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DISSERTATION

Evaluation of the suitability of *in vitro* bioassays for the genotoxicity assessment of food contact materials

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I confirm that this work is original and has not been submitted elsewhere for any examination, nor is it currently under consideration for a thesis elsewhere.

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"Without patience, you will never conquer endurance."

Yiannis Kouros

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English Abstract

The toxicological safety assessment of food contact materials (FCMs) is an ongoing challenge, especially for non-intentionally added substances (NIAS), as a variety of substances with unknown toxicological characteristics could migrate from the FCM into the foodstuff. For NIAS, a non-targeted gas chromatography mass spectrometry (GC-MS) screening is usually applied and will lead to chromatograms with so called 'forest of peaks'. Identifying and characterising every peak is considered to be unfeasible as it is time-intensive and laborious. Instead it is suggested to combine chemical analysis with *in vitro* bioassays, which allows an effect based testing approach, where the overall mixture of the substances can be tested. Therefore, the effect of the substance mix as a whole can be determined. Genotoxic substances are considered to be the most important, as already very small amounts might lead to an adverse health effect. Through the combination and analysis with *in vitro* bioassays, the threshold of toxicological concern (TTC) approach for unknown substances can be followed. In this thesis, commonly used genotoxicity assays were looked into in detail, both in the literature and in the laboratory, and optimised for the assessment of FCMs. Further, new approaches and novel testing systems, such as high content screening (HCS) and reporter gene assays, were analysed and validated for their application for FCM analysis. Finally, several FCM migrates were tested to determine any matrix effect and the assay's ability to function properly in the presence of complex mixtures. Overall, mammalian based systems performed well in the presence of FCM migrates, but lacked the ability to detect most genotoxic substances at sufficiently low concentrations. For this, HepG2 based tests were used, which showed to be independent of an exogenous metabolising system. The bacterial based system, namely the Ames test, showed to be superior, but optimisation experiments were not successful. In a nutshell, this thesis showed that the combination of chemical and biological methods is a promising approach, but both have to be further improved to ensure low concentrations of genotoxic substances migrating from FCM into foodstuff can be detected.

Deutsche Kurzfassung

Die toxikologische Sicherheitsbewertung von Lebensmittelkontaktmaterialien (LMKs) stellt, insbesondere für unbeabsichtigt eingebrachte Substanzen (NIAS), eine Herausforderung dar. Dabei kann es sich um eine Vielzahl an unbekannten Substanzen handeln, welche aus dem LMK in das Lebensmittel migrieren. Mittels Gaschromatographie-Massenspektrometrie (GC-MS) Screening werden so genannte „Forest of Peaks"-Chromatogramme generiert, bei welchen es zeit- und kostenintensiv ist, alle Peaks zu identifizieren und zu charakterisieren. Stattdessen wird der Ansatz verfolgt, chemische und biologische Methoden zu kombinieren und so den Effekt des Substanzgemisches festzustellen. Besonders genotoxische Substanzen sind hierbei von großem Interesse, da bereits kleinste Mengen ausreichen, um einen Effekt auszulösen. Deshalb sollen durch die Kombination chemischer und biologischer Methoden, mehr Informationen über unbekannte Substanzen gewonnen werden und so das „Threshold of Toxicological Concern" (TTC) Prinzip angewendet werden. Für diese Arbeit wurde eine Vielzahl von etablierten Genotoxizitätstests analysiert und für den Einsatz von LMK-Migraten analysiert und optimiert. Dies inkludierte eine umfangreiche Literaturrecherche, sowie direkte Optimierungsversuche im Labor. Außerdem wurden neue Testsysteme entwickelt und validiert, wie zum Beispiel mittels „High Content Screening" (HCS) oder ein Reportergenbasierter Assay mit HepG2 Zellen. Zusätzlich wurden mit den Assays LMK-Migrate analysiert, um Matrix-Effekte nachzuweisen und die Funktionsfähigkeit der Assays, in Anwesenheit von komplexen Mischung, zu evaluieren. Dabei konnte gezeigt werden, dass LMK-Migrate mit humanzellbasierten Testsystemen analysiert werden können, jedoch sind diese nicht in der Lage, genotoxische Substanzen in ausreichend niedrigen Konzentrationen zu detektieren. Der Einsatz der HepG2 Zellen zeigte jedoch auf, dass kein metabolisches Aktivierungssystem zugegeben werden musste, da die metabolische Aktivität der Zellen bereits ausreichend war. Bakterienbasierte Testsysteme wiederum, wiesen bessere Nachweisgrenzen auf, jedoch waren auch diese nicht ausreichend und konnten selbst durch Optimierungsversuche nicht entsprechend verbessert werden. Hierfür wurde der Ames Test in unterschiedlichen Formaten angewandt, welcher sich in der Literaturanalyse als vielversprechendster Assay, in Hinblick auf Nachweisgrenzen herauskristallisiert hatte. Schlussendlich haben die Versuche gezeigt, dass die Kombination aus chemischen und biologischen Methoden zu einem Informationsgewinn führt. Jedoch müssen diese noch verbessert werden, um genotoxische Substanzen in niedrigen Konzentrationen nachzuweisen, sodass diese zuverlässig für die toxikologische Evaluierung von LMKs eingesetzt werden können.

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List of Abbreviations

Abbreviation	Meaning
2AA	2-Aminoanthracene
2AF	2-Aminofluorene
AFB1	Aflatoxin B1
AMP	Ampicillin Trihydrate
ARF	Alternative Reading Frame
ATM	Ataxia-Telangiectasia Mutated Protein
ATR	Ataxia-Telangiectasia and Rad3 Related Protein
BaA	Benzo-a-anthracene
BαP	Benzo-α-pyrene
BTG2	B-Cell Translocation Gene 2
BSA	Bovine Serum Albumin
CALUX	Chemically Activated Luciferase Gene Expression
CAS	Chemical Abstracts Service
CDKN1a	Cyclin Dependent Kinase Inhibitor 1a
CHK1/2	Checkpoint Kinase 1 or 2
CHL	Chinese Hamster Lung
CHO	Chinese Hamster Ovary
CMR	Cancerogen Mutagen Reprotoxic
CYP450	Cytochrome P450
DAPI	4’,6-Diamidin-2-phenylindol
2,4-DAT	2,4-Diaminotoluene
DDB2	Damage-Specific DNA Binding Protein 2
DMEM	Dulbecco’s Modified Eagle Medium
DMNA	Dimethyl Nitrosamine
DMSO	Dimethyl Sulfoxide
cDNA	Complementary DNA
DPBS	Dulbecco’s Phosphate Buffer Saline
DTT	Dithiothreitol
ECACC	European Collection of Authenticated Cell Cultures
ECHA	European Chemicals Agency
ECVAM	European Centre for the Validation of Alternative Methods
EFSA	European Food Safety Authority
ELISA	Enzyme-Linked Immunosorbent Assay
EMA	European Medicines Agency
ENU	N-Ethyl-Nitrosourea
FACS	Fluorescence-Activated Cell Sorting
FBS	Fetal Bovine Serum
FCM	Food Contact Material

FDXR	Ferredoxin Reductase
FHCW	Fachhochschule Campus Wien
G6P	Glucose-6-Phosphate
G6P-DH	Glucose-6-Phosphate-Dehydrogenase
GADD45α	Growth Arrest and DNA-Damage Inducible Protein Alpha
GCF	Global Concentration Factor
GC-MS	Gas Chromatography Mass Spectrometry
GFP	Green Fluorescence Protein
γH2AX	Phosphorylation of Ser139 on the H2A histone family member X
HCS	High Content Screening
HCT116	Human Colorectal Carcinoma Cells
HF	Human Follicular Lymphoma
HepaRG	Human Hepatoma Cell Line
HepG2	Human Liver Carcinoma Cells
HKG	Housekeeping Gene
HPBL	Human Peripheral Blood Lymphocytes
HQ	Hydroquinone
IAS	Intentionally Added Substances
ILSI	International Life Sciences Institute
LMK	Lebensmittelkontaktmaterial
LOD	Limit of Detection
LOBD	Limit of Biological Detection
sLOBD	Surrogated Biological Limit of Detection
MDM2	Murine Double Minute 2
MDM4	Murine Double Minute 4
mES	Mouse Embryonic Stem Cells
MGA	Minimal Glucose Agar
MLA-*hprt*	Mouse Lymphoma Assay Hypoxanthine Phosphorybosyl Transferase
MLA-*tk*	Mouse Lymphoma Assay Thymidine Kinase
MMC	Mitomycin C
MMS	Methyl Methanosulphonate
MN	Micronucleus
2NF	2-Nitrofluorene
NADPH	Nicotinamide Adenine Dinucleotide Phosphate
NIAS	Non-Intentionally Added Substances
Nluc	Nano Luciferase
4NQO	4-Nitroquinoline-N-Oxide
dNTP	Deoxy Nucleoside Triphosphates
OECD	Organization for Economic Co-Operation and Development
OFI	Österreichisches Forschungsinstitut für Chemie und Technik

OML	Overall Migration Limit
PEI	Polyethyleneimine
pPD	p-Phenylenediamine
qPCR	Real-Time Quantitative Polymerase Chain Reaction
REACH	Registration, Evaluation, Authorization and Restriction of Chemicals
mRNA	Messenger RNA
RPMI	Roswell Park Memorial Institute
RRM2b	Ribonucleotide-diphosphate reductase subunit M2b
RT	Room Temperature
SERTAD1	SERTA Domain Containing Protein 1
SML	Specific Migration Limit
SPE	Solid Phase Extraction
tBHQ	Tert-butylhydroquinone
TGIC	Triglycidylisocyanurat
TK6	Human Lymphoblast Thymidine
TP53INP1	Tumour Protein p53-Inducible Nuclear Protein 1
TTC	Threshold of Toxicological Concern
U2OS	Human Bone Osteosarcoma Epithelial Cell Line

1 Introduction and Motivation

Food Contact Materials (FCMs) can be made up of a variety of packaging materials, with the most important being: plastic, paper and board, as well as glass and metal (Muncke et al., 2020). These materials are able to interact via various mechanisms with the packaged foodstuff, such as through migration or scalping. During migration a variety of substances can be transferred from the FCM into the foodstuff and might be later consumed (Barnes et al., 2007). Therefore, it is essential to thoroughly assess FCMs in regards of toxicological safety to ensure no negative effects can be expected towards human health. In previous years, extensive work and research has been done on the presence and effect of endocrine active substances and their possibility to migrate from FCM into the packaged good (Mertl et al., 2014; Muncke, 2009). Recently the focus has shifted towards genotoxic substances and whether they might be migrating in relevant amounts from the FCM. The International Life Sciences Institute (ILSI) has published a recommendation (Schilter et al., 2019), which clearly states the importance of focusing on genotoxic substances, as already small amounts might lead to a health effect and therefore have to be assessed thoroughly. However, the safety assessment is challenging as many unknown substances might be migrating from the FCM, which can include for example: reaction products, contaminations or side products formed during processing and storage. The majority of substances migrating from an FCM are present in small amounts and are difficult and time-intensive to identify using common analytical methods, such as a non-targeted gas chromatography mass spectrometry (GC-MS) screening (Koster et al., 2015). It was therefore suggested by Schilter et al. (2019) and Severin et al. (2017) to use *in vitro* bioassays for the assessment and to obtain more information on the presence of genotoxic substances in an FCM migrate. *In vitro* bioassays measure effects and are not able to identify substances, but could provide important additional information on the toxicological profile of a complex mixture, such as FCM migrates. As there is a great variety of assays available, covering several endpoints of genotoxicity, the most suitable assay(s) have to be determined. Through assessing commonly used and novel assays for genotoxicity, a recommendation can be given on the most promising and reliable method. By combining already established chemical methods and well evaluated biological tests, important information can be gained on the toxicological profile of FCMs and it can be ensured that safe products are placed on the market, which will be discussed and evaluated in detail in this thesis.

1.1. Aim of this thesis

The goal of this thesis is to give an overview on *in vitro* assays that can be used for the genotoxicity assessment of FCMs and to evaluate the most suitable method. Further, within this thesis it should be shown, which methods have appropriate limits of detection and have a good performance in terms of analytical and toxicological sensitivity and specificity.

The following approach will be used to determine suitable methods and to design a new testing system as well as to improve existing methods. An overview of the approach is also shown in Figure 1.

1. *Literature survey:*

Through an initial literature survey, the detection limits and suitability of current *in vitro* bioassays shall be assessed. The major limitations and advantages of the respective assays will be analysed to ensure the most promising approaches can be looked into more detail. This knowledge should then be applied to improve existing genotoxicity assays and to develop a competitive assay, which can be applied for the safety assessment of complex mixtures.

2. *Development of a genotoxicity assay:*

Based on previous research (Pinter, 2017) the p53 pathway in a cell culture based assay was considered to be an appropriate approach, but lacking applicability and analytical sensitivity in some ways. This thesis builds up on these results and takes the use of a different cell line into consideration, which will be more suitable for the assessment of complex mixtures. Through a series of genotoxic substances and by varying assay conditions a new mammalian test system should be developed. For this, different detection systems will be analysed to determine more suitable methods of detection with the most information gain. Specifically, HepG2 cells will be used for a reporter gene assay and a microscopical approach including Nluc measurement and HCS. A specific focus should lie on metabolisation and metabolic activity of the HepG2 cells, as this is considered to be an important aspect of genotoxicity testing.

3. *Screening of appropriate methods and target genes:*

A selective screening through cell treatment and thorough analysis of gene regulation will be performed with real-time quantitative polymerase chain reaction (qPCR) to identify appropriate target genes and their behaviour in the presence of genotoxic substances. This should provide

further knowledge on the suitability of the p53 pathway and mammalian cells for the assessment of genotoxicity.

4. *Optimisation of current methods:*

Besides the development of a novel test system, already established genotoxicity assays shall be assessed in detail and points for optimisation will be considered. This will include alterations of assay conditions and the testing of different formats to ensure the assay is suitable for the assessment of FCM migrates.

5. *Recommendation for a test strategy:*

Finally, in this thesis a recommendation shall be given for the most appropriate system and its applicability for the assessment of FCM migrates. The information gained through the analysis of several assays should provide information on a suitable method and give insight on further necessary steps for the safety assessment of FCMs.

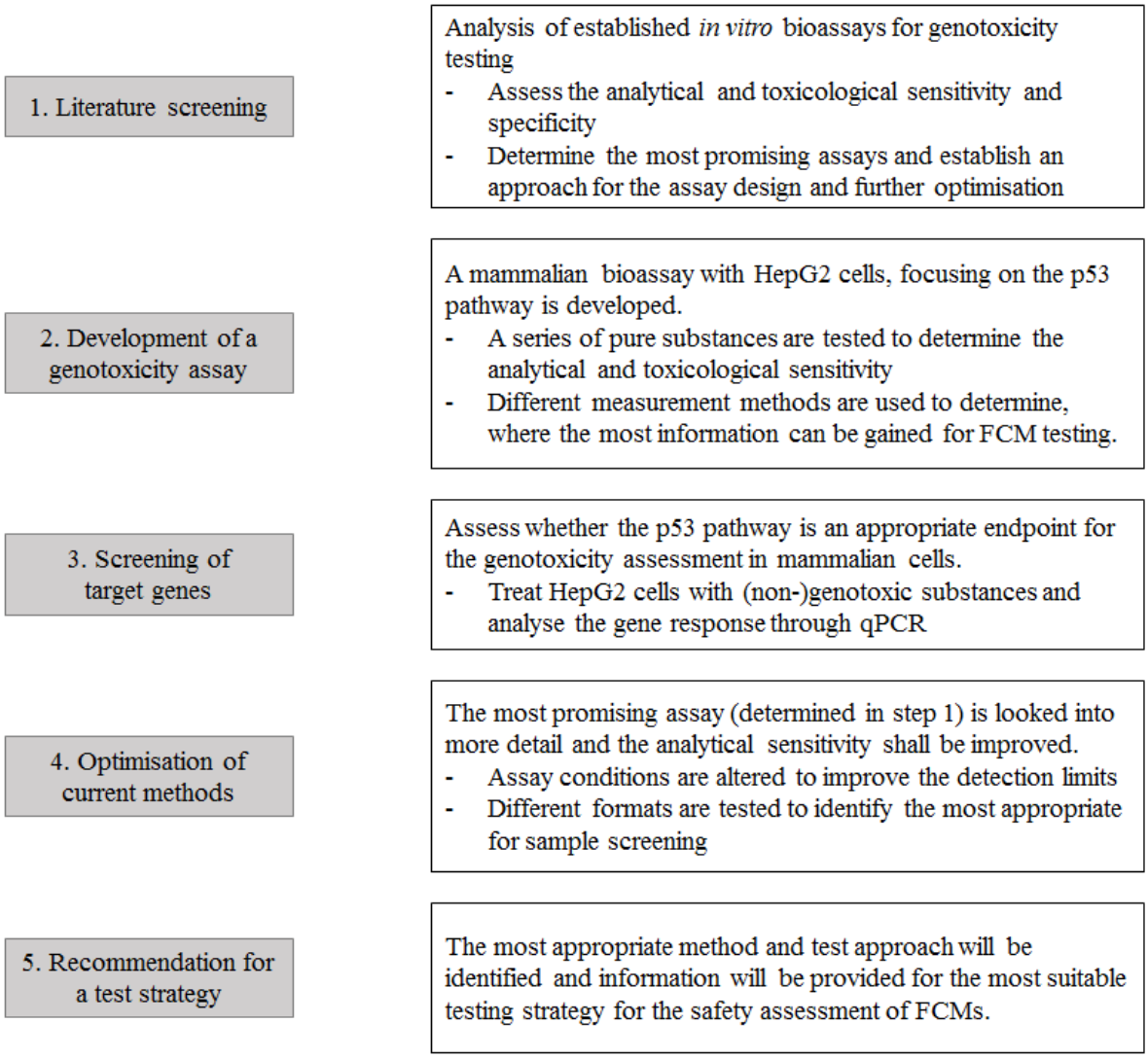


Figure 1: Overview of the planned approaches in this thesis and the expected outcome of this study. Initially a literature study will be performed, followed by practical work in the laboratory to develop a genotoxicity assay and to optimise current methods. Moreover, the activity of certain genes will be measured to gain information on molecular mechanisms. Finally, some recommendations will be made, which assay and approach is considered to be the most suitable.

1.2. Packaging Materials

There are a series of packaging materials available and used depending on the characteristics and requirements of the filling good. Their main role is to protect the food from the environment, from physical damages and to postpone or prevent its spoilage (Muncke et al., 2017). The most commonly used materials are plastic, glass, metal and paper or board. In the European Union the regulation (EC) No 1935/2004 (European Parliament, 2004) on "materials and articles intended to come into contact with food" applies when FCM shall be placed on the market. The regulation deals with all different types of materials and the demands and standards it has to apply with.

Most important, the regulation states that "materials and articles […] do not transfer their constituents to food in quantities which could (a) endanger human health, (b) bring about an unacceptable change in the composition of the food, (c) bring about a deterioration in the organoleptic characteristics thereof" (European Parliament, 2004). Further, the regulation provides a general framework, with which all materials have to comply regarding information, authorisation, traceability and several other aspects.

For most packaging materials, only the (EC) No 1935/2004 applies and some further national legislations and recommendations can be followed, such as the BfR recommendation XXXVI. (BfR, 2019) in Germany, or the DGCCRF in France for paper and board material intended to come into food contact. For plastic packaging, the regulation (EC) No 10/2011 (European Parliament, 2011) is applicable, which deals with "plastic materials and articles intended to come into contact with food". This regulation provides guidance on substances allowed to be used in the manufacturing of plastic materials and on testing procedures to determine the suitability for different applications. Moreover, for testing of packaging materials, a series of guidelines exist by the industries or the standard institutes such as EN645 and EN647 (DIN/EN, 1994b, 1994a) for testing of paper and cardboard materials.

1.2.1. Intentionally Added Substances (IAS) and Non-Intentionally Added Substances (NIAS)

In the European regulation (EC) No 10/2011 (European Parliament, 2011), Annex I provides a list of substances, which are allowed to be used as starting substances, macromolecules, additives and polymer production aids. This list makes up the intentionally added substances (IAS), which can be used during the manufacturing of plastic packaging material. These substances have been risk assessed and their suitability for their application as part of FCMs has been stated. Only these substances may be intentionally added during the manufacturing process of

plastic products. The union list of authorised substances provides a framework for manufacturers and gives information on the overall and specific requirements, when using those substances intentionally.

The so called non-intentionally added substances (NIAS) on the other hand, are not as well assessed and regulated as the IAS. These NIAS can for example be impurities in the used IAS, reaction or breakdown products formed during manufacturing or processing of FCMs. Moreover, they can be contaminations or degradation products. In general, the chemical structure and composition of potential NIAS is unknown, so that they are difficult to classify and assess. Some NIAS can be predicted or estimated as reaction products from IAS used for processing the plastic material. To detect NIAS, a non-targeted GC-MS analysis can be performed, which usually results in a forest-of-peaks, where NIAS make up the majority of substances. Any further assessment of NIAS tends to be laborious and time consuming as one has to deal with several substances of unknown identity (Koster et al., 2015; 2014; Nerin et al., 2013).

1.2.2. Interaction Packaging and Filling Good

An FCM can interact with the packaged food in different ways, provided it is not inert. The most important mechanism is likely to be migration, where substances from the packaging migrate into the food or filling good. On the other hand, substances might migrate from the packaged good into the packaging, which is called scalping. Finally, substances might migrate from its surrounding through the packaging into the food, given the packaging is porous (Barnes et al., 2007). The different scenarios are shown in Figure 2 with the different factors that influence the migration process and the focus of this thesis is on the migration process from the FCM into the food.

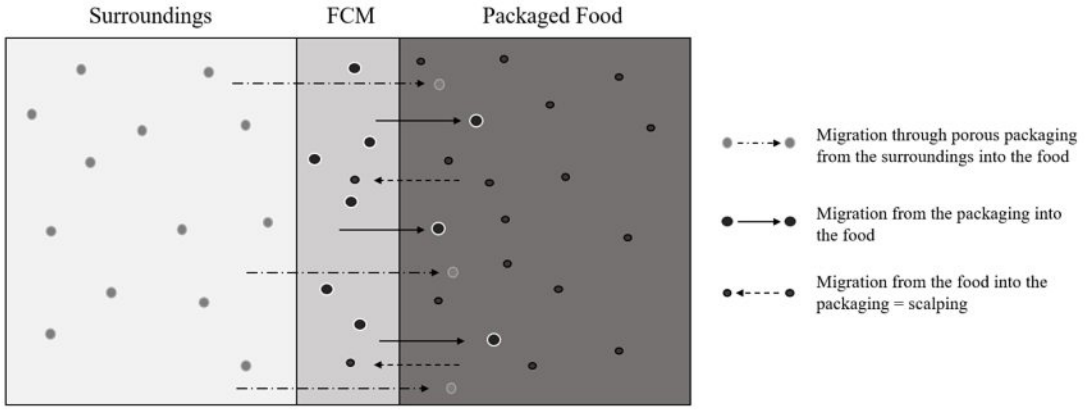


Figure 2: Possible interactions between a packaging, which is not inert, the packaged good and its surroundings. Substances can migrate from the packaging into the food or from the surrounding through a porous packaging into the food. In contrast substances can migrate from the food into the packaging, which is called scalping.

The migration process is dependent on several factors, influencing the amount and extent to which a substance will migrate until it will reach its equilibrium. One of these factors is the molecular diffusion process of a substance, which depends on the time and temperature of the contact, the thickness of the material, the diffusion and partition coefficient, the molecular weight and size of the substance and its concentration (Barnes et al., 2007). The contact duration time and temperature are of great importance for the interaction with the product. For this, different scenarios are given in the European regulation (EC) No 10/2011 (European Parliament, 2011). Moreover, the nature of the filled good is crucial as the compatibility of the solubility of the packaging and the solubility of the packaged good can greatly influence the migration. The properties of the substance itself also influence the migration, as substances with higher molecular weight are less likely to migrate.

In the regulation (EC) No 10/2011 an overall migration limit (OML) is defined, which is the "maximum permitted amount of non-volatile substances released from a material or article into food simulants" (European Parliament, 2011). The OML in the regulation is set at 10 mg per 1 dm^2 corresponding to a migration limit of 60 mg per kg food. This threshold applies to all substances potentially migrating from a plastic packaging into the foodstuff. For IAS, and some substances known to migrate, specific migration limits (SML) apply. These limits only apply to the specific substance, since a higher migration of the chemical might lead to a potential health risk, therefore, the SML of a substance shall not be exceeded. Nevertheless, the sum of all SMLs is not allowed to exceed the OML, to ensure a safe packaging in terms of unspecific migration. In a multi-layer packaging, consisting of several layers of plastic material possibly mixed with other materials, the functional barrier principle applies. The functional barrier is a layer, which is in food contact and prevents the migration from behind the barrier into the foodstuff. Here, for non-authorised substances a threshold of 0.01 mg kg^{-1} applies for migration through the barrier into the foodstuff, given that they are not classified as carcinogen, mutagen or reprotoxic (CMR) (European Parliament, 2011).

To determine the overall and specific migration, experiments have to be conducted to assess the migration potential in specific food simulants under specific conditions. For this, (EC) No 10/2011 Annex V (European Parliament, 2011) proposes migration testing under certain conditions. There the shelf life and the use conditions are taken into account and an accelerated migration is performed in the laboratory. Through predicted contact time and temperature the test parameters are determined. Further, food simulants are used, which are proposed in (EC) No 10/2011 Annex III for different product categories. Depending on the filling good characteristics, different food simulants are used for the migration experiments. Through gravimetric analysis and/or analytical chemistry, the OML and/or SML can be determined for a certain

packaging for a specific application with a specific food type. Specifically for fatty foods, a correction factor has to be taken into account, as here less daily consumption can be assumed compared to other food categories. Finally, the migration testing is necessary to state compliance with the legislation and therefore allowing the packaging to be placed on the European market.

1.2.3. Threshold of Toxicological Concern Concept

For assessment of toxicological risks, the threshold of toxicological concern (TTC) is considered to be an important tool. The application of the TTC for the evaluation of unknown compounds, such as impurities or complex mixtures, has been recommended by various studies (Koster et al., 2015; 2014; 2011; Kroes et al., 2004). In general, the TTC concept is taken into consideration for substances, where full toxicological data is missing. The aim of the TTC concept is to establish threshold values for all chemicals, below which there is no risk towards human health upon exposure. This approach evaluates substances for which toxicity is unknown, by considering the chemical structure and similarities to substances with a full toxicological profile. Further, the TTC concept is suitable for substances at low concentrations, since they are expected to not pose a risk, when present below the respective threshold (Kroes et al., 2004; Munro et al., 2008).

According to Kroes et al. (2004), when applying the TTC decision tree as shown in Figure 3, in a first step any possible genotoxic or high potency carcinogens have to be excluded at a threshold of 0.15 µg per person per day, assuming a bodyweight of 60 kg. Some substance groups are regarded to be of high alert even at such concentrations, which includes aflatoxin-like, N-nitroso- and axozy-compounds. These are the so called cohorts of concern, for which no threshold can be applied below which a negligible risk is expected and are therefore excluded from the TTC concept. When it is proven that there is no genotoxic potential, then the next step in the decision tree in Figure 3 can be made, analysing whether the substance is an organophosphate or N-methyl carbamate associated with neurotoxic effects. For these substances, a threshold of 18 µg per person per day applies. In the next step, the presence of reactive functional groups must be excluded to apply the Cramer Class III with 90 µg per person per day. To conclude, when these cohorts of concern can be excluded, a further analysis based on the Cramer Classes I, II and III can be done. The thresholds for these compounds are 1,800 (Class I), 540 (Class II) or 90 (Class III) µg per person per day.

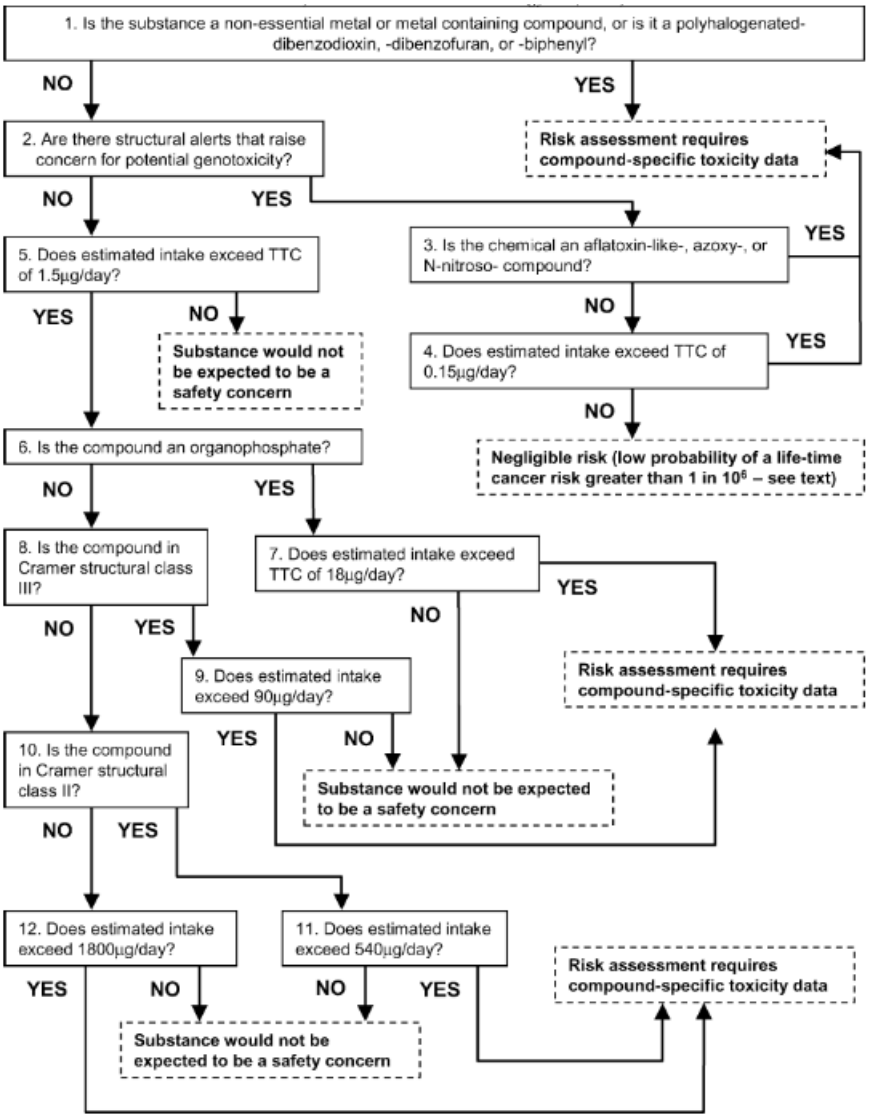


Figure 3: TTC decision tree as presented by Kroes et al. (2004). No adaptions or changes were made to the initial graphic.

A guideline published by the European Medicines Agency (EMA), the ICH M7, focuses on the assessment of impurities in pharmaceutical products regarding DNA reactive substances (ICH, 2017). This guideline deals with the necessity and methods to assess direct DNA-reactive substances inducing genotoxicity, excluding non-DNA-reactive genotoxic mechanisms (Kasper and Müller, 2015). As non-DNA-reactive genotoxins tend to follow a threshold dependent mechanism, they are considered to be of little importance at low concentrations. These can be aneugenic or clastogenic substances, which are considered to indirectly affect the DNA. Direct-DNA-reactive substances on the other hand, pose a carcinogenic risk already at low concentrations and a threshold cannot be applied. Therefore, when assessing impurities the presence of DNA-reactive substances is of interest and has to be evaluated. For this, the ICH (2017) recommends the application of a single assay for mutagenicity: the Ames test. This is possible, as Ames positive substances tend to have a high correlation with rodent carcinogenicity, whereas

Ames negative substances with positive results for clastogenicity or aneugenicity show less correlation (Kasper and Müller, 2015). Other guidelines also recommend focusing on mutagenic impurities in complex mixtures instead of a full genotoxicity assessment, which covers threshold-based mechanisms. For this, the Ames test is again regarded to be sufficient (EFSA, 2011; Schilter et al., 2019).

1.2.4. Risk Assessment of FCMs and the TTC

The European regulation (EC) No 1935/2004 states that any article or material in direct or indirect contact with food shall not endanger human health, bring about changes in the food composition or lead to deterioration of its organoleptic properties, under normal or foreseeable conditions of use (European Parliament, 2004). This requires the material to be safe and basically inert. Any FCM in contact with food has to be thoroughly assessed and evaluated to determine its suitability for use (Bolognesi et al., 2017).

With the European regulation (EC) No 10/2011 (European Parliament, 2011) any plastic material in direct or indirect contact with food is regulated. Here, also a positive list of substances to be used for manufacturing and migration testing conditions are provided. This Union list includes all IAS possible to use for the manufacturing of plastic FCMs. However, a greater concern are those substances, which are not intentionally added, but are impurities or reaction products, the NIAS. These are of great importance for safety assessment as they can migrate into foodstuff in amounts that might be dangerous towards human health (Bolognesi et al., 2017). As it would not be feasible to identify and characterize all NIAS, they are acceptable to migrate in low amounts and below certain detection limits if specific characteristics apply, such as a molecular weight above 1,000 Da or other criterions (European Parliament, 2011).

For risk assessment of FCMs, a proposed approach is the combination of analytical chemical methods with *in vitro* bioassays and the use of the TTC decision tree. For risk assessment of FCMs, in a first step, all IAS used in the manufacturing process of the FCM are assessed and their potential towards human health is evaluated. The finished product is then used for migration testing including several chemical analytical screening methods, such as non-targeted NIAS screening through GC-MS analysis. Here, a variety of substances can be found leading to a “forest-of-peak”, where it is not feasible or possible to identify all substances (Koster et al., 2015). The generation of substance specific toxicological data for every substance migrating out of an FCM into the food is currently not feasible (Munro et al., 2008). Due to the absence of toxicological data but the necessity to thoroughly assess the safety of FCMs it is proposed to use the TTC concept (Munro et al., 2002; Munro et al., 2008; Koster et al., 2015). As discussed

in 1.2.3, the TTC concept can be applied to assess complex mixtures when toxicological data is missing and therefore has been proposed to be used for FCM safety evaluation (Koster et al., 2015; Schilter et al., 2019). In Figure 4, a possible scheme for toxicological safety assessment is shown. Due to lack of information, the TTC decision tree is taken into consideration and after NIAS screening in an initial step, genotoxicity has to be excluded at a threshold of 0.15 µg per person per day. As shown in Figure 4, any substance present below 0.15 µg per person per day is considered not to be problematic if it is genotoxic. Further, the presence of organophosphates and neurotoxin causing substances can be excluded and when no reactive substance groups are present, the Cramer Class III threshold of 90 µg per person per day can be applied. With this approach for a complex mixture, such as an FCM migrate, identification and thorough toxicological assessment has to be done for substances above the threshold of 90 µg per person per day.

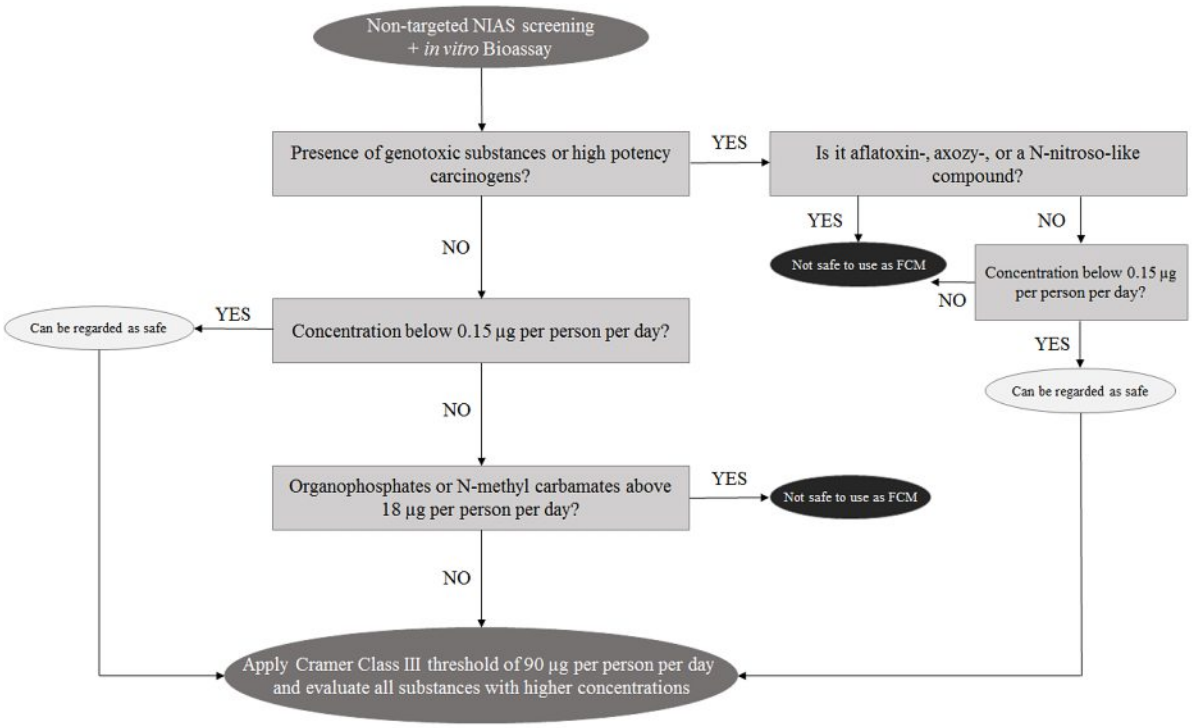


Figure 4: Scheme to assess the toxicological safety of FCMs with a combination of chemical analysis (=non-targeted NIAS screening) and *in vitro* bioassays.

