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Determination of Genotoxicity of Plastic Polymer Recyclates by the Ames-MPF Assay

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Declaration of Originality

I confirm that this assignment is my own work and that I have not sought or used inadmissible help of third parties to produce this work. I confirm that I have clearly referenced all sources used in the work. I have fully referenced all text directly or indirectly quoted from a source. This work has not yet been submitted to another examination institution and has not yet been published.

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Abstract

Using plastic polymers as packaging materials in the food industry has many benefits. However the major disadvantage is that polymers are non-degradable and thus influence the environment badly. In Austria not even 50 per cent of the used polymers can be recycled.

According to the European Circular Economy Strategy, all plastic packaging materials on the market have to be recyclable till 2030. Currently only PET bottles can be recycled for use in food contact. Therefore, the packaging material producing industry has to develop recyclable food packaging solutions for all packaging types.

The biggest problem of recyclable packaging polymers for use in the food industry are the non-intentionally-added substances (NIAS), which can migrate from the food packaging into the food and can be consumed by human beings. Therefore, it is very important to determine the health risk of NIAS on the consumers.

In the project called “Polycycle“, different packaging polymer types have been tested to make sure that the packaging material producing industry can recycle the common packaging polymers. In this master‘s thesis the polymer recyclates have been tested for their genotoxic effect with the Ames MPF assay. For this, the sample preparation of the plastic polymer recyclates is a very important topic. It includes the migration process to extract the substances in the recyclate-samples under defined conditions. This is done with an extracting agent and impact of heat for a certain time. After that the substances are concentrated by rotary evaporation and evaporated under vacuum for solvent change using a Visiprep™ SPE Vacuum Manifold. Then the Ames MPF assay is performed to detect possible genotoxic substances in the polymer recyclate-samples.

Due to the many steps involved in sample preparation and the long impact of heat during the migration process, it is possible that the genotoxic substances are lost or degraded. For this purpose the biological recovery of the genotoxic substances is additionally determined. To do this, aliquots of the extracting agents are spiked with different DNA-reactive substances and are conducted parallel to the recyclate-samples. To assess matrix effects of the recyclate-samples in the assay, the response to a known Ames-positive substance was evaluated. Therefore all polymer recyclate-samples were tested in absence and presence of a positive control spike.

The polymer-recyclate samples which have been tested for the project “Polycycle” clearly show negative results in the Ames MPF assay. It seems as if the results of the biological substance recovery depend on the different pure substances. Furthermore substance losses during sample preparation due to human and technical errors cannot be excluded.

Zusammenfassung

Die Verwendung von Kunststoffpolymeren als Verpackungsmaterial in der Lebensmittelindustrie hat viele Vorteile. Der größte Nachteil ist jedoch, dass Polymere nicht abbaubar sind und somit die Umwelt stark beeinflussen. In Österreich können nicht einmal 50 Prozent der verwendeten Polymere recycelt werden.

Laut der europäischen Strategie zur Kreislaufwirtschaft müssen bis 2030 alle auf dem Markt befindlichen Kunststoffverpackungen recycelbar sein. Derzeit können nur PET-Flaschen für den Lebensmittelkontakt recycelt werden. Daher muss die verpackugsmaterialproduzierende Industrie recyclebare Lebensmittelverpackungslösungen für alle Verpackungsarten entwickeln.

Das größte Problem von recycelbaren Verpackungspolymeren für den Einsatz in der Lebensmittelindustrie sind die nicht absichtlich zugesetzten Substanzen (NIAS), die aus der Lebensmittelverpackung in das Lebensmittel migrieren und vom Menschen verzehrt werden können. Daher ist es sehr wichtig, das Gesundheitsrisiko von NIAS für den Verbraucher zu ermitteln.

In dem Projekt "Polycycle" wurden verschiedene Verpackungspolymertypen getestet, um sicherzustellen, dass die verpackungsmaterialproduzierende Industrie die gängigen Verpackungspolymere recyceln kann. In dieser Masterarbeit wurden die Polymerrezyklate auf ihre genotoxische Wirkung mit dem Ames MPF Assay getestet. Dabei ist die Probenvorbereitung der Kunststoffpolymerrezyklate ein sehr wichtiges Thema. Sie umfasst den Migrationsprozess zur Extraktion der Substanzen in den Rezyklat-Proben unter definierten Bedingungen. Dies geschieht mit einem Extraktionsmittel und Wärmeeinwirkung für eine bestimmte Zeit. Danach werden die Substanzen durch Rotationsverdampfung aufkonzentriert und unter Vakuum zum Lösungsmittelwechsel mit einem Visiprep™ SPE-Vakuum-Manifold eingedampft. Anschließend wird der Ames-MPF-Assay durchgeführt, um mögliche genotoxische Substanzen in den Polymerrezyklat-Proben nachzuweisen.

Aufgrund der vielen Schritte bei der Probenvorbereitung und der langen Hitzeeinwirkung während des Migrationsprozesses ist es möglich, dass die genotoxischen Substanzen verloren gehen oder abgebaut werden. Zu diesem Zweck wird zusätzlich die biologische Wiederfindung der genotoxischen Substanzen bestimmt. Dazu werden Aliquots der Extraktionsmittel mit verschiedenen DNA-reaktiven Substanzen gespikt und parallel zu den Rezyklat-Proben geführt. Um Matrixeffekte der Rezyklat-Proben im Assay zu beurteilen, wurde die Reaktion auf eine bekannte Ames-positive Substanz ausgewertet. Dazu wurden alle Polymer-Rezyklat-Proben in Abwesenheit und Anwesenheit eines positive Kontrollspikes getestet.

Die Polymer-Recyclat-Proben, die für das Projekt "Polycycle" getestet wurden, zeigen eindeutig negative Ergebnisse im Ames MPF Assay. Es scheint so, als ob die Ergebnisse der biologischen Substanzwiederfindung von den unterschiedlichen Reinsubstanzen abhängen. Außerdem können Substanzverluste bei der Probenvorbereitung durch menschliche und technische Fehler nicht ausgeschlossen werden.

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Figures

Glossary

2-AA	2-Aminoanthracene
2-AF	2-Acetylaminofluorene
2-NF	2-Nitrofluorene
4-NQO	4-Nitroquinoline-1-oxide
9-AA	9-Aminoacridine
ADI	acceptable daily intake
BaP	Benzo[a]pyrene
bw	body weight
C- process	process negative control
C-	negative control
C+ process	process positive control
C+	positive control
Da	Dalton
DIN	Deutsches Institut für Normung
DMSO	dimethyl sulfoxide
DNA	desoxyribonucleinacid
ECVAM	European Centre for the Validation of Alternative Methods
EFSA	European Food Safety Authority
ELSD	LC-evaporative light scattering detection
ENU	N-Ethyl-N-Nitrosourea
FCM	food contact materials
G-6-P	Glucose-6-Phosphate
GC-FID	gas chromatography flame ionisation detection
GC-MS	gas chromatography mass spectrometry
GMP	good manufacturing practice
GPPS	general purpose polystyrene
HDPE	high-density polyethylene
HIPS	high impact polystyrene
HPLC-UV	high performance liquid chromatography ultraviolet detection
IAS	intentionally added substances

ICP-MS	inductively coupled plasma with mass spectrometry
k	thermal conductivity
LC	liquid chromatography
LC-MS- Orbitrap	liquid chromatography mass spectrometry with orbitrap
LC-MS	liquid chromatography mass spectrometry
LC-UV	liquid chromatography ultraviolet detection
LDPE	low-density polyethylene
LEC	lowest effect of concentration
LLDPE	linear low-density polyethylene
LOB	limit of blank
LOBD	limit of biological detection
LOCD	limit of chemical detection
LOD	limit of detection
LOI	limit of interest
LOQ	limit of quantification
$MgCl_2$	Magenesium chloride
MMS	Methyl Methanesulfonate
MPF	microplate format
MS-EI	mass spectrometry with electron impact ionization
MS-ESI	mass spectrometry with electrospray ionization
Na_2HPO_4	Disodium phosphate
NaCl	Sodium chloride
NADP	nicotinamidadenindinucleotidphosphat
$NaH_2PO_4 * H_2O$	Sodium Dihydrogen Phosphate Monohydrate
NIAS	non-intentionally added substances
nm	nanometers
NMR	nuclear magnetic resonance
OD	optical density
OECD	the Organisation for Economic Co-operation and Development
OFI	Österreichisches Forschungsinstitut
OM	overall migration
OML	overall migration limit

PE	polyethylene
PET	polyethylenterephthalat
PO_4	phosphate
PP	polypropylen
PPPO	poly(2,6-diphenyl-*p*-phenylenoxid)
PS	polystyrene, polystyrol
rpm	rounds per minute
SD	standard deviation
SM	specific migration
SML	specific migration limit
SMLT	total specific migration limit
SPME	solide phase microextraction
TDI	tolerable daily intake
T_g	glass transition temperature
T_m	softening point
TTC	threshold of toxicological concern

1. Introduction and Research Questions

In this master's thesis polymer recyclate-samples are tested on their genotoxic activity with the Ames MPF assay to find out whether they can be used as food packaging materials without endangering humans. At first the substances are migrated with high and long impact of heat. Then the sample preparation, which includes two main steps is done. To look if substances are lost or degraded during migration process and sample preparation the biological substance recovery is determined. Further experiments for example testing of rPET-samples, different growth-times of the bacteria cultures, testing of other bacteria tester strains in the assay and determination of the biological substance recovery after the harshest migration process (according to the regulation EU10/2011) are also made.

The main part of this master's thesis contains the migration process including the sample preparation of the polymer recyclate samples and the test procedure of the Ames MPF assay.

In this part some research questions are summarized which are dealt with in the practical part of this master's thesis:

- Do the plastic polymer recyclate samples show a genotoxic effect in the Ames MPF assay? Can the different plastic types be recycled for use in foodstuff without hesitation for the consumers?
- Can the genotoxicity of the substances be detected at all after the migration process? Or is it possible that the genotoxicity is influenced/reduced by the migration conditions like high and long impact of heat?
- Is it possible that some substances react with the polymer samples during the preparation steps and thus affect the genotoxicity of the substances?
- How good is the used sample preparation process? Is it possible that substances or part of substances are lost due to human or technical errors?
- Are there polymer samples which cause problems during sample preparation and therefore have to be treated differently?
- Can the mutagenic substances be detected at all after the harshest migration conditions according to the regulation EU 10/2011?
- Do the recycled PET samples really show negative results?
- Do the different growth-times of the bacterial tester strains affect the results? For example, does a longer growth-time of the bacteria culture correlate with a higher amount of revertants in the assay?

1.1. Overview

This master‘s thesis deals with the analysis of different plastic polymer recyclates and their genotoxic effect with the Ames MPF assay. At the beginning the theoretical background is explained. It deals with the food contact materials, the Ames *in-vitro* bioassay, the different types of plastic and the migration process. Then the practical part is presented. It is about the steps of sample preparation and the Ames MPF assay, the results and the interpretation of the analysis. The last two chapters include the discussion of the results and the conclusion.

1.2. Food Contact Materials

Food contact materials are complex mixtures with different chemical and toxicological properties, which are intended to be brought into contact with food[1]. This can happen during the production, processing, storage, preparation and serving. FCM can already be in contact with food, can reasonably be brought into contact with food or can transfer their constituents to the food under normal use or under foreseeable use. The contact can be direct or indirect. FCM include packaging materials, kitchenware and tableware, containers for the transport and machinery for the process of food with different materials such as paper, plastic, metal, glue, coatings. The constituents of FCM should be widely safe, so that there are not any effects on the consumer’s health. Furthermore, the quality of the products should not be influenced by FCM[2].

