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Identification of novel micropollutants in wastewater by a combination of suspect and nontarget screening

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ABSTRACT

To detect site-specific, suspected and formerly unknown contaminants in a wastewater treatment plant effluent, we established a screening procedure based on liquid chromatography–high resolution mass spectrometry (LC–HRMS) with stepwise identification schemes. Based on automated substructure searches a list of 2160 suspected site-specific and documented water contaminants was reduced to those amenable to LC–HRMS. After searching chromatograms for exact masses of suspects, presumably false positive detections were stepwise excluded by retention time prediction, the evaluation of isotope patterns, ionization behavior, and HRMS/MS spectra. In nontarget analysis, peaks for identification were selected based on distinctive isotope patterns and intensity. The stepwise identification of nontarget compounds was automated by a plausibility check of molecular formulas using the Seven Golden Rules, an exclusion of compounds with presumably low commercial importance and an automated HRMS/MS evaluation. Six suspected and five nontarget chemicals were identified, of which two have not been previously reported as environmental pollutants.

1. Introduction

Over the last decades, an increasing number of emerging and unregulated pollutants have been detected in surface waters. The majority of these compounds, such as pharmaceuticals, surfactants, biocides, personal care products, and sweeteners enter surface water mainly via treated and untreated wastewater (Nikolaou et al., 2007; Reemtsma et al., 2006). Many of these compounds are polar and persistent, and not eliminated by conventional wastewater treatment plants (WWTPs) (Clara et al., 2005; Petrovic et al., 2003). Consequently, a large number of compounds and their transformation products are ubiquitously present in surface waters throughout Europe (Loos et al., 2009). Although not all of the 70 000 chemicals registered for commercial application in the European Union (Brack et al., 2005; Schwarzenbach et al., 2006) may enter surface waters, a monitoring of small sets of target compounds misses important site-specific and potentially ecotoxicologically relevant compounds. Thus, novel approaches are required for a comprehensive chemical and biological screening of water samples.

Liquid chromatography–high resolution mass spectrometry (LC–HRMS) offers the possibility to detect hundreds of polar contaminants in targeted approaches without pre-selection of analytes due to its sensitivity and selectivity in full scan analysis. Furthermore, it allows the detection of known compounds suspected of being present in environmental samples (suspect screening) without reference standards, even after measurement (post-target screening) and the screening for yet unknown nontarget chemicals (Hernández et al., 2005, 2012; Krauss et al., 2010).

In suspect screening, chromatograms are analyzed for peaks of an exact mass derived from the known molecular formula of the suspect. Prior studies focused on transformation products, which show a close structural relationship to their parent compounds (Gómez-Ramos et al., 2011; Kern et al., 2009). To confirm the identity of suspects, their retention behavior, MS^n fragmentation, and ionization in different modes were compared to observations on the parent molecules and expert judgment (Gómez-Ramos et al., 2011; Kern et al., 2009).

In true nontarget screening, in which often no initial information on the analytes is available, automated peak detection and spectra deconvolution algorithms are applied, which typically reveal several thousand of peaks in an individual water sample (Diaz et al., 2012; Hollender et al., 2009; Terzic and Ahel, 2011). Subsequently, molecular formulas can be derived for the detected peaks based on accurate mass and isotope patterns. For each

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molecular formula, a large number of candidate structures exist, which have to be ranked or filtered to obtain a useful list of compounds for confirmation by reference standards. To this end, a range of different approaches have been suggested to predict properties of candidates such as MS^n fragmentation energies, product ion spectra, retention times or ion mobility drift times to compare predicted with experimental data (Kind and Fiehn, 2010; Menikarachchi et al., 2012; Ulrich et al., 2011; Wolf et al., 2010). The use of mass spectral libraries for the confirmation of compounds is still limited for LC-(HR)MS data, as libraries are small and the comparability of spectra is limited among different instruments (Zedda and Zwiener, 2012).

The application of both suspect and nontarget screening suffers from the large effort of manual data evaluation. Thus systematic strategies with automated approaches are required to filter suspect compounds to be searched for and "relevant" peaks on which the identification efforts should focus.

Our objective was to apply and critically assess the capabilities of a suspect and nontarget screening workflow for the identification of micropollutants in a WWTP effluent. Starting from a quantitative target screening approach to obtain an overview on the occurrence of well-known micropollutants, we then applied a suspect screening procedure with a stepwise identification scheme, which uses information solely deduced from the structure of the suspects. Furthermore, we applied a nontarget screening approach, which includes a systematic selection of peaks for identification and a stepwise exclusion of candidate structures for each peak. In both cases, manual data processing was supported and accelerated by automated software-based procedures.

2. Materials and methods

2.1. Chemicals

For target screening and a validation of the sample extraction and measurement method 98 target compounds were selected based on their occurrence in water samples (Kolpin et al., 2002; Loos et al., 2009; Richardson and Ternes, 2011; Ternes et al., 2007; Verlicchi et al., 2012). Details about these target compounds and other chemicals used are given in the Supplementary materials.

