Optimization of a pre-metabolization procedure using rat liver S9 and cell-extracted S9 in the Ames fluctuation test

Ying Shao[1,2*]& Andreas Schiwy[3,4,5], Lisa Glauch[1], Luise Henneberger[1], Maria König[1], Marie Mühlenbrink[1], Hongxia Xiao[3,4],Beat Thalmann [4], Rita Schlichting[1], Henner Hollert[3,4,5], Beate I. Escher[1,4,6]

[1]UFZ – Helmholtz Centre for Environmental Research, Department of Cell Toxicology, Permoser Str. 15, 04318 Leipzig, Germany

[2]Key Laboratory of the Three Gorges Reservoir Eco-environment, Ministry of Education, Chongqing University, Shazheng street 174, Shapingba, 400044 Chongqing, China

[3]Department of Ecosystem Analysis, Institute for Environmental Research, RWTH Aachen University, Worringerweg 1, 52074 Aachen, Germany

[4]EWOMIS GmbH, Schießstraße 26c, 63486, Bruchköbel, Germany;

[5]Department of Evolutionary Ecology and Ecotoxicology, Goethe University, Max-von-Laue-Str. 13, 60438 Frankfurt/Main, Germany

[6]Eberhard Karls University of Tübingen, Environmental Toxicology, Centre for Applied Geosciences, 72074 Tubingen, Germany

*Corresponding author

Ying Shao: ying.shao@cqu.edu.cn

College of Environment and ecology, Chongqing University

Shazheng Street 174, Shapingba, 400044 Chongqing, China

Abstract

Many environmental pollutants pose a toxicological hazard only after metabolic activation. *In vitro* bioassays using cell lines or bacteria have often no or reduced metabolic activity, which impedes their use in the risk assessment. To improve the predictive capability of *in vitro* assays, external metabolization systems like the liver S9 fraction are frequently combined with *in vitro* toxicity assays. While it is typical for S9 fractions that samples and testing systems are combined in the same exposure system, we propose to separate the metabolism step and toxicity measurement. This allowes for a modular combination of metabolic activation by enzymes isolated from rat liver (S9) or a biotechnological alternative (ewoS9^{R}) with *in vitro* bioassays that lack metabolic capacity. Benzo(a)pyrene and 2-aminoanthracene were used as model compounds to optimize the conditions for the S9 metabolic degradation/activation step. The Ames assay with *Salmonella typhimurium* strains TA98 and TA100 was applied to validate the set-up of decoupling the S9 activation/metabolism from the bioassay system. S9 protein concentration of 0.25 $mg_{protein}$/mL, a supplement of 0.13 mM NADPH and a pre-incubation time of 100 min are recommended for activation of samples prior to dosing them to *in vitro* bioassays using the regular dosing protocols of the respective bioassay. EwoS9^{R} performed equally well as Moltox S9, which is a step forward in developing true animal-free *in vitro* bioassays. After pre-incubation with S9 fraction, chemicals induced bacteria revertants in both the TA98 and the TA100 assay as efficiently as the standard Ames assay. The pre-incubation of chemicals with S9 fraction could serve for a wide range of cellular *in vitro* assays to efficiently combine activation and toxicity measurement, which may greatly facilitate the application of these assays for chemical hazard assessment and monitoring of environmental samples.

Keywords

Pre-incubation; Effect-based methods; S9 metabolic activation; Detoxification; CYPs

1 Introduction

Bioanalytical tools have been recognized as valuable tools for water quality assessment (Brack et al. 2015). To meet the requirements of the '3R' principles, which were designed to replace, reduce and/or refine animal experiments in teaching, research and testing, arrays of *in vitro* bioassays have been widely utilized to provide toxicity data for aquatic pollutants (Neale et al. 2017a), since their forecast capability allows to reduce costly animal experiments. The application of *in vitro* bioassays is strengthened by the Organization for Economic Cooperation and Development (OECD) in their Guidance document on "Good *in vitro* Method Practice" (GIVIMP)(OECD 2018a). However, the biological effects obtained from *in vitro* bioassays have limitations as to explain the real toxicity of pollutants, since they are often not directly comparable to *in vivo* exposure (Liebsch et al. 2000). One of the most obvious differences between *in vitro* and *in vivo* situations is the absence of Phase I metabolic processes (Yoon et al. 2012). Metabolism is an important factor in determining toxicity of contaminates, since contaminants can be activated or detoxified by Phase I cytochrome P450 monooxygenase (CYP) enzymes (Yu 2020) and Phase II conjugation enzymes (*e.g.*, glucuronosyltransferases, sulfotransferases, and glutathione-S-transferases) (Lepri et al. 2017). *In vitro* systems can only determine the toxicological potential of the parent compounds, unless they have metabolic enzymes that can convert the parent to metabolites (Chitrangi et al. 2017). This is particular important for mutagenicity testing with bacteria assays such as the Ames assay.

To overcome the problem of metabolism absence in *in vitro* methods, liver tissue homogenates from mammalian activation systems have been developed using various fractionation protocols to isolate enzymes from different populations to implement the mammalian metabolism in *in vitro*

bioassays and to detect the toxicity of metabolites (Langsch and Nau 2006, Wienkers and Heath 2005). Various liver derived products have been applied from mice, rats, fish, dogs, monkeys and even humans to conduct metabolization in the *in vitro* models (Yoshihara et al. 2004). The most prominent example for an external metabolization is the homogenate of phenobarbital/beta-naphtoflavone or arochlor 1254 pre-treated rat liver, namely rat liver S9. It is applied to metabolize pro-mutagens which can subsequently be detected by *in vitro* bioassays (Proudlock 2016). The rat liver S9 is obtained as the supernatant fraction after homogenization and centrifugation of the livers at 9000 g (Kwon et al. 2020) . When supplemented with cofactors (S9 mix) energy for the metabolic reactions is produced *via* the generation of reduced β-Nicotinamide adenine dinucleotide phosphate (NADPH) (Reifferscheid et al. 2012a). S9 metabolic activation is widely applied to evaluate the mutagenicity and carcinogenicity of chemical metabolites (Jeong et al. 2014, Richardson et al. 2016) and the *in vitro* intrinsic clearance of a test chemical (OECD 2018b) after a simulated liver passage. In these *in vitro* bioassays, the S9 fraction and the test chemicals were usually directly added into a solution with bacteria or cell suspension (Bernacki et al. 2016, Shao et al. 2018), which combined metabolism and toxicity in the same exposure system. This is a undefined system, and the testing results may be modified by the additional large quantity of proteins introduced by the S9 fraction (Mollergues et al. 2017). Moreover, it has to be noticed that the S9 contains some cytotoxic properties and/or high levels of histidine which could interfere with the bioassays (Kauffmann et al. 2019). Furthermore, it could be a source of contamination *e.g.* by viruses or microorganisms which would interfere with the sterile bioassays or make it necessary to add antibiotics.

On the basis of the previous observations, we hypothesized that 1) the chemical metabolism and toxicity measurement can be separated by a pre-incubation of S9 with reference compounds, and

2) this pre-culture could be modularly combined with different *in vitro* bioassays to make the metabolic activation amenable to testing chemicals and environmental samples in a wide range of *in vitro* assays. In this study, the test chemicals were pre-incubated with the external metabolic activation *via* the S9 fraction before bacteria were exposed. To confirm whether the pre-incubation method and the application of a biotechnological S9 metabolization can be combined with the *in vitro* bioassays for chemical metabolites toxicity evaluation, chemicals after pre-incubation were tested with a robotic platform Ames fluctuation assay to assess the mutagenicity after metabolic activation.

This study can be used as a basis for the development of modular combination of S9 metabolism assays with different bioassays to evaluate effects of chemical metabolites with *in vitro* bioassays that have no or limited intrinsic metabolic capacity.

2 Materials and methods

2.1 Chemicals and reagent

Benzo(a)pyrene (BaP), 2-aminoanthracene (2-AA), nitrofurantoin (NF), 4-nitro-o-phenylenediamine (4-NOPD), L-histidine, beta-nicotinamide adenine dinucleotide phosphate sodium salt (NADP) , β-Nicotinamide adenine dinucleotide 2′-phosphate reduced tetrasodium salt hydrate (NADPH), bromocresol purple, D-glucose-6-phosphate sodium salt (G6P), citric acid, D-glucose, peptone and sodium ammonium hydrogen phosphate-4-hydrate were purchased from Sigma-Aldrich with at least 95 % purity (Sigma-Aldrich, Darmstadt, Germany). HPLC gradient grade acetonitrile was from Th. Geyer (Th. Geyer GmbH, Renningen, Germany). D-biotin was obtained from Invitrogen (ThermoFisher, Darmstadt, Germany). Ampicillin trihydrate was obtained from Fluka (Honeywell, Offenbach, Germany). Potassium chloride and di-potassium

hydrogen phosphate were from Merck (Merck, Darmstadt, Germany). Dimethyl sulfoxide (DMSO), Magnesium chloride and magnesium sulphate were from PanReac Applichem (AppliChem GmbH, Darmstadt, Germany). Sodium chloride was purchased from ROTH (Carl Roth, Karlsruhe, Germany). Meat extract "Lab-lemco" was obtained from OXOID (ThermoFisher, Darmstadt, Germany). Bovine serum albumin (BSA), Gene BLAzer medium and fetal bovine serum (FBS) were from Thermo Fisher (ThermoFisher, Darmstadt, Germany).