Another possibility is to use the technical limit of 10 ppb proposed by the European Union in the regulation (EC) No 10/2011 (European Parliament, 2011). This threshold corresponds to a limit of 0.01 mg L^{-1} for any substance migrating into the FCM, providing they are not CMR (Pinter et al., 2020; Schilter et al., 2019; Rainer et al., 2018). The regulation suggests this limit for any non-authorized substances used behind a barrier layer in an FCM, regarding they do not migrate into the food (European Parliament, 2011). This approach has been widely used for

chemical analysis as part of NIAS screening and is also suggested to be used as a limit of biological detection (LOBD) for genotoxicity assessment of FCMs instead of the TTC threshold of 0.15 µg per person per day by the ILSI (Schilter et al., 2019). The threshold of 0.01 mg L^{-1} is considered a pragmatic approach to prioritize unknown chemicals, which should be regarded more specifically for further assessment.

To exclude the presence of genotoxic substances, several studies and guidelines suggest to assess genotoxicity in FCM migrates by testing with *in vitro* assays (Schilter et al., 2019; Koster et al., 2015; Bolognesi et al., 2017). For this, they recommend the use of an *in vitro* test battery including a gene mutation test, such as the Ames test, in combination with a genotoxicity test for non-DNA-reactive mechanisms. Genotoxic substances that are considered direct DNA-reactive, also called mutagens, possess a risk already at low concentrations. Other non-mutagenic genotoxicants are threshold dependent and do not lead to a risk for humans at concentrations present in impurities (ICH, 2017). Chemicals leading to an oxidative stress response in an organism are considered to be of little concern at low concentrations as protective cellular mechanisms are present and able to deal with small amounts of oxidative acting substances (ICH, 2017). Therefore, the Ames test could be used as a standalone test system, as recommended by Schilter et al. (2019). Non-DNA-reactive impurities are considered to be threshold depended and are therefore covered with the Cramer Class III threshold of 90 µg per person per day (Aardema, 1998). If the Ames test results are clearly negative, then it can be assumed that no genotoxic substances are present in the complex mixture, when following a certain test protocol (Bolognesi et al., 2017). When clear positive results are obtained, in general *in vivo* follow up testing should be performed. However, this is not feasible for FCM migrates and instead a change in material or different articles should be used or a different *in vitro* assay for genotoxicity can be applied for confirmation of the result.

1.2.5. FCM Testing with *In Vitro* Bioassays

Recent studies have already successfully applied *in vitro* bioassays for the safety assessment of FCM migrates (Ozaki et al., 2004, 2005; Bradley et al., 2008; Bach et al., 2013, 2014; Rosenmai et al., 2017). In general chemical analysis is used for the analysis of FCMs, which mostly includes a non-targeted GC-MS screening, headspace-GC-MS or LC-MS (Koster et al., 2015). These analytical methods usually give an overview on the substances migrating from the FCM into the foodstuff and generally substances mentioned in the EU 10/2011 Annex I (European Parliament, 2011) are targeted specifically.

In vitro bioassays have been widely used for the analysis of complex mixtures in the following contexts, such as novel foods (EFSA, 2016a), botanical extracts (EFSA, 2009), ecotoxicology (Eisenbrand et al., 2002), for medical devices (ISO, 2014), or for impurities in the pharmaceutical product testing (ICH, 2017). The application of *in vitro* assays for FCM testing is still a novel approach, but has already been used regularly for the detection of endocrine effects (Rosenmai et al., 2017; Severin et al., 2017; Mertl et al., 2014; Bengtström et al., 2014). Some studies have also been performed regarding genotoxicity assessment of FCMs (Mertens et al., 2017; Nakai et al., 2014; Ubomba-Jaswa et al., 2010; Ozaki et al., 2005, 2004; Monarca et al., 1994). Both Rainer et al. (2018) and Pinter et al. (2020) did a literature review to analyse the studies using *in vitro* bioassays for genotoxicity testing with FCMs. The results for both these studies were a part of this thesis and can be found in the appendix (see Table A 1 and Table A 2 in Appendix). Further, Severin et al. (2017) also analysed the applicability of *in vitro* bioassays for the safety assessment of FCMs focusing on a variety of targets and effects, such as endocrine activity, cytotoxicity and genotoxicity.

The screening of these studies showed that many different assays, protocols and methods were used, which complicates the comparison of the results. Most of these studies tested with different biological test systems and therefore often analysed different endpoints of genotoxicity. Further, sample preparation was often performed to a different extend, where some studies did perform a concentration step and others did not. Some studies even conducted chemical analysis and took cytotoxicity into account. Several studies showed a positive result for the screening of FCM for genotoxicity. However, for most it could not be determined, from where this effect originated and whether it might have been a false positive result (Pinter et al., 2020; Schilter et al., 2019; Rainer et al., 2018).

1.2.6. Sample Preparation

For FCM migrate testing, several steps have to be performed, so that the FCM can be chemically or biologically analysed. The following steps are recommended by the ILSI for the testing of FCM with *in vitro* assays (Schilter et al., 2019):

1. Migration or extraction with a suitable solvent
2. Concentration of the sample
3. Solvent swap
4. Application in the assay

Migration or Extraction

Either a migration process or extraction can be performed with the sample material. A migration tends to be a more realistic approach, where a solvent is used to simulate the migration of substances from the FCM into the foodstuff. For this, the foreseeable use conditions and simulants recommended by the EU 10/2011 Annex III and V (European Parliament, 2011) can be used. Extraction on the other hand is a harsher process, where a solvent is used to extract even more substances out of the FCM, than would normally migrate. This could be considered as a worst-case, however substances might be extracted, which do not possess the ability to migrate into the foodstuff in such amounts. This was described for paper, where extraction is in some cases considered as overestimation of migration (CITPA, 2019).

Depending on the foreseeable use: contact area, temperature, properties of the filling good and storage time are taken into consideration to conduct a migration study. The parameters for this are given in the EU 10/2011 Annex V (European Parliament, 2011). In a guideline published by the ILSI (Schilter et al., 2019), it was recommended to use 96 % ethanol as a simulant with an incubation time of 10 days at 60 °C to simulate a worst-case migration scenario. However, different migration simulants might lead to the migration of different substances, as genotoxic substances are made up of a great variety of characteristics.

Sample Concentration

As stated above, after migration or extraction, usually a concentration step is performed, as the concentration of many substances, especially of NIAS, in the FCM migrate is very low. Moreover, due to a further dilution step in the *in vitro* bioassay application the sample concentration is crucial to indirectly improve the LOBD values.

Different methods can be applied for sample concentration, which also depends on the solvent or simulant used for sample migration. A commonly used method is evaporation, where the solvent volume is reduced. This is usually done by using gentle conditions, such as low temperatures and low pressures, since the mixture should be as little affected by the concentration step as possible. Especially volatile substances are considered crucial, as they might evaporate when too harsh conditions are applied during concentration. This is why the sample is usually not evaporated to dryness, but a certain amount of solvent should remain to ensure as little volatile substances are lost as possible (Schilter et al., 2019; Rainer et al., 2019). In the literature surveys it was summarised by Rainer et al. (2018) and Pinter et al. (2020), which are shown in

Table A 1 and Table A 2 in the appendix, that evaporation was a popular choice for concentration and was conducted by several studies such as by Rosenmai et al. (2017), Riquet et al. (2016), Nakai et al. (2014), Bradley et al. (2008) and Ozaki et al. (2004).

Another commonly used method for concentration is solid phase extraction (SPE). In this approach the migrated or extracted sample is concentrated through a column, where the substances interacting with the column remain. Later an elution step will release the substances again and they are collected in a suitable solvent (Simpson, 2000; Thurman and Mills, 1998). Specifically for the concentration of endocrine substances this method is applied, as they have hydrophobic properties and are rather homogenous in their characteristics (Mertl et al., 2014). For genotoxic substances, using SPE can be challenging, as the substances have a wide spectrum of characteristics from hydrophobic to hydrophilic. Therefore, using just a single column for extraction tends to miss certain substances. Further, applying SPE might lead to a favouring of substances with certain properties and consequently altering the composition of the complex mixture (Andrade-Eiroa et al., 2016). Nevertheless, SPE has been the method of choice also for the testing of FCMs for genotoxicity, as it was applied in several studies by Bach et al. (2014, 2013), Ceretti et al. (2010), Monarca et al. (1994) and Fusco et al. (1990), as listed in Table A 1 and Table A 2 in the appendix.

An approach used less often for concentration is lyophilisation, which is possible only for certain solvents. Here the usually aqueous migrate is concentrated through consequent freezing, leading to a concentration of the remaining substances. This approach is not as commonly used, but has been applied for the sample preparation of mineral water samples, as was done in a study by Biscardi et al. (2003) and Fusco et al. (1990) as shown in Table A 1 in the appendix.

Solvent Swap

For the application in an *in vitro* bioassay, the solvent used for migration or extraction is often not compatible with the biological system. Therefore, a solvent swap is often performed after the concentration step (Schilter et al., 2019; Rainer et al., 2019). A commonly used solvent for this is dimethyl sulfoxide (DMSO), which is a keeper substance, so that the sample can be stored in a stable state. For changing the solvent, the DMSO is added to the concentrated sample and then under gentle air or nitrogen flow the remains of the initial solvent are removed (Schilter et al., 2019). This sample can then be stored at cool temperatures or applied to the *in vitro* test system.

Assay Application

When adding the sample to the assay, a dilution step takes place, as the cells or bacteria are not compatible with higher amounts of organic solvents. Mammalian cells are able to tolerate about 0.5 to a maximum of 5 % (Timm et al., 2013) of organic solvents, such as ethanol or DMSO. Bacteria on the other hand are able to tolerate higher amounts, ranging from 4 to 14 % for ethanol or DMSO (Hakura et al., 1993; Di and Kerns, 2006), without compromising the viability of the bacteria and consequently affecting the test result. For aqueous solvents, the tolerance for both mammalian cells and bacteria is higher, but aqueous solvents are less commonly used for sample preparation of FCMs.

1.3. Genotoxicity

Genotoxicity deals with genetic effects and interactions or damages to the DNA or the cellular apparatus (Custer and Powley, 2015). Substances causing mutations, alterations in nucleic acids, DNA inactivation and changes in the chromosomes, their structure or number, are considered to be genotoxic (Brusick, 1987).

As part of REACH (Registration, Evaluation, Authorization and Restriction of Chemicals) the European Chemicals Agency (ECHA) released a guidance on information requirements (ECHA, 2017) covering the topic and issue of genotoxicity. There genotoxicity is defined as follows:

"*Genotoxicity* is a broader term and refers to processes which alter the structure, information content or segregation of DNA and are not necessarily associated with mutagenicity" (ECHA, 2017). Mutagenicity on the other hand are processes leading to "permanent transmissible changes in the amount or structure of the genetic material of cells or organisms" (ECHA, 2017). To sum up: all mutagenic substances are considered to be genotoxic, but not all genotoxic substances can be classified as mutagenic (OECD, 2015a).

In this context, clastogenicity refers to clastogenic or mutagenic agents leading to chromosomal aberrations, such as chromosomal breaks, loss or rearrangement in chromosome segments. Aneugenicity deals with aneugenic agents causing gain or loss in chromosome number in the cells (ECHA, 2017). In general, genotoxic effects take place at levels, where a substance is not cytotoxic and can interact, resulting in changes to the genetic material (Brusick, 1987).

1.3.1. Genotoxic Effects – Direct/Indirect Acting Genotoxins

Genotoxic substances can lead to a series of effects, as they cover a broad spectrum of substance categories with different properties and structures. Therefore, genotoxic substances are classified into direct and indirect acting agents. The direct acting substances cover those, which lead to point mutations, deletions, insertions or structural damages, such as clastogenicity and they are referred to as DNA-reactive genotoxins. These DNA-reactive effects can be caused by the substance itself or by their metabolites. These effects are usually assessed with a mammalian gene mutation test, such as the MLA-*tk* or MLA-*hprt*, or with the Ames test (Custer and Powley, 2015). For these substances, a non-threshold model is applied, since already small amounts of the DNA-reactive substance can cause changes and alterations to the DNA (Mishima, 2017).

Non-DNA-reactive genotoxins, on the other hand, cover indirect effects. These effects are considered as aneugenicity, including alteration in the number of chromosomes or inhibition of DNA synthesis or spindle function (Custer and Powley, 2015; Stavitskaya et al., 2015). Indirect

effects can be assessed and detected through the use of mammalian assays focusing on aneugenicity, such as the micronucleus (MN). For some of those indirect acting agents, a threshold can be determined and even no genotoxic effect levels can be observed *in vivo* (Custer and Powley, 2015). Further, thresholds can then be calculated from the no observed effect level or lowest-observed effect level as a starting point and then conservatively be evaluated (Mishima, 2017).

The different genotoxic effects and their consequences in the cells and/or genome are demonstrated in Figure 5. Further, the testing system used to detect the effect is also shown and the different tests will be introduced in detail in chapter 1.4.6 on genotoxicity testing systems. Through indirect and direct effects, the DNA-damage response in eukaryotic cells is activated. Some associated genes and proteins are: the p53 tumour suppressor gene, GADD45α, p21 and H2AX (Boehme et al., 2010). These markers are discussed in more detail in the following chapters 1.3.2 on the p53 pathway and 1.3.3 dealing with γH2AX. The DNA-damage response is regarded as a target for genotoxicity, but also non-genotoxicity related effects can activate the cellular response, which is indicated as cellular response in Figure 5. Such stresses include cytotoxicity and oxidative stress, which can lead to activation of the cellular DNA response systems (Pinter et al., 2020).

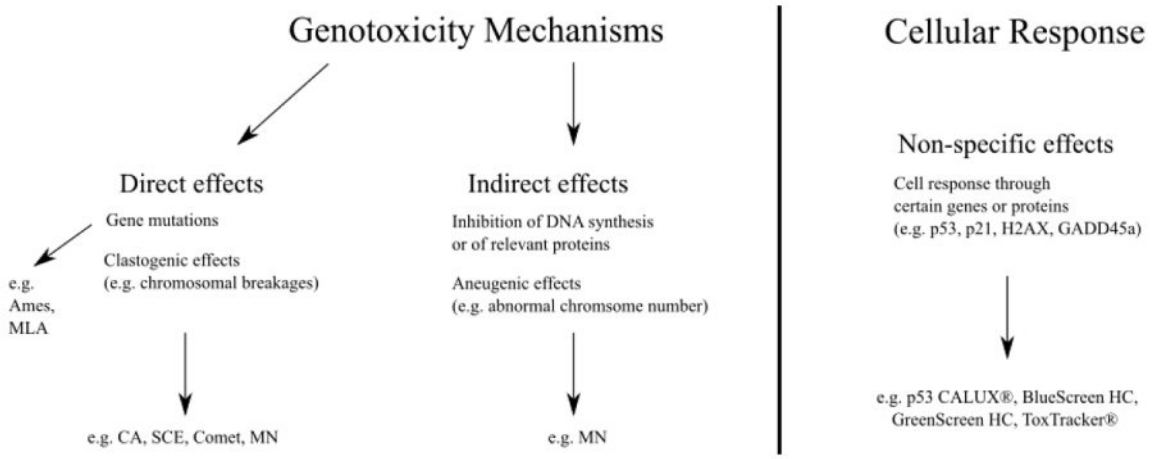


Figure 5: An overview of genotoxic effects and their different categories. Direct effects, such as gene mutations and clastogenicity, indirect effects, such as aneugenicity, and non-specific effects activating the DNA damage response systems are classified. With arrows, the different tests to detect a specific effect are listed.

1.3.2. P53 Pathway

The p53 pathway consists of several genes and proteins that respond to stresses in the cellular environment in human cells. Upon the presence of an activating substance, these responses include apoptosis, cell senescence or cell cycle arrest, by activating the DNA damage response (Levine et al., 2006). Through feedback loops, the activity of the p53 pathway is enhanced or reduced to provide the appropriate responses. The p53 protein is an integral part of the p53 pathway and known as a tumour-suppressor gene, which regulates the p53 activity and function (Levine et al., 2006; Bieging et al., 2014) and is associated with cancerogenesis (Harris and Levine, 2005).

In general, the p53 pathway can be activated by a series of intrinsic and extrinsic stress signals, such as gamma or UV radiation, DNA damage, presence of nitric oxide, certain nutritional deprivations or spindle damages in the chromosomes (Levine et al., 2006; Prives and Hall, 1999). When a stress is present, the pathway will respond by activating the p53 protein through a cascade of events, with different types of stresses leading to different phosphorylation patterns (Harris and Levine, 2005). As can be seen in Figure 6, upon DNA damage the kinases ataxia-telangiectasia mutated (ATM) or ataxia-telangiectasia and Rad3 related protein (ATR) (Levine et al., 2006) lead to a phosphorylation of the p53 protein and its displacement from its negative regulators murine double minute 2 (MDM2) and 4 (MDM4) (Bieging et al., 2014; Toledo and Wahl, 2007). MDM2 and MDM4 bind to the active site of the p53 protein and prevent its activation. By detaching, the p53 protein is stabilised and activated (Brown et al., 2009). Further, ATM and ATR can phosphorylate checkpoint kinase 1 (CHK1) and 2 (CHK2), which are also able to phosphorylate p53 and stabilise it (Bieging et al., 2014). Activation of the p53 protein through detachment of MDM2 and MDM4 can also take place through hyperproliferative signals. These signals lead to the stimulation of the tumour suppressor alternative reading frame (ARF), which inhibits MDM2 and therefore stabilises and activates p53 (Toledo and Wahl, 2007).

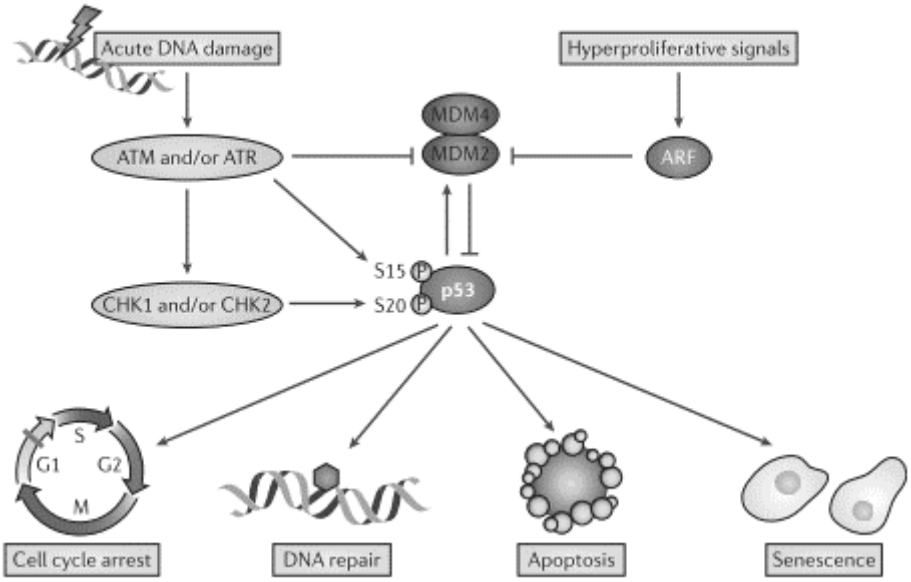
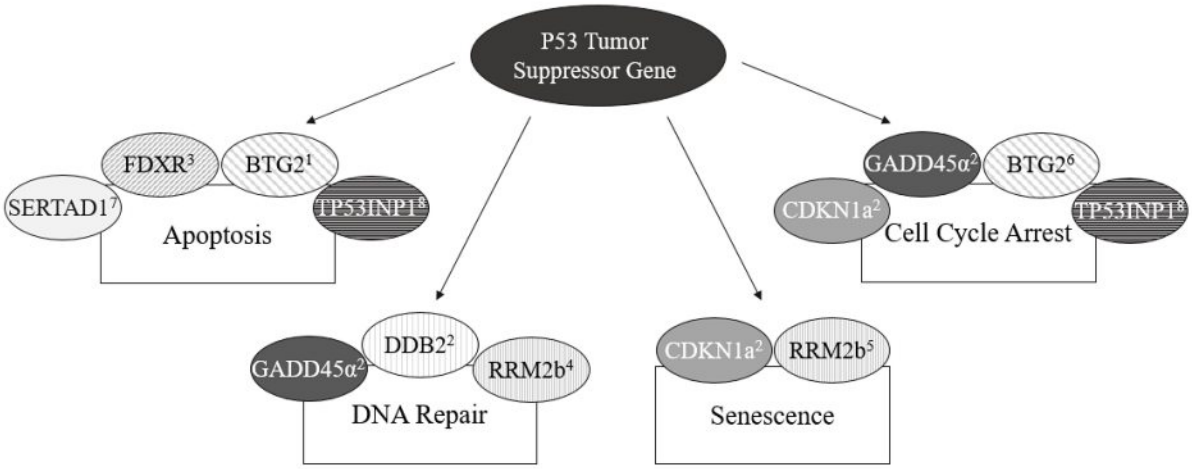


Figure 6: The image from Bieging et al. (2014) shows the feedback loops controlling the p53 pathway. Upon DNA damage, the kinases ATM and ATR interact with MDM2 and MDM4, CHK1 and CHK2, which interact with p53 and lead to its phosphorylation and therefore stabilisation and activation. This then results in various scenarios including cell cycle arrest, senescence, apoptosis and DNA repair. The pathway can also be activated by hyperproliferative signals, which activates the tumour suppressor ARF, which affects MDM2 and MDM4 and thus further interacting with p53 phosphorylation (Bieging et al., 2014).

With the human p53 pathway, several target genes are associated, which are bound and regulated within the pathway. Some important target genes, which were first discovered are for example: GADD45α, CDKN1a (such as p21) and the already mentioned MDM2 (Fischer, 2017). In Figure 7 an overview of some target genes of interest are shown, which are known to be activated by p53 and are associated with a series of possible cellular outcome. The further presented genes will be of interest in this thesis and are, therefore shown here. However, there are more than 300 target genes associated with p53 regulation (Fischer, 2017) so this only focuses on a mere fraction of a great variety of genes. Especially of interest in this thesis are: B-cell translocation gene 2 (BTG2), cyclin dependent kinase inhibitor 1a (CDKN1a), damage-specific DNA binding protein 2 (DDB2), ferredoxin reductase (FDXR), growth arrest and DNA-damage inducible protein alpha (GADD45α), ribonucleotide-diphosphate reductase subunit M2b (RRM2b), SERTA domain containing protein 1 (SERTAD1) and tumour protein p53-inducible nuclear protein 1 (TP53INP1) shown in Figure 7. Most of these genes are associated with several stress responses and the assignment of the genes to the various endpoints is not complete, but only thought to show some prominent and unambiguous relation between genes and effects. Examples of genes linked with apoptosis are: BTG2, FDXR and SERTAD1. P53 can upregulate BTG2 expression, which can promote apoptosis and can cause cell cycle arrest in the G1 phase to enable DNA repair (Mao et al., 2015). The target gene SERTAD1 is involved in regulating cellular signalling responses connected to apoptosis to promote cell survival (Mongre et al., 2019; Biswas et al., 2010). FDXR is further associated with metabolism control,

as it plays an essential role in iron homeostasis, but also for apoptosis regulation (Liu et al., 2019). Some of these target genes are known to induce cell cycle arrest and control cell cycle checkpoints, such as BTG2, CDKN1a, GADD45α and TP53INP1. These genes lead to a blocking of certain genes and proteins important for DNA replication or are required for G2/M phase arrest (Fischer, 2017; Shahbazi et al., 2013). Especially, CDKN1a and GADD45α have a prominent role in the p53 pathway, as they participate in multiple further stress response scenarios such as senescence and DNA repair, where DDB2 also participates (Bieging et al., 2014). RRM2b is known to be expressed during senescence and further partakes in DNA damage repair, as it is induced in a p53 dependent manner (Kuo et al., 2012).



[1](Mao et al., 2015), [2](Bieging et al., 2014), [3](Zhang et al., 2017), [4](Liu et al., 2019), [5](Kuo et al., 2012), [6](Rouault et al., 1996), [7](Biswas et al., 2010), [8](Shahbazi et al., 2013)

Figure 7: Important genes involved in the p53 pathway, which are of interest in this study, are displayed. The target genes are distributed to the various pathway endpoints they are generally associated with. This display is not exhaustive and several genes are associated with more endpoints. Here only the most prominent examples are shown for reasons of simplification. The most important target genes identified in this thesis, which are known to be linked to the p53 pathway, are BTG2, CDKN1a, DDB2, FDXR, GADD45α, RRM2b, SERTAD1 and TP53INP1. The genes were distributed among endpoints they are known to be associated with such as apoptosis, cell cycle arrest, senescence and DNA repair.

In mutated cancer cells, the p53 pathway often experiences mutations and alterations leading to a dysfunction of its regulatory purpose. This leads to proliferation of the cells without senescence or apoptosis resulting in degenerated cells (Frank, 2012). The role of the p53 pathway associated with genotoxicity has given it the name to be the 'guardian of the genome' and emphasizes its importance in cancer and drug research (Brown et al., 2009).

1.3.3. γH2AX-Marker

DNA double strand breaks are important markers for genotoxicity and cancerogenesis and are key-players in pathways for DNA damage response. Some of the key proteins involved in response to DNA double strand breaks are the previously mentioned p53 tumour suppressor protein and the histone H2AX (Frank, 2012). In eukaryotes, the DNA is arranged in chromosomes, which is made up of nucleosomes. The nucleosome itself consists of eight core histone proteins, which can be classified into four histone protein families namely H4, H3, H2B and H2A. The histone families of H4, H3 and H2B differ only in few amino acid residues, with H2A being considerable distinguishable (Rogakou et al., 1998; Baas et al., 2014). The histone family H2A can be further subdivided into H2A1-H2A2, H2AZ and H2AX, which is a very early marker for DNA double strand breaks in mammalian cells (Burma et al., 2001; Nakada et al., 2008; Ando et al., 2014). When a DNA double strand break occurs the serin139 position is phosphorylated in an ATM-kinase dependent matter and the phosphorylated H2AX can now recruit proteins necessary for DNA damage response. Not just DNA double strand breaks, but also reactive oxygen species and DNA topoisomerase inhibitors are known to phosphorylate H2AX to, so called, γH2AX (Ando et al., 2014).

H2AX phosphorylation is preferably monitored via immunohistochemistry to determine the presence of DNA double strand breaks (Frank, 2012). Antibodies can be used specifically against γH2AX detection and its presence in the chromatin can be determined (Ivashkevich et al., 2012). Current measurement methods for γH2AX formation include enzyme-linked immunosorbent assays (ELISA), fluorescence-activated cell sorting (FACS) or via microscopy. At the moment, microscopic assessment of γH2AX formation in cell nuclei is regarded as the most sensitive method to detect DNA double strand breaks and therefore DNA damages. Here the amount of γH2AX foci is counted and their intensity in fluorescence marked antibodies. Measurements can be performed in *in vitro* studies or *in vivo* in a variety of human tissues or cells (Ivashkevich et al., 2012).

1.3.4. Human Liver Carcinoma Cells (HepG2) and Human Colorectal Carcinoma Cells (HCT116)

The human liver carcinoma cells, also called HepG2 are commonly used cells for *in vitro* testing concerning genotoxicity (Boehme et al., 2010; Steinberg, 2013). They were obtained from a 15 year old male's liver carcinoma and are an adherent cell line (ATCC, last visited 01.02.2021, https://www.lgcstandards-atcc.org/products/all/HB-8065.aspx?). HepG2 cells possess 50 to 60 chromosomes, are a highly differentiated cell line and are p53 competent (Ando

et al., 2014). As HepG2 cells are a liver carcinoma cell line, they tend to have some active P450 cytochromes, which are not lost during culturing (Dawson et al., 1985; Hewitt and Hewitt, 2004). Further, HepG2 cells are considered to be more resistant towards cytotoxicity (Schoonen et al., 2005) and have been part of several drug metabolizing studies (Doostdar et al., 1988) due to the unique capability of their endogenous metabolism. Since HepG2 cells possess both activating Phase I enzymes and detoxifying Phase II enzymes, they are suitable candidates for *in vitro* studies regarding direct and indirect acting genotoxic substances (Knasmüller et al., 1998; Westerink et al., 2013).

The human colorectal carcinoma cells (HCT116) were derived from a male's colorectal carcinoma and has a karyotype of about 45 chromosomes making it nearly diploid (ATCC, last visited 01.02.2021, https://www.lgcstandards-atcc.org/products/all/CCL-247.aspx?). HCT116 cells is a stable adherent cell line, with p53 competence (Bakhanashvili et al., 2008) and a popular choice for carcinogenicity studies. Further, HCT116 cells have proven to be metabolically competent by possessing some cytochrome P450 activity (Hasinoff and Begleiter, 2006), however, their metabolic status has not been as rigorously assessed compared to HepG2 cells.

1.4. Genotoxicity Testing *In Vitro*

The ICH S2 R1 (ICH, 2012) defines genotoxicity tests as "*in vitro* and *in vivo* tests designed to detect compounds that induce genetic damage by various mechanisms". Testing of genotoxicity tends to be more exhaustive, as it covers a broad spectrum of mechanisms and several modes of action (see 1.3 Genotoxicity). In comparison to mutagenicity, a series of endpoints has to be addressed to fully evaluate genotoxicity, which is done by using multiple tests as no single test has proven to be sufficient as a standalone test (OECD, 2015a; Pfuhler et al., 2007; EFSA, 2011). Therefore, in general a test battery is recommended to assess genotoxicity. Which *in vitro* test and the combination of which test systems are the most reliable and representative is still under discussion and expert opinions tend to differ. However, regulatory bodies have made recommendations on which tests are to be preferred and which should be used together to assess genotoxicity as comprehensive as possible.

For drug screening in the pharmaceutical industry, compounds are routinely screened for their mutagenic and genotoxic effects. At first, *in vitro* studies are conducted to determine the effect of the compounds and to screen for promising substances. The most interesting substances are then analysed *in vivo* to gain more information on the mode of action and to assess the risk of cancerogenesis (ICH, 2012).

One approach to determine genotoxicity is to use the bacterial reverse mutation assay, known as the Ames test, for mutagenicity assessment, in combination with a mammalian test specific for aneugenicity, such as the micronucleus (MN) (Pfuhler et al., 2007; Kirkland et al., 2011; EFSA, 2011). This aims to cover mutagenicity, aneugenicity and chromosomal aberration in combination through testing in a prokaryotic and eukaryotic system (Pfuhler et al., 2007). The ICH S2 R1 (ICH, 2012) considers a test battery approach to be reasonable, as no single *in vitro* assay can cover all mechanisms of genotoxicity. The use of the Ames test together with a mammalian *in vitro* assay proves to be more sensitive towards detecting rodent carcinogens. However, the application of further mammalian *in vitro* assays also lowers the specificity and potentially leads to an increase in false positive results (EFSA, 2011). Therefore, the ICH S2 R1 (ICH, 2012) recommends to use the Ames test for mutagenicity complemented with a mammalian *in vitro* assay to fully assess genotoxicity. For the assessment of genotoxicity of pharmaceutical impurities, the ICH M7 (ICH, 2017) considers the Ames test to be sufficient as a standalone test system. However, only if it is conducted with all five strains, with and without metabolic activation and leads to clearly positive or negative results, with an appropriate protocol including positive and negative controls.

When assessing chromosomal damages in a test battery: the MLA-*tk*, the MLA-*hprt*, the chromosomal aberration test and the micronucleus are interchangeable. Some guidelines recommend the additional use of *in vivo* tests to assess the suitability of some substances to cause genotoxicity (ICH, 2012). However, this is not feasible for complex mixture such as FCMs, but could be conducted with the corresponding pure chemical of interest (Schilter et al., 2019).

1.4.1. Test Interpretation

The ICH S2 R1 (ICH, 2012) offers guidance on how a positive or negative result of an *in vitro* genotoxicity assay can be interpreted. Ideally, reproduction of an experimental test should lead to the same result. However, if this is not the case then test results are considered to be equivocal. A repetition of an equivocal result might lead to different scenarios. Firstly, if the repetition is then clearly positive the equivocal result is overruled and must be considered as positive. Secondly, if the next result is negative then the previous result is not reproducible and the final conclusion is negative. Thirdly, if the repetition is again equivocal, then the overall result must be considered equivocal.

When the Ames test is used as a standalone test system, as recommended by the ICH M7 for pharmaceutical impurities (ICH, 2017) and equivocal results are obtained, then the test should be repeated with adapted dose-levels of the substances or with a modified testing protocol. The same procedure is recommended for equivocal results when testing with mammalian *in vitro* assays (ICH, 2012).

1.4.2. Toxicological Sensitivity and Specificity

In general, genotoxicity testing systems are evaluated according to their ability to correctly predict carcinogenicity and the reliability of their predictions (EFSA, 2011). For this, the total number and modes of action of the substances analysed is of importance, as it can influence the predictability of an assay. To describe the toxicological predictability of an *in vitro* bioassay, the terms sensitivity and specificity are used as follows:

Toxicological sensitivity is defined as the proportion of substances giving a positive result in a pool of given known true-genotoxic substances in a specific *in vitro* test. Specificity, on the other hand, is the proportion of non-genotoxins giving a negative result from a pool of non-genotoxic substances (Walmsley and Billinton, 2011; Diaz et al., 2007). When an assay is considered to be of low sensitivity, this means that several known genotoxic substances could not

be detected correctly by this assay. Therefore, assays with low specificity tend to classify genotoxins incorrectly as non-genotoxins, leading to false or misleading negative results. A low specificity on the other hand leads to the wrong detection of non-genotoxins as genotoxins, resulting in false or misleading positive results.

To avoid a multitude of false positives in mammalian *in vitro* assays several parameters have to be considered, which were evaluated in detail in several publications by the European Centre for the Validation of Alternative Methods (ECVAM; Kirkland and Fowler, 2010; Pfuhler et al., 2007). An evaluation by Kirkland and Fowler (2010) showed that for determining the genotoxicity of substances with mammalian *in vitro* assays a maximum concentration of 1 mM or 500 µg mL^{-1}, whichever is higher, should be applied. This was concluded, since higher concentrations up to 10 mM tended to cause cytotoxicity or lead to false positive results. Further, for several genotoxic substances an increase of the maximum applicable concentration above 1 mM or 500 µg mL^{-1} did not lead to a positive signal, if they were negative before. Further, any change in pH in the medium through a testing substance, or extreme pH values should also be noted and taken into consideration, when evaluating a result (Kirkland and Müller, 2000). Moreover, when testing is performed with a mammalian cell line then it should be p53 competent, as this leads to the generation of less false positive results.

1.4.3. Analytical Sensitivity

For the analysis of complex mixtures, such as FCM migrates, the assessment of the presence of potentially harmful substances at low concentrations is of great importance. Therefore, the detection limits of certain substances are of interest and have to be taken into account when assessing the toxicological potential of a mixture and their compounds. When assessing these limits the term ‘analytical sensitivity’ is used, which has to be distinguished from the term ‘toxicological sensitivity’ (Pinter et al., 2020). An assay with a high analytical sensitivity is able to detect substances at very low concentrations. A high toxicological sensitivity, on the other hand, means an assay is able to correctly detect a high percentage of substances from a given pool of genotoxic substances as correctly positive. High toxicological sensitivity is regardless of the concentration the substances can be detected, whereas for analytical sensitivity the limit where the substances can be detected is of uttermost importance and should be as low as possible for a high analytical sensitivity. To determine the ability of an *in vitro* assay to detect substances at certain concentrations, the so-called limit of biological detection (LOBD) is used (Schilter et al., 2019). The LOBD is defined as “the lowest concentration, where a substance can be reliably detected with a significant distinction from the background” (Pinter et al., 2020).

As the data in the literature is given in regard to the assay and the protocol conducted, the results have to be normalised. In context to FCMs, this means that the global concentration factor (GCF) has to be taken into account (Pinter et al., 2020; Schilter et al., 2019; Koster et al., 2015). The GCF for FCM is derived by considering the sample preparation protocol and the consecutive assay application. This makes it possible to determine the concentration a substance would need to have in an FCM migrate so that it can be detected in the respective *in vitro* genotoxicity assay and therefore providing important information on the analytical sensitivity of an assay in this context. As described in 1.2.6 Sample Preparation, first migration takes place out of the FCM by use of a suitable solvent and suitable migration conditions. Then the FCM migrate is concentrated and transferred into a solvent, which is suitable for a bioassay, if the initial migration solvent is not. The concentration step can be done by evaporation, solid phase extraction, liquid-liquid extraction or lyophilisation and is then possibly followed by a solvent swap to be compatible with the bioassay. Commonly used solvents for the bioassays can be aqueous or organic, such as dimethyl sulfoxide (DMSO), ethanol, ethyl acetate or water (Di and Kerns, 2006). Subsequently the sample is then applied in the assay and diluted by a factor of 25 for prokaryotic assays (= 4 % final DMSO concentration in the assay medium) and 100 for mammalian assays (= 1 % final DMSO concentration).

