Comparison of pre-assay clean-up procedures for unveiling the mutagenic potency of sediment extracts

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Introduction

Usually, one or several clean-up steps are necessary before applying in vitro bioassays to extracts from complex environmental samples like sediments. If raw extracts are tested or if the clean-up step is insufficient masking effects may occur.

This contribution compares the influence different clean-up procedures have on the determination of mutagenicity. Spiked and real sediments were extracted by Accelerated Solvent Extraction (ASE) and subjected to different clean-up procedures, e.g. the newly developed Rapid Dialysis Procedure (RDP) or gel permeation chromatography (GPC). The mutagenicity of the extracts was measured using the Ames II assay.

Mutagens are affected in different way by masking effects (Brack, 2003). Matrix compounds may

1. reduce the bioavailability of the mutagen.
2. affect the test organisms (e.g. cytotoxicity).
3. interfere with the activator for indirect mutagens.

Hypotheses

Masking effects of sediment constituents on mutagenicity were investigated by spiking sediment extracts with three known mutagens of different properties:

1. benzo[a]pyrene (BaP) - a lipophilic indirect mutagen requiring enzymatic activation in order to develop mutagenic activity
   → a clean-up is expected to be necessary for obtaining a positive test result
2. pyrene-1,6-quinon (PQ) - a highly mutagenic and reactive compound
   → might be bound to sediment constituents so that no or a decreased effect occurs
3. 1,6-dinitropyrene (DNP) as a strong direct mutagen
   → no effect of matrix components on DNP is expected, but masking due to impairing the test organism may occur

Regarding the different clean-up procedures, a stronger effect should be observed in purified than in raw extracts at the same concentration level. No hypotheses were set up concerning differences of GPC and RDP, since the latter is a newly developed method.

Materials & Methods

Experimental design

Sediment extracts were measured either as raw extract or after a clean-up procedure in the Ames II mutagenicity assay. The clean-up was done in three different ways: Using RDP, GPC, or a combination of both (Fig. 1).

Four different experiment series were accomplished:

1. Without spiking the sediment extracts.
2. Spiking with benzo[a]pyrene
3. Spiking with pyrene-1,6-quinone
4. Spiking with 1,6-dinitropyrene

The mutagenic potential of the spiked extracts was compared to the spike compounds in pure solvent. All samples were dissolved in DMSO.

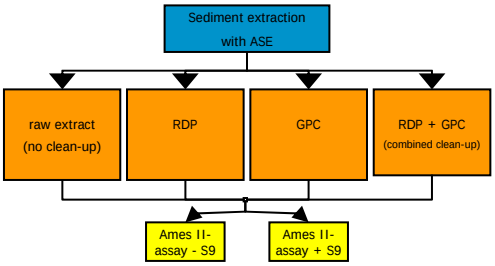
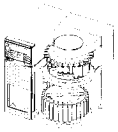


Fig. 1: Study design: sediment extracts were either tested for mutagenicity as raw extracts or cleaned up using RDP, GPC or a combination of both methods

Extraction

Sediments were collected in the year 2005 in the River Elbe above the weir of Přelouč near Pardubice (Czech Republic). Sediments were freeze dried, sieved to 63 µm and extracted by means of an ASE 300 device.
two-step extraction method:

- 1st extraction with hexane:dichloromethane at 80 °C
- 2nd extraction with toluene at 140 °C
- 3 cycles for each extraction
- 10 min static extraction time
- flush volume of 60 % of cell volume
- N_2-purging time of 60 sec



GPC

Clean-up by GPC was carried out according to a slightly modified standard procedure established by US-EPA (Method 3640A) applying a gel-permeation-column (35 cm length, 3 cm i.d) filled with Biobeads SX3 (BioRad, München) and dichloromethane as solvent. The flow rate was adjusted with a HPLC-device to 2.5 ml/min.

RDP

The Rapid Dialysis Procedure (RDP) is a new clean-up technique based mainly on the principle of size exclusion. It combines a dialysis procedure using a semi-permeable membrane and pressurised liquid extraction.

The RDP consists of the following steps:

1. Transfer of the spiked/un-spiked sediment extract (0.5 ml) into a membrane bag made of a polyethylene foil.
2. Placing the membrane, sealed with a welding apparatus, together with a stainless steel mesh into a 33 ml-ASE cell. The steel mesh prevents the membrane from clinging to the inner wall of the extraction cell.
3. The dialysis is carried out using an ASE 200 device pressing solvent into the cell. The cell is heated, the solvent kept under pressure.
4. The membrane separates molecules by size. Analytes diffuse through the membrane into the solvent.
5. Collecting the solvents containing the analytes in ASE-vials. The dialysis procedure is repeated several times using fresh solvent.

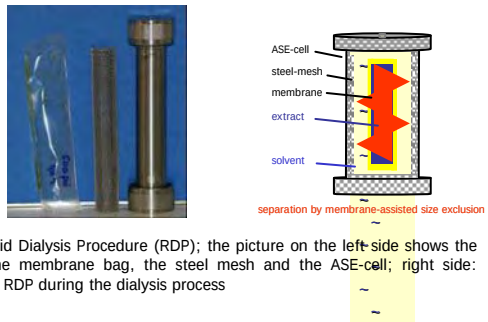


Fig. 2: Rapid Dialysis Procedure (RDP); the picture on the left-side shows the polyethylene membrane bag, the steel mesh and the ASE-cell; right side: principle of RDP during the dialysis process

The RDP was performed using the following parameters and materials

- an ASE 200 device
- a polyethylene membrane with a thickness of 80 µm
- a dialysis temperature of 40 °C
- a pressure of 3.45 MPa
- 16 cycles with a duration of 10 min each
- hexane:dichloromethane 50:50 (v:v) as dialysis solvent

Bioassay

Mutagenicity of the extracts was measured with the Ames-II assay using the strain TA98 of *Salmonella typhimurium*. The Ames-II assay is a liquid microtiter modification of the Ames test (Flückiger et al., 2004).
The Ames-II assay was performed with and without metabolic activation. Aroclor 1254-induced rat liver homogenate S9 was used as activator.
The compounds 2-nitrofluorene (2 µg/ml) and 2-aminoanthracene (5 µg/ml) were used as positive controls in the test sets without and with S9 activation, respectively.
Every extract was diluted resulting in four different concentrated solutions. Determination of mutagenic activity of each solution was repeated three times.

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