Deliberately used chemicals are called intentionally-added substances (IAS). They are needed because of functional and technical reasons in the manufacturing process or the final product. IAS are regulated and their toxicological properties on the consumers have been assessed. Due to the chemical and physical composition of the FCM and food it is possible that chemical reactions take place. This could be degradations, breakdown, side-reactions and migration of constituents. The unknown substances which arise in food because of chemical reactions are called non-intentionally-added substances (NIAS)[3].

1.2.1. Intentionally- and Non-intentionally added Substances

IAS are specifically added substances which are needed in the production process of food or in the final product. Substances such as monomers, additives, production supplies, solvents and so on are considered as IAS. The regulation of EU 10/2011 includes in annex 1 a list of all authorized monomers, starting substances, additives and production supplies which are

[1] (Pinter Elisabeth, 2020)
[2] (EuropeanCommission, 1995-2021)
[3] (Grob K., 2010)

needed to the production of plastics. The substances of the list are toxicologically assessed and are researched as harmless for the human health[4].

Especially, problematic are the NIAS, which are a result of an interaction between constituents of the FCM and the constituents of food. According to the Regulation of EU 10/2011, NIAS are defined as: “non-intentionally added substance means an impurity in the substances used or a reaction intermediate formed during the production process or a decomposition or reaction product”. NIAS always represent a part of the full composition of the FCM. Raw chemicals and pesticides used to synthesis additives are also seen as NIAS. The most substances in this class have unknown chemical structures, therefore they are not identified and toxicological assessed. If the substances migrate to the food, they can lead to negative effects on the consumer’s health. For this reason, it is absolutely necessary to include NIAS in the safety assessment of FCM[5]. For this master’s thesis the substances in the polymer recyclate-samples are migrated and extracted under defined conditions so that they can be detected with the Ames MPF assay.

1.2.2. Legal Basics

The food & packaging industry in the European Union must follow three important regulations: EG 1935/2004, EG 2023/2006 and EU 10/2011. The regulation EG 1935/2004 is about materials and objects which are specified for the contact with food. It includes definitions, limitations and demands about FCM. The materials must not pose a risk to human health. Furthermore, they are not allowed to change the compounds and to impair the organoleptic characteristics of food. They must be authorized and succumb a hazard assessment. The materials must be traceable via production, manufacture and distribution[6]. The regulation EG 2023/2006 includes the good manufacturing practice of materials and objects which are specified for food contact. The GMP means guidelines of quality management and quality control of all stages of production in food industry. This process should guarantee the safety for the consumers[7]. A special regulation is the regulation EU 10/2011 about plastic implementation measure. It includes general guidelines about materials and objects made of plastics for food contact. For example, there is a complete list of all monomers, additives and raw materials which can be used by the production of plastics for food contact. All substances of this list, including the reaction and degradation products which can arise by the use of the

[4] (Regulation EU 10/2011 of 14 January 2011 on plastic materials and articles intended to come into contact with food, 2011)
[5] (Regulation EU 10/2011 of 14 January 2011 on plastic materials and articles intended to come into contact with food, 2011)
[6] (VERORDNUNG (EG) Nr. 1935/2004 über Materialien und Gegenstände, die dazu bestimmt sind, mit Lebensmitteln in Berührung zu kommen und zur Aufhebung der Richtlinien 80/590/EWG und 89/109/EWG, 27. Oktober 2004)
[7] (VERORDNUNG EG Nr. 2023/2006 über gute Hygienepraxis für Materialien und Gegenstände,die dazu bestimmt sind, mit Lebensmitteln in Berührung zu kommen, 22. Dezember 2006)

substances have to be risk assessed. The specific migration limit describes the maximum permitted amount of a specific substance that can migrate from the packaging material into food. It illustrates a safety limit from toxicological studies. If an SML does not exist, a default limit of 60mg/kg food can be used for individual substances. Because of the SML no health risk for the consumers is expected. The migration limit of non-licensed substances is fixed to 0,01mg/kg in food. Mutagenic, cancerogenic and reprotoxic substances are not allowed to be used in food without a licence[8].

The regulation EU 10/2011 also includes the NIAS but there are not any specific regulations nowadays. NIAS can also be used in other materials beside plastic, which are used for food contact[9].

1.2.3. Risk Assessment of FCM

The risk assessment consists of four steps. These steps are hazard identification, hazard characterisation, exposure assessment and risk characterisation. Hazard identification means the identification of biological, chemical and physical agents which are present in food and can have effects on the human health. In the second step, the hazard characterisation, the effects of these hazards are researched[10]. The toxicological classifications numbers ADI and TDI can be determined. The ADI and TDI declare the amount of a substance which can be absorbed daily a lifetime without any health damage[11]. This is possible when the substances are known. Toxicity studies are necessary for the authorization of the known substances and the migration value must be under 0,05mg/kg body weight. But usually only limited toxicity data or no toxicity data are available for the detection and identification of NIAS. This makes the hazard identification and characterisation very challenging[12]. In this case the TTC-value is used. The TTC is a pragmatic screening and prioritisation tool in food safety assessment. It is able to evaluate the risk of substances which are present in food in very low concentrations. A value below the TTC-value means that the substance is harmless for the human health. If the value is above the TTC-value it is necessary to make more tests to determine the toxicity. For substances that have the potential to be DNA-reactive mutagens and/or carcinogens the relevant TTC value is 0,0025 µg/kg body weight per day[13]. In the third step, the exposure assessment, the current exposition of the potential hazards in the population is regarded. Scientists use data on chemicals in food and food consumption from the population across Europe. Finally, all data of the three steps are used for the risk characteri-

[8] (VERORDNUNG (EU) Nr. 10/2011 über Materialien und Gegenstände aus Kunststoff, die dazu bestimmt sind, mit Lebensmitteln in Berührung zu kommen, 14. Januar 2011)
[9] (EuropäischeKommission, VERORDNUNG (EU) Nr. 10/2011 über Materialien und Gegenstände aus Kunststoff, die dazu bestimmt sind, mit Lebensmitteln in Berührung zu kommen, 14. Januar 2011)
[10] (EFSA - European Food Safety Authority)
[11] (AGES - Österreichische Agentur für Gesundheit und Ernährungssicherheit GmbH)
[12] (Koster Sander, 2016)
[13] (EFSA Scientific Committee, 2019)

sation. Scientists try to make statements about likelihood, incidence and intensity of the known or potential negative effects on the human health[14].

1.2.3.1. Chemical Analysis of NIAS

People are exposed to a mixture of chemicals migrating from FCM into food. In practice FCM can include more than 8.000 substances. At the moment toxicological testing of FCM is focused only on single substances because it is difficult to extract and analyse all the substances. The scientists are working with migrates made from FCM, which have the best possible simulation to foods. These migrates are prepared, analysed and tested on their genotoxicity[15].

NIAS with a molecular weight up to 1000 Da need to be analysed. NIAS with a molecular weight exceeding 1000 Da are generally not considered for risk assessment because it is assumed that no migration takes place. The assessment of predicted and unpredicted NIAS in food is performed in different ways. The FCM is disassembled down to the raw material and each step during production is considered. It has been demonstrated for several plastic materials that many NIAS can be predicted based on theoretical chemistry, analytical experience and literature search. Predicted NIAS can be analysed by targeted analytical methods of the known substances. The unpredicted NIAS that have not been detected before are analysed with non-targeted screening analytical methods. The methods must be able to detect substances with a wide range of physical-chemical properties. The scope of targeted analytical methods and non-targeted screening methods should be at the low µg/kg food level[16].

Targeted analytical methods for quantification of predicted NIAS need the use of one or more internal standards. This can be the same or structurally very similar compared to the NIAS. The standards should be added at a level in the range of the expected migration of the NIAS. It may also be considered whether a worst-case scenario is reseted, where 100 per cent transfer to the foodstuff takes place[17].

Screening analysis detects unpredicted NIAS but can also detect predicted NIAS and IAS. To do this a starting substance is extracted with an extraction solvent. After the sample preparation the analysis is done. The analytical methods for volatile compounds are for example the headspace/SPME, GC-FID and GC-MS. To detect semi-volatile substances the GC-FID and GC-MS are used. The methods for nonvolatile and polar compounds are the LC-UV, ELSD and LC-MS. For trace elements the ICP-MS and for general screenings the NMR are used. The use of internal standards by screening methods is a big challenge. A wide range

[14] (AGES - Österreichische Agentur für Gesundheit und Ernährungssicherheit GmbH)
[15] (Ksenia J. Groh, 2017)
[16] (Koster Sander, 2016)
[17] (Koster Sander, 2016)

of NIAS is present in FCM. It is important to choose a detector which gives a relatively comparable response for the large spectrum of the NIAS. Furthermore, the response of the internal standard should be similar to that of the NIAS. For the identification of the substances, a calibration range with the standards is made to determine the accurate concentration of the NIAS. It is not always possible to fully identify every substance. Full identification is necessary when substances exceed the limit of interest (LOI). Substances which fall below the LOI need not to be identified[18].

1.2.3.2. In-Vitro Bioassays

Next to the analytical methods there are *in-vitro* bioassays to test the overall migrate samples from FCM. The assays have been applied to screen the three main toxicological endpoints: cytotoxicity, genotoxicity and endocrine disruption potential. So they play an increasing role in the toxicological hazard identification. The tests may provide a picture of the toxicological effects elicited by the mixture of known and unknown substances which can migrate from FCM[19]. By analysing complete mixtures of FCM and NIAS, the *in-vitro* assays have to acquire additional hazard information on the biological activity of the migrates. This is because of the potential interaction between the NIAS and IAS and other components in the migrates. The results of *in-vitro* assays directly show the effect of compounds which represent a toxicological hazard. In this case the mentioned compounds are analysed with chemical methods or even *in-vivo* assays for further risk assessment. If the toxicity is at very low dose levels, the health relevance of possible cumulative effects is considered also very low. In this case the use of a correction factor to cover possible cumulative effects is very low to absent. For some known contaminants for examples endocrine disruptors and genotoxic carcinogens, it is necessary to consider a combination effect also at low dose[20].

Compared to *in-vivo* assays the *in-vitro* assays are cost-effective, have short processing times, need lower requirements and are able to achieve high throughput. By using *in-vitro* assays a reduction of animal use is reached. Thus *in-vitro* tests represent an economic solution to screen the toxicity of FCM. On the other hand the big limitation of *in-vitro* tests is the lack of complete understanding of toxicokinetic processes, lack of xenobiotic metabolism capacity and absence of interactions present within a complex multicellular organism. Furthermore, the substances tested *in vitro* are not be subject to influences from food components and from the human digestion processes[21].

In the practice the *in-vitro* bioassays are often used. For example, Frederic D.L. Leusch et al. analysed the endrogenic, progestagenic, glucocorticoid, thyroid and estragenic activity suita-

[18] (Koster Sander, 2016)
[19] (Ksenia J. Groh, 2017)
[20] (Koster Sander, 2016)
[21] (Ksenia J. Groh, 2017)

ble for drinking water and environmental water with *in-vitro* assays[22]. In another study L. Connolly et al. used *in-vitro* assays to determine the endocrine-disrupting in food additives and contaminants[23]. The main advantage of *in-vitro* tests is that they wholly investigate the contamination and can detect a hazard of unidentified, not quantified and not detected chemicals. In relation to FCM, the *in-vitro* assays are a helpful tool in hazard identification for the first screening of the substances and after that they can be used in conjunction with further chemical analyses[24].