Further, the following assumptions were made by Pinter et al. (2020), Schilter et al. (2019) and Rainer et al. (2018), when calculating the GCF regarding the FCM migration protocol: a theoretical concentration factor of 1,000, no substances are lost during the process and the sample is swapped to 100 % DMSO. Moreover, it is assumed that no artefacts are present in the FCM migrate that could negatively affect the cell's viability and consequently the LOBD and analytical sensitivity. This leads to a GCF of 10 for mammalian assays and a GCF of 40 for bacterial based tests, which can be used to calculate the surrogate LOBDs (sLOBD) (Pinter et al., 2020; Schilter et al., 2019; Rainer et al., 2018). For this, the LOBD from the literature is divided by the respective GCF, resulting in the sLOBD for the substance regarding the assumptions made above for FCMs.

In literature surveys by Pinter et al. (2020) and Rainer et al. (2018), the sLOBDs were determined for a series of substances and for several assays, including the Ames test, the MLA-*tk*, the MLA-*hprt*, the MN, the comet assay, the p53 CALUX® and the BlueScreen™ HC. For this, a substance set was used, which was recommended by the ECVAM (Kirkland et al., 2016) for the validation of *in vitro* genotoxicity assays. These substances are not likely to be found in FCM migrates, but instead should give an estimation of the assay's analytical sensitivity to generally detect genotoxic substances. The sLOBDs for DNA reactive substances were then compared to a threshold of 0.01 mg L^{-1}, which corresponds to the technical limit proposed by

the EU 10/2011 (European Parliament, 2011) for analytical methods. For indirect acting genotoxic substances, the sLOBDs were set against the TTC threshold for the Cramer Class III, corresponding to 0.09 mg L^{-1}. In conclusion, the studies found the assays to be insufficient to reliably detect the analysed substance set at concentrations below the proposed thresholds (Pinter et al., 2020; Rainer et al., 2018). However, they concluded that by improving the assays LOBDs the thresholds could be within reach for several more substances. Especially the Ames test was considered to be within close reach and Rainer et al. (2018) proposed an improvement of the LOBDs by a factor of 10 to be promising.

1.4.4. Metabolic Activation

Some genotoxic substances are already reactive in their present state and form, while others have to be metabolised to unleash their genotoxic potential. Initially when genotoxins enter the body, they will be deactivated so that they can be cleared. However, some substances are not detoxified but instead are bioactivated and reactive products are formed, which can possess a genotoxic potential (Guengerich, 2006; OECD, 2014a). For drug metabolising, the most important enzymes are comprehended in the family of cytochromes P450 (CYP450). Depending on the substance and mechanisms they are following, different CYP450 are active. The most important CYPs regarding the response to xenobiotics (=chemicals not normally found in the body) are: CYP1A1, CYP1A2, CYP2B6, CYP2C9, CYP2C19, CYP2D6, CYP2E1, CYP3A4, CYP3A5 and CYP3A7. These CYPs are mainly localised in the liver, but are also found in other organs, such as the colon (Guengerich, 2006). The detoxification system of the body consist of phase I and phase II enzymes. Phase I mainly consists of CYP450s and other oxidants. Other enzymes, causing hydrolysis are generally classified as phase II enzymes and will transform the metabolites from phase I to water soluble products, which can be segregated (Jacobs et al., 2013). Especially phase II enzymes are considered to be detoxifying and can lead to the deactivation of some potentially genotoxic substances. Further, the stability of the reactive product formed in phase I can vary greatly depending on the initial substance (Guengerich, 2006).

The European Food Safety Authority (EFSA) recommends performing all *in vitro* tests for genotoxicity testing with and without an appropriate metabolic activation system (EFSA, 2011). For this, EFSA suggests the use of a liver homogenate, typically rodent-based, which consists of several enzymes and cofactors, which are part of the phase I and II liver enzymes. Commonly used rodent-based S9 liver homogenates are aroclor 1254 induced or a combination of phenobarbital and β-naphthoflavone induced S9. Standard rodent-based S9 has a higher activation

capability than human S9 and lacks phase II specific detoxification enzymes (ICH, 2012). Therefore, a correlation with *in vivo* results might not occur as a substance might be detoxified *in vivo*. Excretion or production in low quantities in the human system might also be a reason for disagreeing *in vitro* and *in vivo* results (EFSA, 2011). Despite their various limitations, rodent-based S9 is still the method of choice for metabolic activation in genotoxicity testing, as it is easier to obtain than most other S9 sources.

Alternatively, to exogenous metabolisation systems through S9 mix engineered cell lines, which are able to express specific enzymes for metabolisation can be used. A more practical approach suggested by EFSA (2011) is using metabolically competent, stable cell lines, such as HepG2 or HepaRG cells. These cells are known to possess a series of cytochromes P450, which play a crucial part in metabolisation. However, it is not entirely undisputed how well these cell lines represent the human situation, concerning phase I and II enzymes and the presence of cofactors (EFSA, 2011; Westerink and Schoonen, 2007a). Anyhow, the use of cells with an internal metabolisation system is especially of interest, as the application of an S9 mix is generally regarded as toxic towards mammalian cells, so that only small amounts of S9 can be used for a limited period of time (Valentin-Severin et al., 2003). Further, the activity of S9 batches can differ and therefore the activity of each batch has to be tested individually, before it can be used for routine genotoxicity testing (Bigger et al., 1980). This instability is not the case for stable cell lines with an internal metabolism, however, the passage number should be kept low, as higher passages might lead to changes in the cells characteristics.

Overall, the appropriate use of an exogenous or endogenous metabolisation system is of great importance for the *in vitro* assessment of genotoxicity, as inadequate application can lead to the rise of false positive or false negative results due to lack of detoxification or bioactivation (OECD, 2014a).

1.4.5. Cytotoxicity

Cytotoxicity deals with cell viability and several causes of cell death, such as destruction of cell membrane, prevention of protein synthesis or irreversible binding to receptors. Assays for the assessment of cytotoxicity can be categorised based on their detection principles: dye exclusion, colorimetric, fluorometric and luminometric, and should be fast and cheap screening tools. Some commonly used assays are the trypan blue test, the MTT assay, the alamarBlue assay or the ATP assay (Aslantürk, 2018). Cytotoxicity is often assessed simultaneously to genotoxicity or mutagenicity. Some genotoxic substances tend to give a positive signal, while already being cytotoxic (ICH, 2012). However, when cytotoxicity increases this can lead to a false negative or false positive signal in a mammalian *in vitro* assay for genotoxicity, which can be misleading (Kirkland et al., 2007). Therefore, a threshold for cytotoxicity should be introduced, depending on the assay as not every substance that is cytotoxic is also acting genotoxic (Kirkland and Müller, 2000). A study by Henderson et al. (1998) with TK6 cells in the comet assay showed that cytotoxicity led to the generation of false positive results and should therefore be assessed simultaneously to genotoxicity. Caution has to be taken when choosing the appropriate cytotoxicity assay for a genotoxicity testing system, since different assay principles and cells can lead to different results for cytotoxicity (Niles et al., 2008).

1.4.6. Commonly Used *In Vitro* Genotoxicity Assays

In the following chapter, some important and commonly used *in vitro* assays for genotoxicity are listed and described in detail. The tests were chosen according to their importance for genotoxicity testing, as they are recommended and discussed in detail by multiple guidelines and recommendations (EFSA, 2011; Kirkland et al., 2011; OECD, 2015a). Further, some of these assays are looked into more closely because of their actuality, novelty and importance for this thesis. Additionally, some use-cases for genotoxicity testing of FCMs are given to each assay. A summary of the applications of the assays for FCM testing was given in the publications by Pinter et al. (2020) and Rainer et al. (2018), which were prepared as part of this thesis. Table A 1 and Table A 2 in the appendix summarize the results for genotoxicity testing for FCM with *in vitro* bioassays for mammalian assays (Table A 1) and for the Ames test (Table A 2).

MLA-*tk* and MLA-*hprt*

The mouse lymphoma assay (MLA) is a gene mutation assay, which is mammalian based and can detect gene mutations, such as base-pair substitution and frame-shift. Commonly used cell lines are L5178Y, CHO, V79 or TK6 (EFSA, 2011). The assays are similar in principle but

focus around either the thymidine kinase (*tk*) or hypoxanthine-guanine phosphoribosyl transferase (*hprt*). Nevertheless, both assays are able to reliably detect direct DNA-reactive mutagens and can be used for the assessment of mutagenicity. The OECD guideline TG 476 (OECD, 2014e) gives a protocol on how the MLA-*hprt* assay should be conducted and the same exists for the MLA-*tk* with the guideline TG490 (OECD, 2015b). Both MLA tests are time consuming, laborious and prone to contaminations with an overall assay duration of several days to weeks.

The MLA is a forward mutation assay on the respective gene for *tk* or *hprt*, where a mutation will destroy the functionality of the gene. To determine the mutation and loss of the function, a toxic analogue of *tk* or *hprt* is added when the cells are grown after treatment with a genotoxic substance. Cells that possess a mutation are then able to grow in the media in the present of the toxic analogue and can be seen as viable colonies. With this, a broad range of mutagenic compounds can be detected, as this assay reacts to any kind of mutation (Johnson, 2012).

Micronucleus (MN)

With this assay, structural and numerical chromosome changes can be measured and therefore the effect of aneugenic or clastogenic substances is determined. A great variety of cells can be used for the MN, such as rodent cell lines, human cell lines or primary cell cultures. The stability of the karyotype is of great importance when choosing a cell line, as instable chromosomes can lead to higher background rates for micronuclei (OECD, 2014b; Parry et al., 1996). The MN is explained in detail in a guidance document by the OECD TG 487 (OECD, 2014b). The assay can also be conducted as part of *in vivo* studies, where micronuclei are detected, which is summarized in the OECD guideline TG 474 (OECD, 2014d). The assay focuses on the counting of micronuclei, which are induced due to the presence of a genotoxic substance affecting the chromosomal structure or number within the cells. Micronuclei can be formed through acentric chromosome fragments, or whole chromosomes that stay together during cell division (Avlasevich et al., 2011). For practical performance, the cells are treated with a genotoxic compound and after a certain incubation time, damages can occur. Then cells are harvested and stained and micronuclei are counted of cells, which have undergone cell division (OECD, 2014b).

As the counting of micronuclei is time consuming, high throughput methods have been developed with improved speed and robustness. Some of these novel approaches focus on flow cytometry sorting via fluorescence marking of micronuclei or microscopic analysis with automated imaging of fluorescence stained cells (Avlasevich et al., 2011; Bryce et al., 2016; Collins et al., 2008; Shibai-Ogata et al., 2011; Tilmant et al., 2013). With these, a fast screening method

is available, able to provide information on the presence of aneugenic and clastogenic substances and their effect.

The MN has been used in several studies for the genotoxicity assessment of complex mixtures and especially for FCM testing. Studies by Bach et al. (2014, 2013) conducted with PET and glass were performed with the MN. These experiments used water as a migration solvent to determine the different effects of temperature and exposure to sunlight affecting the possible migration of genotoxic substances. The MN tests were conducted with HepG2 cells and did not lead to any cytotoxic or genotoxic effects upon sample testing.

Comet Assay

With the comet assay, DNA single and double strand breaks are made visible and damages in an individual cell can be shown. The comet assay can be applied *in vitro* and *in vivo* with any cell line or tissue, as it does not require cell growth, but only viable cells (Ross et al., 1995). Currently, there is only an OECD guideline for the *in vivo* application of the comet assay, which is TG 489 (OECD, 2014c). For the *in vitro* method, cells are treated with the genotoxic substance and after exposure, the DNA is lysed and treated with an alkaline or neutral buffer, so that it can unwind. Then the DNA is separated by gel electrophoresis and the results are scored via image analysis. DNA strand breaks form a tail, as the DNA fragments travel away from the so called head. The tail length is measured and compared to the negative control and the total length reflects the amount of DNA breakage (Ashby et al., 1995; Fairbairn et al., 1995).

Generally, the alkaline comet assay detects DNA single and double strand breaks, but under modified experimental conditions also DNA-DNA or DNA-protein crosslinks can be detected, but for this no standard protocols exists (OECD, 2014c). The effect of a substance on single cells are measured with the comet assay and this can provide information, whether a substance affects the cells heterogeneously or individually, which is helpful in the prediction of tumour response (Fairbairn et al., 1995).

In a literature survey as part of this thesis and published by Pinter et al. (2020), the comet assay was successfully used for several genotoxicity tests for FCMs. With the comet assay a variety of materials such as paper and board, recycled paper, PET and polypropylene were analysed with different solvents and migration scenarios. Both Bradley et al. (2008) and Riquet et al. (2016) used HepG2 cells for testing and found some cytotoxicity, but no genotoxic results. A study by Ozaki et al. (2004) analysed recycled paper and resulted in some positive results when using HL-60 cells, but did not evaluate cytotoxicity. Biscardi et al. (2003) and Ceretti et al. (2010) used human leukocytes for testing of PET bottles with mineral water. The study by

Biscardi et al. (2003) found positive results, however, these were concluded to be caused by a contaminated pipe and not the PET material itself. Ceretti et al. (2010) also found some positive results, but those were considered not to be statistically significant.

Ames Test

The bacterial reverse mutation test, also called Ames test, is a prokaryotic mutagenicity test, focusing on *Salmonella enterica ssp. enterica typhimurium* (short form *salmonella typhimurium*) and *Escherichia coli*. For pharmaceutical testing and as part of a genotoxicity test battery, it has been the method of choice for many years. A guideline for this assay by the OECD can be found under TG 471 (OECD, 1997), which describes the procedure in detail. The basic principle, which is visualized in Figure 8, follows the bacteria's inability to produce histidine, which is an essential amino acid for their growth. When a mutagen is present, this can lead to changes in the bacteria and they are no longer histidine auxotroph, but are now able to produce it themselves. To determine the effect on the bacteria, they are grown in a medium lacking histidine, so that only bacteria with a mutation are able to grow. After incubation, the formed colonies are counted and compared to the background to determine, whether a substance has a mutagenic effect or mainly spontaneous background mutations occur (Proudlock, 2016; OECD, 1997).

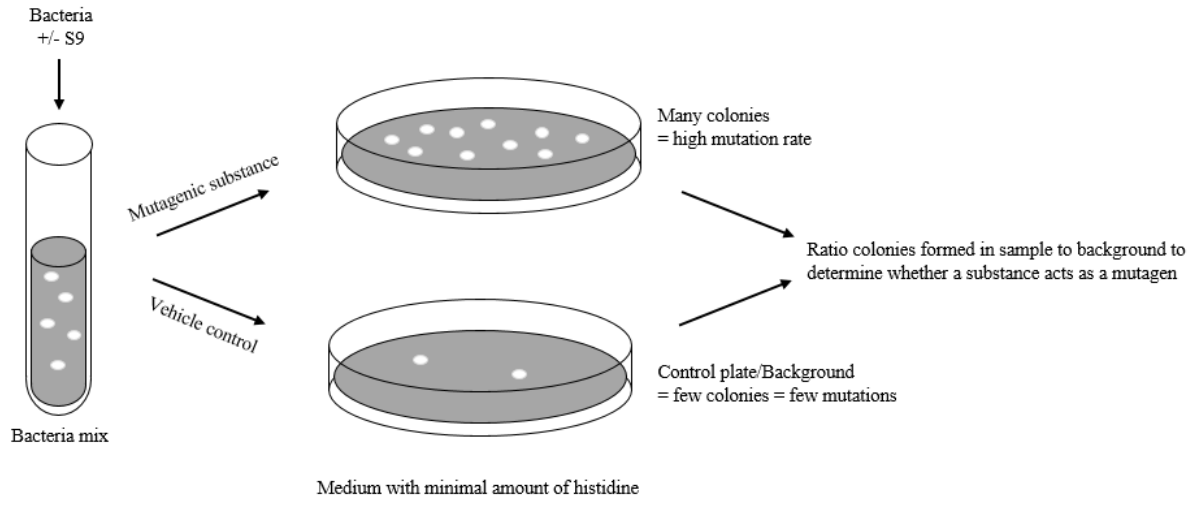


Figure 8: Principle of the bacterial reverse mutation test (=Ames test). Bacteria are treated with a mutagenic substance, with or without the presence of an exogenous metabolism. Then the bacteria are plated and the formed colonies are counted. The colony number of the sample is compared to the vehicle control, to determine if more bacteria were able to grow in the histidine lacking medium, when a mutagenic substance was added, compared to the background control.

The Ames test, which was developed by Bruce Ames (1973), can detect mutagenic effects such as point mutations, frameshift or cross-linking, depending on the bacteria strain used. The

OECD (1997) recommends the use of five strains for a full mutagenicity assessment with and without the addition of an exogenous metabolisation system. These strains are four salmonella strains: TA1535, TA1537 or TA97 or TA97a, TA98 and TA100, and one of the following strains: *E.coli* WP2 uvrA, *E.coli* WP2 uvrA (pKM101) or TA102. The standard Ames test can either be performed as plate incorporation or pre-incubation assay. For the plate incorporation test, the bacteria and the tested substance are plated together and incubated. In the pre-incubation test, on the other hand, the bacteria are incubated with the substance for a certain amount of time in a liquid medium and are then poured onto an agar plate. For any kind of testing, a background control has to be included for every strain, as the spontaneous mutation of each strain can differ greatly, but has to be within the historical control range (OECD, 1997). Moreover, for interpretation of test results, the formation of colonies due to contamination with amino acids or other bacteria has to be excluded. Further, the presence of amino acids or their pre-cursors can make the assay unsuitable for assessing this compound (ICH, 2012).

Several high-throughput and automatized versions of the Ames test exist on the market and an OECD guideline for miniaturised versions is in preparation. Smaller versions of the standard Ames test have been performed in 6-well, 12-well or 24-well formats (Pant et al., 2016; Proudlock and Evans, 2016; Zwarg et al., 2018), or even as liquid version in 384-well plates (Flückiger-Isler and Kamber, 2012). For automation, an imaging software can be used for colony counting (Stewart Houk et al., 1989) or fluorescence and luminescence measurement (Aubrecht et al., 2007; Côté et al., 1995).

A drawback of the Ames test is that it is based on a prokaryotic organism, which differs from mammalian cells in terms of metabolism, uptake of substances, chromosome structure and DNA repair process (EFSA, 2011). However, it is considered to be more reliable in correctly predicting mutagenicity than most other *in vitro* assay (Kirkland et al., 2005). Further, the Ames test is able to detect only mutagens, which are only an aspect of genotoxicity. Therefore, only direct DNA reactive substances can be detected and no indirect effects on the DNA can be measured (EFSA, 2011). However, this is sufficient, when direct DNA reactive genotoxins or mutagens are of interest in a study (ICH, 2017).

A great amount of studies have been performed with the Ames test to determine the mutagenic potential of complex mixtures and for FCM materials. A summary is shown in Table A 2 in the appendix and the literature survey was performed as part of this thesis and published by Rainer et al. 2018). Of the 14 studies analysed only four lead to a positive result. For these, different protocols for sample preparation and for the Ames test were used ranging from the pre-incubation for recycled paper (Binderup et al., 2002), plate incorporation for paper and board (Bradley

et al., 2008), the standard Ames for PET (Fusco et al., 1990) and the fluctuation Ames test for PET (Ubomba-Jaswa et al., 2010). Further, a recent study by Rainer et al. (2019) showed positive results for three out of 20 tested FCM samples of different material categories. Two composite materials and one paper showed mutagenic results, when harsh migration and concentration protocols were followed.

1.4.7. Reporter Gene Assays

Novel genotoxicity assays are often based on the principle of reporter gene assays, as they focus around a certain gene or protein known to be associated with genotoxicity or the DNA damage response. The use of luminescence based reporter gene systems is especially of interest, as already very small quantity changes of the reporter genes or proteins can be measured. Further, they are simple and enable a fast read-out (Martin et al., 1996). In general, in the cells the gene of interest is linked to an easily detectable gene or protein, such as the green fluorescence protein (GFP) or a luciferase (luc), which can then be measured and indirectly the expression of the gene of interest can be determined.

GreenScreen™ HC and BlueScreen™ HC

The GreenScreen™ HC and its successor the BlueScreen™ HC, are genotoxicity tests focused around the gene GADD45α. The assays have been recommended by Koster et al. (2015) for the genotoxicity testing of FCM migrates and are routinely used for the genotoxicity assessment of pharmaceuticals (Hastwell et al., 2009), flavour materials (Etter et al., 2015) and ecotoxicological studies (Zounkova et al., 2007). The GADD45α gene is associated with the p53 pathway as part of the DNA damage response (see 1.3.2 P53 Pathway). Therefore, the assays are able to detect both direct and indirect acting genotoxins, which are associated with the p53 pathway. The GADD45α gene is associated with cell cycle control, DNA repair and apoptosis and is linked to reflect mutagenicity, clastogenicity and aneugenicity (Birrell et al., 2010). The assays are based on the cell line TK6 and use two different detection principles: the GreenScreen™ HC uses GFP (Hastwell et al., 2006) and the BlueScreen™ HC (Hughes et al., 2012) is based on luciferase measurement. In direct comparison the BlueScreen™ HC has proven preferable toxicological predictivity compared to its predecessor. Further, the BlueScreen™ HC can detect substances with auto-fluorescent properties, as a luminescence based detection system is used. However, measuring luminescence is a dead-end measurement, compared to GFP measurement, which can be done repeatedly at different time points (Hughes et al., 2012).

The BlueScreen™ HC is thought to be suitable for the genotoxicity assessment of FCM migrates due to its compatibility with complex mixtures (Koster et al., 2014). Further, both assays are high-throughput systems, where results can be obtained easily and reliably. Moreover, the assays are recognized to be highly toxicologically sensitive and specific, therefore providing important information on the genotoxic potential of a substance (see 1.4.2). For FCM testing, the BlueScreen™ HC was used in a study by Koster et al. (2014) to analyse paper samples. The results were negative and no cytotoxicity could be found, but the assay proved to be suitable for FCM genotoxicity testing.

P53 CALUX®

The p53 Chemically Activated Luciferase Gene Expression (CALUX®) assay was developed to detect all kinds of genotoxic modes of actions. It is based on the osteoblastic osteosarcoma cell line U2OS and revolves around the p53 pathway. This reporter gene assays uses luciferase, which is incorporated into the cells as a reporter construct with the responsive elements (van der Linden et al., 2014). The U2OS cell line is p53 competent, which is essential for genotoxicity testing. The p53 pathway and its importance for DNA damage response and genotoxicity testing has been described in a previous chapter (see 1.3.2 P53 Pathway). This makes the assay able to detect several modes of action related to cell cycle arrest, DNA damage, senescence and apoptosis. As the p53 pathway is activated by both direct and indirect genotoxic substances, the assay can detect mutagenicity, clastogenicity and aneugenicity.

The assay has already been part of a previous study concerning its suitability to detect genotoxic substances in FCM migrates and its ability to do so at low concentrations (Pinter, 2017). There it was found to be of interest, but improvements and modifications would have to be made to use it as a routine screening test. Other CALUX® tests are widely used to assess the presence of dioxins or endocrine active substances in environmental samples (Pieterse et al., 2015; Windal et al., 2005). The p53 CALUX® and several other CALUX® assays for endocrine activity were used in a study by Rosenmai et al. (2017), where paper and board samples were analysed. In this study, out of the 20 samples, six were considered to be positive and the genotoxic substance di-isobutyl phthalate could be identified via GC-QTOF.

1.4.8. High Content Screening (HCS)

High content screening (HCS) is used as an early-stage method in drug-discovery to define the role of genes, proteins and other molecules in normal and abnormal cell processes (Haney et al., 2006; Giuliano et al., 2003; Ye et al., 2007) and for *in vitro* toxicology studies (Li and Xia, 2019). This method enables to rapidly screen preliminary substances and to determine, promising candidates for further drug development. This makes it a high-throughput method, which is simultaneously able to provide important information through cellular imaging (Zanella et al., 2010). For HCS, automated microscopy and image analysis are combined to give information on cell statuses, with possibly improved sensitivity and increased throughput compared to previous methods (Zock, 2009). Especially for drug discovery, the use of HCS to understand and evaluate the effect of certain substances in cell signalling and pathways is of interest (Zock, 2009). Through fluorescence microscopy with auto focusing and sample positioning, HCS has improved to a fast screening method for specific targets (Zanella et al., 2010). Further, important additional information is obtained on the cell's integrity and its cellular state through imaging. Numerous cell stains can be combined to detect several targets at once and to address further points of interest. An advantage of HCS in comparison to reporter assays, is that it obtains information through a collective of individual cells. However, the concordance of an average cell can vary from a whole cell population (Zanella et al., 2010). Another drawback is the great amount of data produced and the still long imaging and measurement time for older devices and tedious sample pre-treatment procedures (Ye et al., 2007).

HepG2 cells have been used for HCS, as they are preferred models to assess hepatotoxicity and metabolic effects (Cole et al., 2014). A typical HCS follows the following workflow (Li and Xia, 2019): first, the treated cells are fixated in the microtiter plates. The quality of an HCS greatly depends on the cells and their morphology. For example, clumping of cells can interfere with the imaging process as the piles cannot be distinguished to individual cells and this will lead to effects on the cell response (Giuliano et al., 2003). In the next step, the cells are stained with appropriate dyes and antibodies, which are specific for the targeted point of interest. Then imaging is conducted with a fluorescence microscope and the results can be assessed with image analysis.

Another important aspect assessed with HCS is genotoxicity and the different modes of action of genotoxic substances. Because multiple endpoints and effects can be measured simultaneously or separately with HCS, the specific mode of action can be determined by measuring, for example γH2AX content or micronuclei formation (Li and Xia, 2019).

2 Materials

Substance	Provider	Identification
Actinomycin D	Sigma Aldrich	A9415
AFB1	Sigma Aldrich	A6636-1MG
Agar Agar Kobe I	Carl Roth	5210.2
Ames Strains TA98, TA100	Xenometrix	
2AA	Carl Roth	AA38800-1
2AF	Sigma Aldrich	A7015-5G
Amitrole	Sigma Aldrich	A8056-10G
AMP	Santa Cruz Biotechnology	sc-254945
Anti-Hu/Mo PH2AX CR55T33 PUR 100 UG	Thermo Fisher	12-9865-42
BaA	Sigma Aldrich	B2209-500MG
BαP > 96 %	Sigma Aldrich	YB1760.250
Biotin	Carl Roth	3822.1
BSA	Sigma Aldrich	A9418
Caps for reaction flasks, 9 mm	VWR	548-1534
Centrifuge	Eppendorf	Centrifuge 5702
	Eppendorf	5417R Refrigerated Centrifuge
	Eppendorf	MiniSpin®
Cisplatin	Santa Cruz Biotechnology	sc-200896
Citric Acid Monohydrate	Carl Roth	5110.3
2-Chloroethyltrimethyl-ammonium chloride	Sigma Aldrich	234435-25G
CO_2-Incubator	Sanyo	MC-18 AIC UV
Coelenterazine (Coel)	Synchem UG & Co, KG	CBP-012-6-A
Colchicine	Sigma Aldrich	C9754-1G
Cy3 AffiniPure Goat Anti-Mo IgG (H+L)	JacksonImmuno	115-295-146

Cryovials Nunc™ (1.8 mL)	Thermo Fisher	10674511
Cuvettes 340 – 900 nm	VWR	634-8112
Cyclophosphamide	Sigma Aldrich	PHR1404-1G
2,4-DAT	Santa Cruz Biotechnology	sc-256319
Diethanolamine	Sigma Aldrich	D8885-25G
7,12-DMBA	Sigma Aldrich	D3254-100MG
DMN solution	Sigma Aldrich	CRM40059
DMSO >99.8 %	Carl Roth	4720.1
Doxorubicin	Santa Cruz Biotechnology	sc-280681
DTT	Sigma Aldrich	10708984001
DMEM Medium	Pan Biotech	P04-03590
cDNA Synthesis Kit	Thermo Fisher	First Strand cDNA Synthesis Kit
PBS – Gibco®	Thermo Fisher	14200-067
EDTA-Trypsin	Pan Biotech	P10-029500
ENU	Sigma Aldrich	N8509-5G
2-Ethyl-1,3-Hexanediol	Santa Cruz Biotechnology	sc-238028
Etoposide	Santa Cruz Biotechnology	sc-3512
Eugenol	Santa Cruz Biotechnology	sc-203043
FBS	Pan Biotech	P40-37500
Fluorescence microscope	Carl Zeiss AG	Cell Observer
Glass flasks 250 mL, 100 mL	Büchi	SJ29/32
Glass reaction flasks 2 mL	VWR	548-1211
D-Glucose monohydrate	Carl Roth	6887.2
Glucose-6-phosphate Monosodium Salt	Carl Roth	5544.1
Glucose-6-phosphate Dehydrogenase	Sigma Aldrich	10127655001
HCT116 cells	Sigma Aldrich	ECACC
HepG2 cells	Sigma Aldrich	ECACC

Hexachloroethane	Sigma Aldrich	185442-5G
Histidine	Carl Roth	7880.1
Hoechst 33342	Sigma Aldrich	H3570
HQ	Sigma Aldrich	H9003-100G
Incubator	Binder	BD115 Avantgarde Line
	Thermo Fisher	MaxQ™ 6000 Incubated Shaker
KCl	Carl Roth	HN02.2
KH_2PO_4	Carl Roth	P018.1
K_2HPO_4	Carl Roth	P018.1
Laminaflow Safety Class II	Heraeus	MSC Advantage
Licrosolve Ethanol > 99.9 %	VWR	1117272500
Luminometer	Thermo Fisher	Luminoskan™ Microplate
$MgCl_2$	Carl Roth	KK36.2
D-Mannitol	Santa Cruz Biotechnology	sc-203020
Melamine	Sigma Aldrich	M2659-5G
D,L-Menthol	Santa Cruz Biotechnology	sc-229843
Methyl Carbamate	Sigma Aldrich	246352-100G
MMS	Sigma Aldrich	129925-5G
$MgCl_2.6H_2O$	Carl Roth	KK36.2
$MgSO_4.H_2O$	Carl Roth	P027.2
MMC	Santa Cruz Biotechnology	sc-3514
NaCl	Carl Roth	0601.1
NADP	Carl Roth	AE13.3
$NaNH_4HPO_4.4H_2O$	Carl Roth	T882.1
2NF	Carl Roth	AN16754.5
4NQO	Sigma Aldrich	N8141-250MG
Nutrient Broth No.2 Oxoid	VWR	OXOICM0067B
Optical Sealing Film	Biozym	BZO Seal Film Adhesive Optical Film 712350

Paraformaldehyde	Carl Roth	0335.1
Petri dishes (Ø 10 cm)	VWR	391-0469
PEI	Sigma Aldrich	78429
Penicillium/Streptomycin 10,000 µg mL^{-1}	Thermo Fisher	SV30010
Petri dish for cell culture	Greiner	66416
Phenformin HCl	Santa Cruz Biotechnology	sc-219590
pPD	Sigma Aldrich	P6001-50G
Plate Reader	Tecan	Infinite®200 Pro
Pyridine	Sigma Aldrich	02486-1ML
Pyromycin	PAA	P11-0
qPCR device and software	Agilent Technologies	Mx3000P and AriaMx Real-Time PCR System
Reagent Reservoir	VWR	613-1176
Resazurin Sodium Salt	Santa Cruz Biotechnology	SC-206037A
mRNA Extraction Kit	Thermo Fisher	Thermo Scientific GeneJet RNA purification kit
Rotary evaporator R300	Büchi	Rotavapor® R-300
RPMI-1640 Medium	HyClone	SH30027.1
S9 Rat Liver 1254 Aroclor	Moltox	M11-101.2
Schott flasks	VWR	215-1594
SA	Sigma Aldrich	S7400-100G
Sodium Saccharin	Santa Cruz Biotechnology	SC-296367
Spectrophotometer	DeNovix Perkin Elmer	DS-11 spectrophotometer PDA UV/VIS Lambda 265
SPE Visiprep 1/8'' PTFE	Supelco	57276
Sulfisoxazole	Sigma Aldrich	31739-250MG
Sulfuric Acid (H_2SO_4) 96 %	Carl Roth	X944.1
SYBR Green 10,000 x DMSO	Sigma Aldrich	S9430-1ML

Taq Polymerase	LabConsulting	LabQ Taq DNA Polymerase LQ-92VPT500U
tBHQ	Sigma Aldrich	112941-5G
Thermocycler	Eppendorf	Thermomixer compact
Tris(2-ethylhexyl)phosphate	Sigma Aldrich	289922-25ML
Triton X-100	Carl Roth	3051.2
TGIC	Santa Cruz Biotechnology	sc-487428
Tubes for Centrifugation	VWR	10025-686
	VWR	525-0610
Urea	Sigma Aldrich	U5378-100G
6-Well Plates	VWR	734-2777
96-Well Plates	Greiner Bio One	651180
	Biozyme	712220
75 cm³ Flasks with Filter Cap – Corning®	Sigma Aldrich	CLS430641

3 Methods

3.1.Sample Preparation

Samples were prepared by the project partner OFI (Österreichisches Forschungsinstitut für Chemie und Technik, Wien) and then send to the University of Applied Sciences FH Campus Wien. There the samples were cooled upon arrival for further testing and immediately stored at -20 °C.

In general, all samples were first migrated at the OFI with 96 % ethanol for 10 days at 60 °C, followed by concentration through a rotary evaporation at 40 °C at approximately 50 mbar. After this, a solvent swap was performed from ethanol to DMSO (Dimethyl sulfoxide) at room temperature (RT) under a gentle vacuum stream. By concentrating 300 mL sample migrate to 1 mL DMSO sample a concentration factor of about 300 was achieved. This sample was then used for the bioassay application (see 3.2.5, 3.4.3 and 3.5.4).

3.1.1. Migration

Sample preparation at the OFI was performed by using 96 % ethanol as a migration simulant. For granulate, double-sided foil or coating migration 30 g of each sample was placed in a Schott flask and filled with 300 mL 96 % ethanol. Other samples such as bottles were filled with the solvent until the typical filling volume was reached. For one-sided migration, so called migration chambers were used, where 10 dm² of the sample were put into the chamber with 100 mL 96 % ethanol to ensure only one side of the sample was migrated. This was of particular interest for printed surfaces, where only the non-printed side should be migrated. Afterwards the filled samples were placed inside an incubator at 60 °C for 10 days. With each preparation a blank consisting of 96 % ethanol without any sample was included, which was treated the same as the samples.

After migration, the samples were concentrated immediately or the sample was removed and the migrate was stored at 4 °C for a maximum of three days.

3.1.2. Sample Concentration and Solvent Exchange

For concentration of the migrate samples, a rotary evaporator was used at 40 °C to ensure gentle evaporation with a limited loss of volatile substances. For evaporation, a maximum of 50 mbar was applied and the sample was not evaporated to dryness, but until about 1 mL of the ethanol sample remained. To exchange the solvent 1 mL of DMSO was added and the DMSO/ethanol

sample was transferred into a vial for further evaporation. To remove the remaining ethanol the sample was treated under a gentle stream of air or nitrogen at RT. The concentrated samples and blanks were transported from the OFI to the University of Applied Sciences FH Campus Wien and stored at -20 °C upon arrival and were later applied in the respective assays.

3.2.Reporter Gene Assay

3.2.1. Cell Line Handling and Treatment

For the reporter gene assay, HepG2 and HCT116 cells were obtained from ECACC (European Collection of Authenticated Cell Cultures) through Sigma Aldrich (MO, US). These were transfected with a p53 reporter construct as shown in Piwonka (2018). For the construct, a pGVL8 backbone was used as described in Mertl et al. (2019) consisting of six times the p53 binding site with the sequence GAACATGTCTAAGCATGCTG. Successful treatment of the cells was verified using puromycin (1 µg mL^{-1}) for selection purposes.

Cell Culture Cultivation: HepG2 Cells

HepG2 cells were cultivated in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10 % Fetal Bovine Serum (FBS) and 1 % Pen/Strep 10.000 U mL^{-1} (=complete DMEM) at 37 °C, 5 % CO_2 in a humid atmosphere, in 75 cm^3 flasks. The cells were split every 2-3 days or until confluence reached 80 %. For this, the media was removed and the cells were washed with 1x Dulbecco's Phosphate Buffered Saline (DPBS) and treated with EDTA-Trypsin for 15 min. Trypsination and detachment of the cells was controlled through observation of movement in a microscope. The trypsination was then stopped by adding DMEM and the cells were homogenised via pipetting and transferred into a centrifugation vial. Then the cells were centrifuged at 900 rpm for 5 min and the supernatant was discarded. Fresh medium was added to homogenate the cells, from which an aliquot was transferred to 75 cm^3 flasks or into 10 dm^2 cell culture dishes.

Cell Culture Cultivation: HCT116 Cells

HCT116 cells were cultivated in Roswell Park Memorial Institute (RPMI)-1640 medium, supplemented with 10 % FBS and 1 % Pen/Strep 10.000 U mL^{-1} (=complete RPMI) at 37 °C, 5 % CO_2 in a humid atmosphere in 75 cm^3 flasks. For cultivation, the cells were passaged every 2-3 days or when confluence reached 80 %. For this, the media was removed and the cells were washed with 1x DPBS and treated for 3-5 min with EDTA-Trypsin. The Trypsination was controlled through microscopic observation and stopped by adding complete RPMI medium to the cells. The cells were then homogenised via pipetting and transferred into a 15 mL centrifugation vial and centrifuged for 5 min at 900 rpm. Then the supernatant was discarded and an aliquot of the HCT116 cell suspension was transferred into fresh RPMI culture medium in 75 cm^3 flasks or 10 dm^2 cell culture dishes.

