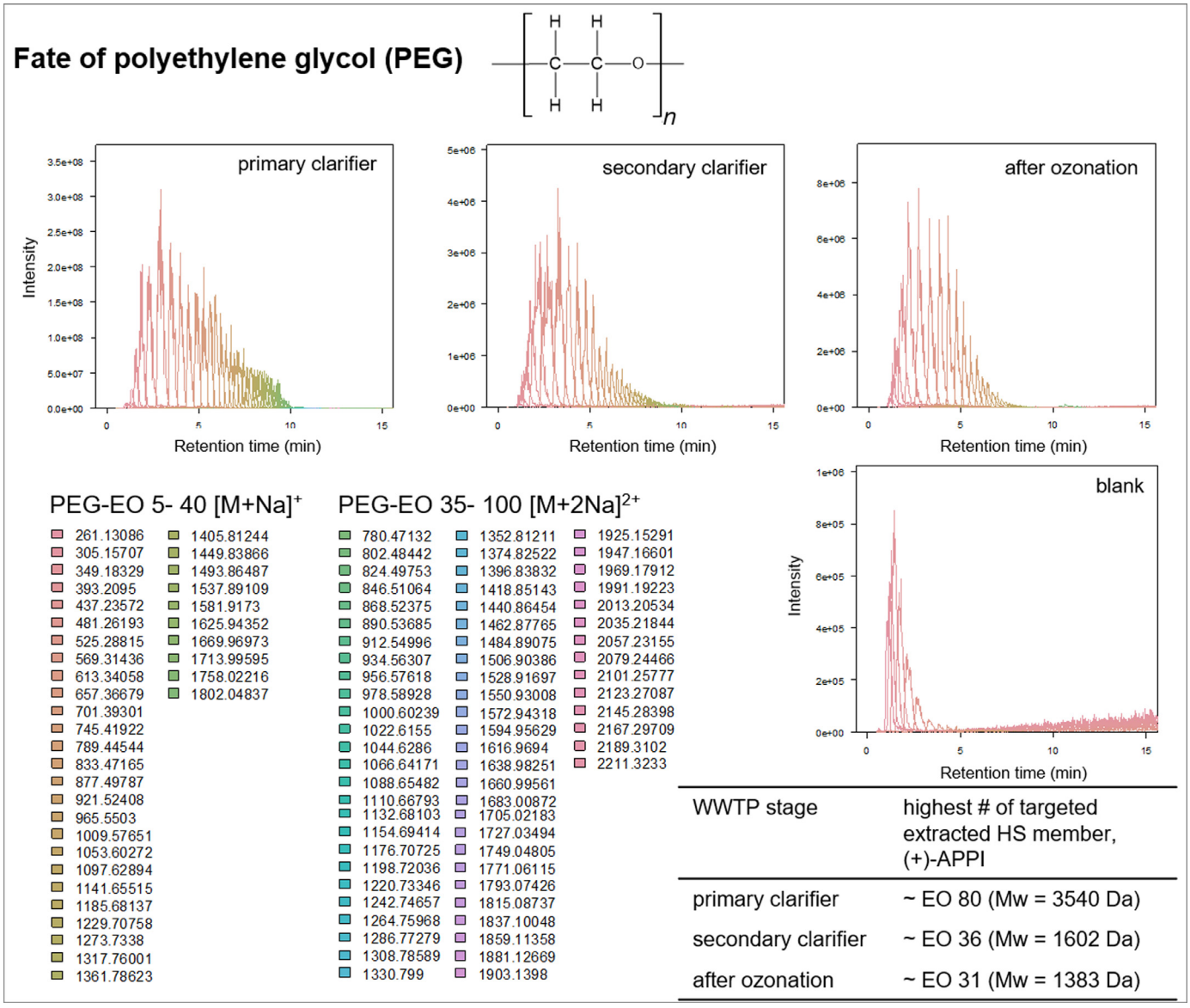


Fig. 2. Changes in PEG-monomethylether (EO*n refers to ethoxy units)* distribution over the different stages of a Swiss WWTP using RPLC-(+)-APPI HRMS/MS analysis.

other HS no MS2 was triggered, due to low intensity (sum of log10(intensities) = 5.7). Checking for potential transformation reactions of the suspect list, indicated an oxidation step from methyl to carboxylic acid of either the $[M+K]^+$ of OPEO18-21 or the $[M+Na]^+$ of PEG-DME-EO18-21. Since no other ion species were grouped to these compounds and both ion species are reasonable for (+)-APPI, this HS exemplifies the lowest stage A of characterization of HS, as described in Fig. 1.