1.3. Toxicology

1.3.1. Genotoxicity

Genotoxic substances have the ability to damage the genetic material in the cells. Under certain circumstances the damage can lead to cancer. DNA damages such as mutagenicity, carcinogenic and teratogenicity belongs to Genotoxicity[25]. If toxic substances damage the cells, reversible and irreversible mutations can arise in the cells. The mutations can be inheritable and passed to the daughter cells. Some chemicals, toxins, rays, bacteria, virus are cancer-causing. Teratogenic substances can have negative effects on the fetus and embryo. They can lead to long-lasting troubles for the unborn child. The dangerous substances are activated in the human body with the metabolism of the enzyme cytochrom-p450 for foreign materials. Some substances interact directly with the DNA in the cells to cause cell mutations, cell changes and cell death. These parameters can finally lead to cancer. Other substances damage the DNA indirectly for example with the activation of oxidative stress in the cell. This process can also cause cancer[26].

In the EU food contact materials must be tested for their genotoxic effect to receive authorisation. This happens with tests which can assess the occurrence of DNA mutations, chromosomal aberrations and alterations in DNA repair processes[27]. According to the OECD-guidelines there are three *in-vitro* assays that could or should be applied for FCM testing: one to detect gene mutation in bacteria (=bacterial reverse mutation test, OECD 471), another to detect gene mutation in mammalian cells (OECD 476) and the third one to detect chromosomal aberrations (OECD 473). There are also two *in-vivo* assays: the mammalian bone marrow chromosome aberration test (OECD 475) and the mammalian erythrocyte micronucleus test (OECD 474)[28]. In this master's thesis the in-*vitro* bacterial reverse mutation test is

[22] (Leusch F.D.L., 2017)
[23] (L. Connolly, 2011)
[24] (Koster Sander, 2016)
[25] (Ksenia J. Groh, 2017)
[26] (Alfred Nordheim, 2018)
[27] (Ksenia J. Groh, 2017)
[28] (Isabelle Severin, 2017)

used as a first screening to detect the genotoxicity of the substances in the polymer recyclate-samples.

1.3.1.1. Mechanism of Gene Mutations

The Gene Mutation is a change of the genetic information in the cell and can be inheritable. Point mutations are the cause of many human genetic diseases and there is fundamental evidence that point mutations in oncogenes and tumour suppressor genes of somatic cells are involved in tumour formation in humans and experimental animals. There are two different types of mutations: gene mutations and chromosomal mutations. Chromosomal mutations are changes in the structure, form or number of chromosomes triggered by five different reasons. The chromosomes can lose a segment (=deletion) or can break and lose a segment which can be built in another chromosome (=translocation). Because of that a segment of a chromosome can be present in double (=duplication). If a chromosome breaks twice, the broken segment can be fixed up inverse (=inversion). A chromosome can have an added segment (=insertion, addition). The gene mutations are limited to changes inside the gene caused by point mutations in the base-pair sequence. A base is added (=insertion, addition) or eliminated (=deletion) inside the base-pair sequence. A silent mutation does not have negative effects because of the degeneration of the genetic code. Accordingly, the amino acid sequence does not change. If an exchange of nucleotides with another amino acid species takes place, the genetic code changes (=missence mutation). If a premature stop codon is present, the translation is stopped prematurely and a shortened and probably not fully functional protein arises (=nonsense mutation). If it comes to an exchange of the pyrimidine nucleotide (cytosine + thymine) with a purine nucleotide (adenine + guanine) or inverse, the process is called transversion. A transition is the exchange of a purine nucleotide with another purine nucleotide or of a pyrimidine nucleotide with another pyrimidine nucleotide. At this point frameshift mutations can develop. These mutations can lead to significant consequences in the genes. In the process of DNA replication always three bases code for one amino acid. If one or more base-pairs are lost (=deletion) or extra attached (=insertion) it comes to a shift in the reading frame of the DNA. The whole reading frame of the DNA is changed and does not work precisely. Consequently, the gene loses its whole function (=loss-of-function-mutation)[29].

1.3.2. Cytotoxicity

Cytotoxicity means the ability of substances being toxic to cells. The substances can damage the cells and tissues, and this can lead to cell death. There are a variety of cell fates for example the cells can start necrosis, lose membrane integrity and die rapidly, the cells can stop

[29] (Alfred Nordheim, 2018)

growing and dividing or the cells can activate apoptosis (controlled cell death)[30]. Cytotoxicity assays are *in-vitro* assays to observe the cell growth, reproduction and morphological effects of the usual physiology of the cells[31]. The tests are used as a primary aid for selecting test concentration. The cells which are used in these assays must in fact undergo cell division. In mammalian cells the cytotoxicity can be determined with the cell viability to assess the cell membrane integrity. The cell viability shows the relationship of living cells to dead cells[32]. The cytotoxicity in bioassays is important to interpret genotoxicity data. Genotoxicity assays should provide cytotoxicity information. But it is possible that cytotoxicity of some components may mask or induce the genotoxicity activity of other components in the assay. Nevertheless it is important to try to produce cytotoxic concentrations in the assay to show that higher concentrations could not have been tested. The sample matrix can have significant effects on the LOBD of the assay. To test matrix-interferences the samples should be spiked with known genotoxic pure substances (directly active substances for example 4-NQO and substances requiring metabolic activator for example 2-AA). If the spike recovery of a sample is below 60% this could be an indication of cytotoxicity or growth inhibiting effects[33]. In this master's thesis the polymer recyclate-samples were spiked with 2-AA in two different concentrations, 4-NQO and 2-NF to assess matrix-interferences.

1.3.3. Limit of Detection, Limit of Qualification, Lowest Effect of Concentration

The Limit of Detection (LOD) is the lowest analyte concentration which can be detected and qualified with the analytical method. It represents the smallest amount which can be differentiated significantly from the LOB. The LOB is the limit of blank. It is the highest apparent analyte concentration expected when replicates of a blank sample containing no analyte are tested. The assumption is that a present analyte produces a signal greater than the analytical noise in the absence of the analyte. With the aim of the LOD the scientists can determine if the analyte is existent. If the amount of the analyte is below the LOD, it can not be determined. Generally, the LOD is calculated three times the standard deviation of the blank[34].

In case of a bioassay the LOD will be termed LOBD (limit of biological detection) and when related to analytical chemistry the LOD will be termed LOCD (limit of chemical detection)[35].

Is the analyte upwards to the LOQ (=limit of quantification) the concentration of the analyte in the sample can be defined. With the aim of the LOQ the scientists can quantify the analyte[36].

[30] (Riss T.L., 2004)
[31] (Weijia Li, 2015)
[32] (Guidance Document on Revisions to OECD Genetic Toxicology Test Guidelines, 31.08.2015)
[33] (Benoit Schilter, 2019)
[34] (David A Armbruster, 2008)
[35] (ICH HARMONISED TRIPARTITE GUIDELINE, Validation of analytical procedures: Text and Methodology, 1996)

The LEC shows the lowest effect of concentration of the analyte which gives a positive effect in the assay. Especially for NIAS no guidelines and limit values in migrates with FCM exist. The upper limiting value for an unlicensed substance is 0,01mg/kg food. Above this value a complete identification and safety assessment is needed[37]. This process is hindered because of the different or unknown structure of the genotoxic substances[38]. According to different studies it can be said that only potential mutagenic substances can be detected in food and FCM-migrates till the value of 0,01mg/kg. For these studies the LOD and LEC are equalized. This means that only 10 per cent of the mutagenic substances can theoretically be detected[39]. But for genotoxic substances the LOD is not adequate to assess the genotoxic potential[40].

Rainer et al. (2018) calculated the target LEC-value for the Ames MPF assay to 0,4mg/L (calculated from the 10ppt-limit). They worked under the following conditions: concentration factor of 1000 during the sample preparation, sample transfer into 100 per cent DMSO as a solvent, final DMSO concentration in the Ames assay is four per cent and the "global concentration factor" is 40, no loss of substances during the sample preparation, no inhibiting/cytotoxic matrix effects negatively affect the LEC and the LEC is assumed to correspond to the LOD. Accordingly, the Ames assay must be able to detect 0,4mg/L (0,01mg/L*40 concentration factor). This means 0,01mg/L of a mutagenic substance in FCM-migrate. As a comparison the LEC-values of standard substances of the ECVAM-list (European Centre for the Validation of Alternative Methods) only 50 per cent of the listed toxins had positive results at the value of 0,4mg/L in the Ames test[41].

In another study the LEC-value of mutagenic substances in pharmaceutical products should be evaluated. For this study literature data of 454 substances are compared with the target-LEC-value of 0,4mg/L. The results show that the Ames assay is not suitable for detecting a level of 0,01mg/kg for most of the tested substances (>80%). Because of the low concentration of the NIAS in the migrates only 10% of the genotoxic substances can be identified. The Ames assay can detect only a small amount of the substances in the low concentration

[36] (ICH HARMONISED TRIPARTITE GUIDELINE, Validation of analytical procedures: Text and Methodology, 1996)
[37] (EuropäischeKommission, VERORDNUNG (EU) Nr. 10/2011 über Materialien und Gegenstände aus Kunststoff, die dazu bestimmt sind, mit Lebensmitteln in Berührung zu kommen, 14. Januar 2011)
[38] (Koster Sander, 2016)
[39] (Bernhard Rainer E. P.-K., 2018)
[40] (Koster Sander, 2016)
[41] (Bernhard Rainer E. P.-K., 2018)

scope that are present in the migrates[42,43]. The TTC-value for genotoxic substances is 0,15µg/person per day. This value also applies to food[44].

1.3.4. Threshold of Toxicological Concern

The TTC is a screening and prioritization tool for the risk assessment of substances with unknown toxicity in food. It is used when the chemical structure of the substance is known but only limited or no chemical-specific toxicity data are available. It should not be used for substances for which EU food/feed legislation requires the submission of toxicity data, when sufficient data are available for a risk assessment or if the substance falls into one of the exclusions categories[45]. For high-potential cancerogenic substances like aflatoxine-related substances, azoxy- and nitroso-compounds, dioxins and steroids the TTC is not suitable. Furthermore, polyhalogens, dibenzodiaxins, dibenzofurans, biphenyls, heavy metals, nanomaterials and proteins are also not appropriate[46].

The TTC-concept uses the classification schema and the decision tree which were proposed by Cramer, Ford and Hall in 1978. The Cramer schema serves as a priority-setting tool which makes expert judgements in food chemical risk assessment more transparent and reproducible. It is based on the chemical structure of the molecules and functional groups and on their toxicity. Due to this, different Cramer classes of chemicals are proposed. The criteria are shown in table 1[47].

genotoxicity alerts	Substances for which there are structural alerts for genotoxicity, but which are not aflatoxin-like, azoxy- or N-nitroso-compounds
organophosphates	Organophosphate structures which may have neurotoxic properties
Class I (least toxic)	Substances with simple chemical structures and for which efficient modes of metabolism exist, suggesting a low order of oral toxicity. This class would include normal constituents of the body (excluding hormones); simply-branched, acyclic aliphatic hydrocarbons; common carbohydrates; common terpenes; substances that are sulfonate or sulfamate salts, without any free primary amines
Class II (intermediate)	Substances which possess structures that are less innocuous than Class I substances, but do not contain structural features suggestive of toxicity like those substances in Class III. This class would include common components of food; substances containing no functional groups other than alcohol, aldehyde, side-chain ketone, acid, ester, or sodium, potassium or calcium sulfonate or sulfamate, or acyclic acetal or ketal and are either a monocycloalkanone or a bicyclic substance with or without a ring ketone

[42] (Bernhard Rainer E. P.-K., 2018)
[43] (Kenyon MO, 2007)
[44] (Kroes R, 2004)
[45] (EFSA Scientific Committee, 2019)
[46] (Koster Sander, 2016)
[47] (EFSA Scientific Committee, 2019)

Class III (most toxic)	Substances with chemical structures that permit no strong initial presumption of safety or may even suggest significant toxicity or have reactive functional groups. This class would include structures that contain elements other than carbon, hydrogen, oxygen, nitrogen or divalent sulfur; certain benzene derivatives; certain heterocyclic substances; aliphatic substances containing more than three types of functional groups

Table 1: Structural classes for chemicals proposed in the Cramer scheme[48]

For every class, a TTC-value is determined. This is shown in table 2.