2.2 Protein determination

The rat liver S9 fraction produced by Molecular Toxicology Inc. (Moltox S9) was purchased from Trinova Biochem (TRINOVA Biochem GmbH, Germany) (the lot number used was 3833). The biotechnological metabolization system ewoS9^{R} was provided by EWOMIS (EWOMIS GmbH, Bruchköbel, Germany) (the lot number used was 1804). The ewoS9^{R} system was chemically induced to show an activated cytochrome pattern with a special focus on cytochrome P450 1A enzymes. Protein concentration of Moltox S9 and ewoS9^{R} were determined with the Lowry assay with slight modifications as described previously (Fischer et al. 2017). Firstly, S9 was homogenized in milliQ water to achieve protein concentrations between 0.1 and 2 $mg_{protein}$/mL. 200 µL of the homogenate was diluted with 200 µL of 0.1 M NaOH solution. Then 200 µL of the Lowry reagent and 20 µL of BSA standard, sample or control were pipetted into a clear bottom 96-well plate. To protect the 96-well plate from light, it was wrapped in aluminum foil. After 5 min of shaking at 900 rpm, 20 µL of the Folin-Ciocalteau-phenol reagent was added into each well. Afterwards, the 96-well plate was shaken at 900 rpm for 30 min. Finally, the protein concentration was measured by absorbance at 750 nm using the BSA standard as the reference for the calibration curve.

2.3 Pre-incubation of BaP or 2-AA with metabolic activation systems

To determine the metabolic activation capability, two metabolization systems were investigated in a pre-incubation approach with the same medium as the subsequent *in vitro* bioassays. Benzo(a)pyrene (BaP) and 2-aminoanthracene (2-AA) were used to develop and validate the combined activation/Ames assay system (Shoukat 2020, Santes-Palacios 2018). An alternative biotechnological metabolization system ewoS9^{R} which had been developed with the aim to reduce the animal-derived products in *in vitro* bioassays, was used in the current study to compare its metabolic competency with the commercial rat liver S9. A rat hepatoma cell line was cultivated in a chemically-defined medium and suspension culture, which exhibits an activated cytochrome pattern with a special focus on cytochrome P450 1A enzymes. Afterwards, the cells were harvested and processed analogous to the animal-derived S9 to produce the biotechnological metabolization system ewoS9^{R}. A NADPH generating system with nicotinamide adenine dinucleotide phosphate (NADP) and glucose-6-phosphate (G6P) was compared to a direct activation with NADPH in its reduced form (Johnson 2010, Reifferscheid et al. 2012b). S9 protein concentration, incubation time and NADPH-generating cofactors were optimized.

Ames medium (0.82 mM $MgSO_4 \cdot 7H_2O$, 9.36 mM $C_6H_8O_7 \cdot H_2O$, 56.45 mM K_2HPO_4, 16.45 mM $NaNH_4HPO_4 \cdot 4H_2O$, 21.82mM $C_{10}H_{16}N_2O_3S$) or Gene Blazer medium (98% Opti-MEM supplemented with 2% FBS) was used as the exposure medium. The cofactor supplementing the external metabolization systems is called S9-mix. The S9-mix consisted of 3.3 mM KCl, 0.8 mM $MgCl_2 \cdot 6H_2O$, 0.5 mM NaH_2PO_4-Buffer (pH7.4), and 4 mM NADP + 5 mM G6P or 4 mM NADPH, and Moltox S9 or ewoS9^{R}. Different protein concentrations of metabolization systems and varying durations of incubation times have been conducted to optimize the pre-incubation condition. S9 concentrations ranged from 0.12 $mg_{protein}$/mL to 0.45 $mg_{protein}$/mL are listed in the Supporting Information (SI), **Table S1**. In addition to the tested chemical of BaP (722.6 nM) or 2-AA (799.6

159 nM), each experiment contained a process control (without S9-mix) and a denatured S9 control
160 (water bath at 80 °C for 3 min).

161 The pre-incubation was carried out at 37°C for 0 to 120 min with shaking at 200 rpm. To quench
162 the metabolic processes, 350 µL of acetonitrile (ACN) was added into each incubation vial at
163 different time points to optimize the S9 activation time. Then a total volume of 450 µL of quenched
164 reaction mixture was centrifuged at 1000 rpm for 10 min. The liquid supernatant was transferred
165 and 350 µL of ACN was added. After centrifugation, a total volume of 800 µL supernatant was
166 stored at -20 °C for chemical concentration analysis. Each metabolic degradation test was repeated
167 in two independent experiments, and tests were only evaluated if no biotransformation was
168 observed in the control incubation (without S9 fraction and without NADPH-generating systems)
169 and denatured incubation (containing denatured S9 fraction and without NADP/G6P or NADPH).

170 The loss of the tested compounds followed first-order kinetics. The natural logarithm of the
171 detected concentrations at time t (C_t), normalized to the concentration at time zero C_0 was plotted
172 against the time to derive the metabolic rate constant k of loss of the parent chemical (BaP or 2-
173 AA) from the negative slope of the linear regression (eq. 1).

174 $$\ln\left(\frac{C_t}{C_0}\right) = -kt \qquad (1)$$

175 The degradation half time ($t_{1/2}$) was derived from k with eq. 2.

176 $$t_{1/2} = \frac{\ln 2}{k} \qquad (2)$$

177 Regressions were performed with Graphpad prism and statistic parameters are given by standard
178 error of fit parameters of r^2 and F.

2.4 HPLC analysis of 2-AA and BaP

A high-performance liquid chromatography (HPLC) system equipped with a fluorescence detector (FLD) was applied to analyze the concentration of 2-AA and BaP after incubation for the metabolic competency evaluation (Sess-Tchotch 2018). Quantification of BaP and 2-AA was performed using a HPLC system (1260 Infinity HPLC, Agilent, Waldbronn, Germany) equipped with a fluorescence detector (FLD) and a C18 column (Kinetex 2.6µm, 100Å, 100×3.0mm from Phenomenex, Aschaffenburg, Germany). For BaP, the injection volume was 10µL for each sample. The following gradient with a flow rate of 1 mL/min was used for BaP: 0 to 1.0 min, 45% acetonitrile in water; 1.0 to 2.5 min, a linear gradient of 45% to 55% acetonitrile in water; 2.5 to 10.0 min, a linear gradient of 55% to 80% acetonitrile in water; 10.0 to 10.5 min, a linear gradient of 80% to 45% acetonitrile in water; 10.5 to 12 min, 45% acetonitrile.

For 2AA, the gradient with a flow rate of 1 mL/min was: 0 to 1.0 min, 20% acetonitrile in water (with 0.1% H_3PO_4); 1.0 to 4.5 min, a linear gradient of 20% to 90% acetonitrile (with 0.1% H_3PO_4) in water (with 0.1% H_3PO_4); 4.5 to 5.5 min, a linear gradient of 90% to 20% acetonitrile (with 0.1% H_3PO_4) in water (with 0.1% H_3PO_4); 5.5 to 6.0 min, 20% acetonitrile(with 0.1% H_3PO_4). The detection of the two compounds was conducted by fluorescence with fluorescence excitation set at 260 nm and the emission was detected at 410 nm for BaP. For 2-AA the fluorescence excitation was set at 254 nm and the emission was detected at 500 nm.