In case of ozonation, 8 HS remained after the filtering step for (+)-APPI and 3 in case of (-) APPI. Again, PEG was characterized in (+)-APPI, however with a shift in mass distribution, namely from 400 to 1190 Da (compared to 525- 1320 Da in the secondary clarifier). This observation can be explained by degradation and sorption processes and is in agreement with findings in literature (Traverso-Soto et al., 2016). Fig. 2 shows this change in HS distribution for all three stages of WWTP for a mass range of 250 up to 5000 Da using (+)-APPI as ionization interface.

Besides, another PEG derivative, namely PEG-MME was characterized (stage D). This HS can either be formed (Kawai, 2005) during the stages of WWTP or was already present in the primary influent or both. Both polymeric substances are used among others as excipient in pharmaceuticals, basis of skin creams or dispersant in toothpastes. Additionally, two other potential transformation products of PEG derivatives were tentatively characterized (stage C). Four HS neither had matches in the suspect list, nor could be explained by the common transformation reactions, however assigning molecular formulae was possible. Aiming at tentatively characterizing the structure of the HS, Fig. 4a) gives an example of the structural variety of branched chain PEGs. Besides, it can be seen how the distribution of the polymeric substance is changing over the course of the WWTP. In case of (-)-APPI, PEG, GES were characterized (stage D). Fig. 4b) shows an example of a stage B characterization: according to known transformation reactions and the exact masses two different PEG derivatives were suggested, however the MS2 spectrum was not indicative of either of the two.

Also, after the described filtering steps, using ESI lead to a significantly higher outcome in HS detection, namely 41 HS for the

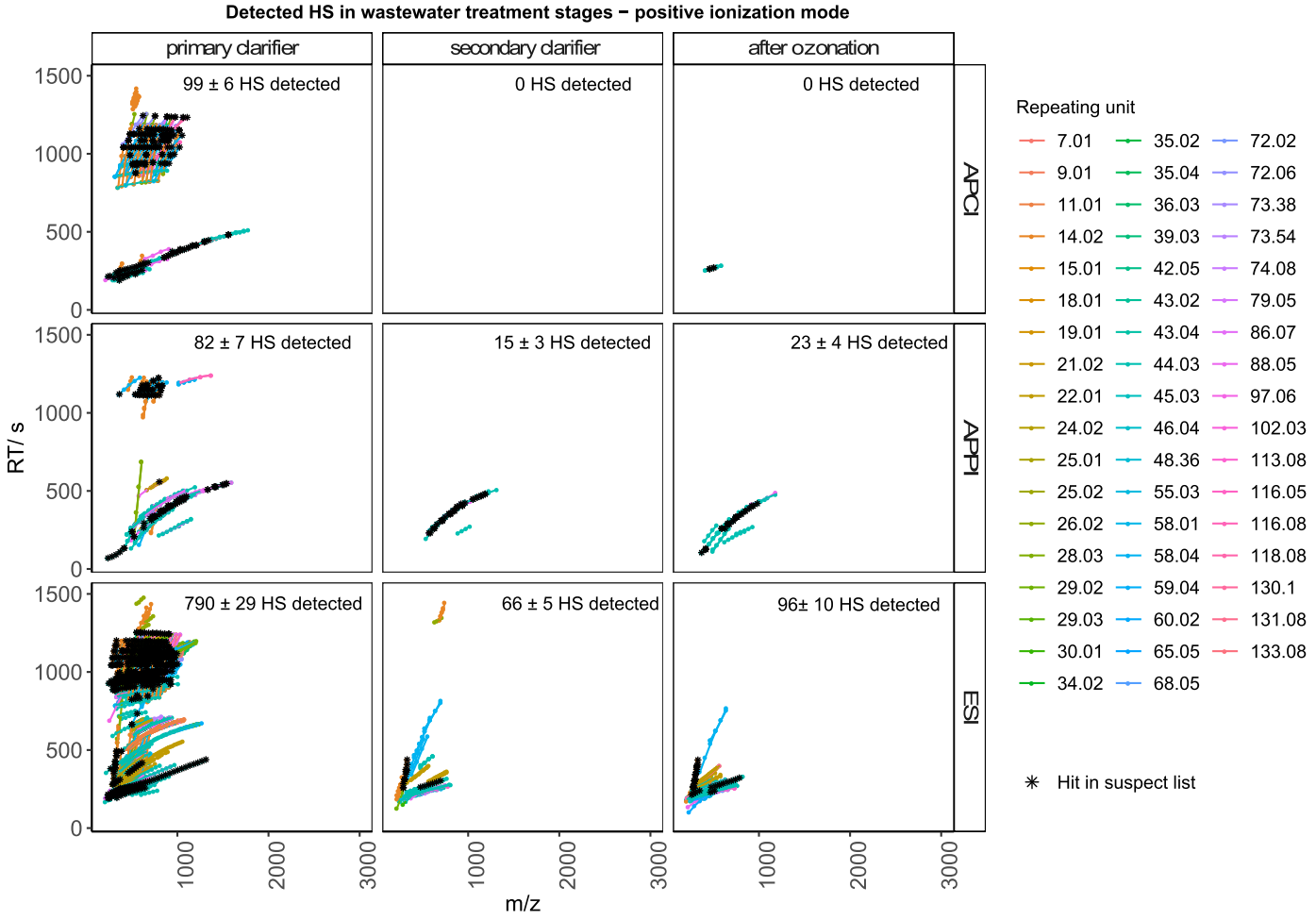
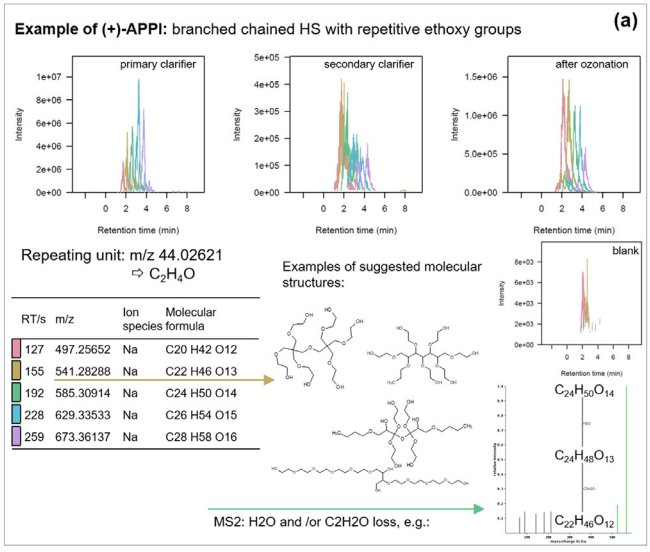