Classification	**TTC value in µg/person per day**	**TTC value in µg/kg bw per day**
potential DNA-reactive mutagenes and/or cancerogenes	0,15	0,0025
organophosphates and carbamates	18	0,3
Cramer class III	90	1,5
Cramer class II	540	9,0
Cramer class I	1,800	30

Table 2: TTC values – classification of the substances[49]

For substances which are potential DNA-reactive mutagens and/or carcinogens the relevant TTC value is 0,0025 µg/kg body weight or <0,15µg/person per day[50,51]. If not all substances can be identified, for example in a complex mixture the substances are classified as "potential DNA-reactive mutagens and/or cancerogens". If the DNA reactivity is excluded and the substance is not an acetylcholinesterase-inhibitor, the substance belongs to the Cramer Class III. The activity of an acetylcholinesterase-inhibitor can be detected with chemical methods and the knowledge of packaging materials. For detecting a DNA reactive substance, the bioassay must be capable of identifying true positives at low levels in mixtures. With the aim of chemical methods, the Ames MPF assay and the knowledge of the processing of the packaging materials a substance can be related to Cramer Class III. The unknown NIAS belongs to Cramer Class III[52].

Picture 1 shows the decision tree based on chemical-specific toxicity data and the known chemical structures of the substances[53].

[48] (EFSA Scientific Committee, 2019)
[49] (EFSA Scientific Committee, 2019)
[50] (EFSA Scientific Committee, 2019)
[51] (Kroes R, 2004)
[52] (Benoit Schilter, 2019)
[53] (EFSA Scientific Committee, 2019)

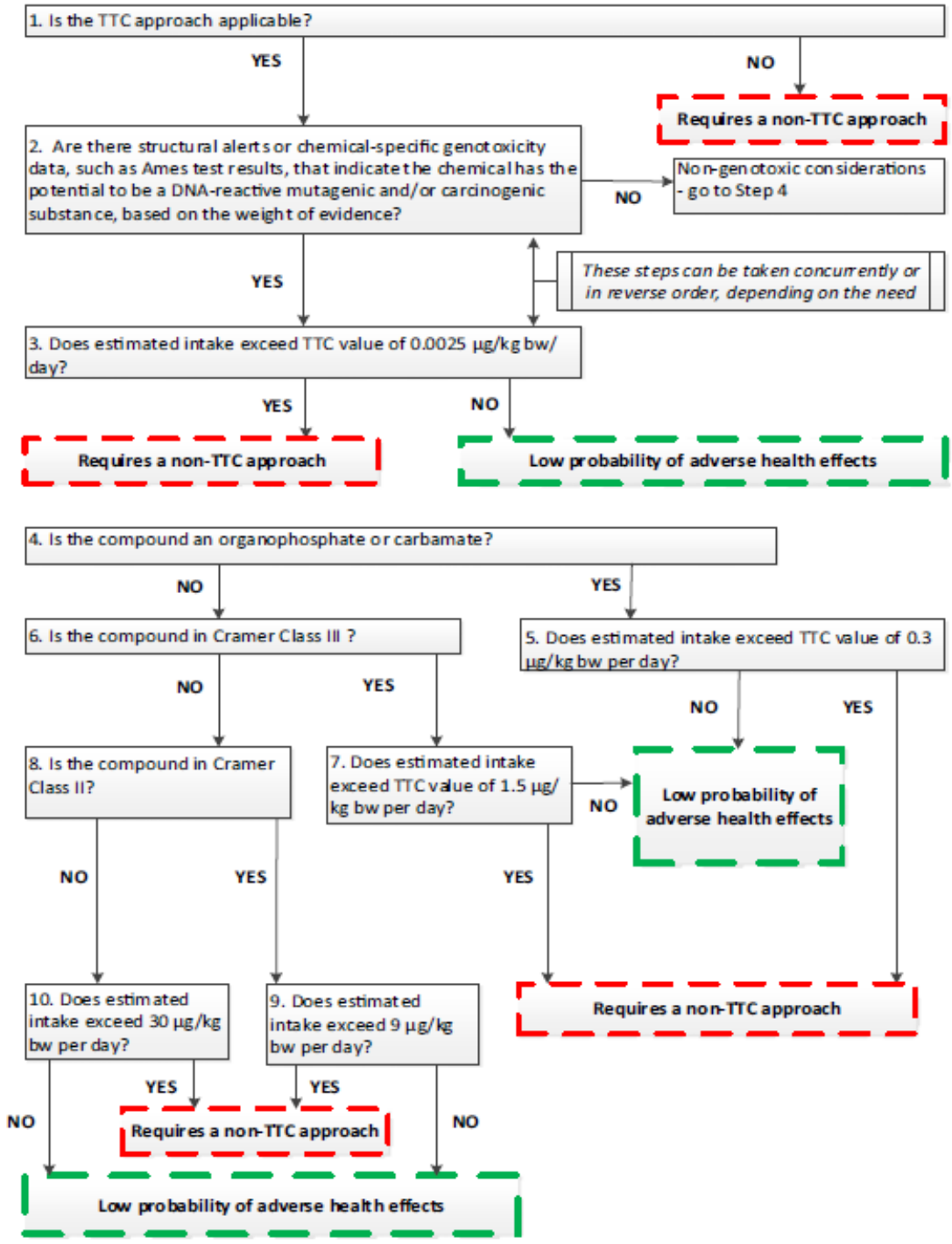

Figure 1: Applying of the TTC decision tree (source: EFSA Scientific Committee, S.J. 2019, Guidance on the use of the Threshold of Toxicological Concern approach in food safety assessment, EFSA Journal)

In step 1 it must be controlled whether the TTC approach is applicable. In step 2 it must be decided whether the substance raises concern for potential DNA-reactive mutagenicity or carcinogenicity. Evidence may come from experimental data, read across from structurally similar chemicals or use of structural alerts. In step 3 the estimated exposure is matched with the TTC-value for DNA-reactive mutagenic or cancerogenic substances. If the estimated exposure is below the TTC-value it can be concluded that there is a low probability of adverse

health effects. If the estimated exposure is higher than the TTC-value, the substances have to be identified in order to determine the toxicity. In step 4/5 it must be checked if the substance is an organophosphate or carbamate and again it must be matched the estimated exposure with the TTC-value for organophosphate and carbamate. In step 6/7 the appropriate Cramer class of the substance is identified. If the substance belongs to Cramer Class III (step 6) and the estimated exposure is below the TTC-value (step 7), it can be concluded that there is a low probability of adverse health effects. If the estimated exposure is higher than this TTC value, a non-TTC approach is required. If the substance belongs to Cramer Class II (step 8) and the estimated exposure is below the TTC-value (step 9), it can be concluded that there is a low probability of adverse health effects. If the estimated exposure is higher than this TTC value, a non-TTC approach is required. If the substance does not belong to Cramer Class II it belongs to Cramer Class I (step 10). Is the estimated exposure below the TTC-value it can be concluded that there is a low probability of adverse health effects. If the estimated exposure is higher than this TTC value, a non-TTC approach is required[54].

Combinative effects of one or more substances cannot be estimated with the TTC-concept. This is critical when substances are absorbed with the same mechanism and the same organs in human body. A special challenge may also occur when several NIAS migrate at very low level. NIAS can migrate in such low concentrations that the identification and the exclusion from the genotoxic effects are not possible. Subsequently the real risk for unknown NIAS cannot be estimated at present[55].

1.4. The Ames *in-vitro* bioassay

The Ames MPF test (Bacteria reverse mutation assay) is a short term bacterial reverse mutation assay. It can specifically detect a wide range of chemical substances that can produce DNA damage and lead to gene mutations. For the determination of the mutagenicity of new substances and FCM the EFSA proposes the Ames test. It is used world-wide as an initial screen to determine the mutagenic potential of new chemicals and drugs in the chemical industry, pharmacy, agriculture and food industry [56].

1.4.1. Historical Background

The Ames assay was developed by Bruce Ames in Berkeley (California) and published in 1973. First the scientists worked with the bacterial tester strain *his*G46 which has a base substitution and the bacteria tester strains C207, C3076 and D3052 which are histidine-requiring frameshift mutants. These tester strains were developed and improved. Later the

[54] (EFSA Scientific Committee, 2019)
[55] (Koster Sander, 2016)
[56] (Kristien Mortelmans, 2000)

test strains were replaced to TA1535, TA1536, TA1537 and TA1538. Finally, the test strains TA97, TA98, TA100 and TA102 were additional added[57,58]. Due to the fact, that bacteria are unable to metabolize chemicals via cytochromes P450 an exogenous mammalian metabolic activation system made of rat liver was needed[59]. According to the OECD guidelines the following standard tester strains are common: *Salmonella typhimurium* TA1535, TA1537 or TA97, TA98, TA100 and *Escherichia Coli* WP2 uvrA or WP2 uvrA (pKM101) or *S. typhimurium* TA102[60].

At first the bacterial tester strains were analysed with the spot-test. A selective agar medium plate which contains the test organism was needed. A small amount of the test chemical was directly put into the center of the top agar. As the test chemical diffuses into the agar a concentration gradient was formed. If the test chemical was a mutagenic substance, it gave rise to a ring of revertant colonies surrounding the area of the test chemical. If the test chemical was a toxic substance, a zone of growth inhibition was also be observed. This process is shown in figure 2. The positive test plate shows the ring of the revertant colonies which decrease towards the margin of the plate and the ring free from colonies directly to the center due to the high concentration of the toxic test chemical[61].

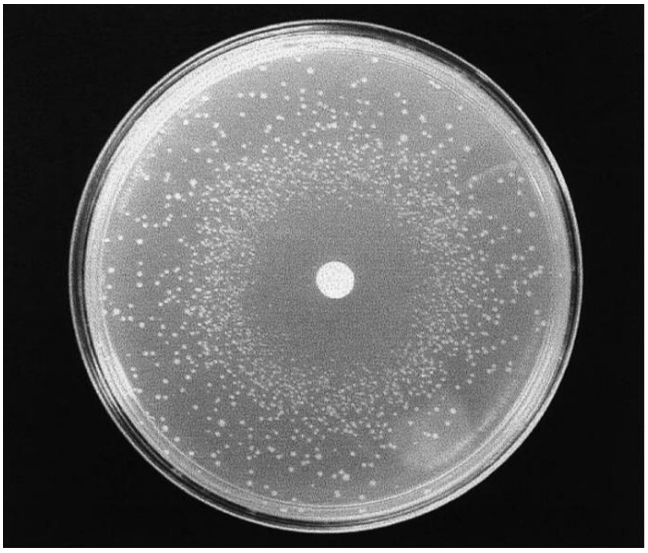

Figure 2: Spot test with bacteria tester strain TA100 and 10µl of the tester chemical substance methyl methanesulfonate[62]

The plate-incorporation-assay was also developed at that time and was based on the spot-test. It is more sensitive and quantitative than the spot-test and more cost efficient. The procedure of the plate-incorporation-assay consists of the fallowing steps: at first the buffer or S-

[57] (Bruce N. Ames C. Y., 1971)
[58] (Dorothy M. Maron, 1983)
[59] (Kristien Mortelmans, 2000)
[60] (OECD GUIDELINE FOR TESTING OF CHEMICALS, Bacterial Reverse Mutation Test, 1997)
[61] (Kristien Mortelmans, 2000)
[62] (Kristien Mortelmans, 2000)

9 mix, the histidine dependent bacteria and test chemical are added to the top agar containing biotin and a trace amount of histidine. Then the mixture is gently mixed and poured on glucose minimal agar plates. When the top agar has solidified, the plates are incubated in the incubator. Then the histidine revertant colonies are counted[63]. Figure 3 shows two plates after the incubation: on the left side is the solvent control plate (negative control) and on the right side is the positive mutagen plate[64].