Freezing and Thawing of Cells

Upon arrival, the HepG2 and HCT116 cells were stored at -196 °C in a nitrogen tank. For thawing of the cells, an aliquot was defrosted and its 1 mL content was transferred into a centrifugation vial containing 9 mL medium. This was homogenised and then centrifuged at 900 rpm for 5 min. Then the supernatant was discarded and the cells were placed in 75 cm^3 flasks or 10 dm^2 cell culture dishes. After 2-3 passages, the cells could be used for testing.

For freezing cells, the cells in culture were washed, trypsinated and centrifuged as described before and then transferred into DMEM or RPMI-1640 (depending on the cell line) with 20 % FBS and 10 % DMSO. After homogenisation, 1 mL of the cell suspension was immediately transferred into 2 mL cryovials and stored at -80 °C for 6 days. Later, the aliquots were placed in a nitrogen tank at -196 °C and stored until further use.

Sample and/or Pure Substance Application

For sample application, 96 well plates were coated with polyethyleneimine (PEI)-Hepes solution and washed two times with 1xDPBS. The coated plates could be used immediately or stored at 4 °C for a maximum of 14 days. Cells were washed, trypsinated and centrifuged as described above and then seeded into coated 96 well plates with 100 µL cell suspension per well and a density of $2*10^4$ cells/well and incubated at 37 °C, 5 % CO_2 in a humid atmosphere. After 24 h cells were treated with samples or pure substances solved in DMSO (or other solvents if indicated) and diluted in DMEM with 5 % FBS, 1 % Pen/Strep (for HepG2 cells) or RPMI-1640 complete (for HCT116 cells), to a total concentration of 1 % DMSO. As negative control 1 % DMSO was used in wells G1-H12.

Viability and Nano luciferase (Nluc) Measurement

After sample application, cells were incubated for 22 h. Then resazurin was added for viability measurement to a final concentration of 5 µM in the well and cells were incubated for another hour. A blank was also prepared consisting of media and resazurin without cells. After this, the metabolisation of resazurin to resorufin was measured using an Infinite®200 Pro (Tecan, CH) with excitation at 544 nm and emission at 590 nm.

Following the viability measurement, the media was discarded and cells were washed with 1xDPBS and lysed using a lysis buffer (25 mM Tris, 0.03 % Triton X-100). After adding the buffer, the plates were shaken for 10 to 15 min and the lysate could then be analysed by luciferase measurement or stored at 4 °C for up to two days. For Nluc measurement, a protocol by

Riegel et al. (2017) was followed with a substrate consisting of coelenterazine and 1 M Tris buffer (pH 7.4) and H_2SO_4 0.3 M for stopping the reaction. The measurement was conducted using a Luminoskan™ Microplate Luminometer (Thermo Fisher, MA, US).

Scoring and Background Determination

For evaluation of results, the fold induction was determined and compared to a threshold, which was determined in experiments on background signals. For this, a 96-well plate consisting only of vehicle controls (1 % DMSO) was incubated and analysed. The mean fold induction was determined and three times the standard deviation was added so that a threshold of 1.7 above the background was established as cut-off for positive or negative results.

To determine the fold induction for Nluc the median intensity was divided by the median background intensity and then compared to a threshold of 1.7. Values above 1.7 were considered to be positive for genotoxicity.

For viability, the resazurin median signal of the sample was divided by the median background signal and compared to a threshold of 0.7. Therefore, the cut off for cytotoxicity was considered 70 % compared to a 100 % cell viability in the background.

3.2.2. HCT116 Experiments

Here the cell line HCT116 was evaluated for its ability to detect genotoxic substances at low concentrations. Particular interest was placed on the cells' ability to detect substances that need to be metabolised to express their genotoxic potential. For this, the four different clones of the cells including the reporter gene, were cultivated as described previously and treated with pure substances. The HCT116 cells were treated with 1:2 dilution steps of 4-NQO (top concentration: 5 µM), MMS (top concentration: 450 µM) and BαP (top concentration: 40 µM). Measurement and scoring was conducted as described.

3.2.3. Optimisation Experiments

For the optimisation experiments, certain parameters of the HepG2 cell assay were varied and compared to the standard conditions. Some of the experiments were conducted together with Christina Friedl and an excerpt of this will be published as part of her master thesis as well (*manuscript in preparation:* Friedl, 2021).

The parameters for variation were: cell concentration in the well, incubation time with samples, media composition with and without S9 testing, FBS concentration in the media, DMSO concentration upon sample treatment and S9 addition. All experiments were performed in triplicates. Cells were treated and viability and Nluc measurement were conducted as previously described. For the experiments, the cells were treated with a 1:2 dilution series of 4-NQO and BαP. 4-NQO was added starting with a concentration of 0.2 µM to a top concentration of 0.6 µM and BαP was added with a starting concentration from 0.16 µM to a top concentration of 5 µM. The alterations in procedures are explained in detail in the following chapter:

Cell Concentration Variation

Different concentrations of cells were seeded in the 96 well plates. The following concentrations were used: $1*10^4$ cells/well, $2*10^4$ cells/well, $4*10^4$ cells/well, $6*10^4$ cells/well, $8*10^4$ cells/well and $1*10^5$ cells/well. A DMSO concentration of 1 % was ensured over the whole plate.

Incubation Time Variation

Here different sample incubation times should be taken into account. After sample treatment, the cells were incubated for 2, 6, 24, 48 and 72 h between sample application and viability and Nluc measurement. A DMSO concentration of 1 % was ensured over the whole plate.

Media Composition Variation 1

Different media compositions were tested to determine the effect on the LEC value. DMEM medium and DMEM supplemented with 10 % FBS was tested. The same composition was also tested in the presence of S9 for 24 h. The S9 mix 2 was used, which is described in more detail in the following paragraph. The pure substances BαP was added at a top concentration of 10 µM, which was diluted in 1:2 steps until 0.31 µM. A DMSO concentration of 1 % was ensured over the whole plate.

Media Composition Variation 2

With a concentration of 2.5 µM 4NQO different media compositions were analysed. The substance was solved in 1xDPBS, 1xDPBS supplemented with 10 % FBS, DMEM and DMEM supplemented with 10 % FBS. Further, the media compositions were also tested in the presence

of S9 mix 2 for 24 h, which is described in more detail in the paragraph on S9 variation. A DMSO concentration of 1 % was ensured over the whole plate.

FBS Variation

For these experiments, the media composition for sample treatment was variated by changing the concentration of FBS. The sample treatment media was varied as following: DMEM with 1 % Pen/Strep and 5, 10 or 15 % FBS was used. A DMSO concentration of 1 % was ensured over the whole plate.

DMSO Variation

The sample concentration in the media should be varied by using different amounts of DMSO. The cells were treated with samples with a final DMSO concentration of 0.25, 0.50, 0.75, 1.00, 1.50 and 2.00 %.

S9 Protocol Variation

For S9 treatment, different protocols were followed and compared to sample treatment without S9 addition. S9 was obtained by Moltox (Moltox, NC, USA) as 1254 aroclor induced rat liver S9. The cofactors NADPH, Glucose-6-phosphate (G6P) and $MgCl_2$ were purchased at Carl Roth (Karlsruhe, GER) and Glucose-6-Phosphate-Dehydrogenase (G6P-DH) at Sigma Aldrich (US).

The S9 mixes were prepared on ice, a maximum of 30 min before treatment of the cells according to the following protocol:

S9 mix 1: 5 mM MgCl2, 3 mM G6P, 0.2 mM NADPH, 0.3 units/mL G6P-DH, 330 µg/mL S9 extract

S9 mix 2: 5 mM MgCl2, 3 mM G6P, 0.2 mM NADPH, 0.3 units/mL G6P-DH, 10 µg/mL S9 extract

Cells were treated with sample and 100 µL S9 mix 1 for 3 h and were then washed with 1xDPBS and incubated in the sample treatment media for 23 h until measurement. Alternatively, the cells were treated with the sample and 10 µL of the S9 mix 2 was added and the cells were incubated for 24 h until measurement. A DMSO concentration of 1 % was ensured over the whole plate.

3.2.4. Validation

Treatment with (Non-)Genotoxic Substances

HepG2 cells were treated as previously described. Measurement of viability and Nluc were conducted as explained above. Experiments were conducted in triplicates. Some experiments were conducted together with Christina Friedl and an excerpt will be shown in her master thesis (*manuscript in preparation:* Friedl, 2021).

For sample application, the cells were treated for 24 h with the given pure substance in 1:√10 dilution series. As a threshold 1.7 fold induction was applied, above which a substance was considered positive. Further, a cytotoxicity threshold of 0.7 fold induction above the background was taken into account. For sample application, 1 mM top concentration was considered as a maximum, or the level of solubility or cytotoxicity was taken into account if observed at lower concentrations. Further, for positive substances the range where the lowest positive signal was expected to be found was used.

The pure substances for genotoxicity testing were preferably solved in DMSO as 100 mM stocks, or in other solvents or concentration if appropriate and as indicated in Table 1 and Table 3. The positive control 0.31 µM 4NQO was added in the wells H1-H12 and a vehicle control or basal control was added as 1 % DMSO in the wells G1-G12.

Further, packaging specific substances were included in the validation as well, which are listed in Table 2 These substances are known to test positive for mutagenicity in the Ames test and are associated with packaging (Zeiger et al., 1992; Malaveille et al., 1975; Watanabe et al., 1990).

Table 1: List of known genotoxic pure substances used for the validation of the HepG2 reporter gene assay. The solvent used for stock preparation and the stock concentration are listed.

	Substance	CAS-Number	Solvent	Stock
Known *in vitro* and *in vivo* genotoxic substance	Cyclophosphamide	6055-19-2	DMSO	1 M
	N-Ethyl-Nitrosurea	759-73-9	DMSO	1 M
	Methyl Methanosulphonate	66-27-3	H2O	1 M
	Benzo-a-pyrene	50-32-8	DMSO	100 mM
	7,12-Dimethylbenzanthra-cene	57-97-6	DMSO	100 mM
	2-Acetylaminofluorene	53-96-3	DMSO	1 M
	2,4-Diaminotoluene	95-80-7	DMSO	1 M
	Aflatoxin B1	1162-65-8	DMSO	3 mM
	Cisplatin	15663-27-1	DMSO	10 mM
	Sodium Arsenite	7784-46-5	H2O	1 M
	Etoposide	33419-45-0	DMSO	10 mM
	4-Nitroquinoline-N-oxide	56-57-5	DMSO	10 mM
	Colchicine	64-86-8	DMSO	100 mM
	Mitomycin C	50-07-7	DMSO	10 mM
	Actinomycin D	50-76-0	DMSO	40 mM
	Doxorubicin	23214-92-8	DMSO	10 mM

Table 2: List of packaging specific pure substances used for the validation of the HepG2 reporter gene assay. The solvent used for stock preparation and the stock concentration are listed.

	Substance	CAS-Number	Solvent	Stock
Packaging specific substances considered to be mutagenic in the Ames test	Benzo-a-anthracene	56-55-3	DMSO	1 mM
	p-Phenylenediamine	106-50-3	DMSO	250 µM
	Triglycidylisocyanurat	2451-62-9	DMSO	250 µM

Table 3: List of non-genotoxic pure substances used for the validation of the HepG2 reporter gene assay. The solvent used for stock preparation and the stock concentration are listed.

	Substance	CAS-Number	Solvent	Stock
Known non-genotoxic substances	Ampicillin trihydrate	7177-48-2	H2O	100 mM
	d-Mannitol	69-65-8	DMSO	100 mM
	Phenformin HCl	834-28-6	DMSO	100 mM
	(2-Chloroethyl)trimethyl-ammonium chloride	999-81-5	DMSO	100 mM
	Amitrole	61-82-5	DMSO	1 M
	Diethanolamine	111-42-2	DMSO	100 mM
	Melamine	108-78-1	DMSO	100 mM
	Methyl carbamate	598-55-0	DMSO	100 mM
	Pyridine	110-86-1	DMSO	1 M
	Tris(2-ethylhexyl)phos-phate	78-42-2	96 % Ethanol	1 M
	Hexachloroethane	67-72-1	DMSO	100 mM
In vivo negative, some-times *in vitro* positive	D,L-Menthol	15356-70-4	DMSO	100 mM
	2-Ethyl-1,3-Hexanediol	94-96-2	DMSO	100 mM
	Sulfisoxazole	127-69-5	DMSO	100 mM
	Urea	57-13-6	DMSO	100 mM
	Sodium Saccharin	128-44-9	DMSO	100 mM
	Eugenol	97-53-0	DMSO	100 mM
	Tert-butylhydroquinone	1948-33-0	DMSO	100 mM

3.2.5. Applicability for Complex Mixtures

Treatment with FCM Samples

The FCM samples were produced as described in 3.1 and the cells for sample application were prepared and scored as described above. Samples were applied in wells A1-F12 with two 1:√10 dilution steps, leading to the testing of three different sample concentrations: 1 % sample, 0.3 % sample and 0.1 % sample in the medium.

As a vehicle control, 1 % DMSO was added in wells G1-G12, 0.31 µM 4-NQO was added as a positive control in the wells H7-H12 and melamine was used as a negative control at a concentration of 250 µM in the wells H1-H6. For a sample experiment to be considered as valid, the positive control had to be above a fold induction of 5 and the negative control had to be below 1.5. Further, the viability in negative and positive control had to be above 0.7, meaning that the cells had a viability above 70 % in the control wells.

Treatment with FCM Samples – Spiking Experiments

For spiking, a duplicate 96 well plate was prepared compared to the normal sample plates, which was then treated with 4-NQO at a concentration of 0.16 µM in the wells. Here the addition of the spike should not lead to a decrease in viability and therefore a lower concentration of 4-NQO was added compared to the positive control. The same thresholds and application procedures as described above were applied.

3.3.qPCR

For qPCR analysis, HepG2 cells were obtained and treated as described in 3.2.1. The cells were treated with pure substances in a previously determined concentration through testing with the reporter gene assay. A selection of pure substances was used for treating the cells in 6-well plates.

3.3.1. Cell Seeding and Sample Treatment

HepG2 cells were seeded in 6-well plates with $2.5*10^5$ cells/well with 2 mL cell suspension per well. After 24 h of incubation at 37 °C, 5 % CO_2 in a humid atmosphere, the cells were treated with 2 mL sample per well. The following substances, which are listed in Table 4, were applied in 1:√10 dilution series resulting in three concentrations. As a vehicle control, 1 % DMSO was used and cells were again incubated for 24 h. Viability was determined by microscopic examination.

Table 4: List of (non)-genotoxic substances used for qPCR analysis. The top and lowest concentration of the substances are shown, which were applied in three concentrations with 1:√10 dilution steps.

Substance	CAS-Number	Solvent	Top Concentration	Lowest Concentration
N-Ethyl-Nitrosurea	759-73-9	DMSO	5,000 µM	500 µM
Methyl Methanosulphonate	66-27-3	H2O	1,000 µM	100 µM
Benzo-a-pyrene	50-32-8	DMSO	5 µM	0.5 µM
7,12-Dimethylbenzanthracene	57-97-6	DMSO	5 µM	0.5 µM
2-Acetylaminofluorene	53-96-3	DMSO	1,000 µM	100 µM
Aflatoxin B1	1162-65-8	DMSO	5 µM	0.5 µM
Cisplatin	15663-27-1	DMSO	5 µM	0.5 µM
Etoposide	33419-45-0	DMSO	5 µM	0.5 µM
Mitomycin C	50-07-7	DMSO	3 µM	0.3 µM
Ampicillin trihydrate	7177-48-2	H2O	1,000 µM	100 µM
d-Mannitol	69-65-8	DMSO	1,000 µM	100 µM
Phenformin HCl	834-28-6	DMSO	1,000 µM	100 µM
D,L-Menthol	15356-70-4	DMSO	1,000 µM	100 µM
Sulfisoxazole	127-69-5	DMSO	1,000 µM	100 µM
Eugenol	97-53-0	DMSO	1,000 µM	100 µM
Tert-butylhydroquinone	1948-33-0	DMSO	1,000 µM	100 µM

3.3.2. RNA Extraction

The total mRNA extraction kit "Thermo Scientific GeneJet RNA purification kit" for 6-well plates was used (Thermo Fisher, MA, US). The medium was removed from the cells and collected in a 2 mL reaction tube and centrifuged for 5 min at 900 rpm and the supernatant was discarded. Cells were directly treated with 600 µL lysis buffer per well, where previously 40 µL 1 M dithiothreitol (DTT) was added per 1 mL lysis buffer. The cells were resuspended in the lysis buffer mix and transferred into the reaction tubes, containing the pellets from the previously centrifuged medium. To this 360 µL of 96 % ethanol was added and mixed by pipetting. Then the suspension was transferred onto spin filters, which were placed inside of receiver tubes and incubated at RT for 1 min. After centrifugation at 10,000 rpm for 1 min, the flow through was discarded and 700 µL Wash Buffer 1 was added onto the filter. The filters were again centrifuged at 10,000 rpm for 1 min and the flow through was discarded and 600 µL Wash Buffer 2 was added. After another centrifugation at 10,000 rpm for 1 min, the flow through was discarded and 250 µL Wash Buffer 2 was added and the filters were centrifuged at 12,000 rpm for 2 min. The receiver tube was then discarded and the spin filters were placed in 1.5 mL reaction tubes and 50 µL nuclease free H_2O was added to the filter and incubated at RT for 2 min. After centrifugation for 1 min at 12,000 rpm, the filter was discarded and the RNA eluate was placed on ice. The RNA content was then quantified using the RNA App on the DeNovix DS-11 spectrophotometer. The RNA elution was then immediately used for cDNA synthesis (described in 3.3.3) or stored at -20 °C until further use.

3.3.3. cDNA Synthesis

Using a First Strand cDNA Synthesis Kit (Thermo Fisher, MA, US) the RNA extract was placed on ice and 2 µL sample RNA was transferred in 1.5 mL reaction tubes. To this 2 µL 10x DNAse I buffer, 2 µL DNAse I and 14 µL nuclease free H_2O was added. The mix was incubated for 30 min at 37 °C and then 2 µL 50 mM EDTA was added and incubated at 65 °C for another 10 min. The tubes were placed on ice and 2 µL Random Hexamer Primer was added to inactivate the DNAse digest. After mixing by pipetting, the mix was spun down for 10 sec on the micro-centrifuge and incubated for 5 min at 70 °C. The mix was immediately put on ice and then spun down again. After this 8 µL 5x RT buffer, 4 µL 10 mM dNTP mix and 2 µL RiboLock RNAse Inhibitor were added on ice. The mix was homogenised by pipetting and then spun down and incubated for 5 min at 25 °C. Then 2 µL Reverse Transcriptase was added and incubated for 10 min at 25 °C. After this, the mix was incubated for 1 h at 42 °C, followed by an

inactivation at 70 °C for 10 min. The lysate was then put on ice and stored at -20 °C until used for qPCR analysis.

3.3.4. qPCR Master Mix and Primers

The master mix was prepared on ice according to the following protocol in Table 5. At first water was put into 5 mL reaction tubes and the substances shown in Table 5 were added under repeated mixing. The primers were added according to the target to be analysed as given in Table 6. As final substance, the Taq Polymerase was added and the mix was vortexed and then 24 µL were applied to each well of a 96 well qPCR plate. On ice, 1 µL of cDNA sample were placed in the respective wells and an optical foil was placed over the 96 well plate. This was then centrifuged and the plates were analysed according to 3.3.5.

Table 5: Master mix recipe for SYBR Green qPCR protocol and GAPDH protocol.

SYBR Green	
Primer (forward + reverse)	110 µL
SYBR Green	110 µL
10x Buffer B	275 µL
2 mM dNTP Mix	275 µL
$MgCl_2$	440 µL
BSA (25 mg/mL)	88 µL
Taq Polymerase	13.75 µL
dH_2O	1328.25 µL

GAPDH	
Primer (forward + reverse)	110 µL
Probe (GAPDH)	41 µL
10x Buffer B	275 µL
2 mM dNTP Mix	275 µL
$MgCl_2$	440 µL
Taq Polymerase	13.75 µL
dH_2O	1385.25 µL

Table 6: List of target genes and the designed forward and reverse primers, which were used for qPCR analysis. The housekeeping genes (HKG) and the primer sequences for the determined targets are shown.

Target	**Forward Primer**	**Reverse Primer**
HKG: β-actin	GGGCATGGGTCAGAAGGATTC	GATTTTCTCCATGTCGTCCCAG
HKG: GAPDH	GGAAGGTGAAGGTCGGAGTCAA	ACCAGAGTTAAAAGCAGCCCTG
GADD45α	AACTTATTTGTTTTTGCCGGGAAAG	GGGAGTAACTGCTTGAGTAAC-TACAAAGG
CDKN1a (=p21)	AAGGCAGGGGGAAGGTGGG	GGGGAGGGACAGCAGCAGA
FDXR	AGAACGGACATCACGAAGGCAG	CGGTAACTGAATCATCTCCCGAAG
DDB2	GTCAGGACCCTCCACCAGCATA	GGATGTAGCCCTCCTGTCAAAGG
SERTAD1	CGTGGCCTCTAGCTCCCTCTTT	GTAAGTTGTCAGCCACACTGGGG
TP53INP1	TACTTGCACTGGTTTCTCAGCAGAA	GCAGGAATCACTTGTATCAGCCAA
RRM2B	TCGACTTATCAAAGGATCTCCCTCA	CCTGACTAAAGCGCTCCACCAAAT
BTG2	CGTGAGCGAGCAGAGGCTTAA	TTGTGGTTGATGCGAATGCAG

As a standard, a previously multiplied cDNA of the specific target gene was used. The standard was diluted in a 1:10 dilution series and applied in seven concentration steps.

3.3.5. qPCR Protocol and Analysis

The qPCR was performed using the Mx3000P and AriaMx Real-Time PCR System (Agilent Technologies, CA, US). The thermal profile for the SYBR Green protocol was: 1 cycle 95 °C for 10 min, followed by 40 cycles of 30 sec at 95 °C, 1 min at 58 °C and 1 min at 72 °C. For GAPDH, 1 cycle at 95 °C for 5 min was followed by 40 cycles of 30 sec at 95 °C and 1 min at 60 °C. In a first experiment, the melting curves were determined to ensure the proper range and function of the primers.

For analysis, a cycle threshold (c_t-value) was determined within the linear range of the signals. This c_t-value was applied to the samples, vehicle control and standard series. As an acceptance criteria, the standard series had to have a correlation above 90 % and the negative control has to be above a c_t of 30 or far enough from the sample values. The sample concentration was determined by comparison with the mean value of the housekeeping gene concentration. Then these were divided by the mean value of the vehicle control and the fold induction had to be above a factor of 2 for the substance to be considered as positive.

3.4.High Content Screening (HCS)

For high content screening, HepG2 cells were obtained and treated as described in 3.2.1. For cell seeding in 96 well plates, $1x10^4$ cells/well were applied and resuspended by using a syringe and needle to avoid any clumping of the HepG2 cells. A lower cell concentration per well was used than for the reporter gene assay, as the formation of clumps should be avoided and the number of cells had to be low enough to ensure reproducible and unobstructed imaging, as a too high cell number can lead to irregularities in HCS.

3.4.1. Concentration of Antibody Solutions

To determine the optimal amount of antibody solutions applied, the antibody solution 1 and 2 were tested in varying concentrations. As the solutions are very costly, different dilutions of antibody solution 1 (AB1) and 2 (AB2) were applied in the ratio 1:1 or 2:1:

AB1 1:1,000 – AB2 1:1,000

AB1 1:2,000 – AB2 1:1,000

AB1 1:2,000 – AB2 1:2,000

Simultaneously viability was measured over the whole plate by using Hoechst 33342 in a 1:6,000 dilution. The pure substance 4NQO was applied in a top concentration of 1.25 µM, which was diluted 1:2 until a concentration of 0.04 µM. The fold induction was calculated by comparison to the basal level without 4NQO, with the respective antibody solution ratios. A DMSO concentration of 1 % over the whole plate was ensured.

3.4.2. Pure Substances Application

For testing, the following substances were analysed, which are shown in Table 7. The substances were applied in the indicated highest concentrations and further diluted by a factor of 1:2 or 1:√10 to reach the lowest concentration in the well. As a vehicle control, 1 % DMSO was used for comparison and for scoring. The HepG2 cells were incubated for 24 h with the substances and were then fixated and treated with antibodies before fluorescence microscopic measurement.

Table 7: List of substances used for screening for the γH2AX target with the packaging relevant substances indicated with an asterisk (*). The highest and lowest concentration applied to HepG2 cells are given, where a 1:2 or 1:√10 dilution series was used.

Substance	CAS-Number	Top Concentration	Lowest Concentration
4-Nitroquinoline-N-Oxide	56-57-5	2.5 µM	0.08 µM
Benzo-α-pyrene	50-32-8	20 µM	0.6 µM
7,12-Dimethylbenzanthracene	57-97-6	50 µM	1.6 µM
Methyl Methanosulphonate	66-27-3	450 µM	14 µM
Cisplatin	15663-27-1	2.5 µM	0.02 µM
Hydroquinone	123-31-9	80 µM	2.5 µM
Etoposide	33419-45-0	20 µM	0.08 µM
Benzo-a-anthracene*	56-55-3	250 µM	8 µM
p-Phenylenediamine*	106-50-3	250 µM	8 µM

3.4.3. Application for Complex Mixtures

Instead of pure substances, the cells were treated with different FCM samples. Cells were seeded and treated as described in 3.2.1 with 1 % DMSO sample concentration in the plate for 24 h and two 1:√10 dilution steps, leading to a sample concentration of 100 %, 30 % and 10 %. Simultaneously, the DMSO concentration on the whole plate was held constant at 1 %. As a positive control 4NQO was used with a concentration of 1.25 µM in the wells and a sample blank at a concentration of 1 % was applied. The basal level was also determined by applying 1 % DMSO.

3.4.4. Fixation and Antibody Treatment

After incubation with the sample, the supernatant was removed and the cells were fixed using a 4 % paraformaldehyde solution for 15 min at RT. The cells were washed with 1x DPBS and were incubated with a permeabilisation solution, consisting of 2.5 % Triton X-100 in 1x DPBS, for 15 min at RT. After washing with 1x DPBS a blocking solution of 10 g BSA L^{-1} DPBS was added and incubated for 1 h at RT. Then the primary antibody solution Anti-Hu/Mo PH2AX CR55T33 PUR 100 UG (Thermo Fisher, MA, US) was added in 1:2,000 dilution and incubated for 1 h at RT. After several washing steps with 1x DPBS the antibody solution 2 was added and incubated at RT in the dark for 1 h. The antibody solution 2 consisted of Cy3 AffiniPure Goat Anti-Mo IgG (H+L) (JacksonImmuno, PA, US) in a 1:1,000 and Hoechst 33342 (Sigma Aldrich, MO, US) in a 1:6,000 dilution. Cells were washed again with 1x DPBS and kept in 1x DPBS to avoid dryness of the cells.

3.4.5. Fluorescence Microscopy Measurement and Scoring

For HCS measurement, a fluorescence microscope (Carl Zeiss, GER) was used with the channels for 4',6-diamidin-2-phenylindol (DAPI), Cy3 and Phase II. The Hoechst dye was detected with DAPI, the γH2AX antibody with Cy3 and the cell contrasts with Phase II with a 1x200 magnification. Exposure time and image plane were adjusted individually with each sample plate. For automatic measurement, six pictures at random positions were taken per well with each fluorescent channel.

For scoring, the "Cell Profiler" software was used. First, the cell nuclei were identified through DAPI and then the signal intensity of the Cy3 for the nuclei was taken into account as shown in Figure 9A and B with an example. For nuclei scoring by DAPI, a minimal and maximum nuclei size range was determined, to prevent the interference of artefacts. For viability measurement, the DAPI signal intensity of samples was compared to the signal intensity of the vehicle control and a threshold of 0.7 was set. For γH2AX measurement, the signal intensity of Cy3 in the respective nuclei was determined in a sample well and compared to the mean Cy3 intensity in the vehicle control wells. A threshold of 2 times above the vehicle control was applied, above which a sample or substance was considered positive for γH2AX induction. To minimise the effect of artefacts in cloudy samples or other contamination, very high and very low Cy3 signal intensities were eliminated. Further, phase II contrasts were monitored and taken into account to gain information on the cell's form. This information was used to further distinguish nuclei from artefacts and any clumped cells and clots decreasing the countable number of nuclei in a picture.

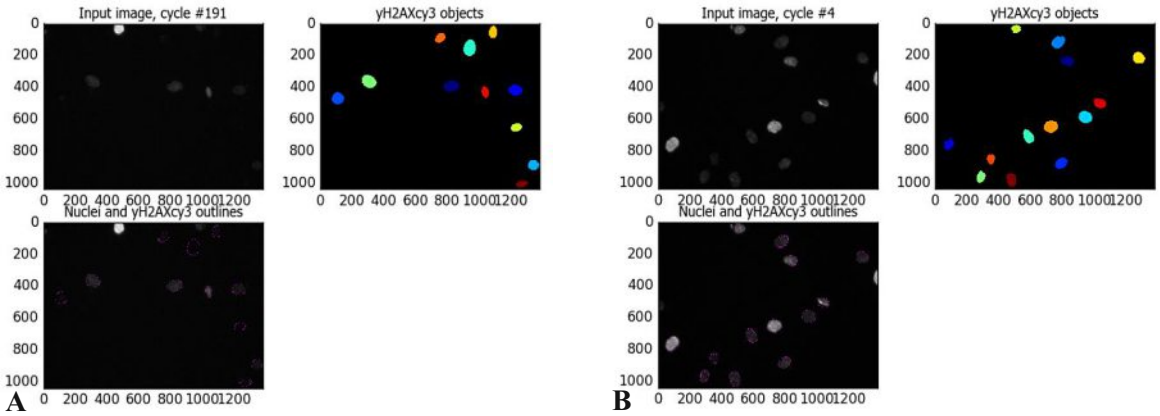


Figure 9: Analysis of cell imaging. The DAPI image was used to identify any nuclei, which was restricted to a specific maximum and minimum nuclei size range to prevent any artefacts from interfering. For the determined nuclei, the Cy3 intensity was measured to determine H2AX phosphorylation. Here also maximum and minimum intensities were eliminated to prevent the interference of artefacts. In both images A and B the nuclei measured with DAPI are shown in the coloured picture ("upper right"). The initial Cy3 measurements are shown in "upper left" and the corrected Cy3 image in "bottom left", where the information on the nuclei is already included.

3.5.Pre-Incubation Ames Test

3.5.1. Experiment Set-up

For conducting the Ames test, TA100 and TA98 strains were obtained through Xenometrix (Basel, CH). The bacteria were grown in Nutrient Broth No. 2 (Thermo Fisher, MA, US) with ampicillin (25 mg mL^{-1}) over night at 37 °C and 250 rpm and stored in aliquots with 20 % glycerol at - 80 °C. Prior to an experiment, 10 µL of the frozen bacteria aliquot was added to 3 mL Oxoid Nutrient Broth No. 2 and 3 µL ampicillin (25 mg mL^{-1} stock solution) and incubated overnight (~16 h) at 37 °C and 250 rpm. For general laboratory use, bacteria solutions were stored at 4 °C and restarted by adding 300 µL of the suspension to 3 mL Oxoid Nutrient Broth No. 2 and 3 µL ampicillin 25 mg mL^{-1} stock. For the pre-incubation Ames test, the bacteria were grown as described at 37 °C and 250 rpm until an OD600 within the range of 2.0-2.8 was reached.

Media and consumables were prepared according to Proudlock (2016) as described in the following and chemicals were obtained by Carl Roth (GER), if not indicated otherwise:

Reagents and Recipes

- *Nutrient broth:* 500 mL dH_2O with 12.5 g of Oxoid Nutrient Broth No. 2 were dissolved and autoclaved at 121 °C for 15 min and then kept at RT.
- *Glucose solution:* 20 % glucose solution was prepared and autoclaved at 121 °C for 15 min. The solution was stored at 4 °C until further use.
- *Phosphate buffer:* for 1 L, 146 mL of NaH_2PO_4 0.2 M and 854 mL Na_2HPO_4 were mixed and the pH was set to 7.3 to 7.5. Then the buffer was autoclaved at 121 °C for 15 min and stored at RT.
- *50xVogler-Bonner Salts (50xVB-salts):* 160 mL dH_2O was heated to 50-80 °C and 2 g of $MgSO_4.H_2O$ was added and solved. Then 20 g citric acid monohydrate, 100 g K_2HPO_4 and 35 g $NaNH_4HPO_4.4H_2O$ was added and stirred until dissolved. The salt solution was filled with dH_2O to 200 mL and then autoclaved at 121 °C for 15 min and stored at RT.
- Preparation of *bottom agar:* Minimal Glucose Agar (MGA) was prepared by autoclaving 6 g agar-agar in 400 mL dH_2O at 121 °C for 15 min. Then 10 mL 20 % glucose solution and 8 mL 50xVB-salts were added while the agar was still liquid. When the MGA had a temperature of about 50 °C, an approximate of 20 to 25 mL of MGA were poured into 10 cm^2 dishes and sored at RT for a maximum of two weeks.

- *Histidine/Biotin solution:* 50 µM His/Biotin solution was prepared by mixing a 0.37 mg mL^{-1} biotin solution and a 5 mg mL^{-1} histidine solution and autoclaving it at 121 °C for 15 min. In general, 500 mL solutions were prepared by using 10.5 mL histidine and 166.5 mL biotin solution. The 50 µM His/Biotin solution was stored at 4 °C.
- Preparation of *top agar*: For 400 mL top agar, 3 g NaCl and 3 g agar were added to 400 mL dH_2O and autoclaved at 121 °C for 15 min. Before solidifying 40 mL of a 50 µM His/Biotin solution was added and mixed thoroughly, leading to a final His/Biotin concentration of 5 µM in the agar mix. The top agar was stored at 4 °C and liquefied before use, or used immediately after preparation.

Pre-Incubation Ames Procedure

MGA plates were placed in the incubator at 37 °C to ensure the plates were at temperature before use and the top agar was melted and 2 mL were portioned in 15 mL reaction tubes. The bacteria strain was incubated until an OD600 range of 2.0-2.8 was reached and then immediately used for the experiment.

Test samples were prepared in 24-well plates. All samples were conducted in biological triplicates and the experiments were performed in independent duplicates or triplicates. The following concentrations are given as concentration in mg mL^{-1} in the plate. As a vehicle control, 7.7 % DMSO was used and the positive control was 0.4 µg mL^{-1} 4-NQO for TA100 and 0.4 µg mL^{-1} 2-nitrofluorene (2NF) for TA98. All plates had a DMSO concentration of 7.7 %, as all samples and controls were solved in DMSO.

For pre-incubation, 100 µL of the grown bacteria solution were added to 500 µL phosphate buffer and 50 µL sample and incubated for 60 min at 37 °C and 250 rpm. Then the bacteria sample mixture was resuspended and mixed in 2 mL top agar and poured onto MGA plates. After drying of the top agar the plates were incubated upside down for 48 h at 37 °C. For scoring, colonies were counted and the mean sample results were compared to the mean vehicle control colonies. A result above the threshold of twice the vehicle control added to twice its standard deviation was considered positive for mutagenicity.

Pre-Incubation Ames Procedure with S9

For S9 addition, an S9-mix had to be prepared prior to incubation. The components of the S9 mix were mixed and the solution itself was then placed on ice and stored for a maximum of 30 min to ensure no activity of the S9 mix was lost.

The cofactors were produced by solving the individual components in dH_2O. A 0.1 M NADP mix was produced by dissolving 76.5 mg mL^{-1} NADP in dH_2O and for 1 M G6P 260 mg mL^{-1} were dissolved in dH_2O. The KMg solution was obtained by dissolving 124 mg KCl and 81 mg $MgCl_2.6H_2O$ in 1 mL dH_2O. For 1 mL of the cofactors master mix: 335 µL dH_2O, 40 µL NADP solution, 5 µL G6P solution and 20 µL KMg solution were mixed and sterile filtered. After filtration, the mix was placed on ice and 1254 aroclor induced rat liver S9 extract (Moltox, NC, USA) was added to a final concentration of 15 %.

The pre-incubation Ames test was conducted as described above, with a few alterations that are mentioned in the following. As a positive control, 0.4 µg mL^{-1} 2-aminoanthracene (2AA) for TA100 and TA98 was used. For the pre-incubation buffer, the S9 buffer mix consisting of co-factors and S9 extract was used. Therefore, the pre-incubation mix was 100 µL bacteria solution, 500 µL S9 buffer mix and 50 µL sample, which was incubated for 60 min at 37 °C and 250 rpm. Pouring of the samples, incubation and scoring was conducted as described above.

3.5.2. Optimisation

Testing for the optimisation of the pre-incubation Ames was conducted together with Tatjana Hasil and an excerpt of the results are shown in her master thesis (Hasil, 2020).

The following factors were considered for optimisation: pre-incubation time, incubation time, histidine concentration in top agar, histidine concentration in the pre-incubation medium, initial bacteria concentration and variation of the amount of S9 liver extract in the S9 mix. For optimisation experiments, for each parameter 7.7 % DMSO was used as a vehicle control and three dilution steps of the positive control were used for the respective strain. The dilution series for TA100 was 0.23 µg mL^{-1} 4NQO with a 1:2 or 1:√10 dilution series. For TA98, 2NF was used at a top concentration of 0.15 µg mL^{-1} with a 1:2 or 1:√10 dilution series. The following sub-chapters describe in detail the change conducted in the method. All other parts of the experiments were conducted as described in 3.5.1 for the pre-incubation Ames without and with S9.