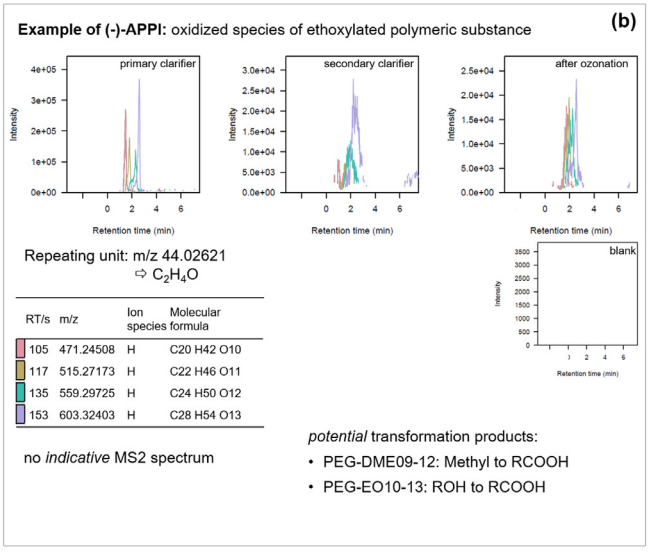
Fig. 3. Detected HS in the different wastewater treatment stages, namely primary clarifier, secondary clarifier and after the ozonation and sand filter step. Samples were pre-concentrated using vacuum- assisted evaporative concentration. Ionization was performed with APCI, APPI or ESI in positive mode (negative mode in Fig. S7). RU are depicted as m/z and indicated in the respective color. Hits from the suspect list are highlighted by a black asterisk.

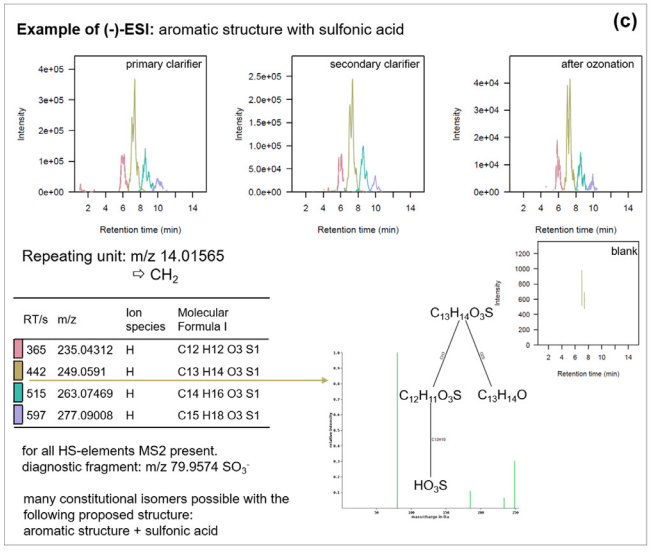
secondary clarifier in positive mode and 27 for negative polarity, and 50 and 13 HS after ozonation, respectively.