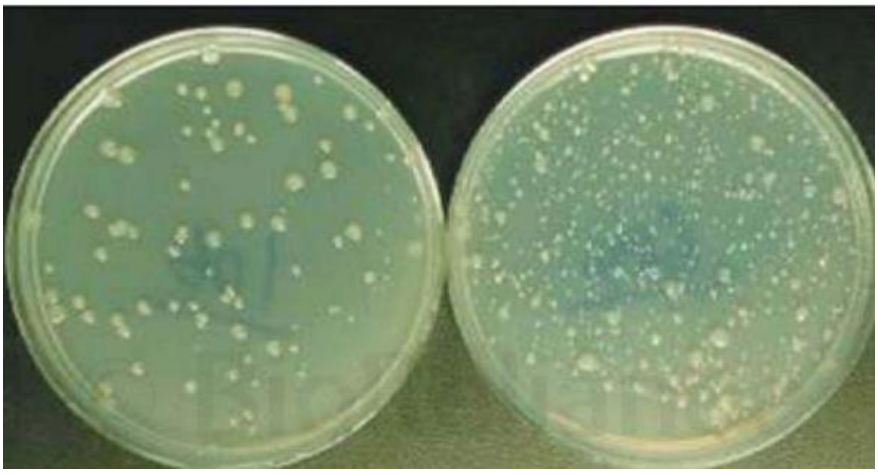

Figure 3: Plate-incorporation-assay: solvent control plate left, positive mutagen plate right[65]

1.4.2. Theoretical Background

The Ames assay is a bacterial reverse mutation test to detect point mutations, including substitution, addition or deletion of one or a few DNA base-pairs. The test uses amino acid requiring strains of *Salmonella typhimurium* and *Escherichia Coli*. The bacteria tester strains carry mutations in genes involved in histidine synthesis. The bacteria tester strains are auxotrophic and require histidine for growing but cannot produce it. In present of a mutagenic substance the bacterial reverse mutation takes place and the bacteria tester strains can synthesize the missing histidine. Consequently, visual revertant colonies arise on the agar. The principle of the assay is the detection of mutations which revert mutations present in the test strain and restore the functional ability of the bacteria to synthesize the essential histidine[66].

The bacteria test strains have several characteristics which make them more sensitive for the detection of mutations. This includes for example a responsive DNA sequences at the reversion site, an increased cell permeability to large molecules, an elimination of DNA repair systems or an improvement of error-prone DNA repair process. The specificity of the bacteria test strains can give useful information on the type of mutations which are induced by the genotoxic substances. Different mutations of the *Salmonella typhimurium* strains arise which

[63] (Kristien Mortelmans, 2000)
[64] (James M. Parry, 2012)
[65] (James M. Parry, 2012)
[66] (OECD GUIDELINE FOR TESTING OF CHEMICALS, Bacterial Reverse Mutation Test, 1997)

operate with different mechanisms. Meanwhile a very large data base of results is available and show a wide variety of testing chemicals with different physic-chemical properties[67].

The bacterial tester strains contain mutations in the histidine operon, so they need histidine for growing. Further mutations and changes in the genetic can improve the sensitivity of the detection and consequently more mutagenic substances can be detected with the test. For technical reasons a deletion excising the *uvrB* gene extends through the bio gene was performed. As a consequence, the bacteria also require biotin for growth. The tester strain TA102 was excluded because it did not contain the *uvrB* mutation. TA102 was constructed for detecting mutagenic substances which require an intact excision repair system. The *rfa* mutation leads to partial loss of the lipopolysaccharide barrier which surrounds the surface of the bacteria and increases the permeability to large molecules[68]. Some tester strains contain the R-factor plasmid, *pKM101*. This plasmid increases chemical and UV-induced mutagenesis and causes a resistance for ampicillin[69]. TA102 also contains the multicopy plasmid, *pAQ1* which carries the *hisG428* mutation. This plasmid increases the detection of DNA-crosslinking agents. The bacteria strains TA97, TA98 and TA1537 are used for the detection of frameshift mutations and the strains TA100, TA102 and TA1535 are used for the detection of point mutations. The *Escherichia Coli* tester strains are also used for the detection of point mutations[70,71,72].

All bacteria test strains are descended from the original *Salmonella typhimurium* strain LT2. Table 3 gives an overview of the different mutations of all bacteria tester strains[73,74,75].

tester strain	mutation	DNA target	repair *uvrB*	LPS *rfa*	plasmid	mutation type
Salmonella typhimurium						
TA97	*hisD6610*	-C-C-C-C-C-C-	+	+	*pKM101*	frameshift
TA98	*hisD3052*	-C-G-C-G-C-G-C-G-	+	+	*pKM101*	frameshift
TA100	*hisG46*	-G-G-G-	+	+	*pKM101*	point
TA102	*hisG428*	TAA	+	+	*pKM101* *pAQ1*	point
TA1535	*hisG46*	-G-G-G-	+	+	-	point
TA1537	*hisC3076*	-C-C-C-C-C-	+	+	-	frameshift

[67] (OECD GUIDELINE FOR TESTING OF CHEMICALS, Bacterial Reverse Mutation Test, 1997)
[68] (Dorothy M. Maron, 1983)
[69] (David E. Levin, 1982)
[70] (Kristien Mortelmans, 2000)
[71] (Dorothy M. Maron, 1983)
[72] (Bruce N. Ames J. M., 1975)
[73] (Kristien Mortelmans, 2000)
[74] (Dorothy M. Maron, 1983)
[75] (Bruce N. Ames J. M., 1975)

Escherichia Coli WP2						
uvrA	*trpE65*	AT	+	-	-	point
uvrA (pKM101)	*trpE65*	AT	-	-	*pKM101*	point

Table 3: Mutations of the bacteria tester strain[76,77,78]

For every tester strain there is a spontaneous mutant frequency characteristic. Normally there is some day-to-day and laboratory-to-laboratory variation in the amount of the revertant colonies. The spontaneous mutant frequency is also depended on the solvent. For this reason, each laboratory has a characteristic range of revertant colonies for every test strain as control values. The bacteria strains are tested for their correctness and spontaneous mutations before they are used[79].

Some carcinogenic substances, for example aromatic amines and polycyclic aromatic hydrocarbons are biologically inactive. They must be metabolized to active forms. In human and lower animals, the cytochrome-based P450 metabolic oxidation system metabolizes many of these carcinogenic substances to DNA-reactive forms. The bacteria do not have this metabolic activator system so an exogenous mammalian organ activation system needs to be added together with the biologically inactive chemicals and the bacteria. The metabolic activation system usually consists of a 9000*g supernatant fraction of a rat liver homogenate (=S-9 microsomal fraction), which is mixed with NADP and cofactors for NADPH-supported oxidation (=S-9 mix). The animals are pretreated with the mixed-function oxidase inducer “Aroclor 1254” or other inducers (phenobarbital, b-naphthoflavone) to increase the level of metabolizing enzymes[80].

1.4.3. Standard Test Methods

According to the OECD guidelines there are two standard-tests: the plate incorporation method and the preincubation method. For the plate incorporation method usually 0,05ml or 0,1ml of the test substance, 0,1ml of fresh bacteria culture, 0,5ml of sterile buffer and eventually 0,5ml of the metabolic activation mixture are mixed with 2ml of the top agar and then the contents are poured over the surface of a minimal agar plate. For the preincubation method the same compounds with the same amount are used. The test substance is preincubated with the test strain, sterile buffer and eventually with the metabolic activation mixture for usually 20 minutes at 30-37 degrees. After the preincubation the mixture is given to the top agar and then the contents are poured of a minimal agar plate. The plates of both assays should be incubated at 37 degrees for 48-72 hours. After the incubation period, the number

[76] (Kristien Mortelmans, 2000)
[77] (Dorothy M. Maron, 1983)
[78] (Bruce N. Ames J. M., 1975)
[79] (Kristien Mortelmans, 2000)
[80] (Dorothy M. Maron, 1983)

of revertant colonies per plate is counted. The negative and positive controls are also included in the results. The result is classified as positive when a concentration-related increase over the tested range and/or a reproducible increase at one or more concentrations in the number of revertant colonies per plate are seen. Otherwise the test substance is classified as non-mutagenic. The most experiments give clearly positive or negative results but in rare cases the data set precludes making a definite assessment. Equivocal results or results which are questionable regardless, for example weak positive results should be clarified by further testing and altered experimental conditions (including concentration spacing, method of treatment, metabolic activator conditions). Positive results from the Ames test represent that a substance induces point mutations by base substitutions or frameshifts in the genome of *Salmonella typhimurium* and/or *Escherichia coli*. Negative results indicate that the test substance is not mutagenic in the tested species under the test conditions[81].

To determine the highest amount of non-cytotoxic and soluble test substance in the final treatment mixture above the concentration of 5mg/plate or 5µl/plate may be considered. If this is not possible for example when the test substance is cytotoxic already below 5mg/plate or 5µl/plate it should be tested up to a cytotoxic concentration. Therefore at least five different concentrations of the test substance should be tested with different intervals between the test points (for example √10). Smaller intervals may be appropriate when a concentration-response is being investigated (for example 1:2 dilution)[82].

1.5. Different Types of Plastic

There are hundred different types of plastics in the packaging industry such as trays, lids, films, pouches and bottles but only very few are utilized in FCM. The basic materials for forming plastic polymers are small hydrocarbon monomers such as ethylene used in different manufacturing processes like addition polymerization, condensation polymerization and synthesis of copolymers. Their basic classification are elastomers, thermoplastics and thermosets. The most common plastic types are polyolefins, copolymers of ethylene, substituted olefins, polyesters, polycarbonate, polyamide (nylon), PET and polypropylene with different barrier materials such as polyvinylidene chloride (PVDC), ethylene-vinyl alcohol (EVOH) or polyethylene[83]. In this chapter the types of plastic which were used for this master's thesis are described.

1.5.1. Polyolefins

Polyolefins are the biggest class of plastic. They are saturated hydrocarbons produced from alkene such as ethylene or propylene. The starting materials are polymerized at high tem-

[81] (OECD GUIDELINE FOR TESTING OF CHEMICALS, Bacterial Reverse Mutation Test, 1997)
[82] (OECD GUIDELINE FOR TESTING OF CHEMICALS, Bacterial Reverse Mutation Test, 1997)
[83] (Kanishka Bhunia, 2013)

peratures, high pressure and catalyser and generate long molecular chains (=polymers). The most important representative of the polyolefins is polyethylene. Typical examples are LDPE, LLDPE, HDPE and PP[84].