Bacteria Concentration Variation

The initial bacteria concentration was varied by diluting the bacteria culture with an OD600 in the range of 2.0-2.8 by a 1:10 or 1:25 dilution in the phosphate buffer. Bacteria were also concentrated by centrifugation. For this, 20 mL of the bacteria culture was centrifuged for 10 min at 10,000 rpm and the supernatant was discarded. The pellet was then dissolved and homogenised in 2 mL phosphate buffer, leading to a 1x10 concentration of the initial bacteria. The bacteria suspension was then used for the experiments as previously described.

Variation of Pre-Incubation Time

For time experiments, either the pre-incubation or the incubation time was varied. For the pre-incubation, the following parameters were applied when the bacteria were treated with sample and shaken at 37 °C and 250 rpm. The buffer-bacteria-sample mix was incubated for 60 min, 90 min, 120 min and 180 min and then poured onto agar plates and incubated as described previously. When the incubation time was altered the plates were scored after 48 h and 72 h.

Histidine Variation During Pre-Incubation and in the Top Agar

For histidine variation, either the concentration of histidine in the top agar or pre-incubation buffer was altered. In the pre-incubation buffer, histidine and biotin were added to a final concentration of 0 µM, 3 µM, 6.4 µM or 10 µM in the buffer mix. For variation of histidine in the top agar, the biotin concentration in the agar mix was constant with 50 µM. The histidine concentration was varied as 0 µM, 5 µM, 16 µM, 50 µM (=standard), 160 µM and 500 µM. Again, otherwise the experiments were conducted as previously described.

Variation of S9 Liver Extract in the S9 Mix

Here the previously described protocol was followed and the final concentration of S9 liver extract in the S9 buffer mix varied at 1 %, 5 %, 10 % and 15 % S9 liver extract. For each, a negative control and the following concentrations of 2AA in the plates were used: 4 µg mL^{-1} of 2AA as a top concentration in a three step 1:√10 dilution series for TA100 and 0.4 µg mL^{-1} 2AA with a three step 1:√10 dilution series for TA98.

3.5.3. Ames Formats Comparison

To determine the optimal format for sample testing, different Ames formats were tested. This was done, because the amount of sample is the limiting factor for testing of complex mixtures and therefore a balance had to be found for low LEC values and little amount of sample necessary for testing.

The following formats were chosen for testing:

- the standard protocol in 10 cm ø petri dishes (as described in 3.5.1)
- 6-well according to Pant et al. (2016) and Diehl et al. (2000)
- 12-well according to Zwarg et al. (2018)
- 24-well according to Proudlock and Evans (2016)

6-well protocol: 5 mL of bottom agar was poured in each well in a 6-well plate. For testing, 10 µL of sample was added to 25 µL of bacteria solution and 100 µL of phosphate buffer. This lead to a final DMSO concentration of 7.7 % in the well and this was kept constant over the whole plate. After pre-incubation in 24-well plates for 60 min, 0.5 mL of top agar was added, mixed and poured onto the bottom agar of a 6-well plate and the plates were incubated for 48 h and scored afterwards.

12-well protocol: 2.8 mL bottom agar was poured in each well of a 12-well plate. 2 µL of sample was added to 25 µL of bacteria solution and 25 µL phosphate buffer, leading to a final DMSO concentration of ~ 4 %. After 60 min of pre-incubation in a 24-well plate, 1 mL of top agar was added and the mix was homogenised through pipetting. It has to be pointed out that this does not make it a true triplicate, but instead a mix is simply split into three wells. The mix was then distributed in three different wells of a 12-well plate, as 250 µL per well was added. Then the plates were incubated for 48 h and scored afterwards.

24-well protocol: 0.5 mL of bottom agar was poured into each well of a 24-well plate. 25 µL of sample was mixed with 25 µL of bacteria solution and 125 µL of phosphate buffer, leading to a final concentration of 14.3 % DMSO. After 60 min of incubation in 24-well plates, 0.5 mL of top agar was added. The top agar sample mix was homogenised through repetitive pipetting and then distributed with 130 µL each into three different wells of a 24-well plate. Again it has to be pointed out that this does not make it a true triplicate, but instead a mix is simply split into three wells. The 24-well plates were then incubated for 48 h and scored afterwards.

For comparability of the formats, the pure substances 2NF for TA98 and 4NQO for TA100 were used, each with a top concentration of 50 µM in the DMSO stock. A 1:2 dilution was applied, resulting in a lowest concentration of 0.05 µM. The tests were conducted simultaneously by one operator and the same bacteria and substance solutions were used for each format.

3.5.4. Applicability for Complex Mixtures

The application of complex mixtures was conducted together with Tatjana Hasil and an excerpt is shown in her master thesis (Hasil, 2020).

To use less amount of sample the experiments were conducted in 6-well plates with a DMSO concentration of 7.7 % and were performed in independent duplicates. To switch from incubation in 10 cm² agar plates to 6-well plates the volumes were adjusted to fit the smaller volumes, all other parameters were not altered. For the 6-well format, 5 mL bottom agar (MGA) was placed in each well and 500 µL top agar was necessary. The pre-incubation mix for each well consisted of 100 µL phosphate buffer, 25 µL bacteria solution and 10 µL sample. As a positive control, 0.4 µg mL^{-1} 4NQO was used for TA100 and 0.4 µg mL^{-1} 2NF for TA98. For S9 experiments, the positive controls were 1.6 µg mL^{-1} 2AA for TA100 and 0.4 µg mL^{-1} 2AA for TA98.

To determine any matrix effects influencing the results, spiking experiments were conducted. For this, the concentration was 0.15 µg mL^{-1} 4NQO for TA100 and 0.15 µg mL^{-1} 2NF for TA98. The spike concentration was lower than the positive concentration to ensure the viability would not be negatively affected by the spike addition. The spike was added during pre-incubation as part of the pre-incubation buffer mix.

Samples were plated in top concentration and two dilution steps in 1:√10 dilutions leading to a sample application of 7.7 %, 2.4 % and 0.77 %, while the DMSO concentration was a constant 7.7 % in each plate or well. Further, a sample blank was also applied to ensure the sample preparation (described in 3.1) did not lead to a false positive result.

For scoring, the mean colonies in each sample concentration was compared to the colonies in the vehicle control. Two times the background colony concentration was considered as threshold for mutagenicity leading to a positive result for samples above the cut off.

4 Results

In this chapter, the results of different *in vitro* assays and analysis methods are shown as a summary. More detailed graphs and results of single experiments are listed in the annex. Further the results of the literature survey as part of this thesis published in Pinter et al. (2020) are shown.

4.1. Results: Literature Survey

As part of this thesis, a literature research on the toxicological sensitivity and specificity of some commonly used prokaryotic and eukaryotic *in vitro* assays was performed. The results are shown in Table 8, adapted from the publication by Pinter et al. (2020) as part of this thesis. The assays are classified according to the different endpoints of genotoxicity they can measure. As prokaryotic assays, the Ames test, the SOS-Chromo test and the Rec-Assay were taken into account. In a thorough analysis, Pinter et al. (2020) concluded that the toxicological sensitivity and specificity depended greatly on the amount of substances analysed and the cell line used for obtaining results. Further, Pinter et al. (2020) considered assays focusing on the DNA-damage response, such as the reporter gene assays listed in Table 8, as promising candidates for the *in vitro* assessment of genotoxicity, as they tend to have a high toxicological sensitivity and specificity compared to other assays. Moreover, the study by Pinter et al. (2020) showed that depending on the substance set and cell line, many mammalian assays were considered lacking in terms of specificity, possibly leading to an increase of false positive results.

Table 8: Adapted from Pinter et al. (2020). Overview of important *in vitro* genotoxicity assays and their toxicological sensitivity and specificity, for a certain set of compounds. Values indicated with □ have a high (> 75 %), with □ a moderate (75 to 50 %), or with □ a low (< 50 %) toxicological sensitivity or specificity.

Endpoint	Assay	Test System	Toxicological Sensitivity	Specificity	Number of Compounds Tested	Source
Gene Mutations	Ames Test OECD No. 471	Salmonella	59%	60%	541	(Kirkland et al., 2005)
			90%	87%	283	(McCann and Ames, 1976)
	SOS-Chromo test	Salmonella	38%	81%	177	(Escobar et al., 2013)
	Rec-Assay	*Bacillus subtilis*	74%	62%	119	(Matthews et al., 2006)
		Escherichia coli	76%	62%	277	(Matthews et al., 2006)
	Mouse Lymphoma Assay (MLA) OECD No. 490	L5178Y	71%	44%	460	(Matthews et al., 2006)
		L5178Y	73%	39%	350	(Kirkland et al., 2005)
Clastogenicity	Chromosomal Aberration (CA) OECD No. 473	CHL [1]	69%	58%	255	(Matthews et al., 2006)
		HPBL [2]	51%	67%	123	(Matthews et al., 2006)
	Sister Chromatid Exchange (SCE) OECD No. 479	CHL and CHO [3]	68%	40%	438	(Matthews et al., 2006)
		HPBL and HF [4]	83%	35%	111	(Matthews et al., 2006)
	Comet Assay OECD No. 489 (for *in vivo*)	HepaRG [5]	44%	100%	16	(Le Hégarat et al., 2014)
		not indicated	88%	64%	95	(Anderson et al., 1998)
Clastogenicity and Aneugenicity	Micronucleus (MN) OECD No. 487	HepaRG	73%	80%	16	(Le Hégarat et al., 2014)
		CHO-k1	80%	88%	62	(Westerink et al., 2011)
		not given	79%	31%	115	(Kirkland et al., 2005)
		TK6 [6]	88%	87%	48	(Thougaard et al., 2014)
		HPBL [2]	79%	33%	38	(Matthews et al., 2006)
DNA-Damage Response	p53 CALUX®	U2OS [7]	82%	90%	60	(van der Linden et al., 2014)
	BlueScreen™ HC	TK6	80%	100%	60	(Hughes et al., 2012)
	GreenScreen™ HC	TK6	90%	96%	43	(Birrell et al., 2010)
		TK6	76%	88%	60	(van der Linden et al., 2014)
		TK6	67%	96%	71	(Hastwell et al., 2009)
	ToxTracker®	mES [8]	85%	79%	27	(Ates et al., 2016)
		mES	95%	94%	54	(Hendriks et al., 2016)

[1] CHL = Chinese hamster lung; [2] HPBL = human peripheral blood lymphocytes; [3] CHO = Chinese hamster ovary; [4] HF = human follicular lymphoma; [5] HepaRG = human hepatoma cell line; [6] TK6 = human lymphoblast thymidine kinase heterozygote; [7] U2OS = human bone osteosarcoma epithelial cell line; [8] mES = mouse embryonic stem cells.

In a next step a literature survey on the biological detection limits (LOBD) of a series of *in vitro* bioassays for genotoxicity assessment was conducted and the results were published in Pinter et al. (2020), partly in Schilter et al. (2019) and Rainer et al. (2018). A summary on the results on LOBDs for some assays is given in Table 9 and Table 10.

Table 9: Limit of biological detection (LOBD) for genotoxic substances that result in gene mutations. Results from a literature survey for commonly used *in vitro* assays for detecting gene mutations with the global concentration factor (GCF) of 10 taken into account for mammalian assays and a GCF of 40 for the Ames test. Adapted from Pinter et al. (2020).

Substance	MLA-*tk* [mg·L^{-1}]	MLA-*hprt* [mg·L^{-1}]	Ames [mg·L^{-1}]
Cyclophosphamide	0.1 (+) (Wangenheim and Bolcsfoldi, 1988)	0.1 (+) (Phillips et al., 1980)	0.02 (+) (Eliopoulos et al., 1995)
ENU (N-Ethyl nitrosourea)	-	0.04 * (Doak et al., 2007)	0.3 (-) (Zeiger et al., 1992)
Methyl Methanosulphonate	0.6 (Clive et al., 1979)	0.6 * (Couch et al., 1978)	0.2 (-) (Kenyon et al., 2007)
Benzo-α-Pyrene	0.1 (+) (Wangenheim and Bolcsfoldi, 1988)	0.01 (+) (Oberly et al., 1993)	0.005 (+) (Kenyon et al., 2007)
7,12-Dimethylbenzanthracene	0.05 (+) (Wangenheim and Bolcsfoldi, 1988)	0.1 (+) (Thompson et al., 1983)	0.2 (+) (Kaden et al., 1979)
2-Acetylaminofluorene	4 (+) (Preisler, 2000)	5 (+) (Oberly et al., 1993)	0.003 (+) (Kenyon et al., 2007)
2,4-Diaminotoluene	20 (Coppinger et al., 1984)	80 (+) (Coppinger et al., 1984)	0.2 (+) (Ames et al., 1975)
Dimethyl Nitrosamine	1 (+) (Clive et al., 1979)	5 (+) (O'Neill et al., 1982)	0.2 (+) (Hakura et al., 2003)
Aflatoxin B1	0.001 (+) (Preisler, 2000)	0.008 (+) (Thompson et al., 1983)	0.00004 (+) (Kenyon et al., 2007)
p-Chloroaniline—free base and HCl salt	19 (-) (Wangenheim and Bolcsfoldi, 1988)	-	3 (+) (Zeiger, 1990)
Cisplatin	-	0.03 (-) (Singh and Gupta, 1983)	0.009 (-) (Zeiger et al., 1992)

(+): value obtained with S9 addition; (-): value obtained without S9; *: no information given whether an exogenous system was used; -: no data was found for a substance with the respective assay.

Table 10: Limit of biological detection (LOBD) for some aneugenic and clastogenic substances. Results from a literature survey for the most commonly used mammalian *in vitro* assays, which cover these endpoints. A global concentration factor (GCF) of 10 was taken into account to normalize the data. Adapted from Pinter et al. (2020).

Substance	p53 CALUX® [mg·L^{-1}] (van der Linden et al., 2014)	BlueScreen™ HC [mg·L^{-1}] (Hughes et al., 2012)	Micronucleus [mg·L^{-1}]	Comet [mg·L^{-1}]
4-Nitroquinoline Oxide	-	0.01 (-)	0.01 (-) (Bryce et al., 2011)	0.001 (-) (Speit and Hartmann, 1995)
Cadmium Chloride	Negative (+/-)	Negative (+/-)	0.0006 (-) (Bryce et al., 2011)	4 (-) (Fotakis et al., 2005)
Etoposide	0.6 (-)	0.01 (-)	0.002 (-) (Bryce et al., 2008)	1 (-) (Lebailly et al., 1997)
Hydroquinone	1 (-)	0.04 (-)	Negative (+/-) (Westerink et al., 2011)	0.05 (-) (Andreoli et al., 1997)
Taxol	0.03 (+/-)	0.003 (-)	0.008 (-) (Bryce et al., 2011)	0.9 (-) (Branham et al., 2004)
Azidothymidine	Negative(+/-)	Negative (+/-)	3 (+) (Westerink et al., 2011)	50 (-) (Zeller et al., 2013)
5-Fluoruracil	-	-	-	0.07 (-) (Kasamatsu et al., 1996)
Sodium Arsenite	0.001 (-)	0.06 (-)	0.01 (-) (Westerink et al., 2011)	3 * (Hartmann and Speit, 1996)
Methyl Nitrosurea	-	-	0.0008 (-) (Bryce et al., 2011)	10 (-) (Kasamatsu et al., 1996)
Doxorubicin	-	-	0.005 (-) (Westerink et al., 2011)	0.07 (-) (Anderson et al., 1997)
Chloramphenicol	32+	Negative+/-	0.3 (+) (Westerink et al., 2011)	-
Bleomycin	-	-	0.5 (-) (Kawaguchi et al., 2010)	1 (-) (Kasamatsu et al., 1996)

(+): value obtained with S9 addition; (-): value obtained without S9; (+/-): value obtained both with and without S9; *: no information given whether an exogenous system was used; -: no data was found for a substance with the respective assay.

The results in Table 9 and Table 10 show a summary of some of the LOBDs found for a series of substances from the ECVAM list (Kirkland et al., 2016). When compared to the proposed thresholds, the survey showed that these were not feasible for most of the substances for mammalian and bacterial assays (Pinter et al., 2020). Table 9 shows that only 11 % of the chosen substances could be detected below the threshold for the MLA-*tk* and 22 % were detectable with the MLA-*hprt*. The Ames test showed to be the most suitable for these substances as it was able to detect 36 % below the threshold of 0.01 mg mL^{-1}. In a more elaborative literature survey focusing on the Ames test it was found that with a broader set the assay could detect 50 % of certain genotoxins below the threshold, but only 10 % of a series of packaging related genotoxic substances (Rainer et al., 2018). Table 10 deals with aneugenic substances, where the Cramer Class III threshold of 0.09 mg mL^{-1} applies. The mammalian assays presented there were able to detect 29 % (p53 CALUX®), 36 % (comet assay), or 63 % (BlueScreen™ HC or

MN) below the threshold. Therefore, in the further progress of this thesis emphasis was put on optimisation experiments and on the development of an assay with sufficient LOBD values to manage these thresholds. The same conclusion was made in Rainer et al. (2018), where the Ames test was looked into more detail and the necessity for the following optimisation experiments in this thesis were determined.

Finally, a comprehensive literature search was conducted to gain information on studies performed with FCM with *in vitro* bioassays. The results were published in the publication by Pinter et al. (2020), where a focus was laid on mammalian assays. A summary of the results is shown in the appendix in Table A 1. The results showed that a variety of protocols were used for sample preparation and the global concentration factor (GCF) was determined to compare the preparation methods. Further, a majority of the studies also performed chemical analysis using GC-MS, LC-MS or HPLC to verify their results and to obtain more information on the samples.

Moreover, in the study published by Pinter et al. (2020) it was found that several genotoxic compounds could be found in the samples through GC-MS analysis. For example, in a study with recycled papers these ranged in concentration from 0.026 mg kg^{-1} for benzophenone to 12 mg kg^{-1} for Michler's ketone. However, these genotoxic substances did not lead to any positive response in the applied comet assay. This is why it is necessary to consider LOBD values, when performing such studies, since Pinter et al. (2020) found that Michler's ketone for example has an LOBD of 31 mg kg^{-1} in the Ames test and would not be detectable there and a similar result is assumed for the applied comet assay. Only few of the studies obtained a positive result, such as by Biscardi et al. (2003), but they concluded that the response was most likely due to a contamination in the distribution of the mineral water used for the testing and the PET bottles themselves have not caused this response. A study by Rosenmai et al. (2017) found a positive response with the p53 CALUX® when analysing a pizza box or a paperboard with printing ink.

In the publication by Rainer et al. (2018) the focus was on the Ames assay and the results are shown in the appendix in Table A 2. There similar conclusions were made to Pinter et al. (2020) and it was found that the LOBD values of the Ames assay and possible matrix effects were rarely discussed in the studies, which conducted FCM analysis with the Ames test. Further, some of the studies found positive results with the Ames test, but identification of the substances through chemical analysis was mostly missing.

Overall, both Pinter et al. (2020) and Rainer et al. (2018) concluded that the result of such studies is greatly affected by the different migration conditions, simulants, sample concentration methods and bioassay applied. Therefore, a comparison of the result is difficult and a standardised method for testing would be advisable.

4.2.Results: Reporter Gene Assay

The results for the HepG2 and HCT116 luciferase reporter gene assay are shown in the following chapters. The optimisation results of the HepG2 cells were taken into account for the conducting of the validation experiments and the sample testing. For all optimisation experiments, the direct acting genotoxic substances 4NQO and BαP were used. BαP was taken into account, as it is a substance that has to be metabolised to reach its genotoxic potential. This is why all effects were tested for genotoxicity where no metabolisation is required and for those substances, where metabolisation is necessary.

4.2.1. HCT116 Experiments

The aim of these experiments was to determine, whether the cell line HCT116 was a suitable candidate to detect a set of genotoxic substances at low concentrations. Especially, the cells ability to detect genotoxins, which need to be activated through a metabolic system was of interest. As no external source of metabolic activity, such as S9 mix, was added, the cells ability to metabolize certain substances, such as BαP could be seen.

In Figure 10, the results for the experiments with the HCT116 reporter gene cells with the substances 4NQO, BαP and MMS are shown. Figure 10A and B show the nano-luciferase (Nluc) induction and the corresponding viability of the cell line with 4NQO which follows a dose-response curve with stable viability. The same could be observed for MMS in the graphs in Figure 10E and F. For BαP, which requires metabolic activation to unfold its genotoxic potential, the results in Figure 10C for Nluc induction show that the substance did not lead to a genotoxic response in the cell line. The viability in Figure 10D for BαP, measured with resazurin is stable and therefore leading to the conclusion that no cytotoxic substances are present.

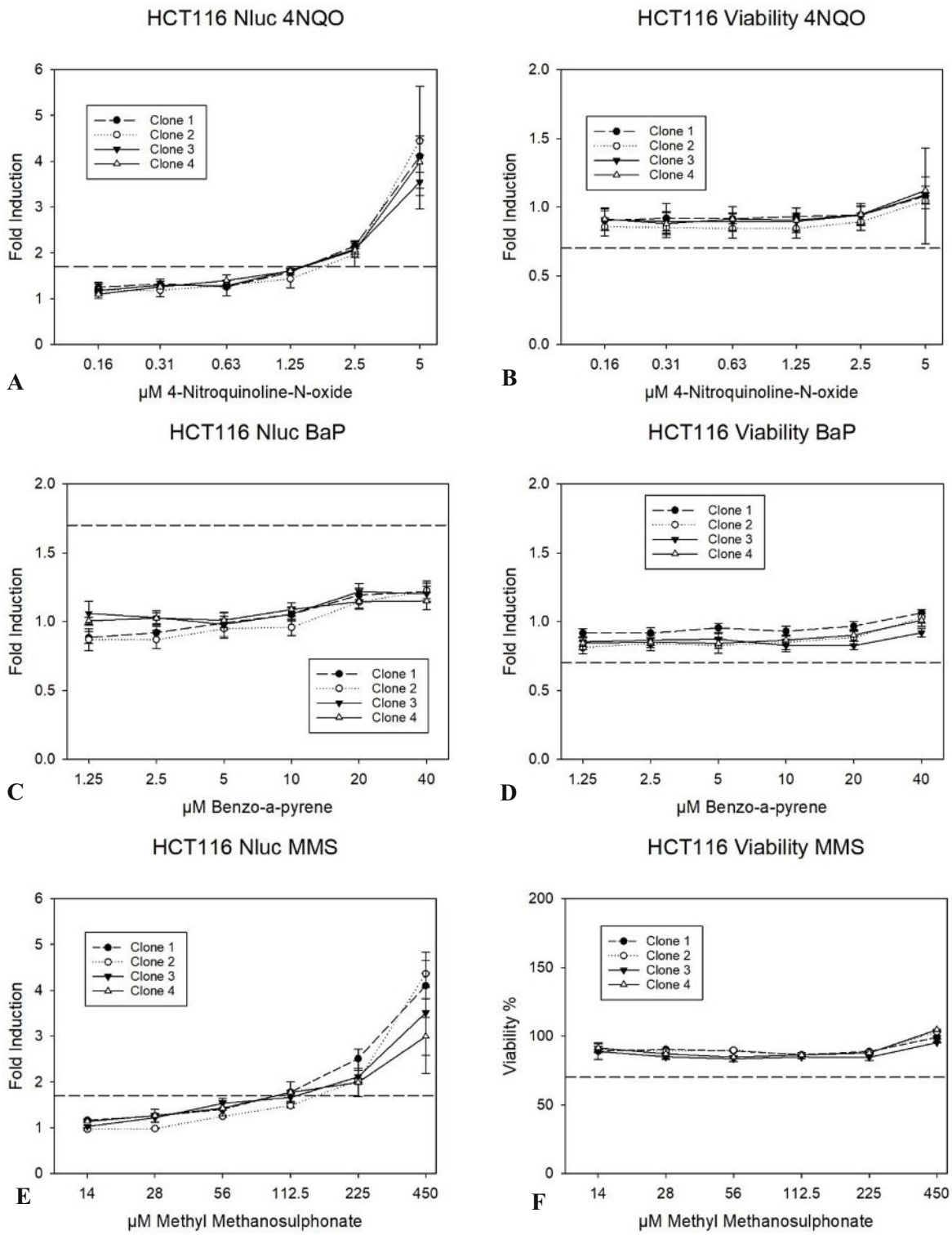


Figure 10: HCT116 reporter gene cells treated with 4NQO (A, B), BαP (C, D) and MMS (E, F). Nluc measurement shows a dose-response for 4NQO (A) and MMS (E) and no response for BαP (C). Viability was stable for all substances measured with resazurin (B, D and F). The fold induction was determined by comparing the Nluc or viability response to the vehicle control.

4.2.2. HepG2: Optimisation

Some of the optimisation experiments were conducted together with Christina Friedl and an excerpt will be published in her master thesis (*manuscript in preparation:* Friedl, 2021). Further, the results will be published in a publication by Pinter et al. (*manuscript in preparation:* 2021).

Cell Concentration Variation

Different concentrations of HepG2 cells were seeded: $1*10^4$ cells/well, $2*10^4$ cells/well, $4*10^4$ cells/well, $6*10^4$ cells/well, $8*10^4$ cells/well and $1*10^5$ cells/well. The genotoxins 4NQO and BαP were used to determine any effect of the cell concentration on the LOBD. The results are shown in Figure 11A-D for Nluc measurement (A, C) and resazurin viability measurements (B, D). For Nluc measurement, the first concentration with a signal above a threshold of 1.7 compared to the background signal was considered as the LOBD. In Figure 11 it can be seen that the lowest cell concentration ($1*10^4$ cells/well) lead to the lowest LOBDs, which are desired. However, the viability at this concentration was lacking upon higher concentrations for the substances 4NQO. This is why the concentration of $2*10^4$ cells/well was agreed upon as a better approach, since here the LOBD was similarly low and the viability was more stable.

The increase in viability upon higher BαP concentration as seen in Figure 11D could be because resazurin measures the metabolisation activity of the HepG2 cells. As BαP is a substance requiring metabolisation this might already activate the cells metabolism and therefore leading to an increased metabolisation of resazurin to resorufin. This can mimic an improved viability and therefore this increase in cell viability has to be approached with caution.

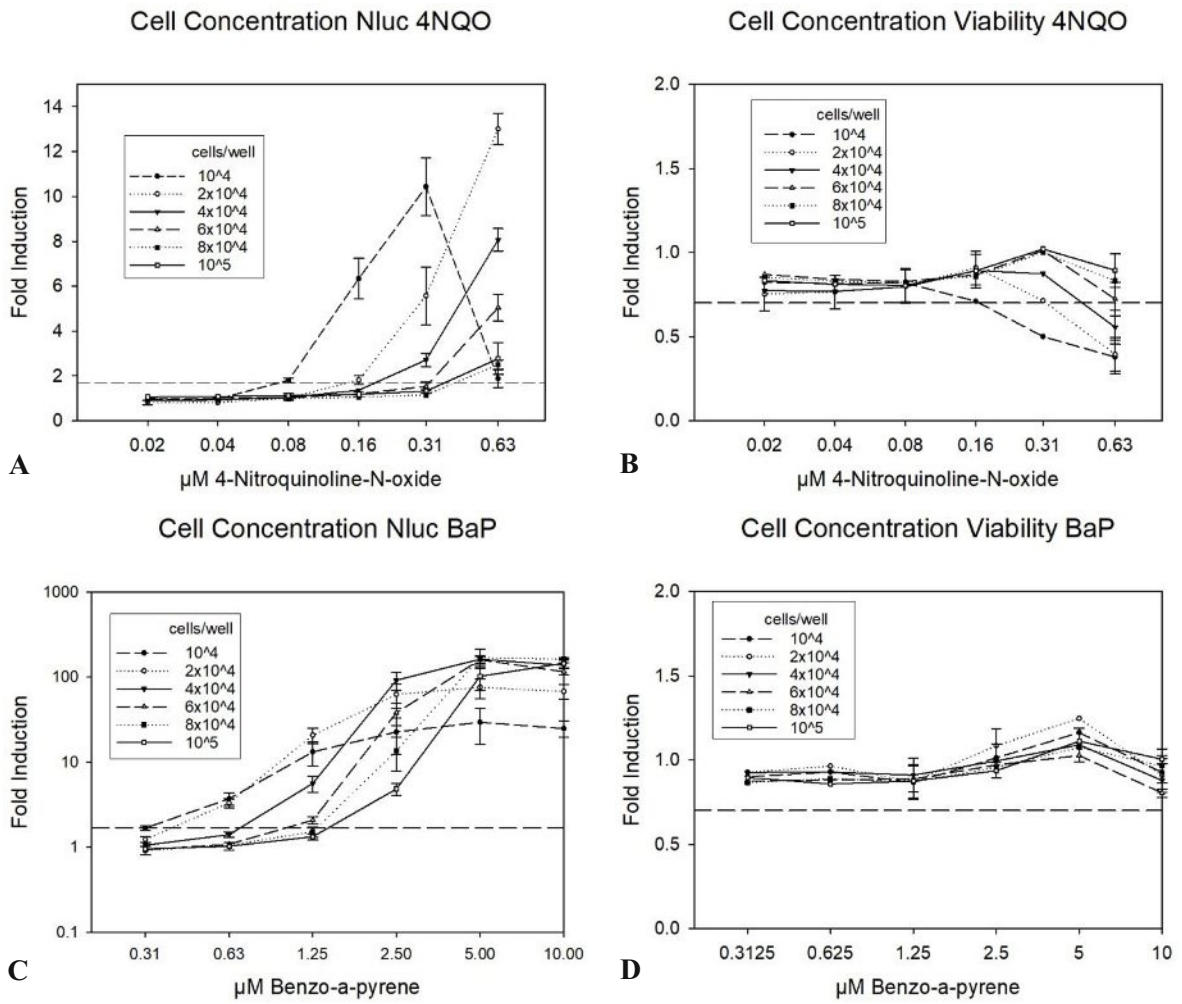


Figure 11: Different cell concentrations 1*10^4, 2*10^4, 4*10^4, 6*10^4, 8*10^4, 1*10^5 cells/well treated with 4NQO (A, B) and BαP (C, D). The Nluc signal for the experiments is given and the fold induction was compared to a threshold of 1.7 above the background value. Resazurin measurements for viability are shown with a threshold of 0.7, where more than 70 % compared to the background can be considered as viable.

Incubation Time Variation

Different incubation times were applied after sample treatment and between Nluc and viability measurement. The following incubation times were used: 2, 6, 24, 48 and 72 h. As can be seen in Figure 12A-D the cells were treated with 4NQO (A, B) and BαP (C, D) and the luciferase signal as well as the viability were measured. For 4NQO, a signal was obtained after 6 and 24 h with the lowest LOBD after 6 h. The viability seemed to be more stable for shorter incubation times (see Figure 12B). For BαP, where metabolisation is necessary, an Nluc signal was obtained after 24 and 48 h, with a decrease in viability after 48 and 72 h, as can be seen in Figure 12D. This led to the conclusion that 24 h incubation is the most appropriate as here both substances requiring metabolic activation and those without can be measured.

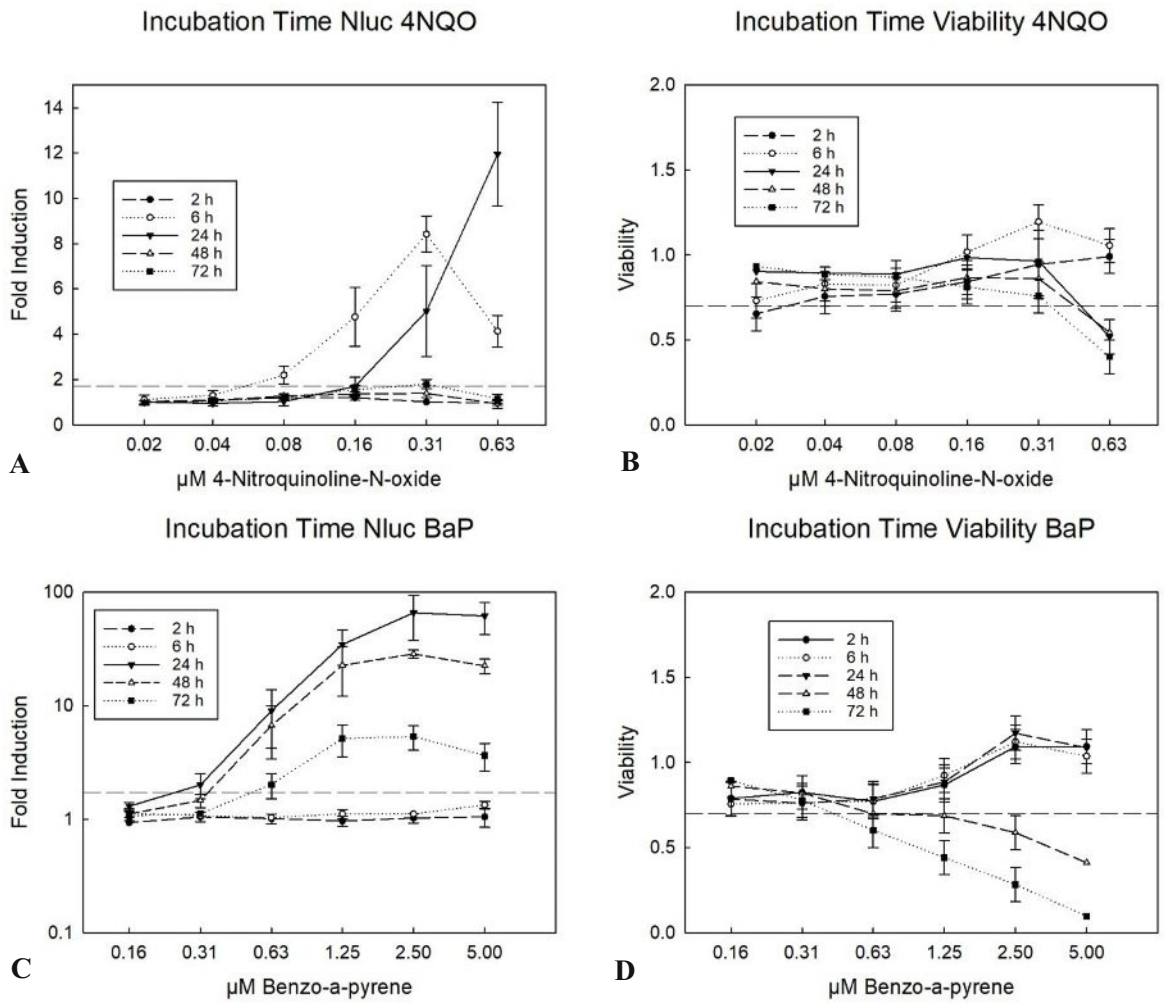


Figure 12: Incubation time variation with the substances 4NQO (A, B) and BαP (C, D). Cells were treated for 2, 6, 24, 48 and 72 h with the genotoxic substances and Nluc and resazurin were measured to determine p53 induction and viability. Nluc induction was considered positive above a threshold of 1.7 compared to the background signal. For viability, measurement with resazurin a cut-off of 0.7 was considered to be the threshold for cytotoxicity.

Media Composition Variation 1

Different media compositions were analysed to determine the effect on the LOBD values. For this, DMEM and DMEM supplemented with 10 % FBS was tested with and without the presence of 10 µg mL^{-1} S9 (for 24 h). The pure substance BαP was used to gain information, whether the presence or absence of proteins, in this case FBS, might lead to a binding of the substance and therefore an increase of the LOBD value. The results in Figure 13 show that the Nluc signal was stronger, when DMEM supplemented with FBS was used, in comparison to a complete lack of FBS. There it is visible, that when the HepG2 cells were treated with DMEM and FBS with or without S9, an LOBD value of 2.5 µM of BαP could be obtained. While without the addition of FBS, the obtained LOBD values with and without S9 were a factor of four higher at 10 µM. When looking at the viability in Figure 13, it can be seen that the cells were viable in all different types of media and sufficient nutrition were available for growth and maintaining

cell functionality. Further, the results show that the presence or absence of S9 did not affect the LOBD value for BαP in either medium composition.

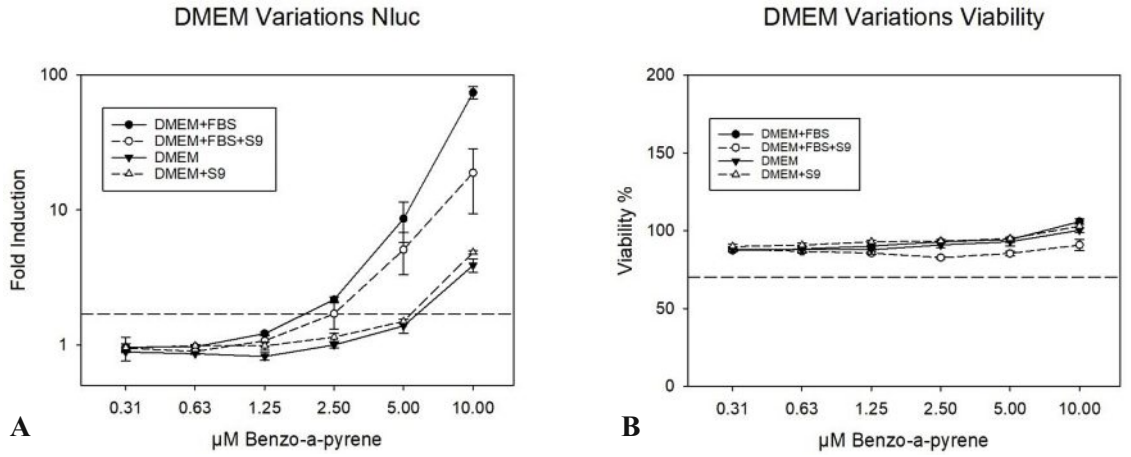


Figure 13: Variation of DMEM composition with and without FBS and/or S9. HepG2 cells were incubated with DMEM with and without the supplementation of 10 % FBS or 10 µg mL^{-1} S9 for 24 h. BαP was used as a pure substance and the fold induction could be determined as comparison of Nluc signal to the respective background. A threshold of 1.7 is used to determine the LOBD values (A). The viability was determined using resazurin and a cut-off of 70 % was used for toxicity (B).

