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Linking mutagenic activity to micropollutant concentrations in wastewater samples by partial least square regression and subsequent identification of variables

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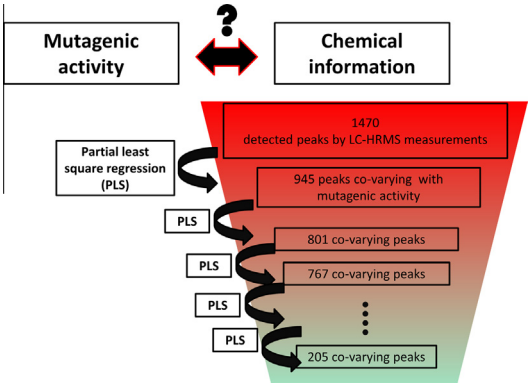
HIGHLIGHTS

- A sequence of wastewater treatment plant effluent samples was analyzed by LC–HRMS and Ames Fluctuation assay.
- Compounds co-varying with the mutagenicity were identified by "virtual" effect-directed analysis.
- Peak lists for identification were reduced by 86% using partial least squares projections.
- Compounds co-varying with mutagenicity were characterized and some identified.
- Identification and characterization of these compounds indicated an industrial source of mutagens.

GRAPHICAL ABSTRACT



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ABSTRACT

We deployed multivariate regression to identify compounds co-varying with the mutagenic activity of complex environmental samples. Wastewater treatment plant (WWTP) effluents with a large share of industrial input of different sampling dates were evaluated for mutagenic activity by the Ames Fluctuation Test and chemically characterized by a screening for suspected pro-mutagens and non-targeted software-based peak detection in full scan data. Areas of automatically detected peaks were used as predictor matrix for partial least squares projections to latent structures (PLS) in combination with measured mutagenic activity. Detected peaks were successively reduced by the exclusion of all peaks with lowest variable importance until the best model (high R^2 and Q^2) was reached. Peaks in the best model co-varying with the observed mutagenicity showed increased chlorine, bromine, sulfur, and nitrogen abundance compared to original peak set indicating a preferential selection of anthropogenic compounds. The PLS regression revealed four tentatively identified compounds, newly identified 4-(dimethylamino)-pyridine, and three known micropollutants present in domestic wastewater as co-varying with the mutagenic activity. Co-variance between compounds stemming from industrial wastewater and mutagenic activity supported the application of "virtual" EDA as a statistical tool to separate toxicologically relevant from less relevant compounds.

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1. Introduction

Over the last decades an increasing number of pollutants has been detected in surface water. The majority of these substances originates from human use and enters surface water via treated and untreated wastewater (Reemtsma et al., 2006; Nikolaou et al., 2007). Simultaneously, the biological characterization of wastewater treatment plant (WWTP) effluents revealed genotoxic, mutagenic, estrogenic, and other effects (Claxton et al., 1998; Miège et al., 2009). Mutagenic and genotoxic effects were reported to trigger long term effects on the survival of species (Anderson and Wild, 1994), but only limited information is available about compounds responsible for these effects.

To identify compounds with adverse effects in the environment, both chemical and biological characterization was combined to establish cause-effect relationships between compounds present and biological effects (Vermeirssen et al., 2010; Smital et al., 2011). To this end, two different approaches have been suggested. (1) Effect-directed analysis (EDA) is based on an experimental reduction of mixture complexity using chromatographic techniques to isolate active fractions for toxicant identification (Brack, 2003). Site-specific effect-relationships between chemical contamination and mutagenicity were investigated in several EDA studies (Reifferscheid et al., 2011; Gallampois et al., 2013). However, EDA is still costly and time-consuming and thus so far its application on a larger scale is limited. (2) An alternative approach using multivariate statistics to reduce the complexity of environmental contamination by correlation of effects with chemical analytical signals has been propagated as "virtual" EDA (Eide et al., 2002, 2004). In contrast to EDA, which focuses on a small number of samples, "virtual" EDA needs to be applied to larger sets of samples in space or time. Although this approach does not provide cause-effect relationships as such it has the potential to extract relevant information out of large datasets to derive hypotheses, which can be confirmed experimentally.

A large number of often co-varying variables (components) and a too small number of samples are major limitations for the application of multivariate statistics to environmental samples. PLS analysis may overcome this limitation since it tolerates data sets with co-varying predictors, large numbers of variables exceeding the number of observations (Kettaneh-Wold, 1992) and strongly co-varying, collinear data matrices (Wold et al., 1984).