The branched-chain structure of LDPE prevents close packing of monomeric units in the polymer chain. Consequently, a relatively low molecular weight (density from 910 to 940 kg/m³) is resulted. The long-chain entanglement prevents crystallization upon cooling, so a low degree of crystallinity (55% to 70%) is formed. LLDPE is another form of LDPE in which the polymer structure has no long molecular chains. This is why LLDPE has a strong and more crystalline structure. The molecules are linearly oriented. HDPE has a relatively simple molecular structure, is nonpolar and linear. It has a relatively high density (941 to 965 kg/m³) and a higher degree of crystallinity (up to 90%). LDPE and HDPE have high water vapor and gas barrier properties[85].

In general, the characteristics of the PE depends on the molecular structure. The molecular structure varies because of the type and amount of the branched-chain structure and the manufacturing conditions (low pressure process or high pressure process). The crystallinity of the polymers depends on the molecular mass and the branching factor. If fewer polymer chains are branched this means that the molecular mass is lower and the crystallinity is higher[86].

LDPE is made by the radical polymerisation with high pressure process thus much short as well as long chain branching arises because many chains transfer reactions take place. Because of the many chain branchings and the weaker intermolecular forces LDPE has a lower tensile strength and a higher resilience than HDPE. The other PEs are made with low pressure. In this process only a few chains transfer reactions take place, so the polymers consist of only short chain branching[87].

PP has a saturated linear polymeric structure and can be classified in atactic, isotactic and syndiotactic PP. Atactic PP has methyl groups randomly distributed on both sides of the polymer mainchain. Due to this distribution it is not crystalline and is amorphous. Syndiotactic PP has the methyl groups positioned on alternating sides of the polymer mainchain thus this form is crystalline. Isotactic PP has the methyl groups on one side of the polymer mainchain and is higher crystalline than the syndiotactic PP (30-60%). The standard PP which is used in general is isotactic[88].

[84] (James L. White, 2005)
[85] (Kanishka Bhunia, 2013)
[86] (Kaiser, 2007)
[87] (Kaiser, 2007)
[88] (MGTPetroil Company, 2016)

PP has a lower density (900 kg/m³) and higher softening point (T_m) than the other PE. Due to the high softening point PP can withstand high temperatures for example at the steamsterilization[89].

LDPE is generally used in bags, films and as coating in packaging materials for milk. HDPE is used in bottles, containers and boxes for food. Yoghurts, margarine, sweet and snacks are often packaged in PP[90].

1.5.2. Polystyrene

Polystyrene is a substituted olefin and produced through the addition polymerization of styrene. Styrene is made through the catalytic dehydrogenation of ethylbenzene. PS has a unique capability of polymerization. It can be polymerised with radical, cationic and anionic catalyser or with metallocene catalyser (=Ziegler-Natta-Polymerisation). The PS can be classified in atactic, syndiotactic and isotactic PS. These three different molecular structures of PS determine the characteristics of the polymer. Atactic PS has phenyl groups randomly distributed on both sides of the polymer mainchain. Due to this distribution it does not have any crystallinity and is amorphous. The atactic form is made through the radical polymerisation. Syndiotactic PS is produced through the Ziegler-Natta-Polymerisation. The phenyl groups are positioned on alternating sides of the polymer mainchain, so this form is highly crystalline. Isotactic PS has the phenyl groups on one side of the polymer mainchain and is half-crystalline. The standard PS which is used in general is atactic[91].

There are another two important types of PS: HIPS (high-impact polystyrene) and GPPS (general purpose polystyrene)

Due to the high permeability to gases and vapors, PS is suitable for perishable food products. The expanded type of PS has a lower thermal conductivity value (0,033) so that the main application of PS is the transportation of frozen food such as coffee, ice cream, yoghurt, creamers. Low-fat content foods are also packaged in PS. The trays and divider for eggs, fruits and vegetables are also made of PS. PS has a high density (1050 kg/m³) and a high softening point[92].

1.5.3. Polyesters

Polyesters are made by condensation-polymerization of carbonyl groups. In this process carbon-oxygen-carbon links are formed[31]. According to the composition of the mainchain Polyesters can be aliphatic, semi-aromatic and aromatic. The aliphatic polyesters are classified in homopolymers (such as polyglycolide, polylactic acid, polyhydroxyalkanoate, polyhy-

[89] (Kanishka Bhunia, 2013)
[90] (PlasticsEurope - Association of Plastics Manufactures)
[91] (Jürgen Maul, 2007)
[92] (Kanishka Bhunia, 2013)

droxybutyrate) and copolymers (such as polyethylene adipate, polybutylene succinate). Semi-aromatic polyesters are copolymers for example polyethylene terephthalate (PET), polybutylene terephthalate and polytrimethylene terephthalate. The aromatic polyesters are also copolymers like vectran[93].

The most important polyester is PET. It is produced during a reaction with ethylene glycol and terephthalic acid (TPA). PET is linear saturated and polar thus strong intermolecular strengths are present. Furthermore, it is semi-crystalline, transparent, has little resistance to water vapor and due to the very high softening point (269°C) PET is stable over a wide temperature range (-60 to 220°C). The glass transition temperature (T_g) ranges from 67 to 80°C. PET is lightweight and has an excellent tensile strength and chemical resistance. In its glassy state PET is hard and ductile and ideal for rigid packaging. Due to the elastic properties it can be further used through molding and extrusion. It is highly impermeable to aromas and gases, so no sensory changes of the packaged food are expected. PET is used to make bottles, films and trays for frozen foods[31]. PET can be recycled into new PET-bottles of nearly 100%. Every year about 23.300 tons used PET-bottles are processed to PET-recyclates for foodstuff in Austria. The PET-recyclates are used in new bottles and replace new plastic materials. In general new PET-bottles consist of about 30 to 40% of PET-recyclates. The good recyclability of PET is related to a high inertness[94].

1.5.4. Summary of the characteristics

Table 4 gives an overview of the most important characteristics of HDPE, LDPE, PP, PS and PET.

	type of polymer	density (kg/m³)	crystallinity (%)	T_m (°C)	T_g (°C)	k (w/m*K)	molecular structure
HDPE	thermoplast	941-965	>90	126-135	-110	0,38-0,51	short chain branching
LDPE	thermoplast	910-940	55-70	110	-125	0,32-0,40	long and short chain branching
PP	thermoplast	900	30-60	176	-18	0,22	atactic, syndiotactic, isotactic
PS	thermoplast	1050	/	240	100	0,033	atactic, syndiotactic, isotactic
PET	thermoplast	1400	semicrystalline	267	67-80	0,14-0,24	linear saturated

Table 4: Characteristics of HDPE, LDPE, PP, PS, PET

The inertness (or sorption ability) of the polymer is the basic parameter which affects the recyclability of the plastic polymers. A high inertness leads to a very low interaction between the packaging polymer and the foodstuff. A good recyclability is related to a high inertness.

[93] (Dominick V. Rosato, 2004)
[94] (PET to PET Recycling Österreich GmbH)

PET has the highest inertness of these five plastic types therefore PET is well-recyclable. The inertness of the other plastics decreases in the following sequence: PET > PS > HDPE, PP > LDPE. Due to the low inertness of PE and PP and consequently high migration between the packaging polymer and the foodstuff, PE and PP pose problems during recycling[95,96].

1.6. The Migration Process

As already mentioned, FCM can be found in the final plastic materials because of the complex formulation of the polymers, manufacturing processes and storages. In addition, physical stress applied to the plastic materials can modify the structure of the chemical ingredients[97]. The FCM are not completely inert and can interact with the filled product or even migrate into foodstuff. Migration means a diffusion-controlled mass transfer from a packaging material or article into food or food simulant. It is influenced by kinetic factors (diffusion coefficient in plastic and food) and thermodynamic factors (equilibrium partitioning coefficient between plastic and food). In the case of highly diffuse materials, such as PE and PP, it is mainly the partitioning coefficient that determines the extent of migration. For low-diffuse materials such as PET and PS the diffusion coefficient determines the extent of migration. The migration is experimentally determined by standardised tests using food simulants but can also be mathematically modelled and predicted. It is useful to base migration evaluations on a worst-case scenario that estimates the total mass transfer based on the starting concentration of each migrant in the plastic. If this calculation exceeds the migration limit it is necessary to refine the evaluation to take account of partitioning and diffusion as the critical parameters for migration[98]. The interactions between the packaging material and the foodstuff start to occur at the point of filling and continue during the regular usage of the product. They may continue even longer if the consumer reuses the empty packaging for another application by filling it with an alternative material. The potential risk of food contamination from recycled packaging plastics is determined by three main factors: the sorption properties of the polymer, the diffusion behaviour of the materials, the time and temperature while the packaging material is in contact with foodstuff[99].

The migration refers to the diffusion of substances from a zone of higher concentration (the food-contact layer) to a zone of lower concentration (usually the food surface) as a distribution process due to the brownian motion of the molecules. Food packaging interactions and temperature often influence the diffusion. It is a very complex process and depends on sev-

[95] (Welle, Food Law Compliance of Poly(ethyleneTerephthalate)(PET) Food Packaging Materials, 2014)
[96] (Welle, Develop a food grade HDPE recycling process, 2005)
[97] (Cristina Bach, 2014)
[98] (Welle, Develop a food grade HDPE recycling process, 2005)
[99] (Cristina Bach, 2014)

eral parameters: concentration of substances in the packaging material and food, nature of food and packaging material, state of polymer matrix, chemical properties of food, temperature of the system and time of contact between the packaging material and the filling. During the process, the compounds enter in another matrix and change their concentration in the packaging material and the food. The degree of solvent stirring, polymer-solvent partition coefficients, swelling of solvents and concentration-dependent diffusivity play an important role. For example, in a liquid, viscous, or solid food, diffusivity may change because the interface between plastics and food material would be different in each of these cases. Furthermore, diffusion in glassy polymers is much lower than in rubbery polymers. The likelihood of migration also increases when the packaging plastic is exposed to high temperatures during thermal processing and when food is stored for very long periods[100].

The migration process can be grouped into 4 major steps: diffusion of chemical compounds through the polymer, desorption of the diffused molecules from the polymer surface, sorption of the compounds at the plastic–food interface and desorption of the compounds in the food. Sorption means a reversible chemical equilibrium partitioning for the accumulation of a substance in an one-phase system (=absorption) or on the interface of two different phases (=adsorption). The reversion process of sorption is desorption[101].

1.6.1. Fick's law of diffusion

The migration process is usually governed by Fick's law of diffusion[102].

Fick's first law: $$N_A = -\mathrm{D}_P \frac{\partial \mathrm{Cp}}{\partial \mathrm{x}}$$

Fick's second law: $$\frac{\partial \mathrm{Cp}}{\partial \mathrm{t}} = -D_P \frac{\partial^2 \mathrm{Cp}}{\partial x^2}$$

N_A= steady state flux
C_P= concentration of migrant in the polymer
D_P= diffusion coefficient of migrant in polymer

It is assumed that the steady state diffusion process indicates no change in the concentration over the time ($\partial Cp/\partial t = 0$), but most of the interactions between packaging materials and food are influenced by nonsteady state conditions[103].