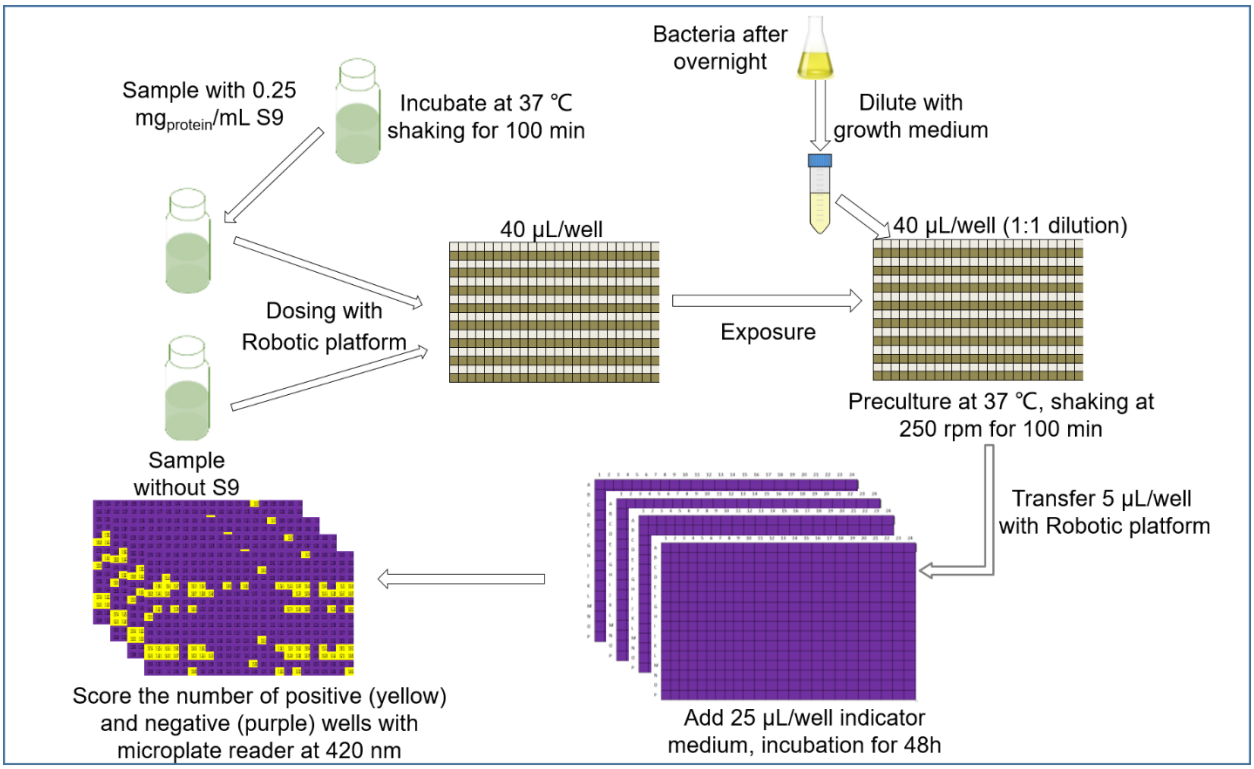
2.5 Application of the S9 metabolic activation in the Ames fluctuation assay

The Ames fluctuation assay was conducted to determine the mutagenic activity of BaP, 2-AA and their metabolites based on the ISO guideline 11350 (ISO11350 2012, Reifferscheid et al. 2012a). Modifications were implemented for the S9 pre-incubation methodology described above and for the use of a robotic platform containing a 384-pipetting unit (**Figure 1**). The Ames test was adapted for the use on a robotic pipetting platform (Hamilton Microlab® STAR, Bonaduz, Switzerland,

Figure 1) with 384-well plates for dilution, 100 min exposure, 48 h incubation and detection. These modifications allowed the easy determination of mutagenicity of a large number of samples in parallel (minimum sample volume: 320 µL per sample per strain for both without S9 test and with S9 test). In brief, cultures of test strains were grown overnight for no more than 10 h in growth medium (7.5 g/L meat extract "Lab-lemco", 7.5 g/L Peptone, 5 g/L NaCl and 50 mg/L Ampicillin) in a shaking incubator at 150 rpm and 37°C.

The samples of chemical and S9-mix were pre-incubated for metabolic activation before exposure. The incubation time, the S9 protein concentration and the NADPH-generating system were determined by the pre-incubation analysis described above. The Ames *salmonella typhimurium* tester strains TA 98 at OD 0.26-0.3 and TA100 at OD 0.05-0.08 were exposed to BaP or 2-AA with S9-mix and without S9-mix in a 384-well plate at 37°C and 200 rpm for 100 min. After exposure, the mixtures were transferred and diluted six-fold with the indicator medium (0.9 mM $MgSO_4 \cdot 7H_2O$, 10.3 mM $C_6H_8O_7 \cdot H_2O$, 62.1mM K_2HPO_4, 18.1 mM $NaNH_4HPO_4 \cdot 4H_2O$, 24mM $C_{10}H_{16}N_2O_3S$, 0.05 mM bromocresol purple and 0.01 mM D-biotin) and distributed into 384-well copy plates (48 wells per replicate). Due to the incubation in a histidine deficient medium, only bacteria that regained histidine prototrophy through mutation show growth. This growth leads to acidification of the indicator medium, which thereby changes color from purple to yellow. Sample-induced reversion from the non-growing to the growing phenotype was detected by measuring OD at 420 nm in a 384-well microplate reader after 48 h of incubation at 37°C. Positive controls included 4-NOPD for the TA 98 strain without S9, NF for TA100 without S9, 2-AA for TA 98 and TA 100 with S9. To match the chemical concentrations for both strains, 4-NOPD (at concentrations from $4.09 \cdot 10^{-6}$ M to $6.54 \cdot 10^{-5}$ M), NF (at concentration from $1.31 \cdot 10^{-7}$ M to $2.10 \cdot 10^{-6}$ M), 2-AA (at concentrations from $6.46 \cdot 10^{-7}$ M to $1.03 \cdot 10^{-5}$ M) and BaP (at concentrations from

225 $4.95 \cdot 10^{-6}$ M to $7.92 \cdot 10^{-5}$ M) were tested in five 1:2 serial dilution steps. Negative control is DMSO
226 for both strains with or without S9. Each exposure test was repeated in two independent
227 experiments. The concentration-response curves were fitted with a log-logistic model for the EC50
228 value calculation.



229

230 **Figure 1.** Ames fluctuation assay on the robotic platform (Hebert et al. 2018)

231 **3 Results**

232 3.1 Degradations kinetics of BaP and 2-AA by S9

233 The concentration of BaP decreased exponentially with time when it was incubated with Moltox
234 S9 and the NADPH-generation systems NADP + G6P (**Figure 2A**) and NADPH (**Figure 2B**). The
235 linear fit of $\ln(C_t/C_0)$ against time (eq.1) yielded high regression coefficients r^2, indicating that the
236 metabolic loss of BaP followed first-order rate law at S9 protein concentrations of 0.12
237 mg$_{protein}$/mL to 0.45 mg$_{protein}$/mL. Similar results were obtained for the tests of BaP degradation

with ewoS9^{R} using NADPH generation system of NADP+G6P (**Figure 2C**) and NADPH (**Figure 2D**).

