Online Resource 1

Details regarding the individual protocols of biomarker analysis and calculation of indices

An attempt to assess the relevance of flood events - Biomarker response of rainbow trout exposed to re-suspended natural sediments in an annular flume

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1. Animal dissection and tissue preparation

After exposure, fish were individually anesthetized in a solution of benzocaine in tap water (Sigma-Aldrich) and then exsanguinated. Subsequently, length and mass were determined for calculation of condition index \((K, \text{equation S1})\), liver somatic index \((LSI, \text{equation S2})\), and visceral index \((VI, \text{equation S3})\).

\[
K = \frac{W_F}{L^3} \times 100 \quad \text{(Equation S1)}
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\[
LSI = \frac{W_L}{W_F} \times 100 \quad \text{(Equation S2)}
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\[
VI = \frac{(W_F - W_C)}{W_F} \quad \text{(Equation S3)}
\]

\(W_F\) is the weight of the fish in mg, \(W_L\) the liver weight in mg, \(L\) the standard length in mm and \(W_C\) the carcass weight in mg, i.e. the weight of the eviscerated animal.

Peripheral blood samples from the caudal vein were taken with heparinized syringes. For each individual, two smears were prepared on separate microscopic glass slides that were previously cleaned with 99\% ethanol (Merck, Darmstadt, Germany). Smears were air-dried and the slides subsequently fixed in methanol (Merck) for 1 min, then stored at room temperature until determination of micronucleus frequencies. A gallbladder bile sample was taken by use of a syringe and transferred to 1.5 ml polypropylene vials (Carl Roth, Karlsruhe, Germany). Bile samples were then stored at -20 °C until determination of PAH metabolite concentrations. The liver was isolated, weighed, cut into four about equally sized pieces, transferred into sterile 2 ml cryogenic vials (Greiner Bio-One, Frickenhausen, Germany) and subsequently frozen in liquid nitrogen. Until determination of 7-ethoxyresorufin O-deethylase (EROD) activity, liver samples were stored at -85 °C.

2. Treatment of bile samples and determination of biliary PAH metabolites

Concentrations of biliary 1-hydroxypyrene and biliverdin were quantified according to a modification of the method published by Kammann (2007). Briefly, 25 µl of the bile fluid was mixed with 95 µl distilled water and 5 µl β-glucuronidase/arylsulfatase solution (30/60 U · ml\(^{-1}\)) and subsequently incubated for 2 h at 37 °C. The reaction was stopped with 125 µl solution of 5 mg ml\(^{-1}\) ascorbic acid in ethanol and the mixture centrifuged (700 \(\times\) g, 5 min). The
concentration of 1-hydroxypyrene was determined by means of high-performance liquid chromatography (HPLC) with fluorescence detection (cf. Kammann 2007).

3. Determination of EROD activity

Pieces of liver explants were thawed carefully and homogenized by use of an electric disperser (VDI 12, VWR, Darmstadt, Germany) at a ratio of 1:10 (w/v) in homogenization buffer (pH 7.4) according to Bonacci et al. (2003), containing 50 mM dipotassium hydrogenphosphate, 0.75 mM sucrose, 1 mM ethylenediamine tetraacetic acid, 0.5 mM dithiothreitol and 0.4 mM phenylmethylsulfonyl fluoride. Subsequently, homogenates were transferred into 1.5 mL micro test tubes (Greiner Bio-One) and centrifuged for 20 min (9000 × g, 4 °C) in a cooling centrifuge (Rotina 420R, Hettich, Tuttlingen, Germany). All steps were carried out on ice. The supernatants (S9 fractions) were carefully transferred to fresh 1.5 mL micro test tubes and stored on ice until measurement of EROD activity and protein concentrations on the same day.

EROD activity was measured in duplicate according to the method described by Burke & Meyer (1974) adopted by Maria et al. (2005). In a semi-micro quartz cuvette (Hellma, Müllheim, Germany), 1 mL solution of 7-ethoxyresorufin (Sigma-Aldrich, Deisenhofen, Germany) in Tris-HCl buffer (0.1 M Tris, 0.15 M potassium chloride, pH 7.4) were mixed with 100 µL of the S9 fractions by repeated inversion of the cuvette. Directly prior to the measurement, the reaction was initiated by addition of 10 µL 10 mM NADPH (Sigma-Aldrich) in Tris-HCl buffer. If the activity was too high, S9 fractions were diluted at a ratio of 1:10 (v/v) with homogenization buffer. Fluorescence of the reaction mixture was recorded in 10 s intervals for 5 min (excitation: 530 nm, emission: 585 nm). To correct for spontaneous substrate conversion, blank measurements containing 100 µL homogenization buffer were treated as the samples. A serial dilution series of resorufin in Tris-HCl buffer was used as external standard.

Protein concentrations for the calculation of specific enzyme activities were determined in triplicates using the bicinchonic acid (BCA) method provided as kit (Sigma-Aldrich). Bovine serum albumin (BSA) was used as external standard (0.125 – 1.25 mg mL⁻¹). In 96-well microplates (TPP, Trasadingen, Switzerland), 200 µl of the working solution were added to 25 µl 1:10 dilutions of liver S9 fractions. The extinction at 562 nm was read after 30 min incubation at 37°C, using an Infinite 200 microplate reader (Tecan, Crailsheim, Germany). Protein concentrations were interpolated from the obtained standard curves. The specific EROD activity was then calculated from the relative fluorescence units and the protein concentrations and expressed as pmol resorufin generated per mg protein and minute.
4. Determination of micronuclei in peripheral erythrocytes

The proportion of micronucleated cells in peripheral erythrocytes was determined according to methods published in Rocha et al. (2009). The previously prepared smears were stained by adding a few drops of a 0.2 µm membrane filtered (Millipore Millex, Schwalbach, Germany), 0.004 % acridine orange solution (w/v) in phosphate buffered saline (PBS, Sigma-Aldrich). After 3 min incubation, the staining solution was discarded, the slides rinsed with distilled water and air-dried (Ueda et al. 1992). For each individual fish, 4000 erythrocytes were examined on the two separate smears using an epifluorescence microscope at 1000 × magnification (Nikon Instruments, Düsseldorf, Germany). The following scoring criteria were used for identification of micronuclei: a) cells with oval appearance and intact cytoplasm, b) oval nuclei with intact nuclear membrane, c) micronuclei less than or equal to one third the size of the main nuclei, d) micronuclei clearly separated from the main nuclei (Huber et al. 1983, Titenko-Holland et al. 1998). In order to avoid experimenter’s bias, the slides were coded. Results were expressed as micronucleated cells relative to the total number of cells counted.
References


Kammann U (2007): PAH metabolites in bile fluids of dab (Limanda limanda) and flounder (Platichthys flesus) - spatial distribution and seasonal changes. Environ Sci Pollut Res 14, 102-108