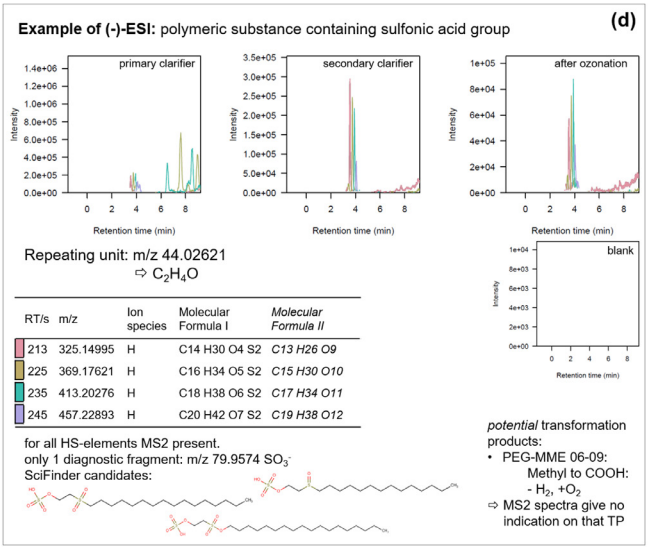
As for the secondary clarifier, the majority (around 75% (32 HS) for positive and 55% (15 HS) for negative mode) were characterized as unknowns. However, for each polarity 9 unknown HS allowed for a characterization stage C. Molecular formula generation of detected HS in negative mode revealed pre-dominantly sulphur (see exemplarily Fig. 4c) and d)) as well as phosphorus containing molecules. Interestingly, Fig. 4d) shows an example, where the m/z difference would suggest an ethoxy group as RU, however no polymeric substances in databases comprising two sulfur atoms and ethoxy as RUs fit to the respective masses. In positive mode suggested molecules often centered around sugar derivatives with alkyl residues (see exemplarily Fig. 4e)). The latter polymeric substances are also known to be used as surfactants in personal care products (Plat and Linhardt, 2001).

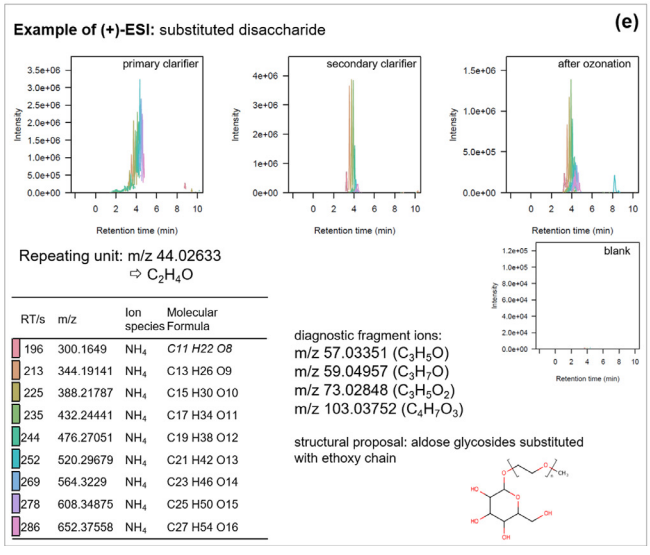
Generally, AEOs and PEGs are removed with high efficiency in WWTP by sorption and degradation (Traverso-Soto et al., 2016). In case of aerobic degradation of AEOs, pathways involve the cleavage of the ether bond between the alkyl and ethoxy chain, resulting in the formation of fatty acids and PEGs (Marcomini et al., 2000). Being consistent with findings in literature (Traverso-Soto et al., 2016; Bernhard et al., 2008), in positive mode, for both, biological treatment (secondary clarifier) as well as ozonation, some detected HS could be potentially explained by known transformation reactions of suspects, here dealkylation, dehydration as well as carboxylation reaction of different PEG derivatives. It was possible to assign molecular formulas, however, again the majority of the MS2 spectra were not conclusive enough to reveal the head group and certainly not the exact molecular structure. This is in agreement with findings of Schymanski et al., showing very few clean MS/MS spectra in positive mode (Schymanski et al., 2014b). Regarding the negative mode, sulphur containing suspect polymeric substance were potentially transformed via (de)hydration, hydroxylation, decarboxylation, de(hydrogenation). An example for the lowest stage of characterization, *i.e.* A, is depicted in Fig. 4f). As shown, the chromatographic behavior, i.e. non-baseline separated HS only detected after ozonation, suggests a constitutional isomer and transformation processes, as described in (Lara-Martin et al., 2011) for ethoxymers of NPECs in pore water samples. Even when using a mass spectrometer with excellent mass accuracy and stability, the molecular formula assignment was not unequivocal and neither the isotopologue pattern nor the MS2 spectra allowed for a reduction in number of candidates. Notably for the latter, fragmentation spectra showed only very few ions and hence were not conclusive.