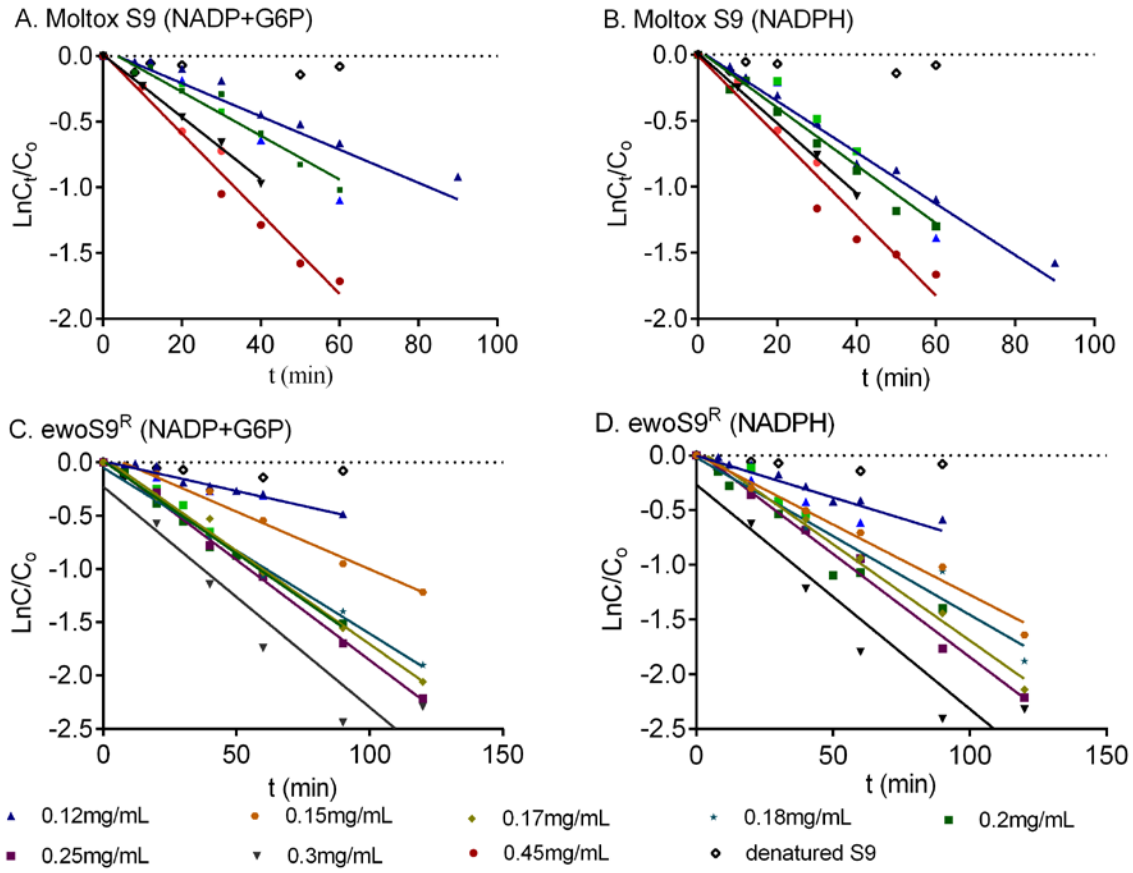


Figure 2. Phase I metabolism of BaP (starting concentration C_0 723 nM) by Moltox S9 using NADPH generation system of (A) NADP+G6P and (B) NADPH or by ewoS9^{R} using NADPH generation system of (C) NADP+G6P and (D) NADPH at a protein concentration of 0.12 $mg_{protein}$/mL (dark and light blue trianglesthe two colors represent two independent experiments), 0.15 $mg_{protein}$/mL (orange circles), 0.17 $mg_{protein}$/mL (bluish yellow rhombi), 0.18 $mg_{protein}$/mL (turquoise blue pentagram), 0.2 $mg_{protein}$/mL (light and dark green squares, the two colors represent two independent experiments), 0.25 $mg_{protein}$/mL (purple squares), 0.3 $mg_{protein}$/mL (black triangles), 0.45 $mg_{protein}$/mL (light and dark red circles, the two colors represent two independent experiments) and denatured S9 (empty black rhombi). BaP degradation at S9 concentrations 0.12

250 $mg_{protein}$/mL, 0.2 $mg_{protein}$/mL and 0.45 $mg_{protein}$/mL were conducted in duplicates, and 0.15 $mg_{protein}$/mL,
251 0.17 $mg_{protein}$/mL, 0.18 $mg_{protein}$/mL, 0.25 $mg_{protein}$/mL, 0.3 $mg_{protein}$/mL and denatured S9 were
252 conducted in a single experiment. The solid lines correspond to fits of the first order kinetics with
253 eq.1.

254 3.2 Decay half-times as a function of S9 concentration

255 The decay half times of BaP and 2-AA decreased with increased protein content of the S9 fractions
256 for both Moltox S9 and ewoS9^R in the Ames medium (**Figure 3A and B**). The protein content
257 quantified in this study with the Lowry assay was slightly different from the protein content
258 reported by the suppliers (**Table S2**). Since protein content is only operationally defined, it is
259 important to compare protein contents determined with the same method.
260 **Figure 3** shows that the NADPH generation system of NADP+G6P and NADPH yielded equal
261 $t_{1/2}$ for both BaP and 2-AA (**Table S3**), indicating that both systems could be used directly with
262 S9 fractions for chemical activation.
263 The different metabolic activities of Moltox S9 and ewoS9^R are illustrated by differences in the
264 slope of the linear regression of $t_{1/2}$ vs. S9 protein concentration, but both lines overlap in the range
265 of 0.2 to 0.3 $mg_{protein}$/mL with a similar $t_{1/2}$ of approximately 50 min.
266 In addition, BaP and 2-AA degradation tests using GeneBLAzer cell culture medium showed
267 similar first order decay kinetics (**Figure S2**) and thereof derived decay half times $t_{1/2}$ (**Table S4**)
268 were similar to the results of degradation tests using the Ames medium (**Figure 3C and D**).

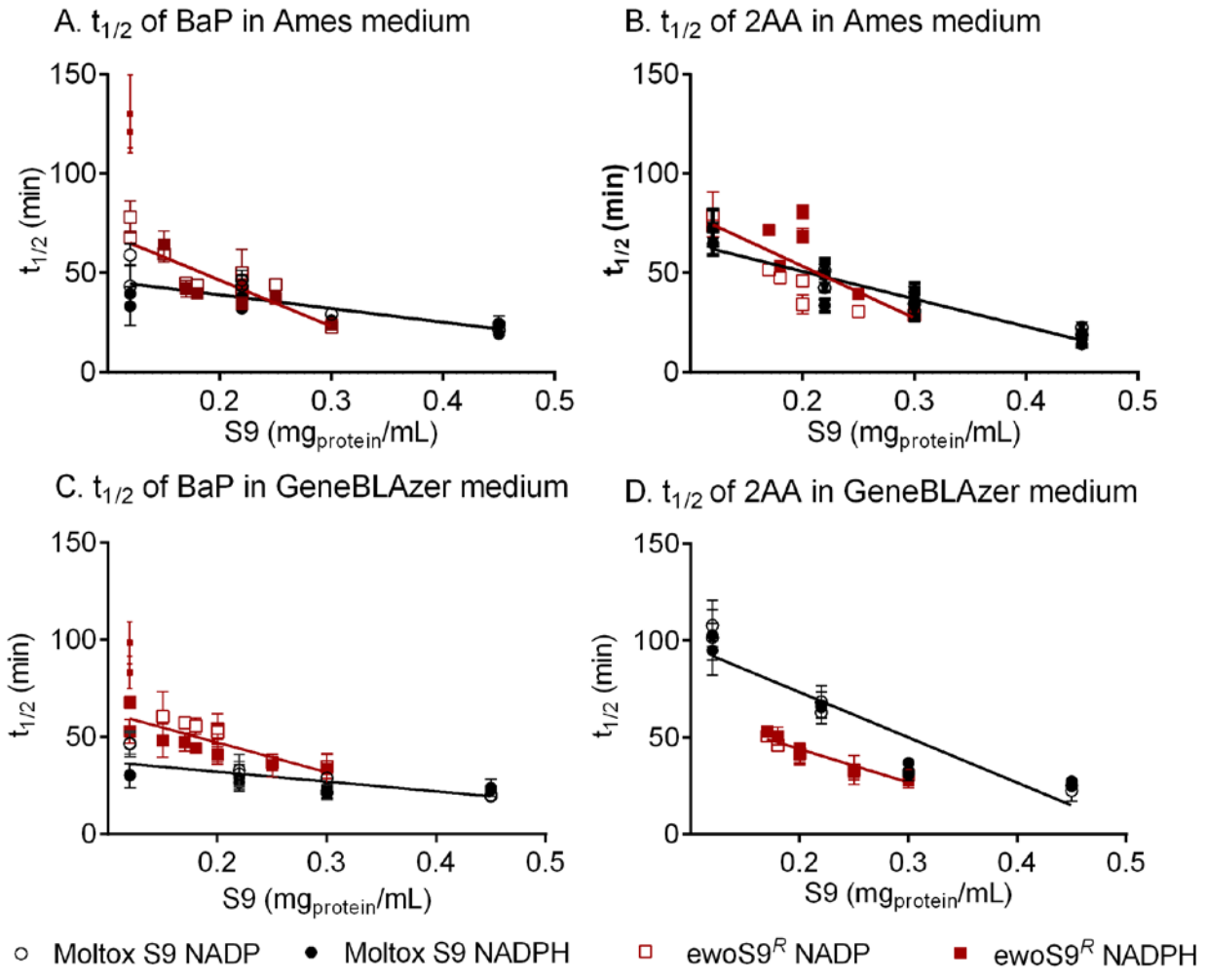


Figure 3 Half time ($t_{1/2}$) vs S9 protein concentration for (A) BaP and (B) 2-AA degradation with Moltox S9 and ewoS9^R using Ames medium and for (C) BaP and (D) 2-AA degradation with Moltox S9 and ewoS9^R using GeneBLAzer medium. The lines are linear regressions. The smaller symbols are experiments that were outliers (visually identified) and are not included in the regressions.

3.3 S9 pre-incubation combined with the Ames assay

Moltox S9 or ewoS9^R at a protein concentration of 0.25 $mg_{protein}$/mL were pre-incubated with BaP or 2-AA for 100 min to obtain mammalian metabolism derived metabolites for bacteria exposure. Afterwards, the pre-incubated BaP or 2-AA solutions were tested with the Ames fluctuation assay

with the tester strains TA98 and TA100 to evaluate their mutagenicity potential. As shown in Figure 4, after activation by S9 both BaP and 2-AA caused mutagenicity in the tester strains TA98 and TA100. Moltox S9 and ewoS9^{R} yielded the same concentrations causing 50% of revertants (EC_{50}, **Table 1**). The EC_{50} values for 2-AA on both strains TA98 and TA100 were similar, but TA98 was slightly more sensitive than TA100 for BaP.