Furthermore diffusion and partition coefficients (k_p) are assumed to be constant[104]. There are many models for the theoretical estimation of diffusion coefficients in polymers but today

[100] (Kanishka Bhunia, 2013)
[101] (Eddo J. Hoekstra (Ed.), 2015)
[102] (Welle, Develop a food grade HDPE recycling process, 2005)
[103] (Welle, Develop a food grade HDPE recycling process, 2005)

these models are too complicated for the practical application. A simple approach was developed in the past by Piringer in 1994, Hamdani et al. in 1997, Limm and Hollifield in 1996. The diffusion coefficients were correlated with the relative molecular mass of the migrant by a temperature dependent polymer specific constant and the absolute temperature based on empirical relationships and experimental data[105].

The partition coefficient determines the migration at the polymer-solvent boundary and can be written as[106]:

$$k_P = \frac{C_S}{C_P}$$

k_P= partition coefficient
C_S= concentration of migrant in food/food simulant
C_P= concentration of migrant in polymer

Is the k_P-value low it means that more migrant is absorbed into the food. According to a study (Piringer et al., 2007) fatty food has a low k_P-value (<1) and water has a very high k_P-value (>1000)[107]. The partition coefficient is heavily dependent on the temperature. This can be described with the Arrhenius equation. The Arrhenius type equation (Bastarrachea et al., 2010) correlates the diffusion coefficient and the temperature as follows[108]:

$$D_P = D_0 e^{-\frac{E}{RT}}$$

D_0= pre-exponential factor
E= activation energy for diffusive molecules
R= gas constant
T= absolute temperature

1.6.2. Migration Tests

Testing the migration of chemical compounds from food packaging materials into food simulants involves two different steps. The first step is to allow the substances to migrate from the packaging material into the food simulant. The second step is the quantification of the migrants transferred to a food simulant with the overall migration (OM) or specific migration (SM)[109,110].

The overall migration represents the total amount of nonvolatile substances which can possibly migrate from the packaging plastics into the food. It is a regulatory requirement in the EU and has established migration limitations for substances from FCM. According to the European Plastics Regulation 10/2011 the OM for plastics is limited to 10mg/dm² on a contact

[104] (Kroes R, 2004)
[105] (Eddo J. Hoekstra (Ed.), 2015)
[106] (Eddo J. Hoekstra (Ed.), 2015)
[107] (Piringer O., 2007)
[108] (Bastarrachea L., 2010)
[109] (L.L., 1996)
[110] (Castle L., 2007)

area basis and 60mg/kg in the food simulant or food (=overall migration limit, OML). To analyse the OM from the food-contact plastic layer the common food simulants are used[111]:

- simulant A with 10% ethanol (v/v): for aqueous food
- simulant B with 3% acetic acid (w/v): for acidic food which has a hydrophilic character and is able to extract hydrophilic substances
- simulant C with 20% ethanol (v/v): for food which has a hydrophilic character and is able to extract hydrophilic substances, alcoholic food with an alcohol content of up to 20 % and food which contains a relevant amount of organic ingredients that make the food more lipophilic
- simulant D1 with 50% ethanol (v/v): for alcoholic food with an alcohol content above 20 % and fatty food
- simulant D2 also with 50% ethanol (v/v): for vegetable oil and food which contains free fat at the surface
- simulant E with an adsorbent material named Tenax (PPPO): for dry food[112]

The OM of chemicals which could possibly migrate during heat exposure or other types of physical stress can be determined with a simple gravimetric method. The residue of specimen is weighed after the evaporation of the volatile simulants (for example alcohol, simulant C), or the mass loss of the plastic specimen is weighed before and after exposure (for example olive oil and nonvolatile fat simulants)[113].

Table 5 shows the classification of types of food and food simulants recommended for FCM reproduced from chemical migration by Barnes et al[114].

Type	Description	Classification	recommended simulant
I	nonacid aqueous products which may contain salt, sugar, or both (ph>5)	aqueous	10% ethanol
II	acid aqueous products which may contain salt, sugar, or both and include oil-in-water emulsion of low or high fat content	acidic	10% ethanol
III	aqueous, acid or nonacid products which contain free oil or fat, may contain salt, include oil-in-water emulsion of low or high fat content	fatty	food oil, HB307, Miglyol812
IV	fatty products and modification a: water-in-oil emulsion low or high fat b: oil-in-water emulsion low or high fat	 fatty aqueous	 food oil, HB307, Miglyol812 10% ethanol
V	low-moisture fats and oils	fatty	food oil, HB307, Miglyol812
VI	beverages a: containing up to 8% alcohol b: nonalcoholic c: containing more than 8% alcohol	 low alcohol aqueous high alcohol	 10% ethanol 10% ethanol 50% ethanol

[111] (European Plastics Regulation 10/2011)
[112] (Piringer O., 2007)
[113] (Bradley EL, 2009)
[114] (Barnes KA, 2007)

VII	bakery products a: containing free fat or oil on the surface b: containing no free fat or oil on the surface	 fatty aqueous	 food oil, HB307, Miglyol812 10% ethanol
VIII	dry solids containing no free fat or oil on the surface	dry	Tenax
IX	dry solids containing free fat or oil on the surface	fatty	food oil, HB307, Miglyol812

Table 5: Classification of food types and food simulants for FCM[115]

Several standard testing conditions for the determination of the OM are recommended by EU Directive 10/2011[116]. These conditions are listed in table 6.

Intended food-contact condition	simulant contact time and temperature
frozen and refrigerated products	10 days at 20°C
long-term storage at or below room temperature, including heating process up to 100°C for 15 minutes or 70°C for up to 2h	10 days at 40°C
products heated up to 70°C for up to 2h, or up to 100°C for up to 15 minutes with no long-term room storage or refrigerated temperature storage	2h at 70°C
high-temperature applications up to 100°C for all food simulants	1h at 100°C
high temperature applications up to 121°C	2h at 100°C or at reflux or alternatively 1h at 121°C
food-contact conditions with simulants A, B or C at temperature exceeding 40°C	4h at 100°C or at reflux
high temperature applications with fatty food exceeding heating process up to 121°C	2h at 175°C

Table 6: Standard testing conditions for the overall migration[117]

The specific migration is the amount of a specific compound which migrates from the packaging plastic material into the food or food simulant. In the European Plastics Regulation 10/2011 the specific migration limit (SML) is set for each potential migrant by using one of the simulants. If no SML is set a default limit of 60mg/kg food can be used for individual substances. Another concept is the total specific migration limit SMLT, which indicates a group of similar substances in food or food simulants[118].

Today the OML of 60mg/kg is no longer considered adequate because highly toxic compounds can represent already a risk at far lower concentrations. Furthermore, an overall migration below this value does not necessarily ensure safety. The OML is mainly used to control the total amount of substances migrating from packaging plastics into foodstuff, rather than for determining the toxicity of the substances and for reducing the SM experiments. The concept of the OML is merely considered as a restriction against excessive food contamination[119].

[115] (Barnes KA, 2007)
[116] (Bradley EL, 2009)
[117] (Bradley EL, 2009)
[118] (Kanishka Bhunia, 2013)
[119] (Grob K., 2010)

1.6.3. Substitute Tests

In some cases the migration test is not feasible because of technical reasons connected with the test method, interferences, incomplete extraction of the agents, absence of stability of the mass of the plastics, excessive absorption of the food simulant and reaction of components. Then the use of substitute tests may be appropriate. The test media in substitute tests are iso-octane, 95% ethanol in aqueous solution or a modified polyphenylene oxide (MPPO) under the conventional substitute test conditions[120]. The harshest migration conditions according to the regulation EU10/2011 are: 95% ethanol as extracting agent, 60°C heat impact for 10 days. It is possible that due to the long and high heat impact the substances lose their mutagenic activity. For this purpose the biological substance recovery is determined to look if the mutagenic substances are degraded during the harsh migration process. For the project "Polycycle" weaker migration conditions (40°C for 24 hours) are used to test the plastic polymer recyclate-samples.

1.6.4. Chemical Analysis for detection of migrants

After the migration under defined conditions appropriate analytical methods are needed to identify and quantify the potential migrants from the packaging material into the food simulant. There have been many modifications of the technique depending on analytes, matrices, instrumentation and analyst preferences. There are specified analytic methods for some, but not for all potential migrants. It is necessary to validate and verify the analytical test methods to ensure the precision and accuracy of the experimental test. Three approaches are used for experimental method validation applicable to FCM: "full" single laboratory validation protocol, "standard level" of single laboratory validation and "basic level" of single laboratory validation. In terms of migration from new polymeric materials without well-characterized properties, mathematical models are used frequently to predict the migration of low-molecular-weight compounds. The "full validation" protocol is usually used for a new developed method by thorough analysis and characterization of the experimental parameters and system performance. The" standard level" shows the minimum of demands to establish the criteria for noncompliance of an analysis. The "basic level" is the starting point from which the update and improvement of the test procedures must be performed. This shows that the method can be validated under a set of standard conditions[121].

Although the detection and identification of migrants using chemical analysis is important, this process seems to be difficult because the substances represent a very heterogenous group surrounding a diverse set of different chemical properties. With the use of *in-vitro* bioassays such as the Ames MPF assay the presence of contaminants in migrants descended

[120] (Materials and articles in contact with foodstuffs - Plastics - Part 1: Guide to the selection of conditions and test methods for overall migration)
[121] (Bratinova S, 2009)

from FCM can be determined easily. The application of *in-vitro* bioassays has been proposed as a possible avenue to tackle this issue[122].

[122] (Bernhard Rainer E. M.-K., 2019)

2. Materials

In this chapter all materials and chemicals are listed which were used for the experimental procedure of this master's thesis. Table 7 shows all the equipment, table 8 includes the expendables materials, table 9 shows the chemicals and in table 10 the pure substances are listed.

2.1. The Equipment

Equipment	Producer
Autoclave, 85 liters	HMC Europe
Autoclave, CertoClav®, 18 liters	VWR
Drying oven	Thermo Scientific
Eppendorf Research® plus pipette, one-channel, 100 – 1.000 µL	Eppendorf
Eppendorf Research® plus pipette, one-channel, 2 – 20µL	Eppendorf
Eppendorf Research® plus pipette, one-channel, 20 – 200µl	Eppendorf
Freezer -80°C, CryoCube F101h	Eppendorf
Fridge -4°C, -20°C	Liebherr
Incubator BINDER BD 115 Avantgarde.Line	Binder
LAMBDA™ 265 UV/Vis Spectrophotometer	Perkin Elmer
Multi-channel pipette, mechanical, 5 - 50µl	VWR
Orbital Shaker MAXQ6000	Thermo Scientific
pH-meter Lab 845	SI Analytics
Picus® electronic multi-channel pipette, 8-channel, 50 – 1.200µl	Sartorius
Rotary Evaporation: Recirculating Chiller F-305, Vacuum Pump V-300, Heating Bath B-300, Interface I-300, Rotovapor R-300	Büchi
Scale, analytical balance	Ohaus Pioneer
Scale, precision balance	Ohaus Pioneer
Visiprep™ SPE Vacuum Manifold	Supelco
Vortex	VWR
Water Purification, Sartorius arium® comfort	Sartorius
Water Purification, small	Sartorius

Table 7: List of the used equipment

2.2. The Expendable materials

Materials	Producer
24-well-plates	VWR
384-well-plates	VWR
96-well-mikrotiterplates	Brand
Beaker, various size	VWR
Boiling flakes with round bottom, various size	Büchi
Centrifuge tubes, 15ml, 50ml	VWR
Clear sample vials, 20ml	Thermo Scientific
Combitips advanced, 50ml	Eppendorf
Cuvettes, semimicro, single-use	VWR
Flip flops, sterile, ventilated lock, 14ml	Greiner
Membrane filter, sterile, 0,2µm cellulose	VWR
NORM-JECT® syringe, single-use, 12ml, 24ml	VWR
Pipette sterile, single-use, 5ml, 10ml	Sarstedt