Media Composition Variation 2

To determine whether DMEM or DPBS is a suitable medium, experiments were conducted with the two media with and without the supplementation of 10 % FBS or 10 µg mL^{-1} S9 for 24 h. 2.5 µM of 4NQO was used as a spike to determine if the different media have a visible effect on the obtained LOBD values. The results in Figure 14 show that the use of DPBS without S9 led to a very low viability of the cells (< 70 %) and the genotoxin 4NQO could not be detected. However, the viability was more stable upon the addition of S9 mix. This might be due to the enzymes and co-factors present in the S9 mix. When DPBS supplemented with FBS was used, the viability increased, but was still lower than the 70 % threshold. Again the viability increased in the presence of S9. Nevertheless, the signal intensity of 4NQO was greatly lowered compared to the results obtained with DMEM with and without FBS. The results for DMEM supplemented with 10 % FBS in Figure 14 show that the viability is stable both with and without S9 or the presence of a spike. The 4NQO signal is highest when using DMEM without FBS, however the viability was highly out of the expected range with 200 % increase compared to the background. In repetitive and preliminary experiments this was also the case. However, it was concluded that the high signal intensity is worth looking into more detail. Therefore, the presence of FBS was necessary to obtain a stable viability, but the amount of FBS could be reduced, possibly leading to a higher Nluc signal intensity. This is why consecutive FBS experiments were conducted in the following paragraph.

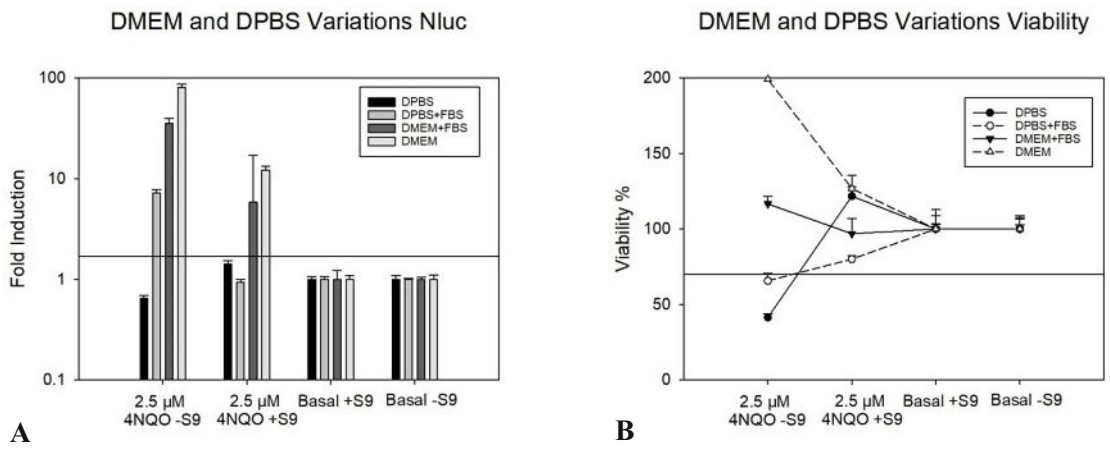


Figure 14: Variation of DMEM and PBS with and without FBS and/or S9. The HepG2 cells were treated with 4NQO in DPBS or DMEM with and without 10 % FBS. 2.5 µM of 4NQO was added and incubated for 24 h in the presence or absence of 10 µg mL^{-1} of S9. Nluc was measured and a threshold of 1.7 compared to the background signal was used to determine a positive result (A). For viability, resazurin was measured and the basal levels were used to compare the viability to a threshold of 70 % (B).

FBS Variation

The amount of FBS in the media during sample treatment was varied to the concentrations 5, 10 or 15 % FBS with the substances 4NQO and BαP. In a preliminary experiment, (data not shown) complete depletion of FBS led to a great loss of Nluc signal and decrease in viability, so that this was not further pursued. As shown in Figure 15A and B the variation of FBS concentration did not affect the LOBD greatly. However, a higher signal intensity for Nluc measurement was determined for 5 % FBS, when using BαP as can be seen in Figure 15B. The viability was slightly negatively affected by lowering the amount of FBS, when taking 4NQO into account, but this could not be seen for BαP. In the Figure 15A and B the results are shown in the range of the LOBD concentration for better comparability. For this, a 4NQO concentration of 0.16 µM and for BαP a concentration of 0.62 µM was chosen.

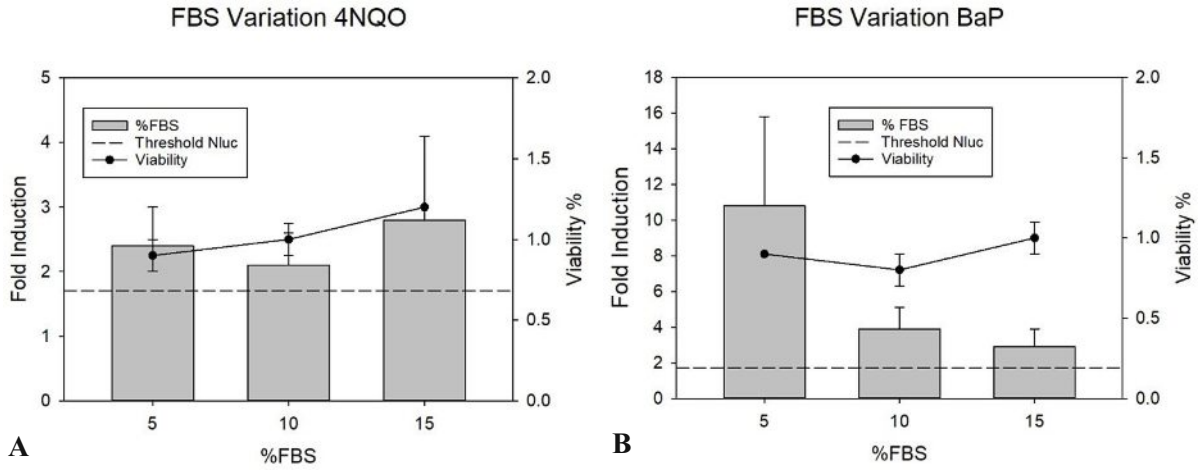


Figure 15: Variation of FBS in the media during sample treatment with the concentrations 5, 10 and 15 % FBS. The cells were treated with the substances 4NQO (A) and BαP (B) and the Nluc signal and resazurin were measured. For 4NQO, a concentration of 0.16 µM and for BaP a concentration of 0.62 µM is shown as representative. A threshold of 1.7 compared to the background control was used for p53 activation. For viability, a cut-off of 0.7 was taken into account when assessing for cytotoxicity.

DMSO Variation

In a preliminary experiment (data not shown) a quenching of the signal with increased DMSO concentration was found. To determine the effect of DMSO on the Nluc signal intensity and the viability different concentrations of DMSO were added during sample treatment, namely 0.25, 0.50, 0.75, 1.00, 1.50 and 2.00 % DMSO in the well. A respective vehicle control of 0.25, 0.50, 0.75, 1.00, 1.50 and 2.00 % DMSO was used to compare the results. The results in Figure 16A and B show that an increase in DMSO concentration led to a decrease of signal intensity for Nluc induction. For comparability, a 4NQO concentration of 0.16 µM and a BαP concentration of 0.63 µM was chosen to display the signal intensity in the range of the LOBD for these substances in detail. The experiments show that the quenching of the signal might lead to an undesired increase of the LOBD. Especially upon BαP addition (see Figure 16B) the signal intensity greatly decreased and therefore possibly increasing the LOBD, but the viability was not affected by higher DMSO concentration. However, the application of as much sample as possible is a necessity for FCM testing and therefore the highest DMSO concentration, where the impact on the signal is minimal and the viability is most stable is required. This was considered to be with 1 % DMSO as here the Nluc signal and the LOBD were still considered appropriate and the viability was seen as stable.

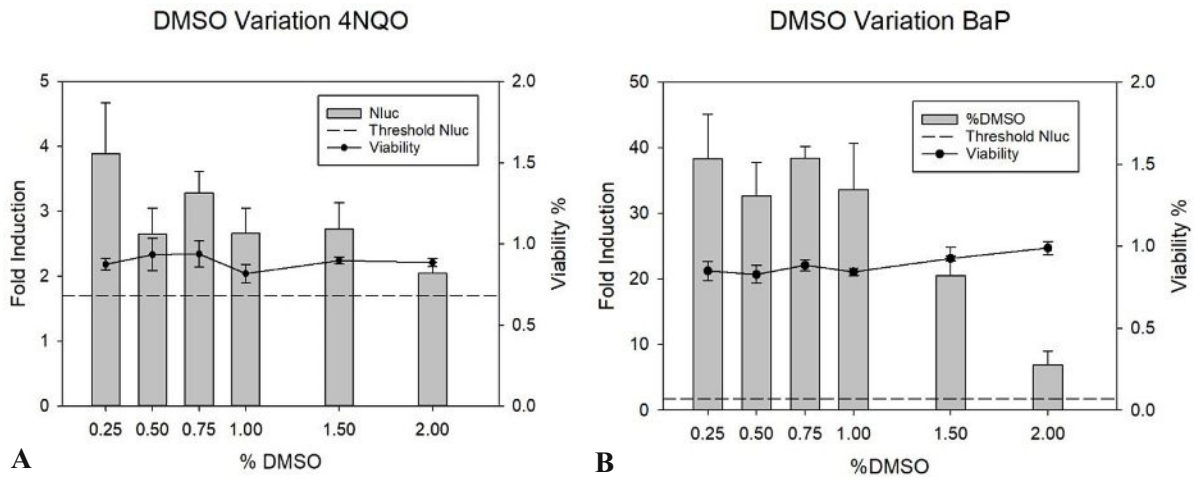


Figure 16: Variation of DMSO during sample treatment with 0.25, 0.50, 0.75, 1.00, 1.50 and 2.00 % DMSO in the well. Nluc signal intensity was compared to a threshold of 1.7 compared to the background control and a cut-off of 0.7 was taken into account for viability. The cells were treated with the substances 4NQO (A) and BαP (B) for 24 h. For better comparability, only the concentration 0.16 µM for 4NQO and 0.63 for BαP are shown, which are in the range of the LOBD value.

S9 Protocol Variation

Different S9 protocols were applied to determine whether the addition of an exogenous metabolisation system affected the toxicological and analytical sensitivity. Two different S9 protocols (incubation for 3 h with 330 µg mL^{-1} or 24 h with 10 µg mL^{-1}) were compared to the treatment without S9 addition. The results for the substances 2AF, AFB1, BαP, cisplatin, cyclophosphamide, ENU, 2,4-DAT and etoposide are shown in Table 11 for Nluc and viability measurement. The substance 2AF was negative irrelevant, whether an exogenous system was used or no external metabolisation occurred. For the substance AFB1, the LOBD could be lowered upon addition of 330 µg mL^{-1} for 3 h from 0.20 to 0.10 µg mL^{-1}. In none of the experiments, the addition of 10 µg mL^{-1} S9 for 24 h lead to an improvement of the LOBD, as all substances were negative with this protocol. The viability was considered to be slightly affected by the exogenous system with a decrease in stability when 330 µg mL^{-1} S9 extract was added for 3 h as can be seen regarding the viability values at the LOBD or at the highest concentration a substance was added, if no LOBD was obtained in Table 11.

For the substance cyclophosphamide, the addition of 330 µg mL^{-1} S9 extract for 3 h lead to the generation of a positive signal, which would have to be considered negative without S9 addition. The other substances ENU and etoposide experienced an undesired decrease for the LOBD when S9 was added, leading to superior results without the application of an exogenous system. Solely the substances cisplatin and 2,4-DAT were negative upon S9 addition, but positive without any exogenous system.

Based on these results, the decision was made to perform sample testing without the addition of an exogenous system, as this did not lead to an improvement of the LOBD for most substances and even led to a decrease or negative result for some other substances. Since S9 extract, is a costly and controversial substance the goal was to use as little as possible in the experiments, so that the decision to omit S9 in the following experiments was reassured.

Table 11: Results of a series of known genotoxic substances with different S9 protocols. The HepG2 cells were treated with the substance without S9, with 330 µg mL^{-1} S9 for 3 h or with 10 µg mL^{-1} S9 for 24 h. The Nluc induction was measured and compared to the background to determine the fold induction. The LOBD was the first concentration above a threshold of 1.7 compared to the background. Viability was determined with luciferase measurement and compared to untreated HepG2 cells as 100 %.

Substance	S9 Protocol	LOBD Result [µg mL^{-1}]	Viability for LOBD value or highest concentration
2-Acetylaminofluorene	No S9	Negative	80 %
	3 h S9	Negative	70 %
	24 h S9	Negative	60 %
Aflatoxin B1	No S9	0.20	90 %
	3 h S9	0.10	60 %
	24 h S9	Negative	70 %
Benzo-α-pyrene	No S9	0.16	100 %
	3 h S9	0.32	60 %
	24 h S9	Negative	80 %
Cisplatin	No S9	0.38	90 %
	3 h S9	Negative	70 %
	24 h S9	Negative	90 %
Cyclophosphamide	No S9	Negative	50 %
	3 h S9	175	90 %
	24 h S9	Negative	70 %
2,4-Diaminotoluene	No S9	300	100 %
	3 h S9	Negative	30 %
	24 h S9	Negative	30 %
N-Ethyl-Nitrosourea	No S9	75	90 %
	3 h S9	75	110 %
	24 h S9	Negative	100 %
Etoposide	No S9	1.50	60 %
	3 h S9	Negative	100 %
	24 h S9	Negative	60 %

4.2.3. Validation

Some of the experiments for the validation were conducted together with Christina Friedl and an excerpt will be shown in her master thesis (*manuscript in preparation:* Friedl, 2021). Further, the results will be published in a publication by Pinter et al. (*manuscript in preparation* 2021).

For validation, a series of pure substances was tested and the LOBD values were determined. For this experiment, the results from the optimisation experiments were taken into account and $2*10^4$ cells/well were seeded and sample treatment was conducted for 24 h with 5 % FBS in the media and 1 % DMSO sample concentration. The results for the validation with 16 known genotoxic substances, 11 known non-genotoxic substances, 7 known non-genotoxic substances that give positive results in mammalian *in vitro* assays and 3 packaging specific potentially genotoxic substances are shown in Table 12, Table 13 and Table 14. The LOBD values range from 183 µg mL^{-1} for 2,4-DAT to 0.03 µg mL^{-1} for doxorubicin. The substances 2-AF and Colchicine, which are known true genotoxic substances, were negative, as they did not reach a signal above the threshold of 1.7 Nluc fold induction, in comparison to the background signal. All non-genotoxic substances (see Table 14) were negative, as their Nluc signal was below the threshold, with the exception of tBHQ. This substance was positive with an LOBD of 10 µg mL^{-1}, however, no dose-response curve could be obtained for tBHQ. Further, some experiments did not lead to a positive signal for tBHQ so that it should be considered as "equivocal" and results for tBHQ have to be handled with caution (see Table 12). The packaging relevant substance BaA was negative and its Nluc signal was below the threshold of 1.7. The results for the other packaging relevant substances ranged between 73 µg mL^{-1} for pPD and 4 µg mL^{-1} for TGIC as can be seen in Table 13.

Table 12: Result for the validation of the HepG2 reporter gene assay for known genotoxic substances. The lowest concentration, where a positive response above a threshold of 1.7 Nluc induction was found is given as LOBD in µg mL^{-1}. For substances where the signal intensity did not reach the threshold compared to the background, it is indicated as negative.

	Substance	CAS-Number	Solvent	LOBD [µg mL^{-1}]
Known *in vitro* and *in vivo* genotoxic substances	Cyclophosphamide	6055-19-2	DMSO	88
	N-Ethyl-Nitrosurea	759-73-9	DMSO	73
	Methyl Methanosulphonate	66-27-3	H2O	69
	Benzo-a-pyrene	50-32-8	DMSO	0.2
	7,12-Dimethylbenzanthracene	57-97-6	DMSO	0.4
	2-Acetylaminofluorene	53-96-3	DMSO	Negative
	2,4-Diaminotoluene	95-80-7	DMSO	183
	Aflatoxin B1	1162-65-8	DMSO	0.2
	Cisplatin	15663-27-1	DMSO	0.2
	Sodium Arsenite	7784-46-5	H2O	13
	Etoposide	33419-45-0	DMSO	0.8
	4-Nitroquinoline-N-oxide	56-57-5	DMSO	0.04
	Colchicine	64-86-8	DMSO	Negative
	Mitomycin C	50-07-7	DMSO	0.1
	Actinomycin D	50-76-0	DMSO	1.6
	Doxorubicin	23214-92-8	DMSO	0.03

Table 13: Result for the validation of the HepG2 reporter gene assay for packaging specific substances, which are considered to be mutagenic. The lowest concentration where a positive response above a threshold of 1.7 Nluc induction was found is given as LOBD in µg mL^{-1}. For substances where the signal intensity did not reach the threshold compared to the background, it is indicated as negative.

	Substance	CAS-Number	Solvent	LOBD [µg mL^{-1}]
Packaging specific substances considered to be mutagenic in the Ames test	Benzo-a-anthracene	56-55-3	DMSO	Negative
	p-Phenylenediamine	106-50-3	DMSO	8
	Triglycidylisocyanurat	2451-62-9	DMSO	1.2

Table 14: Result for the validation of the HepG2 reporter gene assay for non-genotoxic substances. The lowest concentration where a positive response above a threshold of 1.7 Nluc induction was found is given as LOBD in µg mL^{-1}. For substances, where the signal intensity did not reach the threshold compared to the background, it is indicated as negative.

	Substance	CAS-Number	Solvent	LOBD [µg mL^{-1}]
Known non-genotoxic substances	Ampicillin trihydrate	7177-48-2	H2O	Negative
	d-Mannitol	69-65-8	DMSO	Negative
	Phenformin HCl	834-28-6	DMSO	Negative
	(2-Chloroethyl)trimethyl-ammonium chloride	999-81-5	DMSO	Negative
	Amitrole	61-82-5	DMSO	Negative
	Diethanolamine	111-42-2	DMSO	Negative
	Melamine	108-78-1	DMSO	Negative
	Methyl carbamate	598-55-0	DMSO	Negative
	Pyridine	110-86-1	DMSO	Negative
	Tris(2-ethylhexyl)phosphate	78-42-2	96 % Ethanol	Negative
	Hexachloroethane	67-72-1	DMSO	Negative
In vivo negative, sometimes *in vitro* positive	D,L-Menthol	15356-70-4	DMSO	Negative
	2-Ethyl-1,3-Hexanediol	94-96-2	DMSO	Negative
	Sulfisoxazole	127-69-5	DMSO	Negative
	Urea	57-13-6	DMSO	Negative
	Sodium Saccharin	128-44-9	DMSO	Negative
	Eugenol	97-53-0	DMSO	Negative
	Tert-butylhydroquinone	1948-33-0	DMSO	10 (Equivocal)

With the results in Table 12 and Table 14, a toxicological sensitivity and specificity could be determined. For the toxicological sensitivity, the amount of correctly identified known genotoxins is set in relation to the total amount of known genotoxic substances analysed. Therefore, with this substance set of 16 known genotoxins, two substances classified as false negative and this results in a toxicological sensitivity of 87.5 % (14/16 true-positive genotoxic substances). For the specificity, the correctly identified non-genotoxic substances in comparison to the total amount of non-genotoxins tested is determined. With one substance being false positive in the substance set of 18 non-genotoxic substances, this leads to a specificity of 94 % (17/18 true-negative non-genotoxic substances).

Comparison of LOBDs

Finally the results are compared to the LOBDs of commonly used *in vitro* bioassays, which were determined as part of the exhaustive literature survey (see 4.1). The results obtained in 4.2.3 shown in Table 12, Table 13 and Table 14 were compared with OECD approved assays and are given in Table 15, which is from the planned publication by Pinter et al. (*manuscript in preparation* 2021). There the known genotoxins, which were analysed with the HepG2 reporter gene assay were compared to commonly used assays, where an OECD guideline exists, namely the MN and the comet. From the 16 substances the HepG2 assay was able to detect 25 % of the substances at lower LOBDs than the compared assays. Especially for cisplatin the assay was 500 times more sensitive than the others. For 50 % of the substances, the assay was within a similar range as the others, where the LOBDs were within a biological deviation factor of two- to tenfold. Finally, for 19 % of the substances the assay was less analytically sensitive than the compared OECD approved assays. The remaining 6 % were negative with the assay, so that the LOBDs could not be compared.

Table 15: Comparison of the HepG2 p53 reporter gene assay to regulated and OECD approved (OECD, 2014b, 2014c) mammalian genotoxicity assays. Adapted from Pinter et al. (*manuscript in preparation* 2021).

Substance	HepG2p53 Assay [µg mL^{-1}]	Micronucleus [µg mL^{-1}]	Comet [µg mL^{-1}]
Cyclophosphamide	88	9 (-) (Westerink et al., 2011)	70 (+) (Hartmann et al., 1995)
N-Ethyl-nitrosourea	73	73 (-) (Le Hégarat et al., 2014)	250 (-) (Kawaguchi et al., 2010)
Methyl methanosulfonate	69	11 (-) (Westerink et al., 2011)	8 (-) (Pfuhler and Uwe Wolf, 1996)
Benzo-a-pyrene	0.2	3 (-) (Westerink et al., 2011)	1.3 (+) (Speit and Hartmann, 1995)
7,12-Dimethyl-benzanthracene	0.4	2 (-) (Le Hégarat et al., 2014)	0.3 (+) (Speit and Hartmann, 1995)
2-Acetylaminofluorene	Negative	58 (-) (Le Hégarat et al., 2014)	Negative (-) (Valentin-Severin et al., 2003)
2,4-Diaminotoluene	76	39 (-) (Westerink et al., 2011)	178 (-) (Séverin et al., 2005)
Aflatoxin B1	0.2	0.08 (-) (Le Hégarat et al., 2014)	9.4 (+) (Corcuera et al., 2011)
Cisplatin	0.2	95 (-) (Westerink et al., 2011)	Negative (-) (Pfuhler and Uwe Wolf, 1996)
Sodium arsenite	8	0.1 (-) (Westerink et al., 2011)	26* (Hartmann and Speit, 1996)
Etoposide	0.8	2 (-) (Westerink et al., 2011)	10 (-) (Lebailly et al., 1997)
4-Nitroquinoline-n-oxide	0.03	0.6 (-) (Le Hégarat et al., 2014)	0.01 (-) (Speit and Hartmann, 1995)
Colchicine	Negative	5 (-) (Parry et al., 1996)	N/A
Mitomycin C	0.1	N/A	Negative (-) (Henderson et al., 1998)
Actinomycin D	1.6	N/A	N/A
Doxorubicin	0.03	0.05 (-) (Westerink et al., 2011)	0.05 (-) (Anderson et al., 1997)

(+): value obtained with S9 addition; (-): value obtained without S9; *: no information given whether an exogenous metabolizing system was used to obtain the result; N/A: no LEC data was found in the literature for a substance with the respective assay

4.2.4. Applicability for Complex Mixtures

To determine any matrix effect hindering the assay and to assess whether the reporter gene assay is able to detect genotoxic responses in complex mixtures, sample testing was conducted. For these experiments, a variety of FCM sample migrates were used and added to the HepG2 reporter gene assay and the Nluc signal was determined. A genotoxic substance was added to the samples as a spike, to determine any matrix effect and to see whether a genotoxic response would be detectable in a complex mixture sample. Figure 17A and B (*manuscript in preparation* Pinter et al., 2021) show the results for sample testing with the addition of different genotoxic substances. The main aim was to determine whether cytotoxicity caused by the complex mixtures and their matrixes might lead to a false negative result when 4NQO or BαP was added. The results in Figure 17A and B show that the addition of a sample did not lead to a negative impact on the viability of the cells. In addition, the present of a complex mixture did not lead to a quenching of the signal, when BαP was added, as can be seen in Figure 17B. The addition of the sample matrix with 4NQO had a slight quenching effect and an impact on the LOBD as shown in Figure 17A. However, the quenching of the signal was minimal and the LOBD varied by one dilution step, which can be seen as biological deviation.

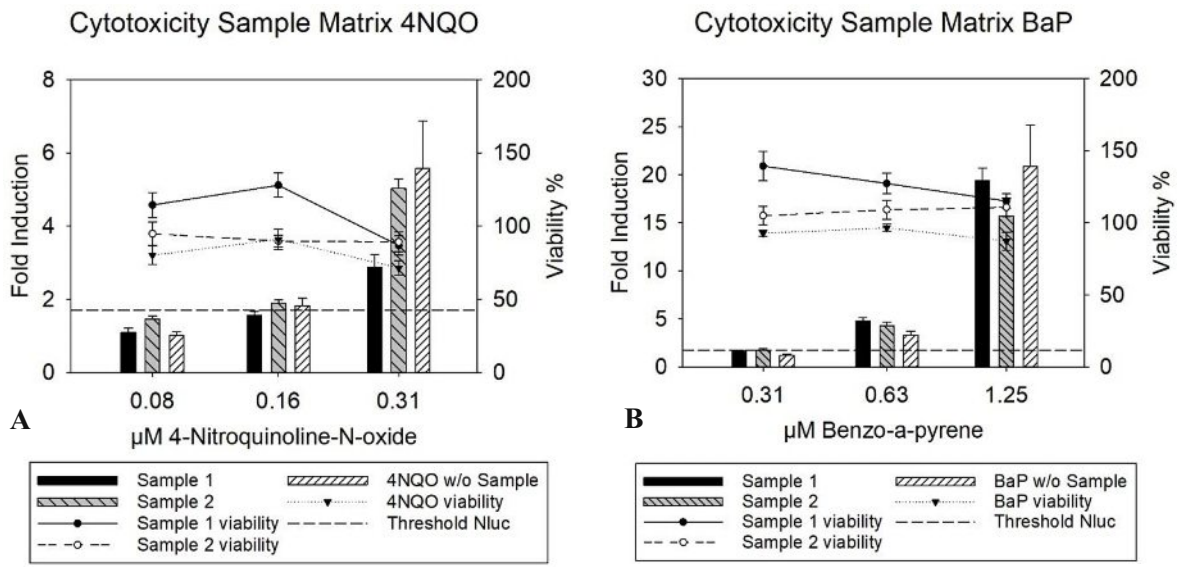


Figure 17: Result of sample testing with different FCM migrate samples with the HepG2 reporter gene assay. The samples were spiked with 4NQO (A) or BαP (B) and the Nluc intensity and the resazurin results are shown. A threshold of 1.7 fold induction compared to the background was used for assessing whether a sample was positive for Nluc. For viability, a cut-off of 0.7 compared to the background was used to determine any cytotoxic effects.

4.3.Results: qPCR

4.3.1. Gene Target Screening

For the determination of gene targets for qPCR analysis, the most relevant genes were identified by screening a database for marker genes associated for genotoxicity (screening conducted by Anja Friedrich, *manuscript in preparation*). The most relevant genes, GADD45α, p21 (CDKN1a), FDXR, DDB2, SERTAD1, TP53INP1, RRM2B and BTG2, were screened by conducting a qPCR and the result was considered positive above a threshold of two times the vehicle control.

In Figure 18 some exemplary results for the pure substances BαP, DMBA, ENU and MMC are shown, for the genes GADD45α, CDNK1a and the incorporated Nluc gene. Overall it can be seen that the Nluc signal corresponds well to those of the two genes associated with genotoxicity and the signal curve is very similar. For BαP shown in Figure 18A, the Nluc signal differs from that of GADD45α and CDNK1a. Nevertheless, a good induction can be observed, even if it is at a slightly higher concentration. The results in Figure 18 show a good concordance between the integrated Nluc gene and the expression of the “classical” genes associated with genotoxicity.

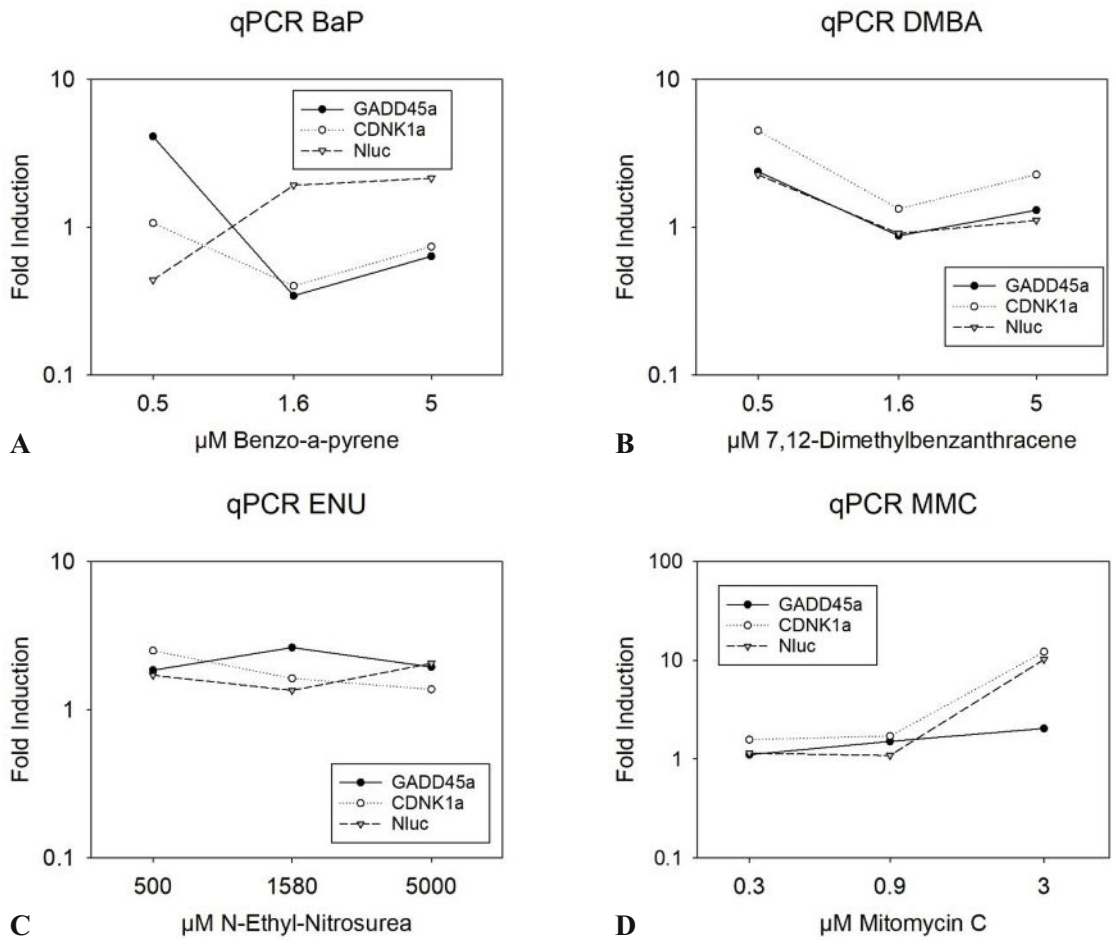


Figure 18: qPCR results for the genes GADD45α, CDNK1a and the incorporated Nluc with the substances BaP (A), DMBA (B), ENU (C) and MMC (D). The qPCR results were compared to the vehicle control consisting of 1 % DMSO. Beforehand, the gene concentration was determined through the concentration of the HKGs GAPDH and β-actin.

In Table 16 the qPCR results are shown for the respective genes and for comparison also the results for the reporter gene assay are given, which was an indicator for the p53 gene. The results show that some targets are more appropriate for correctly distinguishing genotoxic from non-genotoxic substances. The substance MMS proofed to be an issue for all qPCR targets as it was consistently below the threshold and had to be considered as negative. In terms of correctly identifying true positives the target gene SERTAD1 was the most effective, but was severely lacking for correctly detecting true negative substances. Other genes with a good predictability were CDKN1a and RRM2B, which could detect most true positives well. The other targets, GADD45α, FDXR, DDB2, TP53INP1 and BTG2, did not perform as well for correctly detecting true positive genotoxins.

For detecting true negatives as such, the target gene CDKN1a was the most promising, closely followed by FDXR for which only tBHQ proofed to be a problem. The other genes' predictability of true negatives was not as good and led to several false positive results.

By comparing the results from the qPCR target gene analysis with the result for the p53 gene in the reporter gene assay in Table 16, it is obvious that the reporter gene assay has a high predictivity. The p53 pathway response is both specific for correctly detecting true positives and true negatives, with only the substances 2-AF incorrectly classified as negative and tBHQ incorrectly as positive.

Table 16: qPCR results for the genotoxicity gene targets for some relevant genotoxic and non-genotoxic substances.

Substance		CAS Number	qPCR Gene Target								Reporter gene assay
			GADD45α	CDKN1a	FDXR	DDB2	SERTAD1	TP53INP1	RRM2B	BTG2	p53
Known *in vitro* and *in vivo* genotoxic substances	2-Aminofluorene	53-96-3	negative	positive	negative	negative	positive	negative	positive	positive	negative
	Aflatoxine B1	1162-65-8	positive	positive	positive	positive	positive	positive	positive	negative	positive
	Benzo-a-pyrene	50-32-8	negative	negative	positive	negative	positive	negative	negative	negative	positive
	Cisplatin	15663-27-1	positive	positive	positive	positive	positive	positive	positive	positive	positive
	7,12-Dimethylbenzanthracene	57-97-6	positive	positive	positive	positive	positive	positive	positive	positive	positive
	N-Ethyl-Nitrosourea	759-73-9	positive	positive	positive	negative	positive	positive	positive	positive	positive
	Etoposide	33419-45-0	negative	positive	negative	positive	positive	positive	positive	negative	positive
	Mitomycin C	50-07-7	positive	positive	positive	positive	positive	positive	positive	positive	positive
	Methyl Methanosulphonate	66-27-3	negative	negative	negative	negative	negative	negative	negative	negative	positive
Known non-geno-toxic substances	Ampicillin Trihydrate	7177-48-2	positive	negative	negative	negative	negative	negative	positive	negative	negative
	D-Mannitol	69-65-8	positive	negative	negative	negative	negative	positive	negative	negative	negative
	Phenformin HCl	834-28-6	positive	negative	negative	negative	positive	positive	negative	negative	negative
In vivo negative, sometime *in vitro* positive	Eugenol	97-53-0	negative	negative	negative	negative	positive	negative	negative	positive	negative
	D-Menthol	15356-70-4	negative	negative	negative	negative	negative	positive	negative	negative	negative
	Sulfisoxazole	127-69-5	negative	negative	negative	positive	positive	positive	positive	positive	negative
	Tert-butylhydroquinone	1948-33-0	positive	negative	positive	negative	positive	negative	negative	negative	positive

4.4.Results: High Content Screening (HCS)

With HCS measurement, the phosphorylation intensity of H2AX was determined for a series of pure substances. In Figure 19 exemplary results are shown of HepG2 cells treated with the genotoxic substance 4NQO. The images in Figure 19 show the Cy3 intensity (A) in red, DAPI measurements to determine cell nuclei (B) and Phase II results (C) for contrast measurement to prevent the interference of artefacts.

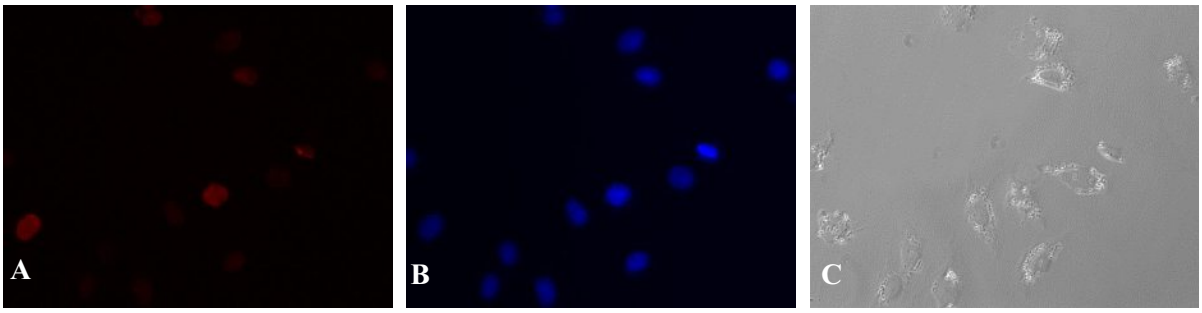


Figure 19: Exemplary HCS results for HepG2 cells treated with 4NQO. Cy3 measurements to determine the γH2AX induction (A), cell nuclei measured with DAPI (B) and contrast determined via Phase II (C) to distinguish cells from artefacts interferring with the signals.