This active concentration range is consistent with the previous standard mutation test using 24-well plates, where metabolic activation of BaP at 5.07 µM and 2-AA at 25.9 µM was mediated by a rat liver derived S9 fraction (Proudlock and Evans 2016). Thus, pre-incubation of chemicals via an external metabolization system derived from rat liver S9 or a biotechnological process to simulate the metabolic activation was successfully applied to the Ames fluctuation assay.

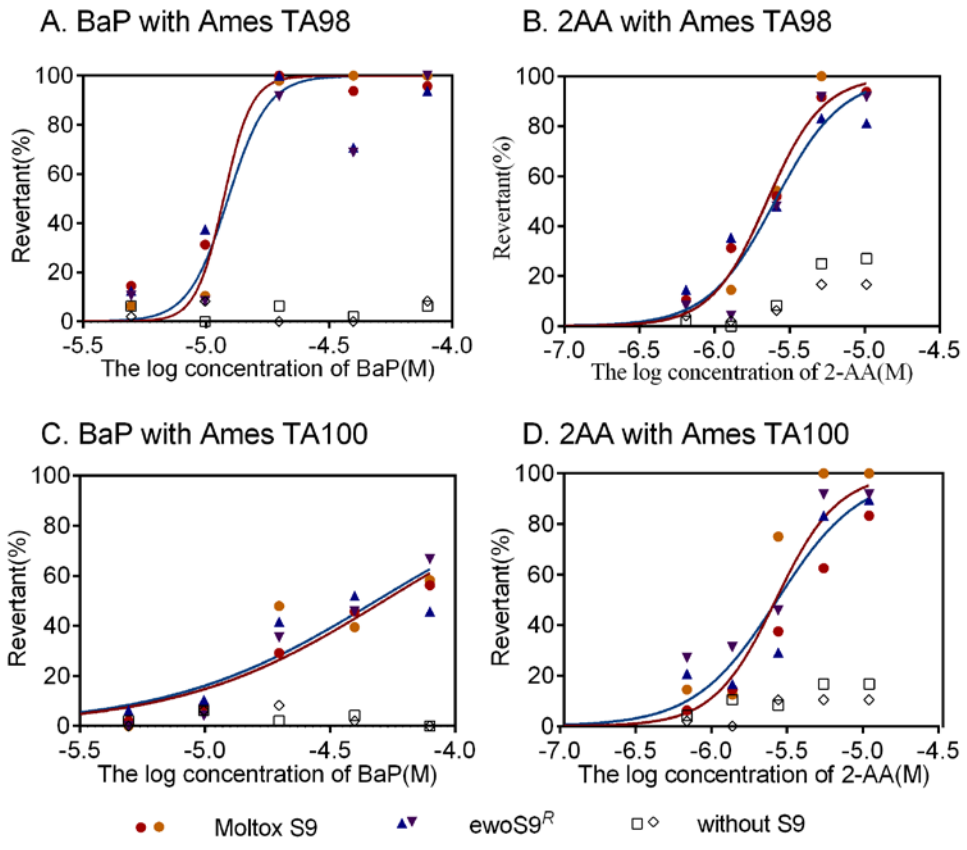


Figure 4. Concentration-response curves from the Ames fluctuation assay with TA 98 and TA 100 strain for the reference compounds BaP and 2-AA. Different symbols of a similar color stand for independent experiments using Moltox S9 or ewoS9^R. (A) Ames strain TA98 revertant percentage for BaP pre-incubated with Moltox S9 (red and orange the colors represent two independent experiments) and ewoS9^R (blue and purple) and without S9 (black rhombus and squares, the shapes represent two independent experiments); (B) Ames strain TA98 revertant percentage for 2AA pre-incubated with Moltox S9 (red and orange) and ewoS9^R (blue and purple) and without S9 (black rhombus and squares); (C) Ames train TA100 revertant percentage for BaP pre-incubated with Moltox S9 (red and orange) and ewoS9^R (blue and purple) and without S9 (black rhombus and squares); (D) Ames train TA100 revertant percentage for 2AA pre-incubated with Moltox S9 (red and orange) and ewoS9^R (blue and purple) and without S9 (black rhombus and squares). The lines are log-logistic fits.

Table 1. Effect concentrations causing 50% of revertants (EC_{50}) for BaP and 2-AA after activation with Moltox S9 and ewoS9^R.

		BaP		2-AA	
		log EC_{50}(M)	EC_{50} (M)	log EC_{50}(M)	EC_{50} (M)
TA 98	Moltox S9	-5.65±0.03	2.24×10^{-6}	-4.93±0.04	11.8×10^{-6}
	ewoS9^R	-5.60±0.04	2.53×10^{-6}	-4.91±0.05	12.3×10^{-6}
TA 100	Moltox S9	-5.60±0.06	2.5×10^{-6}	-4.29±0.07	52×10^{-6}
	ewoS9^R	-5.59±0.06	2.52×10^{-6}	-4.32±0.07	48.2×10^{-6}

4. **Discussion**

4.1 Standardizing metabolic activation

The adequate optimization of the exogenous metabolic system is a prerequisite for the realistic assessment of chemicals and environmental samples for a wide range of cellular *in vitro* assays. Previously the addition of S9 for metabolic activation was determined by its percentage in

mediums (1% S9 suspension irrespective of its activity), so that differences between different S9 batches and S9 origin could be expected. In this study, we have demonstrated that S9 protein concentration of 0.25 $mg_{protein}$/mL, a supplement of 0.13 mM NADPH and a pre-incubation time of 100 min is an optimized protocol for the activation of samples and reference chemicals before dosing them in the Ames assay, irrespective of the source of S9 as evidenced by comparison of the animal-derived Moltox S9 and the cell-derived ewoS9^{R}.

S9 fractions isolated from animals allow to activate substances that require phase I enzymatic biotransformation to express toxicity effects (Mollergues et al. 2016). A level of 1% S9 is recommended in literature to yield a clearly enhanced response in effect-based methods like mammalian or bacterial test systems for metabolites toxicity investigation (Natsch and Haupt 2013), However, this study has demonstrated that the S9 content should be adjusted depending on the protein content instead of the percentage of the S9 used. This is due to the fact that the protein concentrations of S9 products vary strongly between different production lots. For instance, the concentration of current S9 is 45.01 $mg_{protein}$/mL for commercial rat liver Moltox S9 and 12.15 $mg_{protein}$/mL for cell-extracted ewoS9^{R} (**Table S2**). The addition of 1% S9, which is often recommended, would result in a final protein content of 0.45 $mg_{protein}$/mL for Moltox S9 and 0.12 $mg_{protein}$/mL for ewoS9^{R}. Moreover, the species-, strain- and sex-specific differences also influence the protein concentration and enzymatic composition at the same percentage of S9 fraction, even though the molecular biology of xenobiotic-metabolising enzymes is widely conserved across mammals (Callander et al. 1995, Cox et al. 2015, Elliott et al. 1992, Rudeck et al. 2018b). This was reflected by a study where the concentration of liver S9 was 20 $mg_{protein}$/mL from 35 male trouts and 26 $mg_{protein}$/mL from 6 mixed-sex trouts (Laue et al. 2014). Thus, relating the dose to

the protein concentration, instead of the percentage of S9 in the media, is recommended in future S9 studies.

To improve this situation, metabolism studies should focus more on the desired phase I activity, cytochrome P450 content or even the single cytochrome P450 activity (Burke et al. 1994, Welters et al. 2017).The BaP degradation rate was found to increase with increasing S9 protein concentration (**Figure 3**), which is in agreement with a previous study on BaP metabolism characteristics (Prough et al. 1979). Thus, the percentage related S9 concentration may give misleading results for the enzymatic efficiency, since the metabolic activity of the S9 was clearly related to its protein content (**Figure 3**).

S9 fraction containing both phase I and phase II enzymes, is a robust source of CYPs, which play important roles not only in chemical activation, but also in chemical detoxification by different P450 isoforms (Wang et al. 2017). A BaP pharmacokinetics study showed that the detoxification of BaP is also predominantly mediated by CYPs, and BaP can be oxidized by several P450 isoforms (CYP1A1, CYP1A2, and CYP1B1) along with epoxide hydrolase, yielding phase I metabolites, further conjugated with Phase II enzyme to form more hydrophilic metabolites to excretion (Nebert et al. 2013). In the current study, only approximate 20% of BaP/2-AA was left in the media after 100 min incubation, which was found to be sufficient for chemical activation, and yielded a good balance between activation and detoxification (**Figure 2**). This 100 min incubation time is aligning with the required exposure time of the ISO 11350 (ISO11350 2012). These results are in contrast to the 3 h contact time that was recommended for genotoxicity testing in the mammalian cell assays (Natsch and Haupt 2013). Our decay half-times clearly indicate that the 100 min should be sufficient for the Ames assay. Too long incubation time could lead to deactivation by further degradation.