The major goal of PLS analysis is the discrimination of variables co-varying with the response from those not co-varying. PLS has been demonstrated to provide meaningful correlation of chemical fingerprints of exhaust particle extracts and mutagenicity, but also of biotic indicators of surface water quality and landscape conditions (Eide et al., 2002; Nash and Chaloud, 2011).

This study presents "virtual" EDA as a tool for the exploitation of combined chemical and toxicological monitoring data for the characterization of chemicals correlating with observed effects. The exercise was conducted using a sequence of six weekly taken effluent samples from a mixed industrial and municipal WWTP effluent exhibiting varying mutagenicity in the Ames Fluctuation Test after activation with S9. To this end, mutagenicity testing was combined with a LC–HRMS non-target screening (Hug et al., 2014) supplemented with a suspect screening for a set of (pro)-mutagenic aromatic amines since this compound group requires S9 activation and has been made responsible for mutagenicity in surface waters in the past (Kataoka et al., 2000; Fukazawa et al., 2001). Mutagenic activity and all detected peaks were subjected to a PLS regression to filter out those peaks co-varying with the mutagenicity by a stepwise dimensionality reduction of the model. Differences between peaks identified as co-varying with the mutagenicity and all detected peaks were determined and evaluated for their plausibility.

2. Materials and methods

2.1. Sampling and extraction

The effluent from the WWTP Bitterfeld-Wolfen, Saxony-Anhalt, Germany was grab-sampled at the outlet into the river Mulde once a week for six weeks using 5 L aluminum containers. Samples were named as sample 1 to sample 6 based on their order of sampling and stored for up to 5 days at −20 °C before extraction.

After filtration through glass fiber filters (GF/F Whatman), a volume of 20 L of WWTP effluent (pH 7–7.8) was extracted by solid-phase extraction (SPE) using 4 g of Chromabond HR-X sorbent (Macherey-Nagel) in Omnifit columns (Diba Industries Ltd) and a preparative HPLC pump (NoraPrep 200, Merck). After percolation through the SPE cartridge the aqueous phase was adjusted to pH 3 with formic acid and extracted a second time. The sorbent from both extractions was 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 combined extracts were neutralized, evaporated to dryness and redissolved in methanol to a concentration factor of 1000. The ability of the SPE method to extract compounds with a wide range of physico-chemical properties has been shown before (Hug et al., 2014). Defined aliquots of samples were dried under nitrogen and re-dissolved in dimethyl sulfoxide (DMSO) for biological assessment. Prior to LC–MS analysis an aliquot of the methanol extract was filtered using a PTFE syringe filter (0.45 μm, Macherey-Nagel) and diluted with three parts of bidistilled water. A blank sample from 10 L of bidistilled water was prepared applying the described extraction procedure.

2.2. Mutagenicity testing and prediction

The Ames Fluctuation Test (AFT) was performed as described in Reifferscheid et al. (2012) with slight modifications using a TA98 tester strain with and without metabolic activation by S9 on 384-well microplates. Mutagenic samples induced a reversion from the auxotrophic to the prototrophic genotype indicated by a color change of the pH indicator bromocresol purple. Spontaneous reversion was evaluated in eight replicates with DMSO as negative control. The sensitivity of the TA98 strain was monitored with a dilution series of 2-nitrofluorene for tests without and 2-aminoanthracene for tests with metabolic activation. The mutagenic activity (given as revertants per L wastewater in L methanolic extract) was determined by fitting the number of positive wells to an exponential equation, see equation S1 and Supplementary Material (SM) S1.6. (Gallampois et al., 2013). The mutagenic activity was determined in triplicates with eight dilutions in three independent tests.

2.3. LC–MS/MS analyses and data processing

The liquid chromatography separation was performed with an Agilent 1200 UPLC system equipped with a Kinetex™ Core-Shell C18 column (100 mm × 3.0 mm; 2.6 μm; Phenomenex) with a linear gradient elution with water and methanol both containing 0.1% formic acid with a flow of 0.2 mL/min. The initial fraction of 10% methanol was increased after 3.2 min to 95% within a linear gradient in 17.8 min. Menthanol was maintained at 95% for 20 min followed by a re-equilibration for 9 min. The LC system was connected to an ion trap-Orbitrap hybrid instrument (LTQ Orbitrap XL, Thermo Scientific). Analytes were ionized by electrospray ionization in positive (ESI+) and negative (ESI−) ion mode.