2.2. Sampling site and sample extraction

Grab water samples were collected at the outlet of the WWTP Bitterfeld-Wolfen, Saxony-Anhalt, Germany. Details on the WWTP and the treatment train are given in the Supplementary materials, S1.2. The sample analyzed by suspect and nontarget screening in this study was taken on 25 October 2010. To additionally monitor the repeated occurrence of nontarget chemicals, one sample per week was taken over six weeks between 25 May and 29 June 2011. Due to the need for large volumes for a subsequent biological assessment in an accompanying study, we sampled 20 L of WWTP effluent into aluminum containers. After filtration through glass fiber filters (GF/F, Whatman) the water was solid-phase extracted (SPE) with 4 g of Chromabond HR-X sorbent (Macherey–Nagel) in Omnifit columns (Diba Industries Ltd.). The sample was pumped through preconditioned columns at a flow rate of 20 mL/min by a preparative HPLC pump (NoraPrep 200, Merck). After washing with 400 mL of bidistilled water/methanol (90:10; v:v) and drying in a nitrogen stream, analytes were eluted with 300 mL of methanol, 200 mL of methanol containing 0.2% formic acid and 200 mL of methanol/acetone (80:20; v:v). The sample which percolated through the SPE cartridge was collected, adjusted to pH 3 with formic acid, and extracted a second time as described above. Combined eluates of both extractions were neutralized, evaporated to dryness and redissolved in methanol to a concentration of 1 mL of extract corresponding to 1 L of effluent (i.e., a concentration factor of 1000). Extracts were stored at −20 °C. A blank sample was prepared from bidistilled water and processed by the same procedure. Prior to LC–HRMS analysis an aliquot of extract was filtered using a PTFE syringe filter (0.2 μm, Macherey–Nagel) and diluted with three aliquots of bidistilled water.

2.3. LC–HRMS analyses

The reversed-phase liquid chromatographic separation was performed on a core–shell C18 column using a water-methanol gradient (both with 0.1% formic acid), for details see Supplementary materials (S1.3). To determine the number of exchangeable hydrogen atoms in deuterium exchange experiments eluents were replaced by deuterium oxide (99.9 atom % D, Sigma–Aldrich) and by tetradeuteromethanol (99.8 atom % D, Sigma–Aldrich). The LC system was connected to an ion trap-Orbitrap hybrid instrument (LTQ Orbitrap XL, Thermo Scientific). Full scan HRMS spectra (m/z 100–1000) were acquired using positive and negative ion mode electrospray ionization (ESI+/ESI−) in separate runs with a nominal resolving power of 60 000. Based on the results of the full scan data evaluation, data-dependent high resolution product ion spectra (HRMS/MS) were recorded in separate runs based on parent mass lists including (i) the masses of all detected target compounds, (ii) all masses assigned to a suspect detected in the chromatogram, and (iii) all masses of peaks picked for identification in nontarget screening. Collision induced dissociation (CID) at normalized collision energies of 35 and 50% and at higher-energy collisional dissociation (HCD) at 50, 70, 90, and 120% at a nominal resolving power of 15 000 were used. Further details on instrument settings are given in the Supplementary materials (S1.3).

2.4. Data processing and evaluation

For the target screening we searched exact masses of 98 compounds at known retention times and integrated the peak areas using the QuanBrowser of the XCalibur software (Thermo Scientific). The identity of detected targets was confirmed by HRMS/MS spectra and isotope peaks evaluated in the QualBrowser. Target compounds were quantified by standard addition, pentobarbital and the suspect secobarbital by isotope dilution (details in Supplementary materials, S1.7).

Accurate mass ion chromatograms and peak lists were generated from full scan spectra using the software MZmine v2.9 (Pluskal et al., 2010). The individual processing steps and settings are detailed in the Supplementary materials (S1.4). For the suspect and nontarget screening peak lists with accurate mass, retention time, peak intensity and area were exported from MZmine and further processed in Microsoft Excel and using the package R nontarget (Loos, 2012; RDevelopmentCoreTeam, 2010). Retention time prediction was conducted using a linear solvation energy relationship (LSER) model introduced by Ulrich et al. (2011); Supplementary Materials S1.8. HRMS/MS spectra were compared with those predicted for candidate structures using MetFrag (Wolf et al., 2010). To determine the number of exchangeable hydrogen atoms of compound peaks, we compared data from measurements in water/methanol gradients with those from D_2O/tetradeuteromethanol gradients in the QualBrowser.

2.5. Method validation

Details on the validation of the SPE method and the LC–HRMS measurement using 98 target compounds are given in the Supplementary materials.

3. Results and discussion

3.1. Performance of the extraction and LC–HRMS analysis

The method validation focused on the determination of the domain of chemicals extracted from water samples with an acceptable recovery and detected with a high sensitivity. This information is indispensable for the selection of suspect chemicals, and an important prerequisite for a nontarget screening analysis covering a wide range of structures and physicochemical properties.

Relative recoveries in WWTP effluent were between 75% and 135% for 64 of 98 compounds. Other 22 compounds were recovered between 50% and 75% and twelve below 50% (Table S3). While most of the nonionic compounds of intermediate hydrophobicity showed good recoveries >75%, recoveries of some ionic compounds such as fluoroquinolones (zwitterions) or verapamil (cation) and of more hydrophobic compounds such as octocrylene and musk compounds ($\log K_{OW} > 5$) were lower. Highly hydrophilic nonionic or ionic compounds were not tested and are not likely to be retained on HR-X or similar material such as Oasis HLB (Waters) (Huntscha et al., 2012). The lowest detectable concentrations (LDCs) in WWTP effluent for 93% of the compounds ranged from 0.5 to 50 ng/L, and were above 100 ng/L for only 4% of the compounds due to a high background signal for the particular ion (Table S3). The performance of the extraction and the detection was considered transferable to most of the other target compounds and the 2160 suspects, since the properties of about 95% of target and suspect compounds overlapped with the validation set with regard to $\log D_{OW}$, charge, and number of H-bond donors and acceptors (Fig. S4).