4.4.1. Validation

In a first step the optimal dilution of the antibody solutions 1 and 2 (= AB1 and AB2) had to be determined. For this, the dilutions 1:1,000 or 1:2,000 of AB1 or AB2 were used. The results in Figure 20 show the different LOBDs, which were obtained for 4NQO with Cy3 measurement. It can be seen that there is only a slight difference in signal intensity, when comparing the different antibody dilution combinations to each other. As the difference in LOBD was considered to be negligible and the antibody solutions are costly, it was decided that a dilution of AB1 and AB2 of 1:2,000 was sufficient for the testing. Further, the viability in Figure 20 steadily decreases with increased 4NQO concentrations, as was expected.

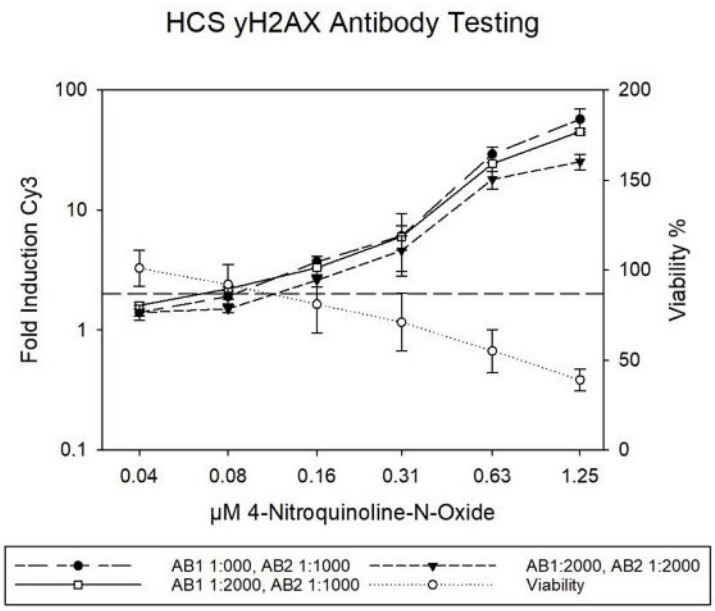


Figure 20: Determination of the optimal dilution of the antibody solutions 1 and 2. The pure substance 4NQO was used to determine the LOBD at different antibody concentrations. The fold induction of the Cy3 signal was obtainded through comparison with a 1 % DMSO vehicle control and a threshold of 2 was used for the LOBD determination. Viability was monitored through DAPI measurement as an overall result for the respective 4NQO concentration.

To determine the suitability of the target γH2AX, a series of known genotoxic substances and two substances, which are known to cause mutagenicity in the Ames test (Watanabe et al., 1990; Malaveille et al., 1975), were tested. The results for the measurement of the γH2AX intensity are shown in detail in Figure 21 and are summarised in Table 17. The results in Figure 21 clearly show a dose response for all pure substances, except for BaA (A), which gave a negative result. Further, the decrease in cell viability with increasing concentration of the respective genotoxins is clearly visible. Especially for 4NQO (Figure 21G) it can be seen that the decrease in viability leads to a decrease in Cy3 signal, when the concentration of 4NQO is increased further. This points out the importance of monitoring genotoxicity and cell viability together, as a decrease in cell viability could mask a genotoxic effect. The substances BαP and DMBA need the presence of a metabolising system to obtain positive results in a genotoxicity test (Kirkland et al., 2016). As can be seen in Figure 21B and D, it was possible to detect these substances through the use of HepG2 cells without the use of an external metabolising system.

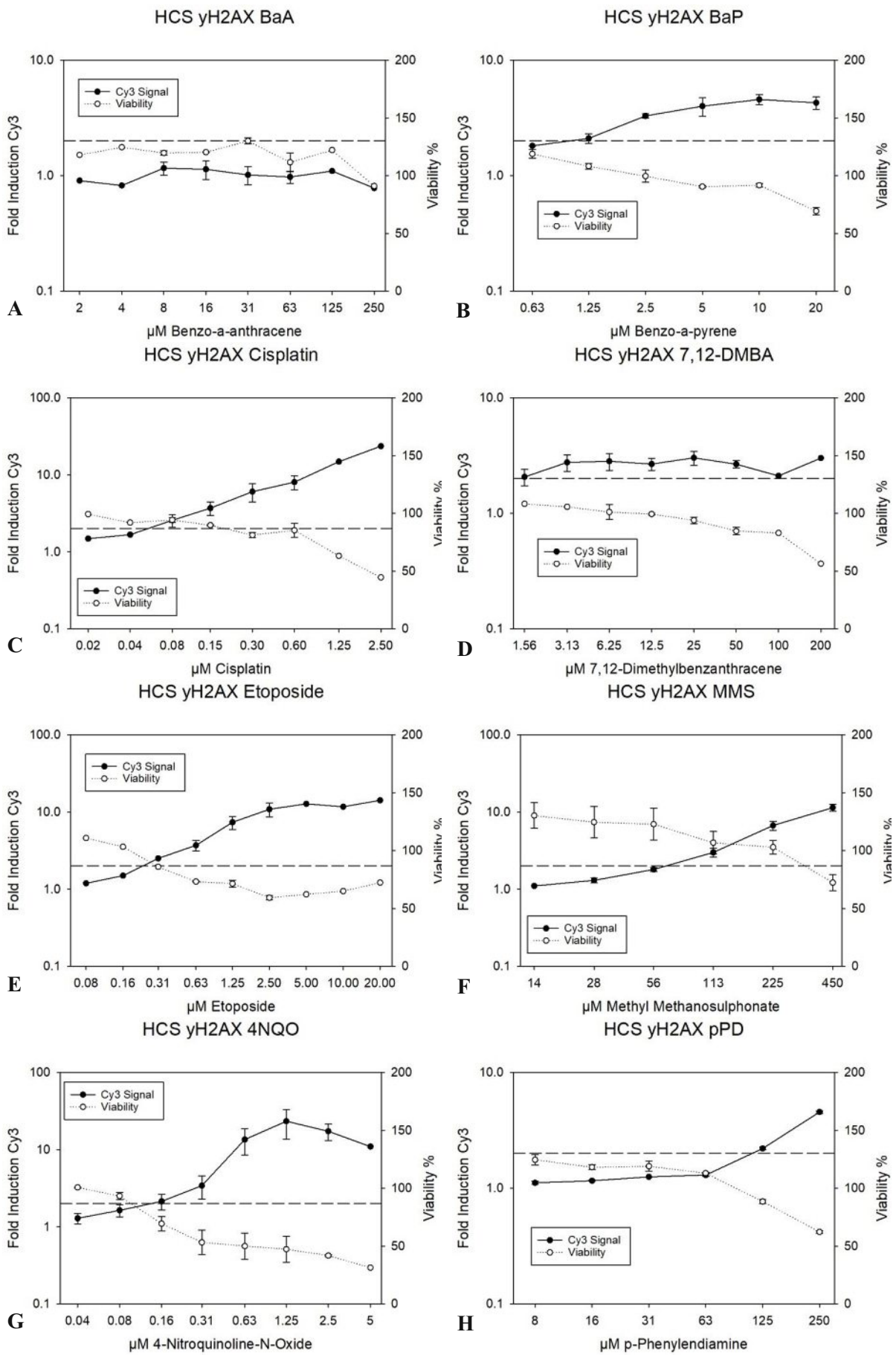


Figure 21: Detailed results for the HCS for the substances BaA (A), BαP (B), cisplatin (C), DMBA (D), etoposide (E), MMS (F), 4NQO (G) and pPD (H). The fold induction was caluclated as Cy3 signal compared to the vehicle control of 1 % DMSO and a threshold of 2 was applied. The viability was measured with DAPI and also compared to the vehicle control.

A summary of the results from the HCS is given in Table 17. The lowest concentration where a positive result could be obtained is shown as resulting LOBD in Table 17. Further, the viability had to be above 70 % to ensure that a genotoxic effect would not be masked by cytotoxicity. Therefore, the viability at the LOBD value is given in the table as well. The HepG2 reporter gene assay is also included as a comparison in Table 17. In general, the LOBDs showed to be slightly higher, than the results for the p53 reporter gene assay, however, this was still within the range of biological deviation.

Table 17: Results of HCS induction for the target γH2AX, which was measured as Cy3 intensity compared to the background value. The viability was measured with DAPI and is the value at which the LOBD was obtained.

Substance	Result – LOBD [µg mL^{-1}]	Viability	LOBD reporter gene assay [µg mL^{-1}]
4-Nitroquinoline-N-Oxide	0.04	75 %	0.03
Benzo-α-pyrene	0.5	102 %	0.2
7,12-Dimethylbenzanthracene	0.9	106 %	0.4
Methyl Methanosulphonate	12	107 %	69
Cisplatin	0.03	93 %	0.2
Etoposide	0.3	79 %	0.8
Benzo-a-anthracene*	Negative	100 %	Negative
p-Phenylenediamine*	14	88 %	8

*Packaging specific substances, which are positive for mutagenicity in the Ames test.

4.4.2. Application for Complex Mixtures

To determine whether the application of complex mixtures might affect the HCS results, different sample migrates were added. Especially the presence of artefacts disturbing the fluorescence microscopy was considered an issue to be addressed with this experiment. The cells were treated with the positive control 4NQO and different FCM sample migrates. As shown in Figure 22A several sample extracts lead to an increase in H2AX phosphorylation. In addition, a variety of sample blanks were measured to determine whether artefacts lead to increasing signals. However, this was not the case as the identification of nuclei through DAPI and Phase II was able to distinguish any artefacts from HepG2 cells. Figure 22B shows an exemplary sample, which had increased γH2AX levels and followed a dose-response curve upon sample dilution. In the

highest sample concentration (100 %), the viability was below the threshold of 70 % making it cytotoxic. Upon dilution, the viability increased but the sample could still be considered as positive.

Nevertheless, the HCS method is unlikely to be a suitable method for high-throughput sample testing, since the assay protocol is very time consuming and only few samples can be tested in a single experiment. However, important information can be obtained on the cells, due to the imaging technique and might be suitable as an additional tool for verification of results.

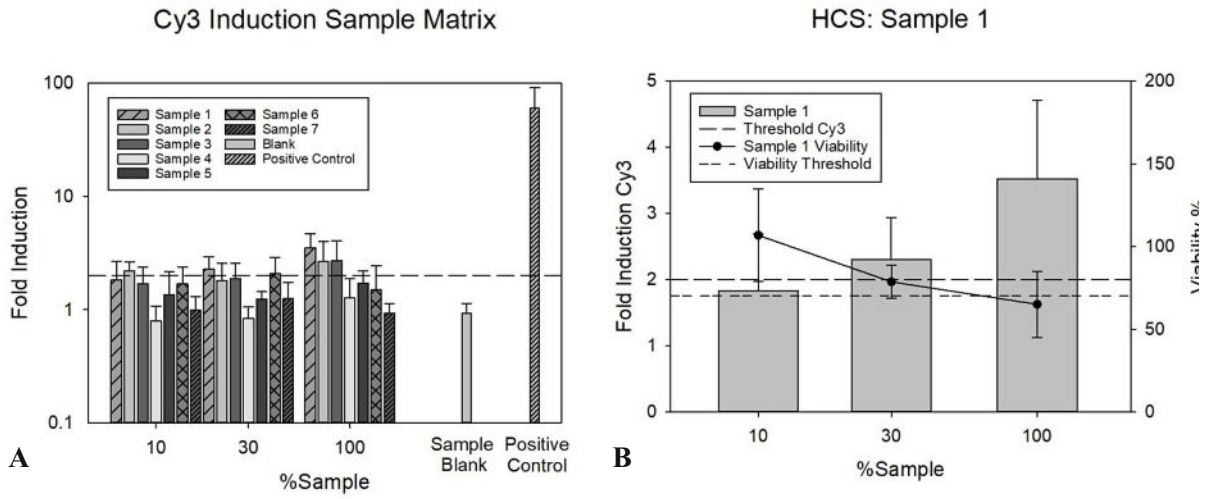


Figure 22: Sample extracts tested with HCS with HepG2 cells and their response towards H2AX phosphorylation as Cy3 intensity and DAPI staining for viability. Sample overview (A) and more detailed information on sample 1 (B) with viability measurement are given. Some samples had high levels of γH2AX with decreasing intensity upon dilution of the samples with values above a threshold of 2 compared to the background. For sample 1, (B) Cy3 intensity decreased with dilution and the viability increased as less sample was applied. For Cy3 intensity and DAPI measurements untreated cells were used for comparison.

4.5.Results: Pre-incubation Ames Test

To determine whether the Ames pre-incubation test is suitable for detecting low concentrations of genotoxic substances in complex mixtures, several experiments were conducted. Further, as discussed by Schilter et al. (2019) and in a review by Rainer et al. (2018), the Ames test lacks the ability to reliably detect all types of genotoxic substances with low LOBDs. Therefore, improvements are necessary concerning the LOBDs of the Ames pre-incubation test. This is why a number of optimisation experiments were conducted to determine the effect of different experiment parameters on the LOBD. Further, the presence of complex mixtures and their effect on the LOBD was analysed. Sample testing was done in 6 well plates, as for this format less sample material was necessary compared to the standard Ames procedure and sample material was limited.

4.5.1. Optimisation

Optimisation testing was done together with Tatjana Hasil and an excerpt of the results is also shown as part of her master thesis (Hasil, 2020).

The concentration and dilution series of 4NQO for TA100 and 2NF for TA98 was chosen in a range closely to the expected LOBD. For this, the highest dilution expected to be negative, the highest concentration to be positive and the middle dilution to be close to the threshold were used. With this procedure, any change in the LOBD should be visible and a positive or negative effect on the analytical sensitivity should be observable.

Further, if an improvement of the LOBD was observed it was only implemented, if a sufficiently great improvement was made. This is the reason because the result would have to be verified with a series of substances and a factor of 2 change in the LOBD is considered to be the biological deviation within experiments. Further, a thorough re-evaluation of the assay would be necessary to ensure its functionality in all aspects is unchanged. This would only be done, if a sufficiently high improvement was observed in the preliminary experiments, which was not the case.

Bacteria Concentration Variation

For these experiments, the bacteria were applied in the initial concentration, 10x concentration, 1:10 dilution or 1:25 dilution of the initial concentration. The experiments were conducted both with the TA100 and TA98 strain without S9 and both results were taken into account for any further changes in the Ames protocol. The results in Figure 23B show that upon dilution the TA98 bacteria where less sensitive towards 2NF. For the TA100 strain, a dilution by 1:10 was promising, but did not lead to any improvement in a consecutive experiment as shown in Figure 24.

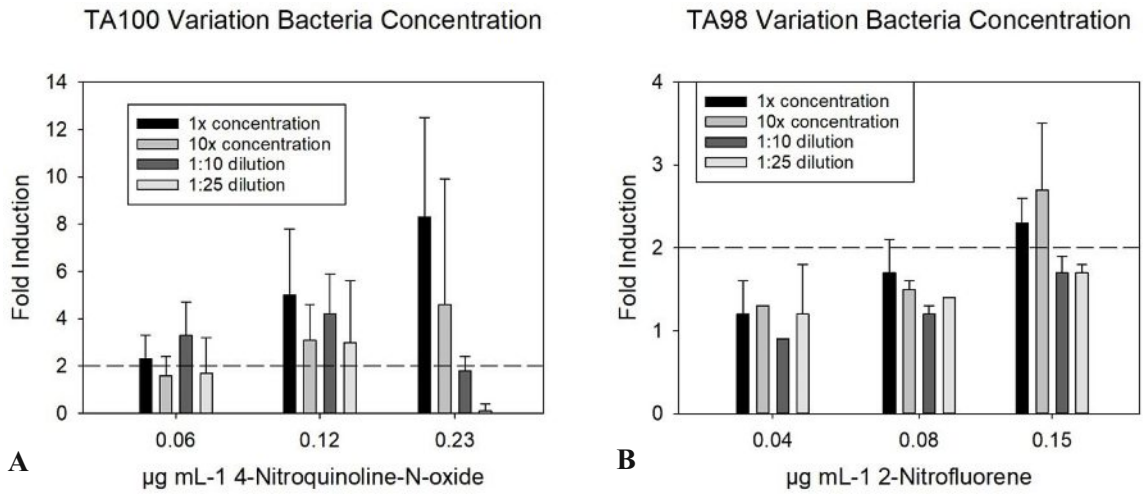


Figure 23: Pre-incubation Ames test with TA100 (A) and TA98 (B) with different bacteria concentration. The bacteria were applied in a 1x concentration, 10x concentration, 1:10 dilution or 1:25 dilution of the initial bacteria concentration. The concentration of the substances 4NQO for TA100 and 2NF for TA98 are given in µg mL^{-1} in the plate and for scoring the mutated bacteria were counted and compared to the background control.

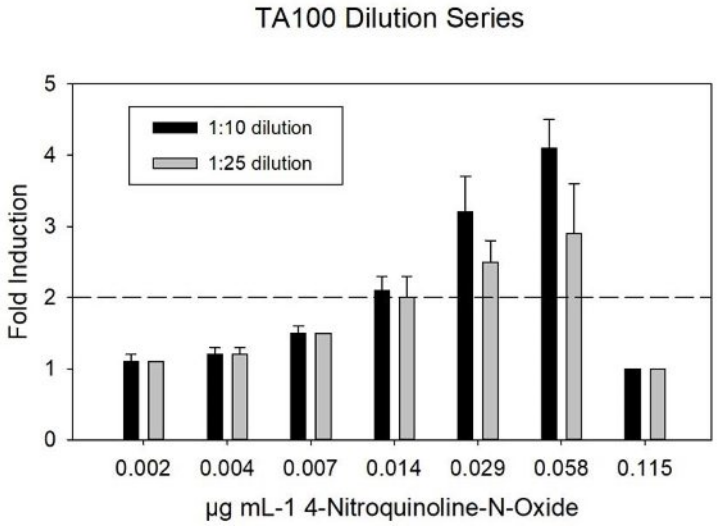


Figure 24: Pre-incubation Ames dilution series with 4NQO with different bacteria concentration. 1:10 and 1:25 dilutions of the initial bacteria concentration were tested and compared to the background control.

Variation of Pre-Incubation Time

Different pre-incubation times were taken into account to determine any effect on the LOBD. The following pre-incubation times were used: 60 min, 90 min, 120 min and 180 min and the bacteria were then incubated for 48 h. Figure 25A and Figure 25B show the results for TA100 with the substance 4NQO and for TA98 for the substance 2NF. The results in Figure 25A show that with increasing pre-incubation time the amount of mutated bacteria in relation to the background mutants decreased. This led to a negative effect on the LOBD and therefore longer pre-incubation times were dismissed and 60 min pre-incubation time was considered suitable for TA100. For TA98 seen in Figure 25B, the increase in pre-incubation time seems to have a slightly positive effect on the LOBD. However, the improvement is minimal and might be a biological deviation. Therefore, a change in pre-incubation protocol was not considered for the TA98.

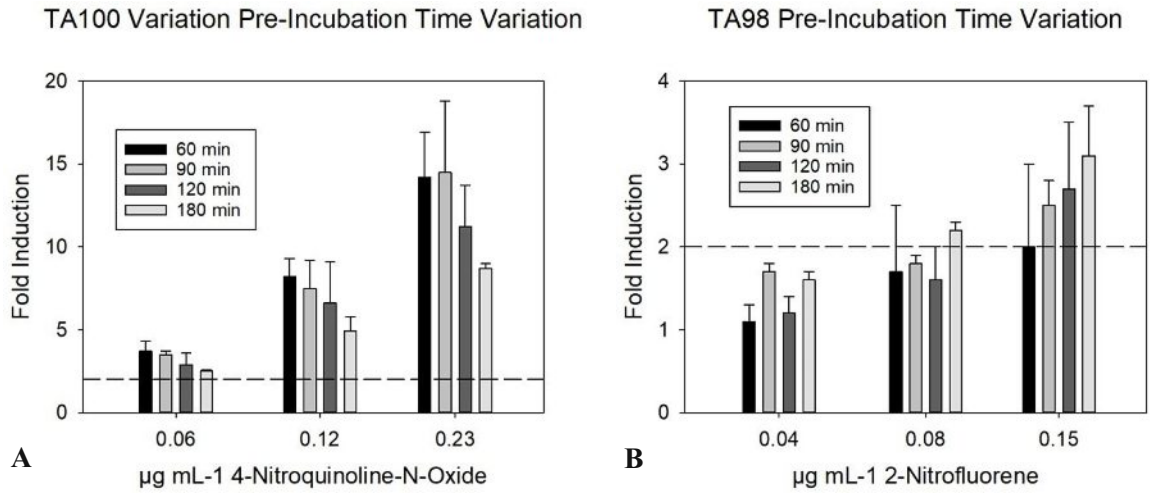


Figure 25: Variation of the pre-incubation time in the pre-incubation Ames test with the strains TA100 (A) and TA98 (B). The bacteria were incubated with the substance 4NQO for TA100 or 2NF for TA98 for 60, 90, 120 or 180 min. The pure substances were applied in three dilution steps, for which the concentration is shown in µg mL^{-1} in the plate. Mutant colonies were counted and compared to the background control.

Histidine Variation during Pre-Incubation and in the Top Agar

The concentration of histidine in the pre-incubation medium and in the top agar was altered independently to determine whether an increase or decrease in available histidine had any effect on the LOBD of the assay. As initial concentration 0 µM histidine in the pre-incubation medium was used and 50 µM histidine in the top agar. The results for the variation of histidine in the pre-incubation medium is shown in Figure 26A for TA100 and in Figure 26B for TA98. For both strains, a lack of histidine in the pre-incubation medium was considered as the most appropriate method leading to low LOBD values. The result of the decrease or increase of histidine in the top agar is shown in Figure 26C for TA100 and Figure 26D for TA98. For TA100,

less histidine seemed to have a positive effect on the LOBD, however, this was within the range of biological deviation (see Figure 26C). The increased addition of histidine in the top agar led to a positive effect for the TA98 strain in response to 2NF as shown in Figure 26D. However, the increase in histidine led to a severe increase of background mutations and a great deviation within the experiments.

Note: the addition of 500 µM histidine in the top agar led to an increase in background mutations so that single colonies could not be counted on the agar. Therefore, the results of the experiments with 500 µM histidine in the top agar are not shown.

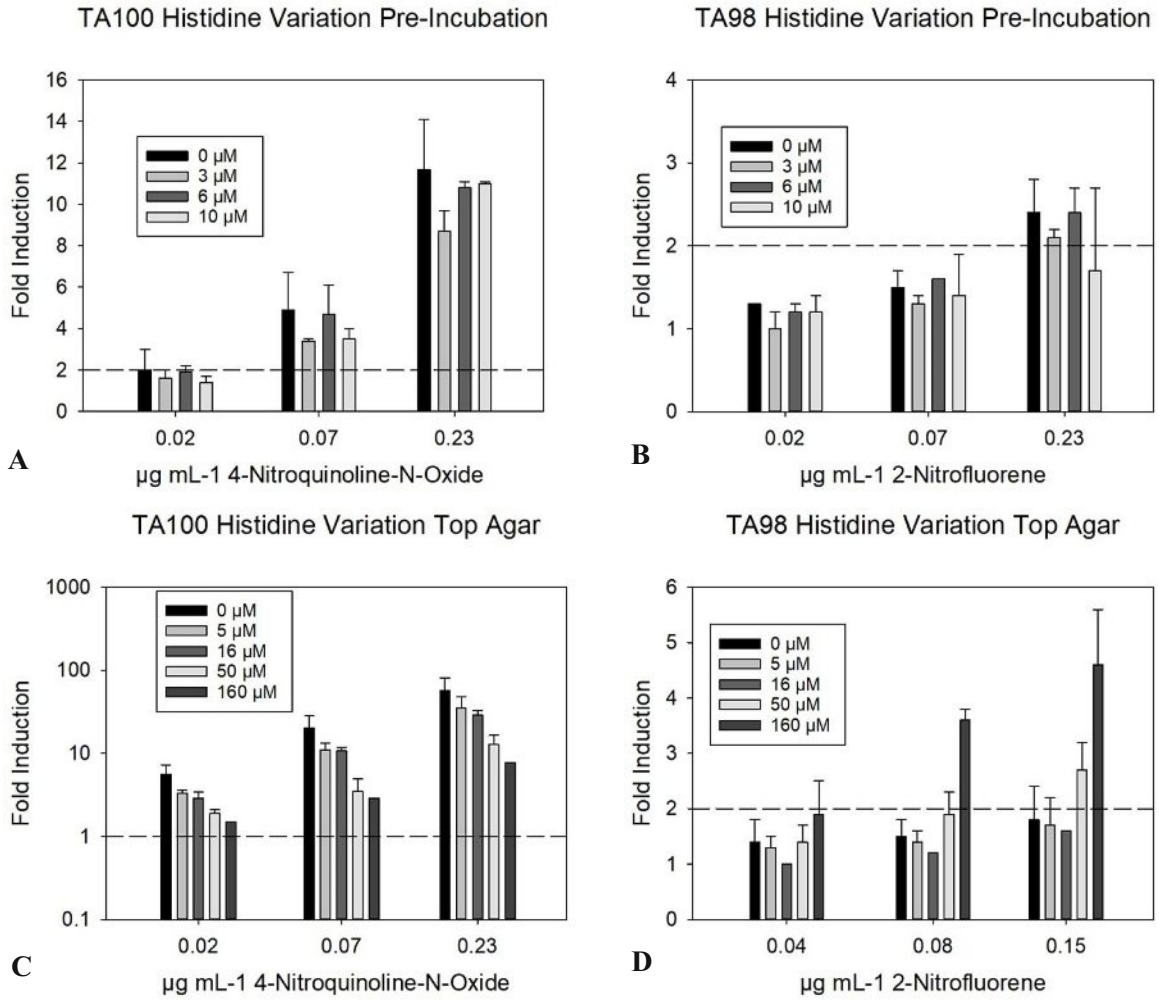


Figure 26: Variation of histidine concentration for the strains TA100 (A, C) and TA98 (B, D) with the pre-incubation Ames test. The histidine concentration in the pre-incubation medium during sample treatment was altered to 3, 6 and 10 µM (A, B) from the initial 0 µM. In the experiments C-D the histidine concentration in the top agar was changed to 0, 5, 16, 50 (initial concentration), 160 and 500 µM. The concentration of 4NQO for TA100 and 2NF for TA98 is given as µg mL^{-1} in the plate and scoring was conducted by counting mutant colonies compared to the background.

Variation of S9 Liver Extract in the S9 Mix

As S9 rat liver extract is a limited resource, experiments were conducted to determine how much S9 extract is necessary to improve the LOBDs. Further, the OECD suggests varying amounts of S9 (OECD, 1997) and with this experiment, the optimal concentration in the mix should be determined. The bacteria were treated with the substance 2AA, which has to be metabolised by addition of an S9 mix. The results for the strains TA100 and TA98 are shown in Figure 27. For both strains, it can be seen that less S9 extract in the mix led to an improved LOBD and higher concentration led to a negative effect on the LOBD. Therefore, it was concluded to use 1 % S9 in any subsequent S9 experiment, as this saves valuable and limited resources and led to improvements of the LOBD.

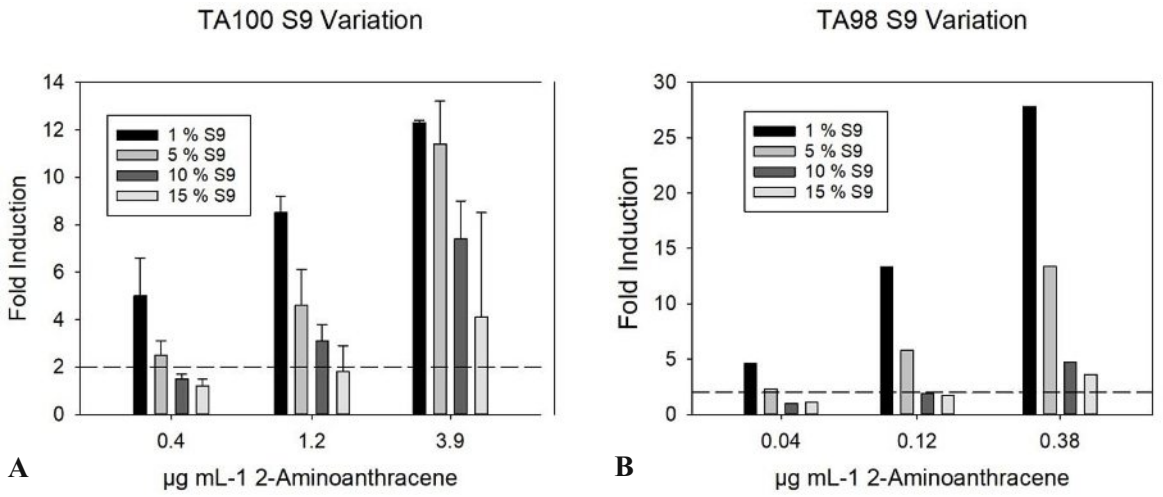


Figure 27: Variation of 1254 aroclor induced rat liver S9 extract in the S9 mix in the pre-incubation Ames test with the strain TA100 (A) and TA98 (B). The substance 2AA was used and the concentration is given in µg mL^{-1} in the plate. The bacteria colonies were counted and compared to the background control.

4.5.2. Ames Formats Comparison

For sample testing, the amount used for the standard pre-incubation Ames test of 50 µL per plate is rather high, as the amount of sample is a limiting factor. Due to this, different miniaturised formats of the pre-incubation Ames test were tested together with the standard petri dish variation. The chosen miniaturised formats have already been studied and evaluated by other authors (Proudlock and Evans, 2016; Pant et al., 2016; Diehl et al., 2000; Zwarg et al., 2018). In this study instead the LOBDs should be compared and its suitability for high-throughput testing with complex mixtures. Due to the lack of a true triplicate the 12-well and 24-well tests were regarded with caution, as the samples were simply distributed between three wells, which did not lead to true biological triplicates.

The results in Figure 28A show varying results for the strain TA98 with the substance 2NF for the miniaturised formats. A high standard variation and deviation could be observed, especially for the smaller 12-well and 24-well format. This is due to the reason that TA98 has a very low spontaneous mutation rate, compared to TA100, and in the smaller formats wells with a value of 0 or 1 could be observed. Therefore, a fold induction could already be crossed with two or more colonies in a well, if the background was very low, leading to the high deviations with the strain TA98. Otherwise the LOBDs were in a similar range, if the outliner for the 24-well at 0.5 µM 2NF is not taken into account. For the strain TA100, which is shown in Figure 28B, the LOBDs were lower for the miniaturised formats with 6-well and 12-well compared to the standard petri dish Ames test and the 24-well variation. For the 6-well variation the highest concentration of 4NQO, 50 µM, was negative and seemed to be cytotoxic with growth of very small deformed colonies in the wells. However, this was considered as negligible since it was of uttermost importance that the LOBD was in a similar range in the miniaturised formats as with the standard petri dish Ames test format.

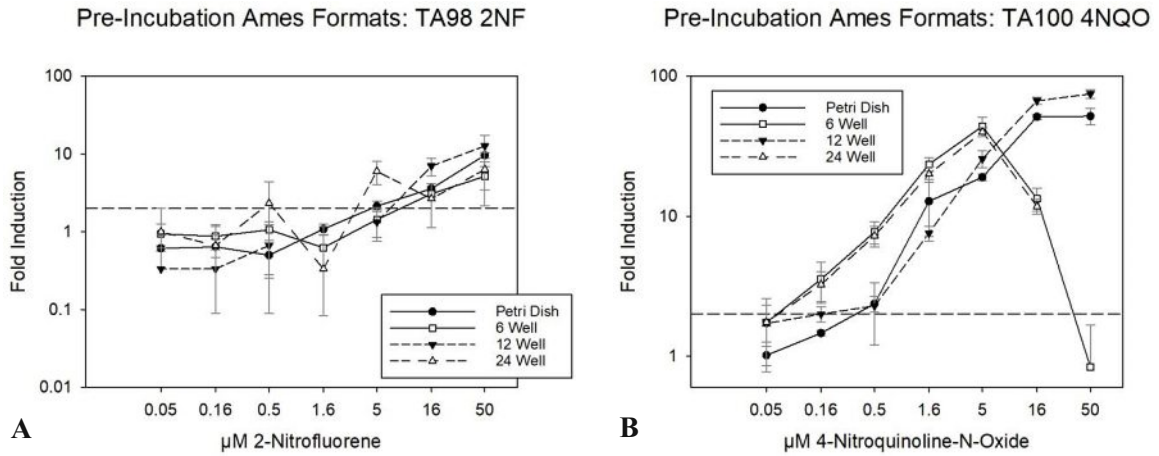


Figure 28: Comparison of different Ames formats including the standard petri dish and miniaturised formats. The standard pre-incubation petri dish Ames test, a 6-well, 12-well and 24-well protocol were performed with the strains TA98 (A) and TA100 (B). The pure substances 2NF for TA98 (A) and 4NQO for TA100 (B) were used and the fold induction compared to the background was determined. A threshold of 2 was used as a cut-off for a positive result.

In a nutshell it was concluded that the 12-well and 24-well formats were less suitable for sample testing as no true biological triplicates were used in these formats. Further, the results with the strain TA98, as presented in Figure 28A, showed a high variation due to very low background values. A background value of 0 or 1 must always be handled with cation, since it cannot be ruled out with certainty that bacteria toxic substances might be present or there was an issue with the test. Finally, the LOBDs were in a similar range for all the formats and dose-response curves could be obtained for both substances 2NF and 4NQO. The high-throughput possibilities and the lower sample volume therefore led to the conclusion that the 6-well format is the most

suitable for sample testing. Consequently the following tests with complex mixtures (see 4.5.3) were performed with the 6-well Ames format instead of the standard petri dish Ames test, where greater amount of sample and consumables would have been necessary.

4.5.3. Applicability for Complex Mixtures

The application of complex mixtures was performed together with Tatjana Hasil and an excerpt of the results is shown in her master thesis (Hasil, 2020).

Several FCM sample migrates were added to determine whether the presence of a complex mixture might affect the response of the assay. The results in Figure 29A and B show a representative experiment, where an FCM sample was added to the bacteria and a spike was used. With this experiment the assays' ability to detect a positive result for genotoxicity in the presence of a sample matrix was evaluated. The results for TA100 (Figure 29A) and TA98 (Figure 29B) show that the bacteria were not negatively affected by the presence of a sample matrix. For TA98, a slight decrease in colony count could be observed at 100 % sample concentration, which was eliminated upon sample dilution. Moreover, a spike was added to determine if the complex mixtures led to a quenching or effect of the bacteria response. The curves for spike recovery in Figure 29A and B show that the bacteria were not affected by the sample and the reference substances could be detected as in the background. Further, the sample was considered negative as no sample concentration led to a fold induction compared to the background above the threshold.

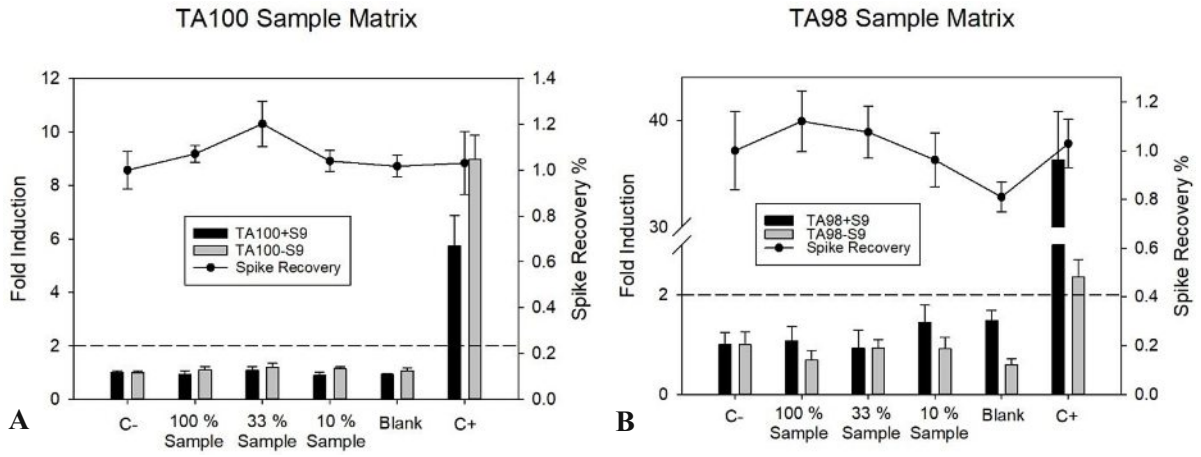


Figure 29: Addition of an FCM sample to determine matrix effects in TA100 (A) and TA98 (B) strains in the Ames test. The samples were solved in DMSO and as 100% sample and diluted with a constant DMSO concentration of 7.7 % in the plates. A vehicle control of DMSO (C-) and a positive control (C+) of 4NQO for TA100, 2NF for TA98 and 2AA for S9 addition were used. For spiking, the positive substance was added to all wells and the recovery was measured with the background value as a reference. The counted colonies were compared to the background colonies to determine the fold induction, which was considered positive above a threshold of 2.

5 Discussion

The safety assessment of FCMs has been an ongoing challenge of great interest. Especially the interaction between the FCM and the foodstuff, such as the migration of NIAS, has been of uttermost importance. Several endpoints have already been exhaustively studied and discussed in the context of migration from FCM and their possible health effects for the consumer (Muncke et al., 2020; Severin et al., 2017). Specifically endocrine activity has been a focus and was exhaustively studied within the past years (Mertl et al., 2014; Muncke, 2009). These substances are usually threshold dependent and often present in higher concentrations, therefore being less of concern then, for example mutagenic substances, which are already a potential risk at low concentrations (Schilter et al., 2019; Severin et al., 2017).