NADPH, as a redox cofactor, plays a critical role during chemical metabolism, which is essential for many enzymatic steps in the biosynthesis of cellular macromolecules (Partow et al. 2017). Moreover, microsomal metabolism studies provided evidences that NADPH was a rate-limiting factor in the oxidation of chemicals in murine hepatoma cells (Paolini et al. 1987). The endogenous NADPH is generated from NADP by a glucose-6-phosphate dehydrogenase (G6PDH) catalyzed oxidation of G6P (Savidov et al. 1998). Short-term tests with metabolic activation therefore generally utilize G6P and NADP for the NADPH-generating machinery (Ortiz et al. 2016). In the current study, the NADPH generation systems of NADP+G6P and NADPH give very similar redox activity at 0.13 mM for BaP and 2-AA metabolism (**Figure 2**), indicating that NADPH could be a convenient and economic alternative to NADP+G6P for short-term *in vitro* metabolic activation tests.

4.2 S9 metabolic activation as pre-incubation in the Ames fluctuation assay

The Ames fluctuation assay has been included in the ISO 11350 in 2012 and has been widely applied to measure the genotoxic potential of chemicals and environmental samples in liquid culture by using small amounts of test items (Berg et al. 2016, Neale et al. 2017b). This bioassay allows the detection of base pair and frameshift mutagenicity in the presence and absence of metabolic activation (Reifferscheid et al. 2012b). In the current study, BaP exhibited mutagenicity with the supplement of Moltox S9 or ewoS9^{R} in the Ames fluctuation assay (**Figure 4**), which is in agreement with a BaP metabolites study where BaP had high mutagenic effects on TA 97, TA 98 and TA 100 in the presence of S9 metabolic activation, and BaP diols were found to be most sensitive BaP metabolites on mutagenicity for *Salmonella typhimurium* (Schoeny et al. 1985). BaP metabolites studies reported that BaP, catalysed by cytochrome P450 1A1 enzymes (CYP1A1) (Willis et al. 2018), could be oxidized into BaP diol epoxide (Jiang et al. 2007) which may bind to

the nucleophilic guanine bases; distorts the DNA and causes mutations (Eaton and Gallagher 1994). Similarly, Moltox S9 and ewoS9^{R} at 0.25 $mg_{protein}$/mL increased the mutagenicity of 2-AA by more than 5-fold in both TA98 and TA100 assays, when compared to the revertants without S9 (**Figure 4**). This result is consistent with a non-genotoxic carcinogens study, in which 2-AA revealed marked increase of revertant colonies in presence of a metabolic activation system (S9) by a plate incorporation method (Kanode et al. 2017). An *in vitro* mammary epithelium study demonstrated that polar metabolites of 2-AA were produced probably via mixed-function oxidation, which may induce mutagenesis in breast tissue (Silva et al. 1985). 2AA is a CYP1A2 dependent promutagen, which requires CYP1A2-mediated oxidation and subsequent O-acetyl or Osulfo conjugation to generate DNA-damaging metabolites (Palma et al. 2016). CYP1A2 is almost exclusively expressed in the liver (Zanger and Schwab 2013), which further proved the metabolic activity of Moltox S9 and ewoS9^{R} for 2AA. In the current study, pre-incubation with Moltox S9 or ewoS9^{R} S9 fraction of the reference compounds, BaP and 2-AA induced bacteria revertants in both tester strains TA98 and TA100. These results are comparable to the standard protocol of the Ames fluctuation assay in which the reference compounds are metabolized during the exposure time of the bacteria. This indicates the validity of modular combination of pre-S9 metabolic activation with *in vitr*o bioassays.

The benefit of the biotechnological system ewoS9^{R} is that no animals were needed for its production. In the production process cells cultivated in a chemically-defined medium were used. A biotechnological metabolization system contributes to the requirements of 3R as a replacement of the animal metabolization systems. Furthermore, the biotechnological approach results in a highly standardized product as all the components of the production process are controlled. This is not the case for the animal systems. In the study by Rudeck et al. (2018a), different animals or

even different lobes of the liver within an animal showed varying levels of protein content and cytochrome activity. In the study, ewoS9^{R} is obtained from a rat hepatoma cell line and standardized, which may avoid the above differences. The cell line does not show a sex specificity. The strain is defined and if desired can be simulated by adopting another rat liver cell line as the foundation for the ewoS9^{R} if needed. Hence, a biotechnological system offers great flexibility.

5 Conclusion

This study demonstrates that the pre-incubation of chemicals with S9 fraction is applicable to separate the chemical metabolism and toxicity measurement in the *in vitro* bioassays. The described pre-incubation procedure can serve for a wide range of cellular *in vitro* assays to standardize the exposure environment, which could improve the bioassay efficacy and comparability between different assays. However, there will always be some limitations when using a metabolic activation/detoxification step. We recommend a S9 protein concentration of 0.25 $mg_{protein}$/mL and an incubation time of 100 min for the Ames test. These corresponds to two decay half times of BaP and 2-AA, which assures that the reaction is advanced but not fully completed. In the proposed set-up, the metabolism kinetics for reference chemicals tested should be evaluated prior to testing environmental samples. In complex mixtures, there will probably be a trade-off between activation and detoxification. The application of a biotechnological metabolization system bears the benefit of reducing animal experiments as it does not require the dosing and killing of animals for the production of S9. Furthermore, due to its chemically-defined medium composition the production process for a biotechnological metabolization system even complies with the rules of pharmaceutical production. In this controlled environment the variability can be greatly reduced and the cytochrome P450 composition can be standardized and defined. In

summary, this technique can be applied to test the real toxicity of chemicals and environmental samples, and may greatly facilitate regulatory toxicity testing whilst reducing animal testing.

Acknowledgements

This study was partially supported by the project SOLUTIONS and is supported by the European Union Seventh Framework Programme (FP7-ENV-2013-two-stage Collaborative project) under grant agreement number 603437. We are grateful to Jung-Hwan Kwon of Korea University for sharing his experience with the S9 metabolism assay and his assay protocol. We thank Fabian Fischer for help with the protein determination. We thank Sandy Schöne for help with the language editing.

Conflict of interest

AS, BT and HH are co-founder of EWOMIS GmbH, a company aimed to commercialize biotechnological metabolization systems.

References

Berg, K., Bischoff, R., Stegmüller, S., Cartus, A. and Schrenk, D. (2016) Comparative investigation of the mutagenicity of propenylic and allylic asarone isomers in the Ames fluctuation assay. Mutagenesis 31(4), 443-451.

Bernacki, D., Bryce, S., Bemis, J., Kirkland, D. and Dertinger, S. (2016) γH2AX and p53 Responses in TK6 Cells Discriminate Promutagens and Non-genotoxicants in the Presence of Rat Liver S9. Environ Mol Mutagen 57(7), 546-558.

Brack, W., Altenburger, R., Schüürmann, G., Krauss, M., Herráez, D., von Gils, J., Slobodnik, J., Munthe, J., Gawlik, B., van Wezel, A., Schriks, M., Hollender, J., Tollefsen, K., Mekenya, O., Dimitrov, S., Bunke, D., Cousins, I., Posthuma, L., van den Brink, P., de Alda, M.,

Barcelo, D., Faust, M., Kortenkamp, A., Scrimshaw, M., Igmatova, S., Engelen, G., Massmann, G., Lemkine, G., Teodorovic, I., Walz, K., Dulio, V., Jonker, M., Jaeger, F., Chipman, K., Falciani, F., Liska, I., Rooke, D., Zhang, X., Hollert, H., Vrana, B., Hilscherovã, K., Kra, e., K, Neumann, S., Hammerbacher, R., Backhaus, T., Mack, J., Segner, H., Escher, B. and de Aragao Umbuzeiro, G. (2015) The SOLUTIONS project: Challenges and responses for present and future emerging pollutants in land and water resources management. Sci Total Environ 503-504, 22-31.

Burke, M.D., Thompson, S., Weaver, R.J., Wolf, C.R. and Mayer, R.T. (1994) Cytochrome-P450 specificities of alkoxyresorufin o-dealkylation in human and rat-liver. Biochem Pharmacol 48(5), 923-936.

Callander, R.D., Mackay, J.M., Clay, P., Elcombe, C.R. and Elliott, B.M. (1995) Evaluation of phenobarbital beta-naphthoflavone as an alternative S9-induction regime to aroclor-1254 in the rat for use in *in-vitro* genotoxicity assays. Mutagenesis 10(6), 517-522.

Chitrangi, S., Nair, P. and Khanna, A. (2017) 3D engineered In vitro hepatospheroids for studying drug toxicity and metabolism. Toxicol in Vitro 38, 8-18.

Yu, C.(2020) Metabolism and in vitro drug–drug interaction assessment of viloxazine. Xenobiotica 1-16. doi: 10.1080/00498254.2020.1767319.