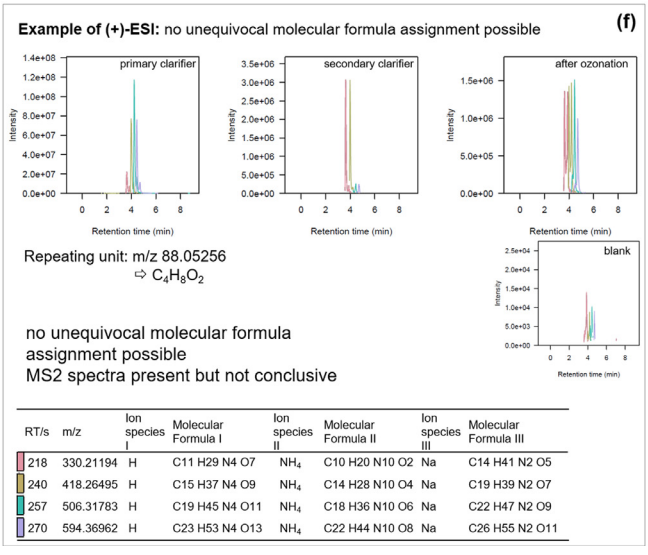


Fig. 4. Examples of characterized HS of different stages of Swiss WWTP. a) gives an example of branched chained HS using (+) APPI having a repetitive ethoxy chain, leading to a characterization stage of C according to Fig. 1. b) shows a HS using (-) APPI as ionization interface and leading to a characterization stage of B. (-)-ESI as ionization technique was used for c) and d), showing an example for characterization stage of C and B. Whereas (+)-ESI was employed for the examples e) and f). e) exemplifies a stage C, whereas f) the lowest level A.

4. Conclusion

- There are clearly many other HS present in wastewater, apart from the known surfactants, which could not yet be explained by common transformation reactions. Using a non-targeted screening approach, in total 146 HS were detected, of which 56 (ca 39%) could be associated with suspect matches or transformation products of suspects.
- Due to high degree of freedom in terms of molecular structure, their characterization was only partly successful, since MS2 spectra do not fully resolve these constitutional isomers. Tentative characterization or probable chemical structure could be assigned to almost half of the findings.
- Clearly, (+)-ESI shows the highest number of detected HS, however, high number of charge states and adducts in (+)-ESI complicate data deconvolution and hence complicate characterization of HS.
- (+) – APPI shows overall good ionization efficiency – easier interpretable mass spectra compared to (+)-ESI, though MS2 interpretation suffers due to preference of Na – adduct formation.
- A combination of the two different ionization approaches, namely APPI and ESI seems to be promising to expand the mass to polarity plane.
- Expanding the mass range towards a higher m/z value proved to be reasonable, especially if studying HS occurring in the primary clarifier is of interest.
- In ESI higher molecular masses come along with higher charge states. Common identification workflows in non-targeted analysis approaches rely on *in silico* MS2 fragmentation spectra interpretation, e.g. MetFrag (Ruttkies et al., 2016) or CSI:FingerID/Sirius (Dührkop et al., 2019). Since these platforms are restricted to singly charged ions, the characterization of HS with multiple charged members is hampered and hence would require a different approach.
- The results demonstrate effective removal of HS during wastewater treatment and formation of some oxidative transformation products.

5. Contributors

T.M. planned and performed the experiment, evaluated the data and wrote the manuscript. M.L. extended the enviMass HS filtering steps for envihomolog, contributed to scientific discussion in terms of data evaluation and manuscript writing. J.H. gave valuable scientific input in all steps including the manuscript writing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.watres.2020.116745.

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