**Revisiting the Mutagenicity in River Rhine: Searching for Mutagenic Aromatic Amines**

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River Rhine is the second largest river in Europe and receives discharges from many pollution sources. Since the 1970s mutagenic activity was observed in many samples from the lower part of the river and although the mutagenicity of the river decreased by time, the activity is still detectable (Alink 2007). The mutagenicity increases significantly with metabolic activation in AMES test (Hendriks 1994) and comet assays (Kosmehl 2004) pointing at the contribution of mutagenic aromatic amines. However to the best knowledge of the authors, the responsible compounds still remain unidentified. Thus, the aim of this study is to detect and identify the cause of mutagenicity in the River Rhine using effect-directed analysis.

Samples were taken in May 2014 at Lobith, Netherlands by an on-site large volume solid phase extraction machine using a polymeric sorbent. The extract was fractionated using a semi-preparative C18 column and minute fractions were collected. The fractions were not combined but used separately for screening in order to obtain the active fractions. Ames fluctuation assay (Ames II test), as described by Perez et al. (2003), with slight modifications, was used to assess the mutagenicity. Tester strain TA98 was employed with and without metabolic activation by S9 on 24-well and 384-well microplates. The raw extract showed a significant mutagenicity in the presence of S9 with TA98 at a relative enrichment factor of 250. In addition to that, strain YG 1024, which has an increased sensitivity for aromatic amines, was used to test the fractions active to TA98.

The raw sample and the active fractions were analyzed using an ion trap-Orbitrap hybrid instrument (LTQ Orbitrap XL, Thermo Scientific) in ESI positive ion mode by a previously developed diagnostic derivatization method to selectively label aromatic amines. The labeling reagent NBD-F (4-fluoro-7-nitro-2,1,3-benzoxadiazole) was used due to its high reactivity and very low ionization by ESI in positive mode. Prior to LC-HRMS analysis, 10 µL of NBD-F solution (0.8 mM in acetonitrile) and 2 µL of 20 mM ammonium acetate buffer (pH 5.7) were added to 30 µL of the fractions (in 70:30 MeOH/H2O). Vials were heated after the addition of NBD-F at 80°C for 30 minutes.

Data evaluation is currently being processed according to the following procedure: Peak lists from the full scan HRMS spectra were obtained using the software MZmine. The peak lists of the original and derivatized extracts were aligned and peaks that appeared in the derivatized sample were considered as derivatives of amines. Derivatives were further confirmed by using the diagnostic neutral losses -34.0055 (-2 OH) and -64.0035 (-2 OH, -NO) after MS/MS fragmentation. The mass shift of 163.0018 was used to detect the precursor amines in the original extract. The molecular formulas were obtained from accurate mass and isotope patterns and searched for candidate structures in the Chemspider database. To narrow down the candidate lists, fragmentation prediction using MetFrag and the presence of a primary or secondary amino group are being used as selection criteria.

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