Cox, J., Fellows, M., Hashizume, T. and White, P. (2015) The utility of metabolic activation mixtures containing human hepatic post-mitochondrial supernatant (S9) for in vitro genetic toxicity assessment. Mutagenesis 31(2), 117-130.

Eaton, D. and Gallagher, E. (1994) Mechanisms of aflatoxin carcinogenesis. Annu Rev Pharmacol Toxicol 34(1), 135-172.

Elliott, B.M., Combes, R.D., Elcombe, C.R., Gatehouse, D.G., Gibson, G.G., Mackay, J.M. and Wolf, R.C. (1992) Alternatives to aroclor 1254 - induced S9 in *in vitro* genotoxicity assays. Mutagenesis 7(3), 175-177.

Escher, B., Dutt, M., Maylin, E., Tang, J., Toze, S., Wolf, C. and Lang, M. (2012) Water quality assessment using the AREc32 reporter gene assay indicative of the oxidative stress response pathway. J Environ Monit 14(11), 2877-2885.

Fischer, F., Abele, C., Droge, S., Henneberger, L., König, M., Schlichting, R., Scholz, S. and Escher, B. (2018) Cellular uptake kinetics of neutral and ionizable chemicals by fluorescence microscopy. Chem Res Toxicol 31, 646-657.

Fischer, F., Henneberger, L., König, M., Bittermann, K., Linden, L., Goss, K. and Escher, B. (2017) Modeling exposure in the Tox21 in vitro bioassays. Chem Res Toxicol 30(5), 1197-1208.

Hebert, A., Feliers, C., Lecarpentier, C., Neale, P., Schlichting, R., Thibert, S. and Escher, B. (2018) Bioanalytical assessment of adaptive stress responses in drinking water: A predictive tool to differentiate between micropollutants and disinfection by-products. Water Res 132, 340-349.

Heldur, H. and Letcher, R. (2004) Metabolism in the toxicokinetics and fate of brominated flame retardants--a review. Environ Int 29(6), 801-828.

Helleberg, H. and Tornqvist, M. (2000) A new approach for measuring protein adducts from benzo a pyrene diolepoxide by high performance liquid chromatography/tandem mass spectrometry. Rapid Commun Mass Sp 14(18), 1644-1653.

ISO11350 (2012) Water quality -- Determination of the genotoxicity of water and waste water -- Salmonella/microsome fluctuation test (Ames fluctuation test). https://www.iso.org/standard/50393.html

ISO16240 (2005) Water quality -- Determination of the genotoxicity of water and waste water -- Salmonella/microsome test (Ames test). https://webstore.ansi.org/Standards/ISO/ISO162402005

Jeong, M., Yang, K., Lee, C., Jeong, D., Park, Y., Choi, Y., Kim, J., Oh, S., Jeong, S. and Jo, W. (2014) In Vitro Genotoxicity Assessment of a Novel Resveratrol Analogue, HS-1793. Toxicol Res 30(3), 211-220.

Jiang, H., Gelhaus, S., Mangal, D., RG, H., Blair, I. and Penning, T. (2007) Metabolism of benzo[a]pyrene in human bronchoalveolar H358 cells using liquid chromatography-mass spectrometry. Chem Res Toxicol 20(9), 1331-1341.

Johnson, B. (2010) An evaluation of a genotoxicity assay with liver s9 for activation and luminescent bacteria for detection. Environ Toxicol Chem 11(4), 473-480.

Kanode, R., Chandra, S. and Sharma, S. (2017) Application of bacterial reverse mutation assay for detection of non-genotoxic carcinogens. Toxicol Method 27(5), 376-381.

Kauffmann, K., Brendt, J., Schiwy, A., Hollert, H. and Buechs, J. (2019) Online Monitoring of Respiration Activity Allows for an Optimized Ames Mutagenicity Test. Environ Mol Mutagen 60, 86-86.

Khan, M.K.A., Akhtar, S. and Arif, J.M. (2018) Structural Insight into the Mechanism of Dibenzo[a,l]pyrene and Benzo[a]pyrene-Mediated Cell Proliferation Using Molecular Docking Simulations. Interdiscip Sci 2017.10(4), 653-673.

509 Kiefer, F., Cumpelik, O., Reen, R., Doehmer, J. and Wiebel, F.J. (1994) Arylamines suppress their
510 own activation and that of nitroarenes in V79 Chinese-hamster cells by competing for
511 acetyltransferases. Environ Health Persp 102, 95-97.
512 Kwon, J.H., Lee, H.J., Escher, B.I.(2020) Bioavailability of hydrophobic organic chemicals on an
513 in vitro metabolic transformation using rat liver S9 fraction. Toxicology in Vitro. 66,1-8.
514 Langsch, A. and Nau, H. (2006) Metabolic activation for in vitro systems. Altex 22(2), 354-358.
515 Laue, H., Gfeller, H., Jenner, K., Nichols, J., Kern, S. and Natsch, A. (2014) Predicting the
516 bioconcentration of fragrance ingredients by rainbow trout using measured rates of in vitro
517 intrinsic clearance. Environ Sci Tech 48(16), 9486-9495.
518 Le Vee, M., Kolasa, E., Jouan, E., Collet, N. and Fardel, O. (2014) Differentiation of human
519 placental BeWo cells by the environmental contaminant benzo(a)pyrene. Chem-Biol Interact
520 210, 1-11.
521 Lepri, S., Ceccarelli, M., Milani, N., Tortorella, S., Cucco, A., Valeri, A., Goracci, L., Brink, A.
522 and Cruciani, G. (2017) Structure-metabolism relationships in human-AOX: Chemical
523 insights from a large database of aza-aromatic and amide compounds. P Natl Acad sci USA
524 114(16), 3178-3187.
525 Liebsch, M., Traue, D., Barrabas, C., Spielmann, H., Uphill, P., Wilkins, S., Mcpherson, J.,
526 Wiemann, C., Kaufmann, T. and Remmele, M. (2000) The ECVAM Prevalidation Study on
527 the Use of EpiDerm for Skin Corrosivity Testing. Atla-Altern Lab Anim 28(3), 371-401.
528 Mollergues, J., Van Vugt-Lussenburg, B., Kirchnawy, C., Bandi, R., van der Lee, R., Marin-Kuan,
529 M., Schilter, B. and Fussell, K. (2017) Incorporation of a metabolizing system in biodetection
530 assays for endocrine active substances. Atla-Altern Lab Anim 34(3), 389-398.

Natsch, A. and Haupt, T. (2013) Utility of rat liver S9 fractions to study skin-sensitizing prohaptens in a modified KeratinoSens assay. Toxicol Sci 135(2), 356-368.

Neale, P., Altenburger, R., Ait-Aissa, S., Brion, F., Busch, W., de Aragao Umbuzeiro, G., Denison, M., Du Pasquier, D., Hilscherovã, K., Hollert, H., Morales, D., Novak, J., Schlichting, R., Seiler, T., Serra, H., Shao, Y., Tindall, A., Tollefsen, K., Williams, T. and Escher, B. (2017a) Development of a bioanalytical test battery for water quality monitoring: Fingerprinting identified micropollutants and their contribution to effects in surface water. Water Res 123, 734-750.

Neale, P., Munz, N., Aït-Aïssa, S., Altenburger, R., Brion, F., Busch, W., Escher, B., Hilscherová, K., Kienle, C., Novák, J., Seiler, T., Shao, Y., Stamm, C. and Hollender, J. (2017b) Integrating chemical analysis and bioanalysis to evaluate the contribution of wastewater effluent on the micropollutant burden in small streams. Sci Total Environ 576, 785-795.

Nebert, D., Shi, Z., Gálvez-Peralta, M., Uno, S. and Dragin, N. (2013) Oral benzo[a]pyrene: understanding pharmacokinetics, detoxication, and consequences--Cyp1 knockout mouse lines as a paradigm. Mol Pharmacol 84(3), 304-313.

OECD (2018a) Guidance Document on Good In Vitro Method Practices (GIVIMP) https://www.oecd.org/env/guidance-document-on-good-in-vitro-method-practices-givimp-9789264304796-en.htm.

OECD (2018b) OECD guideline for testing of chemicals - determination of *in vitro* intrinsic clearance using rainbow trout liver S9 subcellular fraction (RT-S9).

Ortiz, C., Moraca, F., Medeiros, A., Botta, M., Hamilton, N. and Comini, M. (2016) Binding mode and selectivity of steroids towards glucose-6-phosphate dehydrogenase from the pathogen Trypanosoma cruzi. Molecules 21(3), 368,1-14.