For a thorough toxicological safety assessment, *in vivo* studies would have to be conducted with substances regarded to be of concern towards human health (Bolognesi et al., 2017). However, the replacement of animal studies is of great interest and the conducting of *in vivo* studies for complex mixtures, such as FCMs would not be ethically feasible (Schilter et al., 2019). Alternatively, *in vitro* assays have gained importance to replace *in vivo* studies for the prediction of toxicological effects. The ECVAM has spared no expenses or effort to propagate and push forward the development and thorough evaluation of promising alternatives (Pfuhler et al., 2007).

Due to this reason, in this thesis the safety assessment of FCMs focused on the presence of genotoxic substances and how they can be detected reliably at low concentrations. In a first step, a series of commonly used mammalian assays were analysed through performing a literature survey. Both the toxicological sensitivity and specificity and the analytical sensitivity were taken into account. For the analytical sensitivity, a threshold of 0.00015 µg mL^{-1} was of interest, as the TTC uses this threshold for the exclusion of genotoxicity (Munro et al., 2008; Kroes et al., 2004). The rational was that assays with LOBDs below the TTC threshold could be used to exclude the presence of genotoxicity in a complex mixture. As this threshold was considered to be conservative and technically challenging, the proposed limit of detection by the EU regulation (EC) No 10/2011 (European Parliament, 2011) was further considered. This is a pragmatic limit used for the detection and handling of unknown substances migrating from FCMs. Here the limit is set at 0.01 µg mL^{-1} and detection levels for CMR substances would need to be lower (Schilter et al., 2019). The results showed (Pinter et al., 2020) that the mammalian assays covered a variety of endpoints, but were lacking in analytical sensitivity to do so at the proposed thresholds. Instead the Ames test showed to be more suitable for detecting genotoxic substances

at low concentrations (Rainer et al., 2018). However, Pinter et al. (2020) and Rainer et al. (2018) concluded that both the Ames test and the mammalian assays need to be improved to be used for the genotoxicity assessment of FCMs.

Mammalian Assays – p53 Pathway

An assay which has already been studied in this context is the P53 CALUX® (van der Linden et al., 2014), which was analysed in detail by Pinter (2017) for its suitability for the analysis of FCM migrates. It was thought to be an appropriate assay as it revolves around the p53 pathway, however it was greatly lacking when taking metabolic activation into account. Already Yang and Duerksen-Hughes (1998) investigated the possibility of p53 associated proteins as a marker for genotoxicity and suggested to develop *in vitro* assays based around this. Therefore, by using a different cell line in this thesis, which was suitable for metabolic activation, the principle was considered appropriate for FCM safety assessment, compared to the previously used P53 CALUX® (Pinter, 2017). In this study HepG2 cells were used, as they are considered to have some metabolic activity (Hewitt and Hewitt, 2004; Dawson et al., 1985) and are less prone towards cytotoxicity (Schoonen et al., 2005). Already Bigger et al. (1980) recommended the use of intact cells with their own metabolic activation system instead of using an external metabolisation system, such as liver homogenates. In addition, it was suggested to use stable cell lines, since they are considered to be more reliable than primary hepatocytes, which tend to be polymorphic (Valentin-Severin et al., 2003). Experiments by Sassa et al. (1987) also found HepG2 cells to be capable to correctly detect BαP without S9 addition, as the cells possess a metabolisation capacity. This was also seen in this study for the substances BαP, 7,12-DMBA, AFB1 and 2,4-DAT, which would normally not be positive without the addition of an external metabolising system.

The results with the HepG2-based reporter gene assay, showed a good toxicological sensitivity of 87.5 % and a specificity of 94 %, tested with substances from the ECVAM list (Kirkland et al., 2016). This is well within the range of other newly developed assays, such as the BlueScreen™ HC (Hughes et al., 2012), the GreenScreen™ HC (Hastwell et al., 2006) or the ToxTracker (Hendriks et al., 2016). The analytical sensitivity was also within the range of other assays, but it was not sufficient to reach the proposed threshold of 0.00015 µg mL^{-1} for genotoxic substances in the TTC decision tree or the technical limit of 0.01 µg mL^{-1} by the European regulation (EC) No 10/2011 (European Parliament, 2011).

Mammalian Assays – Target Genes and yH2AX

Besides the p53 pathway, other gene targets were looked into more detail by conducting a screening of several genes associated with genotoxicity. The HepG2 cells were again treated with genotoxic substances and the gene regulatory response was monitored by performing qPCR. The results showed, that the target protein p21 (cDKN1A) performed well, however the p53 protein was superior and therefore considered to be a suitable approach.

Another endpoint of interest concerning genotoxicity was the occurrence of DNA double strand breaks, due to the presence of genotoxic substances. For this, HepG2 cells were again used, since they had shown to be suitable and metabolically active when treated with a series of genotoxic substances. DNA double strand breaks can be detected in histones, such as the histone family H2AX, which tends to be phosphorylated (yH2AX), upon the presence of genotoxic substances (Nikolova et al., 2014; Rogakou et al., 1998). Already a study by Ando et al. (2014) found yH2AX to be a suitable marker to determine genotoxicity. However, they concluded that H2AX phosphorylation has to be assessed together with cell cycle arrest markers, as several genotoxic compounds can cause apoptosis through cell cycle arrest, leading to DNA double strand breaks. This gives important information whether a substance attacks the DNA directly or interferes with mitosis.

In this study, H2AX phosphorylation was analysed through HCS, which is an imaging technology applying fluorescent markers and has been used to study genotoxicity (Bryce et al., 2016; Baas et al., 2014; Bryce et al., 2008). An increased signal in phosphorylated H2AX is directly proportional to a higher fluorescent signal and therefore suggests the presence of a genotoxic substance. The HCS provided important information on the mechanisms of the different genotoxic substances.

A study by Garcia-Canton et al. (2013) validated a HCS γH2AX assay using a group of known genotoxic and non-genotoxic compounds in a human bronchial epithelial cell line BEAS-2B. This assay showed high accuracy (86 %) with 86 to 92 % sensitivity and 80 to 88 % specificity (Garcia-Canton et al., 2013). HCS micronucleus assays were developed with the rodent cell line, CHO-K1, and the human hepatoma cell line, HepG2, used in regulatory genotoxicity assays. The sensitivity and specificity was 80 % and 88 % for the CHO-K1 cells, and 60 % and 88 % for the HepG2 cells, respectively (Westerink et al., 2011). Recently, an HCS micronucleus assay in a 384-well plate format was developed for evaluating genotoxicity in CHO-K1 cells (Shahane et al., 2016). Further, Clewell et al. (2014) conducted the micronucleus in a microarray approach with a focus on p53 activation and regulation and on yH2AX, as did Mishima

(2017) regarding the distinction of clastogenicity and aneugenicity through γH2AX activation characterisation.

Similarly to the listed studies, in these experiments the HCS γH2AX assay showed some promising results for a good toxicological sensitivity and specificity. However, the aim of this thesis was to identify genotoxicity assays, which are able to detect genotoxic substances correctly at low concentrations. Again the HCS γH2AX assay did not lead to improved LOBD values and was considered to be lacking sensitivity for the assessment of FCM migrates. Moreover, it is a very time-consuming assay and therefore less suitable for a high-throughput screening than other assays analysed in this thesis.

Bacterial Assays – Ames Test

To determine the presence of genotoxic substances, mammalian assays are considered to be of great importance, as they cover all types of genotoxicity, such as aneugens and clastogens. Bacterial test systems on the other hand, are only able to detect mutagenicity, as they do not possess chromosomes for e.g. DNA double strand break detection or aneugenicity. However, when it comes to the detection of genotoxicity, those substances leading to mutagenicity are regarded as most crucial, as here no threshold can be applied and already very low amounts might lead to a health effect (Schilter et al., 2019). Aneugenic and clastogenic substances on the other hand, can be covered with the Cramer Class III threshold of 0.09 µg mL^{-1} as they are threshold dependent (Boobis et al., 2017; Nohmi, 2018; EFSA, 2016b). This is due to the fact, that they are regarded as less potent compared to DNA reactive substances, since a critical number of target sites must be occupied before a biological effect occurs (Elhajouji et al., 2011).

For mutagenic impurities, the ICH M7 (ICH, 2017) states that the application of the Ames test as a standalone assay is sufficient, since direct DNA reactive mutagenic substances are non-threshold dependent. Due to this reason, the recommendation by the ILSI (Schilter et al., 2019) and the historic importance of the Ames test, the assay was assessed in detail in this study. Already a recommendation by Schilter et al. (2019) and a literature survey by Rainer et al. (2018) showed that the Ames test was a promising candidate for the safety assessment of FCM migrates, but its LOBDs had to be further improved.

In this study, the pre-incubation Ames test protocol was altered to determine the effect on the LOBDs and whether improvements were possible. A series of changes led to slight improvements, however, they were considered to be in the range of biological deviation. The most promising approach was the alteration in S9 concentration applied in the S9 mix. The rat liver S9 1254 aroclor mix could be reduced to 1 %, while the concentration of cofactors remained

constant. For some substances, this led to an LOBD decrease by a factor of 10, suggesting that this could be a suitable optimisation. Overall, however the optimisation did not led to a sufficient improvement of LOBD values to apply the threshold of 0.00015 µg mL^{-1} by the TTC or the threshold of 0.01 µg mL^{-1} by the EU for the safety assessment of FCM migrates.

Metabolic Activation

When assessing genotoxicity the metabolisation is a crucial step, as some substances will only develop their potential when being metabolised (EFSA, 2011). For example, BαP itself is not genotoxic, but when it is metabolised in the liver through a series of enzymes and proteins, such as cytochromes P450 (CYP450), it unfolds its genotoxic characteristics (Brinkmann et al., 2013). This is why, the addition of an external metabolisation system is of great importance, when testing for genotoxicity with cells or bacteria incapable of metabolisation. Depending on the S9 mix and protocol used, different results might be obtained (Billinton et al., 2010). Also various types of cells have a different tolerance towards S9 liver extract, affecting the result (Kirkland et al., 1989). This shows that the addition of an external metabolism is a very sensitive equilibrium, which can greatly influence test results.

Due to this, the use of cells, capable of internal metabolisation are of interest for *in vitro* genotoxicity testing. Further, the application of an external metabolisation system using rat, human or hamster liver S9 does not entirely comply with the approach of animal free testing, which is often an important aim of *in vitro* testing systems. Primary hepatocytes are considered to represent the human metabolisation system the most accurately, however, they tend to be inhomogenous in their characteristics and depend greatly on the donor and sources (Westerink and Schoonen, 2007b). HepG2 cells are in general considered to perform well in terms of metabolisation (Valentin-Severin et al., 2003), as already mentioned above. Several protocols tested in this study with the addition of different amounts of S9, such as a protocol proposed by Mollergues et al. (2016), compared to no S9 addition, showed that the cells performed as good as when greater amounts of S9 were added. Therefore, the addition of S9 was no longer considered for experiments carried out with the HepG2 cells. A study performed by Valentin-Severin et al. (2003) also showed that HepG2 cells were able to detect a variety of genotoxic effects through internal metabolisation.

Some tests were performed with HCT116, which are human colorectal carcinoma cells, and also have been known to possess some metabolisation activity (Hasinoff and Begleiter, 2006). However, experiments in this study with a series of genotoxic substances showed that the metabolisation capability was not sufficient. Substances such as BαP did not lead to a positive

signal in the absence of an external metabolism and therefore the cell line was not considered for further testing.

For bacterial tests, it is not possible to rely on an internal metabolisation system, as bacteria do not possess the appropriate enzymes and an exogenous metabolisation system has to be added (Tejs, 2008). For this, the OECD recommends the application of 5 to 30 % (v/v) of S9 rat liver homogenate (OECD, 1997). However, in this study it could be shown that a reduction of the overall S9 amount during incubation was possible and led to a slight improvement of LOBDs. This was also concluded in a study by Belser et al. (1981) where it was shown that the Ames test was able to detect BαP in lower concentrations with less S9 (2 to 4 % (v/v)) present in the mix. Similar results were found in a study by Zeiger et al. (1979) for the substances BαP and 2AA, as was concluded in this study as well.

Testing of Complex Mixtures - FCM Migrates

To determine the ability of the assays to correctly detect genotoxic substances in the presence of a complex mixture, spiking experiments were performed. As the FCM matrix might interfere with the detection, the impact of the presence of an FCM migrate has to be considered (Schilter et al., 2019). In this study, FCM migrates consisting mainly of plastic samples were used and known amounts of a genotoxic substance were added. In all these experiments the assays performed well and no distinct diminishing in signal intensity could be detected, due to the presence of a complex mixture matrix.

The presence of cytotoxic substances in the complex mixture might lead to false negative results and spiking experiments can provide important information for this. HepG2 cells are considered to be less sensitive towards cytotoxicity (Schoonen et al., 2005) and little impact of cytotoxicity could be detected within the experiments of this study. For the Ames test, the detection of cytotoxicity is somewhat more challenging and can be found through a thin background haze (OECD, 1997). However, most FCM migrates did not lead to a cytotoxic effect, as the bacteria are very robust towards such factors.

Overall, the *in vitro* assays showed to be compatible with FCM migrates and reliable results could be obtained. Nevertheless, it must be kept in mind that the literature study showed that most *in vitro* bioassays are not able to detect genotoxic substances at sufficiently low concentrations (Pinter et al., 2020; Rainer et al., 2018). Even sample preparation to increase the concentration of the genotoxic substances only led to a slight improvement. Other studies have found some positive results (Rosenmai et al., 2017; Ubomba-Jaswa et al., 2010; Bradley et al., 2008; Ozaki et al., 2004; Biscardi et al., 2003), but did not take the LOBDs of the assays used

into account. Therefore, perhaps also leading to false negative results. A recent study by Rainer et al. (2019) found some positive results for some FCM materials, though they were using exaggerated worst-case scenarios for the sample preparation.

The findings in this study show, that *in vitro* bioassays can provide important additional information for the safety assessment of FCMs. However, to reliably detect genotoxic substances below a threshold of 0.00015 µg mL^{-1} proposed by the TTC approach, they still need to be improved. Nevertheless, current chemical methods used for analysis of FCM migrates use a detection limit of 0.01 µg mL^{-1} and are therefore also not able to certainly rule out the presence of any genotoxic substances as considered by the TTC. Anyhow, the combination of the two methods can provide important toxicological information on an FCM to make a thorough safety assessment of unknown substances possible.

6 Summary - Conclusion

The results of this thesis showed, that the combination of chemical and biological methods for the toxicological safety assessment of FCMs is promising but not sufficient. Especially, the information gained with *in vitro* bioassays in addition to commonly used methods was considered as very valuable. However, the results also showed that, whilst the toxicological sensitivity and specificity is already well studied, data on the analytical sensitivity is often missing. Further, it could be concluded that the assays were lacking analytical sensitivity for several genotoxic substances. This holds true for both mammalian and bacterial assays, which have to be improved and optimised, so that a reliable detection of a majority of genotoxic substances at the proposed thresholds is possible. As only a small amount of genotoxic substances could be looked at in detail, more substances migrating from FCM should be analysed and toxicologically assessed to obtain more information in this context. However, not just the biological methods lack analytical sensitivity, but the chemical methods need to be improved as well to reliably exclude genotoxic substances in a routine testing. With all this considered, the additional application of *in vitro* bioassays for the safety assessment of FCM provides important information, which is necessary to ensure safe packaging being placed on the market.

7 Appendix

Table A 1: Summary of genotoxicity testing studies of FCM with mammalian *in vitro* assays as published in Pinter et al. (2020). The studies are classified by the different conducted tests and their sample preparation, concentration method, sample solvent and results for genotoxicity and cytotoxicity testing are shown. Further, it is indicated whether S9 was used or not.

Assay	Material	Simulant	Migration protocol	Concentration method	Sample solvent	Result	Cell type	+/-S9	Source
BlueScreen™ HC	Paper	Tenax	10 d at 60 °C	-	Aqueous Water	0/3 positive Not cytotoxic No genotoxic substances in GC-MS and LC-MS analysis	TK6	yes	(Koster et al., 2014)
Chromosomal Aberration Test	Polystyrene	Acetone	1 h at 40 °C, then addition of Methanol for 1 h at 40 °C	Evaporation GCF[1] = 1.5	Acetone	0/1 positive Not cytotoxic No genotoxic substances in GC-MS	CHL	yes	(Nakai et al., 2014)
Comet Assay	Paper & Board	Water, 95% Ethanol, Tenax	Water: EN 645 (cold water extraction) and EN 647 (hot water extraction) 24 h 20 or 80 °C 95 % Ethanol: 24 h at RT Tenax: 24 h at RT, 5 d at 50 °C, 10 d at 20 °C	Evaporation GCF = 10	95% Ethanol	0/20 positive Some samples cytotoxic No genotoxic substances in GC-MS	HepG2	no[2]	(Bradley et al., 2008)
	Paper (recycled)	Ethanol	Refluxed for 2 h	Evaporation	DMSO	6/8 positive Cytotoxicity not evaluated Several genotoxic substances identified with GC-MS	HL-60	no info	(Ozaki et al., 2004)
	Polyethylene-terephthalate (PET)	Mineral water	Storage for 1 to 12 months	Lyophilisation and evaporation	DMSO	6/12 samples positive for mineral water 5/12 samples positive for carbonated mineral water Cytotoxicity not evaluated No genotoxic substances in GC-MS	Human Leukocytes	no info	(Biscardi et al., 2003)
	PET	Mineral water	10 d at 40°C and at RT	Solid Phase Extraction	DMSO	some positive results, but not statistically significant	Human Leukocytes	no info	(Ceretti et al., 2010)

						Cytotoxicity not evaluated No genotoxic substances in GC-MS			
	Polypropylene	-	Dissolving of the sample in ethyl acetate	Evaporation GCF = 0.1	DMSO	0/6 positive some cytotoxic effects No genotoxic substances in GC-MS and HPLC	HepG2	no info	(Riquet et al., 2016)
Micronucleus	PET & glass	Water	10 d at 40, 50, 60°C	Solid Phase Extraction GCF = 5	Ethyl acetate	0/4 sample pools positive Not cytotoxic No genotoxic substances in GC-MS	HepG2	no info	(Bach et al., 2013)
	PET & glass	Water	Exposure to sunlight for 2, 6, 10 d	Solid Phase Extraction GCF = 5	Ethyl acetate	0/4 sample pools positive Not cytotoxic Low concentrations of formaldehyde and acetaldehyde in GC-MS	HepG2	no info	(Bach et al., 2014)
P53 CALUX®	Paper & board	Ethanol	Refluxed for 4 h	Evaporation GCF = 1.3	Ethanol	6/20 positive Cytotoxicity not evaluated in one positive sample di-isobutyl phthalate identified with GC-QTOF	U2OS	no info	(Rosenmai et al., 2017)
Sister chromatid exchange	PET	Mineral water	8 weeks RT	-	Water	0/4 sample pools positive some cytotoxic effects No chemical analytical method conducted	Human Lymphocytes	no info	(Ergene et al., 2008)

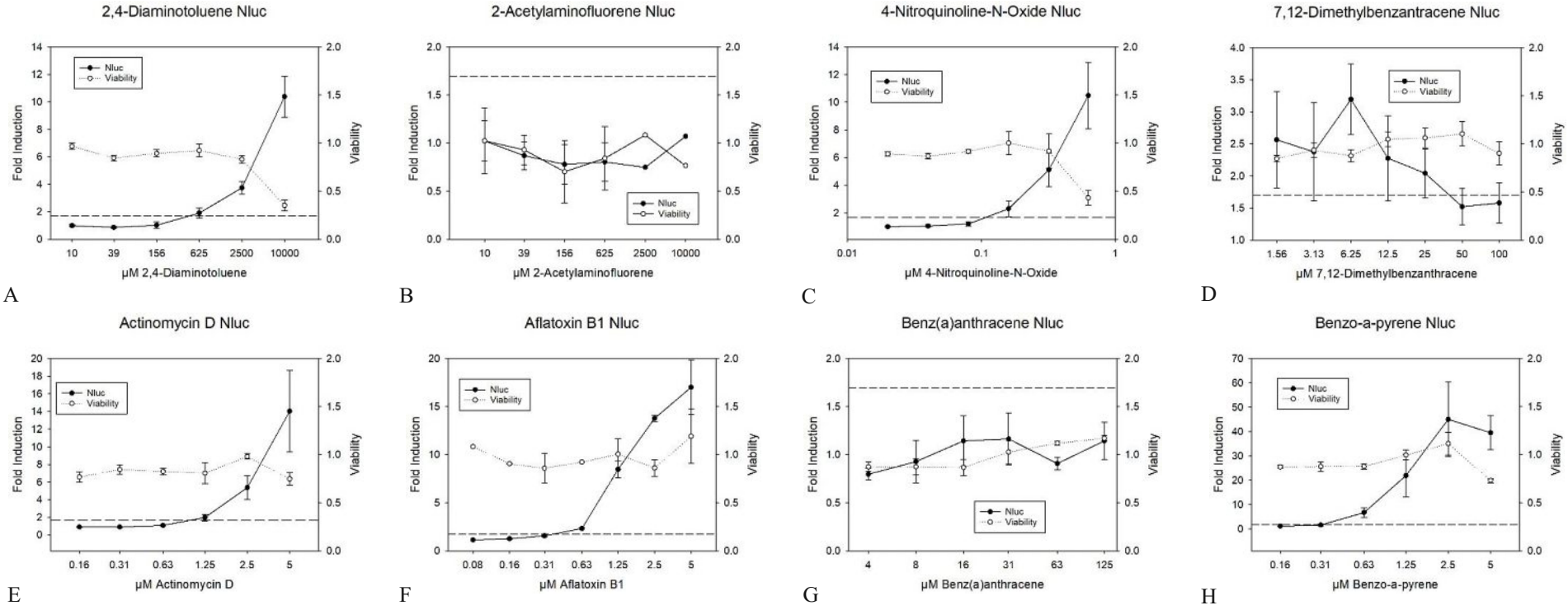
[1]: GCF = Global Concentration Factor; [2]: HepG2 cells were used which proofed to be metabolic competent, so that the addition of a further metabolic system was not necessary (Bradley et al., 2008).

Table A 2: Overview of genotoxicity testing of FCM with the Ames test, as published in Rainer et al. (2018). The studies are arranged according to the material analysed and the used assay type (pre-incubation, plate incorporation, fluctuation Ames test, Ames MPF or Ames II). Moreover, the sample preparation and concentration methods are shown in detail and the results are given for each test.

Sample material	Ames variation	Result	Strain[1]	S9[2]	Sample preparation	Author/Year
Recycled paper	Pre-incubation	2/4 samples equivocal, not reproducible	TA98, one more strain not specified	no	EN 645 cold water extraction; 2.5 g sample to 150 mL 99% Ethanol at 23°C, Pooling of extracts and evaporation	(Binderup et al., 2002)
Paper & board	Plate incorporation	1/20 samples positive (ethanolic extracts)	TA98	no	1. Extraction in 95% Ethanol for 2h/24h at 40/60 °C, concentration through evaporation 2. EN 645 cold water extraction and EN 647 hot water extraction 3. Tenex migration 24 h at RT	(Bradley et al., 2008)
Paper adhesives	-	0/6 samples positive			Extraction with water (50 °C) or Ethanol (RT) for 24 h, later evaporation	(Störmer and Franz, 2009)
Plastic (PET)	Standard Ames	1/1 batch of PET bottles positive after one month storage	TA98	yes	Mineral water at 40 °C for 24/48 h, later concentration with SPE	(Fusco et al., 1990)
Plastic (PET)	Standard Ames	0/1 batch of PET bottle positive	-	-	1. Distilled water at 40 °C for 10 d or 120 °C for 2 h, later evaporation or lyophilisation 2. Mineral water at RT for 1 to 6 months, concentration through SPE and evaporation	(Monarca et al., 1994)
Plastic (PET)	Fluctuation test	1/1 batch of PET bottles positive after two month storage	TA100	no	Mineral water at RT for 1, 2, 3, 4, 5 or 6 months exposed to sunlight, unconcentrated samples	(Ubomba-Jaswa et al., 2010)
Plastic (PET)	Plate incorporation	0/2 batches of PET bottles positive	-	-	Bottled water at 20/40/50/60 °C for 10 d, concentration through SPE	(Bach et al., 2013)
Plastic (PET)	Plate incorporation	0/2 batches of PET bottles positive	-	-	Bottled water exposed to sunlight for 2/6/10 d, concentration through SPE	(Bach et al., 2014)
Plastic (PET)	Ames MPF™	0/1 batches of PET bottles positive with distilled water, partly positive with different water sources	TA98, TA100	yes/no	Water exposed to sunlight for 6 h to 90 d, no concentration	(Moraa, 2015)
Plastic (PS)	Pre incubation	0/1 samples positive	-	-	Pellets in acetone for 1 h at 40 °C, mixed with Methanol at RT, filtered and evaporated, dissolved in acetone	(Nakai et al., 2014)
Clay, PLA nanocomposites	Plate incorporation	0/2 samples positive	-	-	Distilled water or cell culture medium as simulant at 40 °C for 10 d, concentration through evaporation	(Maisanaba et al., 2015)
Can coatings	Ames II	0/2 samples positive	-	-	Extraction in 95% Ethanol for 4 h at 60 °C, evaporation to dryness and dissolved in DMSO	(Mittag et al., 2006)
Coating (on PC)	Plate incorporation	0/2 samples positive	-	-	Isooctane or 10/95% Ethanol as simulant for 1 h at 100 °C, 3 h at 60 °C or 1 h at 60 °C (Isooctane), then evaporated to dryness and dissolved in Ethanol (95 or 10%)	(Séverin et al., 2016)

[1] tester strain/s for which the LEC was found

[2] this column shows, whether an external metabolic activation system (S9) was used to achieve the positive result



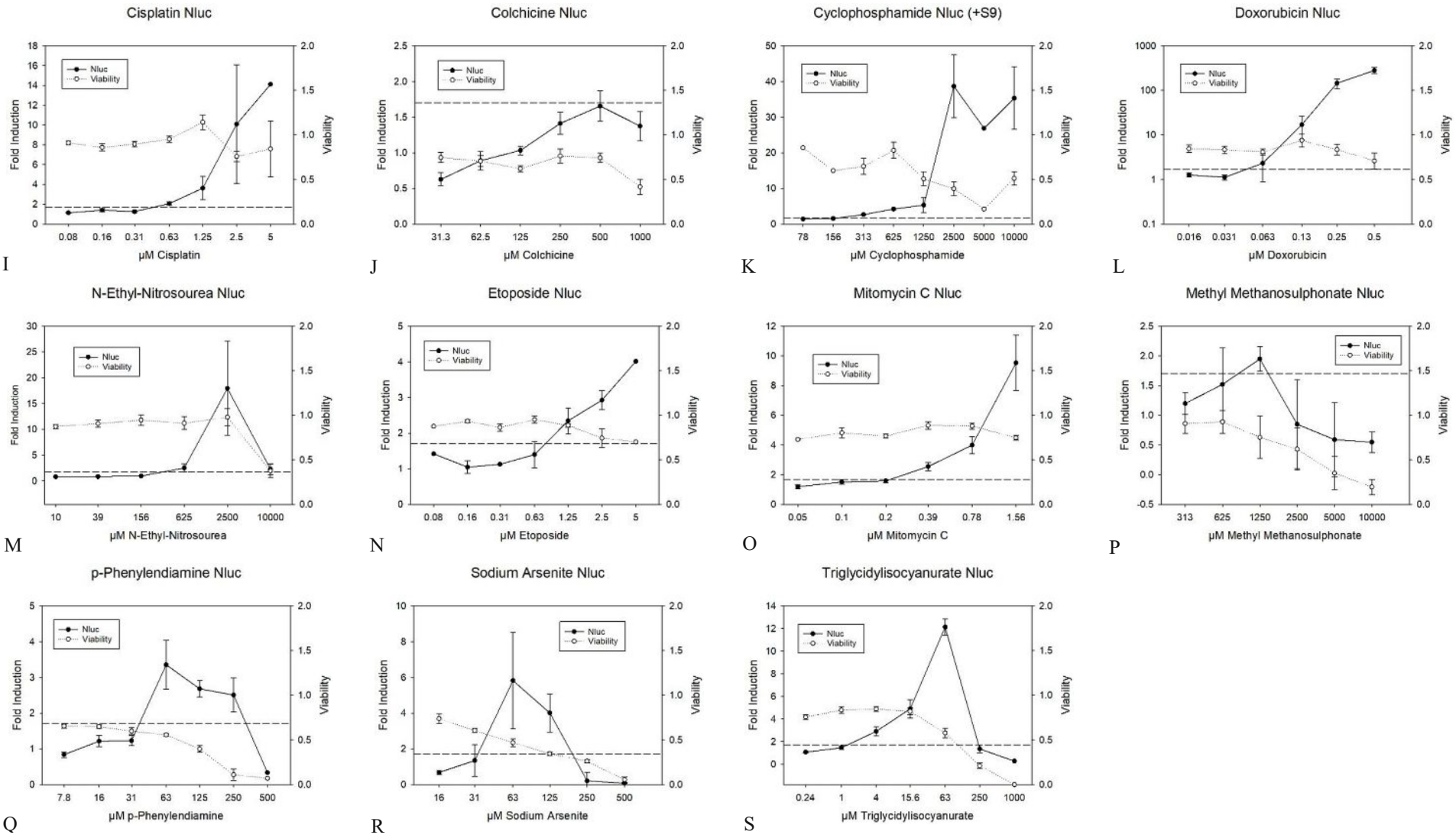


Figure A 1: Detailed results for known genotoxic substances for pure substances tested with the HepG2 reporter gene assay. The Nluc response is calculated in relation to the mean background value and a threshold of 1.7 is used to determine a positive or negative response. The viability threshold is set at 70 %, below which the response is considered as cytotoxic.

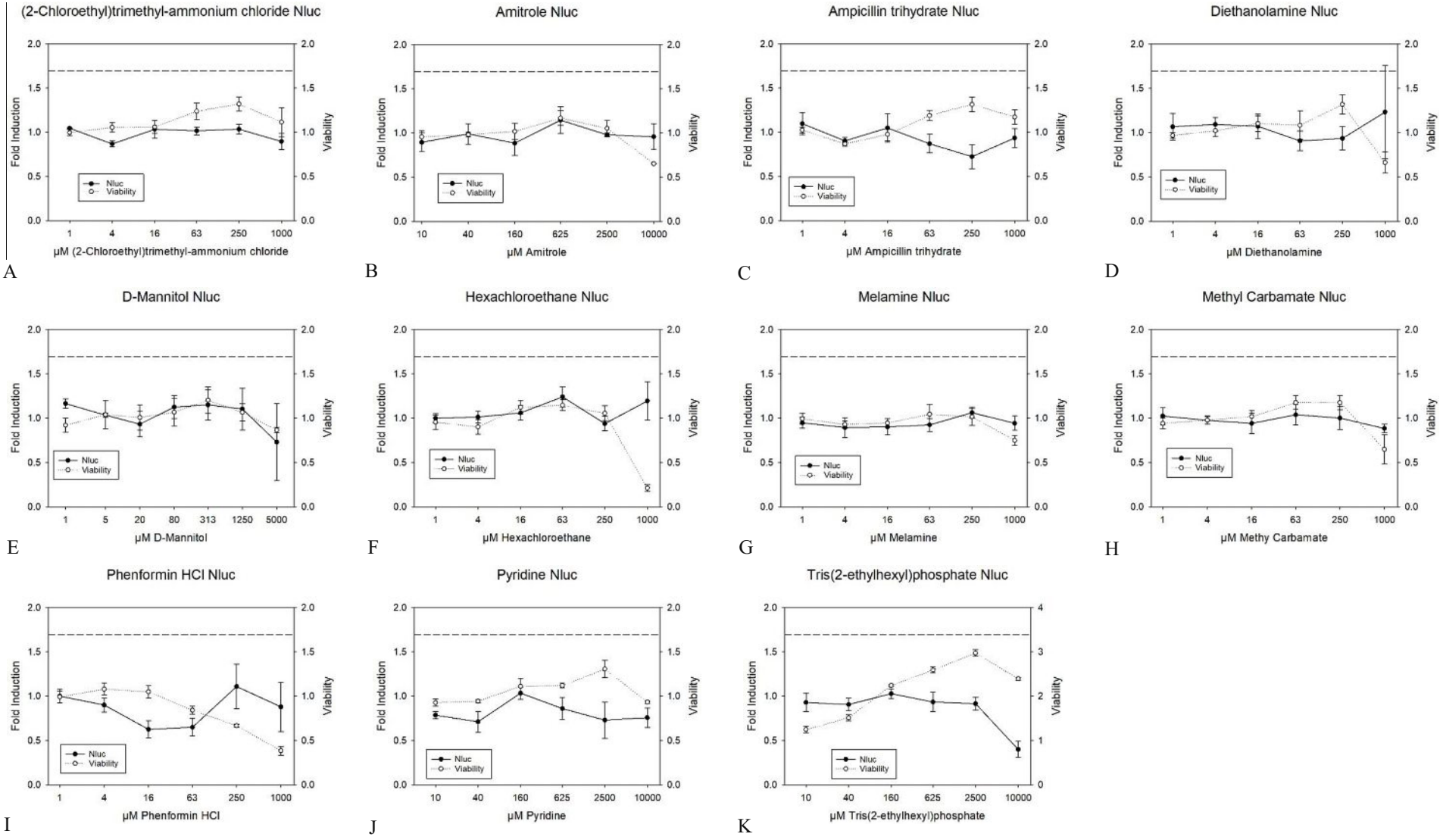


Figure A 2: Results of the HepG2 reporter gene assay for the non-genotoxic substances. The Nluc response is calculated in relation to the mean background value and a threshold of 1.7 is used to determine a positive or negative response. The viability threshold is set at 70 %, below which the response is considered as cytotoxic.

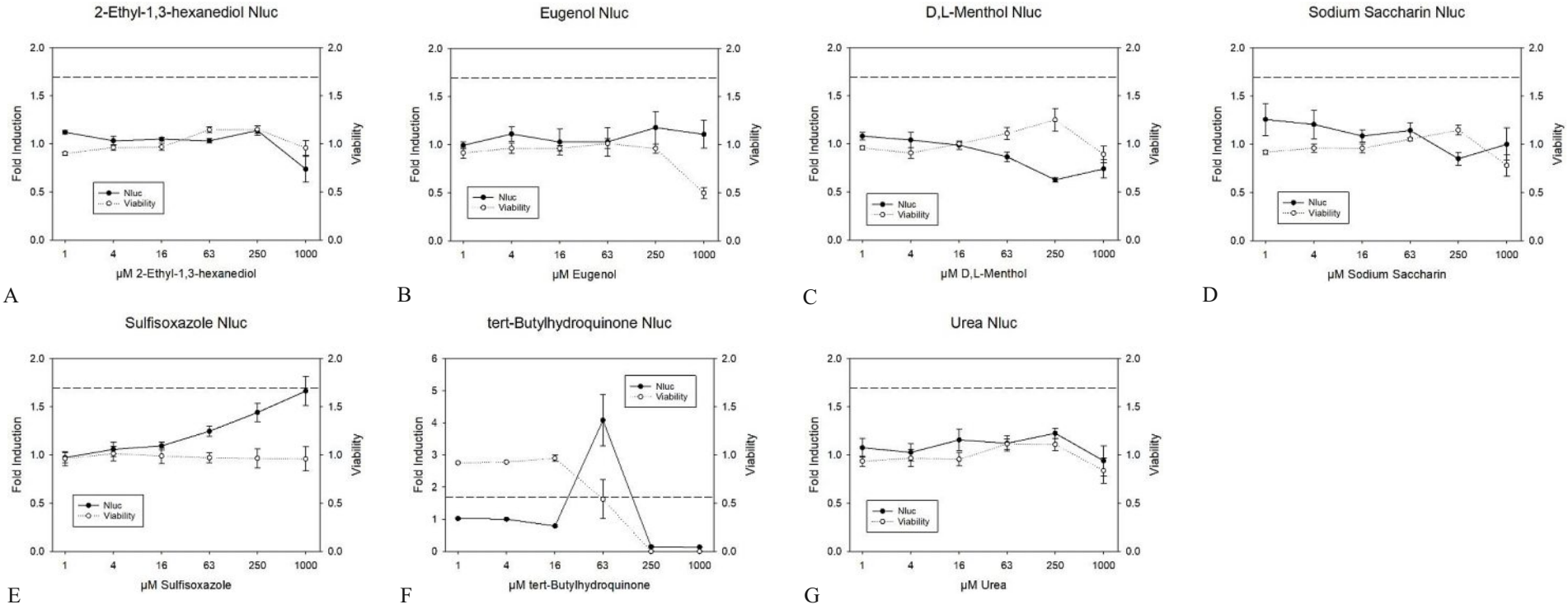


Figure A 3: Results of the HepG2 reporter gene assay for non-genotoxic substances *in vivo* that are sometimes *in vitro* positive. The Nluc response is calculated in relation to the mean background value and a threshold of 1.7 is used to determine a positive or negative response. The viability threshold is set at 70 %, below which the response is considered as cytotoxic.

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