554 Palma, B.B., Moutinho, D., Urban, P., Rueff, J. and Kranendonk, M. (2016) Cytochrome P450
555 Expression System for High-throughput Real-time Detection of Genotoxicity: Application to
556 the Study of Human CYP1A2 Variants. Mutat Res-Gen Tox En 806, 24-33.
557 Paolini, M., Hrelia, P., Corsi, C., Bronzetti, G., Biagi, G. and Cantelli-Forti, G. (1987) NADPH as
558 rate-limiting factor for microsomal metabolism. An alternative and economic NADPH-
559 generating system for microsomal mono-oxygenase in in vitro genotoxicity studies. Mutat
560 Res 178(1), 11-20.
561 Partow, S., Hyland, P. and Mahadevan, R. (2017) Synthetic rescue couples NADPH generation to
562 metabolite overproduction in Saccharomyces cerevisiae. Metab Eng 43(Pt A), 64-70.
563 Pflaum, J., Schlosser, S. and Muller, M. (2014) p53 Family and Cellular Stress Responses in
564 Cancer. Front Oncol 4, 1-15.
565 Prieto-Alamo, M., Jurado, J., Abril, N., Díaz-Pohl, C., Bolcsfoldi, G. and Pueyo, C. (1996)
566 Mutational specificity of aflatoxin B1. Comparison of in vivo host-mediated assay with in
567 vitro S9 metabolic activation. Carcinogenesis 17(9), 1997-2002.
568 Proudlock, R. and Evans, K. (2016) The micro-Ames test: A direct comparison of the performance
569 and sensitivities of the standard and 24-well plate versions of the bacterial mutation test.
570 Environ Mol Mutagen 57(9), 687-705.
571 Prough, R., Patrizi, V., Okita, R., Masters, B. and Jakobsson, S. (1979) Characteristics of
572 benzo(a)pyrene metabolism by kidney, liver and lung microsomal fractions from rodents and
573 humans. Cancer Res 39(4), 1199-1206.
574 Reifferscheid, G., Maes, H., Allner, B., Badurova, J., Belkin, S., Bluhm, K., Brauer, F., Bressling,
575 J., Domeneghetti, S., Elad, T., Flueckiger-Isler, S., Grummt, H., Guertler, R., Hecht, A.,
576 Heringa, M., Hollert, H., Huber, S., Kramer, M., Magdeburg, A., Ratte, H., Sauerborn-

Klobucar, R., Sokolowski, A., Soldan, P., Smital, T., Stalter, D., Venier, P., Ziemann, C., Zipperle, J. and S, B. (2012a) International round-robin study on the Ames fluctuation test. Environ Mol Mutagen 53(3), 185-197.

Reifferscheid, G., Maes, H.M., Allner, B., Badurova, J., Belkin, S., Bluhm, K., Brauer, F., Bressling, J., Domeneghetti, S. and Elad, T. (2012b) International round-robin study on the Ames fluctuation test. Environ Mol Mutagen 53(3), 185-197.

Richardson, S.J., Bai, A., Kulkarni, A.A. and Moghaddam, M.F. (2016) Efficiency in Drug Discovery: Liver S9 Fraction Assay As a Screen for Metabolic Stability. Drug Metab Lett 10(2), 83-90.

Rudeck, J., Bert, B., Marx-Stoelting, P., Schönfelder, G. and Vogl, S. (2018a) Liver lobe and strain differences in the activity of murine cytochrome p450 enzymes. Toxicology 404-405, 76-85.

Rudeck, J., Bert, B., Marx-Stoelting, P., Schoenfelder, G. and Vogl, S. (2018b) Liver lobe and strain differences in the activity of murine cytochrome P450 enzymes. Toxicology 404, 76-85.

Santes-Palacios, R., Camacho-Carranza, R., Javier Espinosa-Aguirre, J. (2018) Bacterial mutagenicity of selected procarcinogens in the presence of recombinant human or rat cytochrome P4501A1. Mutat Res-Gen Tox En 835:25-31.

Savidov, N., Alikulov, Z. and Lips, S. (1998) Identification of an endogenous NADPH-regenerating system coupled to nitrate reduction in vitro in plant and fungal crude extracts. Plant Sci 133(1), 33-45.

Schoeny, R., Cody, T., Radike, M. and Warshawsky, D. (1985) Mutagenicity of algal metabolites of benzo(a)pyrene for Salmonella typhimurium. Environ Mutagen 7(6), 839-855.

599 Sess-Tchotch, D.A., Kedjebo, K.B.D., Faulet, B.M., Fontana-Tachon, A., Alter, P., Durand, N.,;
600 Grabulos, J., Montet, D., Guehi, T.S. (2018) Analytical Method Validation and Rapid
601 Determination of Polycyclic Aromatic Hydrocarbons (PAHs) in Cocoa Butter Using HPLC-
602 FLD. Food Anal Method 11(11): 3138-3146
603 Shao, Y., Hollert, H., Tarcai, Z., Deutschmann, B. and Seiler, T. (2018) Integrating bioassays,
604 chemical analysis and in silico techniques to identify genotoxicants in surface water. Sci Total
605 Environ 650, 3084-3092.
606 Shoukat, S. Potential anti-carcinogenic effect of probiotic and lactic acid bacteria in detoxification
607 of benzo[a]pyrene: A review. Trends Food Sci Tech 99:450-459
608 Silva, M., Petrakis, N., Musker, W. and Talcott, R. (1985) Metabolism of 2-aminoanthracene by
609 BALB/c and C57BL mammary epithelium in vitro. Carcinogenesis 6(1), 53-58.
610 van der Linden, S., von Bergh, A., van Vught-Lussenburg, B., Jonker, L., Teunis, M., Krul, C. and
611 van der Burg, B. (2014) Development of a panel of high-throughput reporter-gene assays to
612 detect genotoxicity and oxidative stress. Mutat Res-Gen Tox En 760(1), 23-32.
613 van Vugt-Lussenburg, B., van der Lee, R., Man, H., Irene, M., Brouwer, A., Besselink, H. and van
614 der Burg, B. (2017) Incorporation of metabolic enzymes to improve predictivity of reporter
615 gene assay results for estrogenic and anti-androgenic activity. Reprod Toxicol 75, 40-48.
616 Wang, L., Xu, W., Ma, L., Zhang, S., Zhang, K., Ye, P., Xing, G., Zhang, X., Cao, Y., Xi, J., Gu,
617 J. and Luan, Y. (2017) Detoxification of benzo[a]pyrene primarily depends on cytochrome
618 P450, while bioactivation involves additional oxidoreductases including 5-lipoxygenase,
619 cyclooxygenase, and aldo-keto reductase in the liver. J Biochem Mol Toxic 31(7), 1-7.

620 Wang, X., Hayes, J. and Wolf, C. (2006) Generation of a stable antioxidant response element-
621 driven reporter gene cell line and its use to show redox-dependent activation of nrf2 by cancer
622 chemotherapeutic agents. Cancer Res 66(22), 10983-10994.
623 Welters, T., Horn, T., Ruff, A.J., Schwaneberg, U. and Buechs, J. (2017) Novel technique for high
624 throughput measurement of active monooxygenase concentration. Biotechnol Bioeng 114(4),
625 929-933.
626 Wienkers, L.C. and Heath, T.G. (2005) Predicting in vivo drug interactions from in vitro drug
627 discovery data. Nature Reviews Drug Discovery 4(10), 825-833.
628 Willis, A., Indra, R., Wohak, L., Sozeri, O., Feser, K., Mrizova, I., Phillips, D., Stiborova, M. and
629 Arlt, V. (2018) The impact of chemotherapeutic drugs on the CYP1A1-catalysed metabolism
630 of the environmental carcinogen benzo[a]pyrene: Effects in human colorectal HCT116
631 TP53(+/+), TP53(+/-) and TP53(-/-) cells. Toxicology 398-399, 1-12.
632 Yeh, R., Farré, M., Stalter, D., Tang, J., Molendijk, J. and Escher, B. (2014) Bioanalytical and
633 chemical evaluation of disinfection by-products in swimming pool water. Water Res 59(4),
634 172-184.
635 Yoon, M., Campbell, J., Andersen, M. and Clewell, H. (2012) Quantitative in vitro to in vivo
636 extrapolation of cell-based toxicity assay results. Crit Rev Toxicol 42(8), 633-652.
637 Yoshihara, S.I., Mizutare, T., Makishima, M., Suzuki, N., Fujimoto, N., Igarashi, K. and Ohta, S.
638 (2004) Potent Estrogenic Metabolites of Bisphenol A and Bisphenol B Formed by Rat Liver
639 S9 Fraction: Their Structures and Estrogenic Potency. Toxicol Sci 78(1), 50-59.
640 Zanger, U.M. and Schwab, M. (2013) Cytochrome P450 enzymes in drug metabolism: Regulation
641 of gene expression, enzyme activities, and impact of genetic variation. Pharmacol Ther
642 138(1), 103-141.

643