Pipette tips, various size	Eppendorf
Reaction vials 1,5ml	Carl Roth
Schott glass bottles, various size	Duran

Table 8: List of the used expendables materials

2.3. The Chemicals

Chemicals	Producer	Code	Cas Nr.	Batch Nr.
Aceton 99,5%	Carl Roth VWR	5025.5 20063.412	75-05-8	158269019 15!020524
Ampicillin trihydrat	Santa Cruz Biotechnologies	sc-254945	7177-48-2	I2418-01/19
Dichlormethane 99,5%	Carl Roth	8424.1	75-09-2	29278732
DMSO	Carl Roth	7029.1	67-68-5	58267400
Ethanol >99,9%	Merck	1.11727.2500	64-17-5	K50182327818
Ethanol 70%, denaturated	Carl Roth	T913.2	64-17-5	159282579
Exposure Media	Xenometrix			
G-6-P monosodium salt	Carl Roth	5544.1	54010-71-8	507266202
Indicator Media	Xenometrix			
KCl	Carl Roth	HN02.2	7447-40-7	158224516
$MgCl_2$	Carl Roth	KK36.2	7786-30-3	158267878
Na_2HPO_4	Carl Roth	P030.1	7558-79-4	188270955
NaCl	Carl Roth	0601.1	7647-14-5	407262033
NADP disodium salt 97%	Carl Roth	AE13.3	24292-60-2	78262170
$NaH_2PO_4*H_2O$	Carl Roth	K300.1	10049-21-5	158269947
Nutrient Broth No. 2	Oxoid	CM0067		2378115
S9 rat liver extract	Xenometrix	PRS-AC02		

Table 9: List of the used chemicals

Pure Substances	Producer	Code	Cas Nr.	Charge Nr.
2-Acetylaminofluorene (2-AF)	Sigma Aldrich	A7015-5G	53-96-3	
2-Aminoanthracene (2-AA)	Carl Roth	AA38800-1	613-13-8	STBD3302V
2-Nitrofluorene (2-NF)	Carl Roth TCI	AN16754.5 N0201	607-57-8	G5AXF-DT
4-Nitroquinoline-1-oxide (4-NQO)	Sigma Aldrich	H9003-100G	56-57-5	
9-Aminoacridine (9-AA)	Santa Cruz Biotech	SC-291761A		
Benzo[a]pyrene (BaP)	Sigma Aldrich	YB1760250		
Methyl methanesulfonate (MMS)	Sigma Aldrich	129925-5G	66-27-3	
N-Ethyl-N-Nitrosourea (ENU)	Sigma Aldrich	N8509-5G	759-73-9	

Table 10: List of the used pure substances

2.4. Self-made Materials

The Growth Media is made of “Nutrient Broth No. 2” (which consists of meat-extract, peptone and sodium chloride) and distilled water. The mixture is sterilized by autoclaving at 121°C for 15 minutes.

Growth Medium	
Nutrient Broth No. 2	25g
distilled H_2O	1 litre

Table 11: Procedure of the Growth Medium

For substances which need a metabolic activator system (S9 rat liver extract) co-factors must also be added. They are made of KCl, $MgCl_2$, G-6-P, NADP and NaH_2PO_4-Puffer and were produced with the formula of Maron & Ames (1983).

Co-factors:	per ml
distilled H_2O	0,335ml
KMg-solution	0,02ml
NADP disodium salt	0,04ml
NaH_2PO_4-puffer	0,5ml
G-6-P monosodium salt	0,005ml
NaH_2PO_4-puffer:	
$NaH_2PO_4*H_2O$	4,0296mg/ml
Na_2HPO_4	25,1007mg/ml
KMg-solution for 1ml:	
KCl	124mg
$MgCl_2$	81mg
NADP disodium salt	76,5mg/ml
G-6-P monosodium salt	282mg/ml

Table 12: Composition of the co-factors

In this master’s thesis the *Salmonella typhimurium* strains TA98 and TA100 were predominantly used. For the side experiments the *Salmonella typhimurium* strains TA97, TA1535 and TA1537 were also used. The bacteria stocks were provided by “Xenometrix” and stored in the freezer at -80°C.

3. Methodology

In this chapter the practical part of this master's thesis is explained. The migration conditions and the preparation conditions of the polymer samples were carried out based on the regulations of plastic materials and articles intended to come into contact with food, EU 10/2011 (European Commission 2011) and DIN norm EN 1186 (DIN 2002), on the meeting with Frank Welle (2019) and our industry partner institute OFI. The testing procedure of the Ames MPF *in-vitro* bioassay was conducted according to the publications of Flückiger-Isler and Kamber (2012), Xenometrix (2015) and Rainer et al. (2019).

3.1. Sample Preparation

For this master's thesis different polymer recyclate samples were used. The test items were received directly from industry partners. The sample materials were wrapped in plastic bags to avoid any contaminations from their surroundings and were stored at room temperature under dry conditions. The test items represent different recycled plastic packaging materials. The samples are shown in the common table.

HDPE01	= Re-Granulat from OFI
HDPE02	= Re-Granulat out of Film or grinded material
LDPE02	= Re-Granulat out of Film
PET02	= Re-Granulat out of Bottle Flake
PP02	= Re-Granulat out of Film
PS02	= Re-Granulat out of X-foam
PS03	= Re-GRanulat out of E-foam

Table 13: Overview of the plastic polymer recyclate samples

3.1.1. Migration Process

As migration containers schott glass bottles closed tightly with polytetrafluorethylene coated screw caps were used. The samples (10mg of the polymer recyclate) were stored in contact with dichlormethane (50ml) for 24h at 40°C in the drying oven. For control purpose a solvent blank (= C- process, negative control, only extracting agent) was run with all samples.

The PET-recyclate and PS-recyclate did not work with dichlormethane as extracting agent. The PET-sample became firm during the preparation process and the PS-sample precipitated during the preparation process. Thus both samples were migrated with 95%-ethanol (99,9% diluted to 95%) as extracting agent under the same conditions described above.

3.1.2. Pre-Concentration Step

To obtain higher concentrations of the substances for easier detection by the Ames assay the migrates were concentrated by rotary evaporation under the following conditions: water bath temperature 40°C (ethanol migrates), 35°C-40°C (dichlormethane migrates, at the begin

35°C till maximum 40°C) and vacuum 70mbar (ethanol migrates), 850mbar (dichlormethane migrates). 50ml of migrates were concentrated to 1ml, so a concentration factor of approximately 50-fold was achieved. After the pre-concentration step the migrates were mixed with equal volumes of DMSO (1ml) and evaporated under vacuum for solvent change using a Visiprep™ SPE Vacuum Manifold (Supelco). The samples were stored in the fridge at 4°C.

3.1.3. Determination of substance recovery during the preparation process

Due to many steps in the sample preparation it is possible that substances are lost, interact with the plastic recyclates or even are degarded during the migration process. That is why the biological recovery of the substances is additionally determined.

For this purpose 50ml aliquots of the extracting agents (dichlormethane or rather ethanol) were spiked with three different DNA-reactive substances (= C+ process samples). The final concentrations of the mutagenic substances in the samples are shown in table 14.

mutagenic substance:	final concentration in the sample:
2-Aminoanthracene/1 (2-AA/1)	62,5µg/ml
2-Aminoanthracene/2 (2-AA/2)	25µg/ml
2-Nitrofluorene (2-NF)	50µg/ml
4-Nitrochinoline-1-Oxid (4-NQO)	2,5µg/ml

Table 14: Mutagenic substances and their final concentrations

The concentrations were determined with the aim of the LEC-values of the substances in the Ames assay. The spiked samples were treated as the polymer samples under the described conditions above.

One test run involved the test of one type of polymer recyclate samples (for example HDPE) spiked with the mutagenic substances (final concentrations shown in table 14). This was necessary to see if the polymer samples interact with the mutagenic substances and possibly the mutagenic substances could have lost their effects. For comparison positive control samples (= C+ process samples, shown in table 14) were run with the polymer samples.

3.2. Ames MPF assay

At first the tests for the determination of the substance recovery were made with every polymer recyclate type of our samples and the genotoxic standard substance. Finally only the polymer samples were tested under exact by the same conditions. To make sure that the assay was not defective, spiked plates were run with the polymer sample plates.

3.2.1. Preparation Steps

For every assay an appropriate design was constructed. A bacteria overnight culture had to be made the day before testing. Therefore, 10µl of the frozen bacteria stock was added to

3ml nutrient broth No. 2 and 3µl ampicillin (25mg/ml). The bacteria overnight culture was incubated in the Orbital shaker by 250 rpm up to 16 hours at 37°C. This culture was stored at 4°C for a maximum of 14 days and was regrown as needed. On the testing day the overnight culture was cultivated newly. To do this, 200-400µl of the overnight culture were mixed with 3ml nutrient broth No. 2 and 3µl ampicillin and incubated at the same conditions. The growth of the bacteria was constantly controlled with the photometer and usually took 2-3 hours depending on the bacteria tester strain. Therefore, the bacteria were diluted 1:10 with a PO_4-puffer and the optical density (OD) was measured at a wavelength of 600nm. Cultures with an OD > 2,0 (at maximum 2,8) were used.

Till the bacteria were ready for testing, the essential chemicals and expendable materials were prepared. The samples were stored in the fridge and had to be vortexed before using. The pure substances were stored frozen and had to be defrosted at room temperature before using. The negative control (C-) in the assay was DMSO and the positive controls were accordingly 2-AA, 2-NF and 4-NQO in the same concentrations as in the spiked samples (table 15). The dilution series in the assay was a 1:2 dilution. The bacteria strain TA98 was tested with 2-AA/2 and 2-NF. The bacteria strain TA100 was tested with 2-AA/1 and 4-NQO. The mutagenic substance 2-AA needed a metabolic activator system so S9 and the co-factors had to be added.

The S9-mix consisted of 72,5% Na_2HPO_4-puffer, 2,5% G-6-P, 10% NADP and 15% S9-extract. 15% of these S9-mix were used and added to the bacteria and exposure medium. To test the TA100 tester strain 5% overnight culture, 80% exposure medium and 15% S9-mix were necessary and to test the TA98 10% of the overnight culture, 75% exposure medium and also 15% S9-mix were used. For one plate the amount of the compounds was 7ml in total.

In the following table the amount of the ingredients of the final mix is summarized.

	TA98 -S9 (with 2-NF)	TA98 +S9 (with 2-AA/2)	TA100 -S9 (with 4-NQO)	TA100 +S9 (with 2-AA/1)
Co-factors	/	0,73ml	/	0,73ml
S9 extract	/	0,32ml	/	0,32ml
bacteria culture	0,7ml	0,7ml	0,35ml	0,35ml
Exposure media	6,3ml	5,25ml	6,65ml	5,6ml
Volume	7ml	7ml	7ml	7ml

Table 15: Overview of the test schema, amount of the ingredients of the final mix

3.2.2. Test Procedure

The dilution series of the spiked polymer samples and process positive controls (C+ process) was made in 96-well-plates with DMSO as solvent. The layout of the dilution series is shown

in figure 4. The dilution series was transferred to the 24-well plate. The amount of 10µl of every dilution step was pipetted three times to the 24-well plate. The layout of the 24-well plate is shown in figure 5. The final bacteria mix which is described above and shown in table 15 was added to the 24-well plate. The amount of 240µl of the mix was added to every well of the 24-well plate. The 24-well plate was incubated in the Orbital shaker by 250rpm at 37°C for 90 minutes.

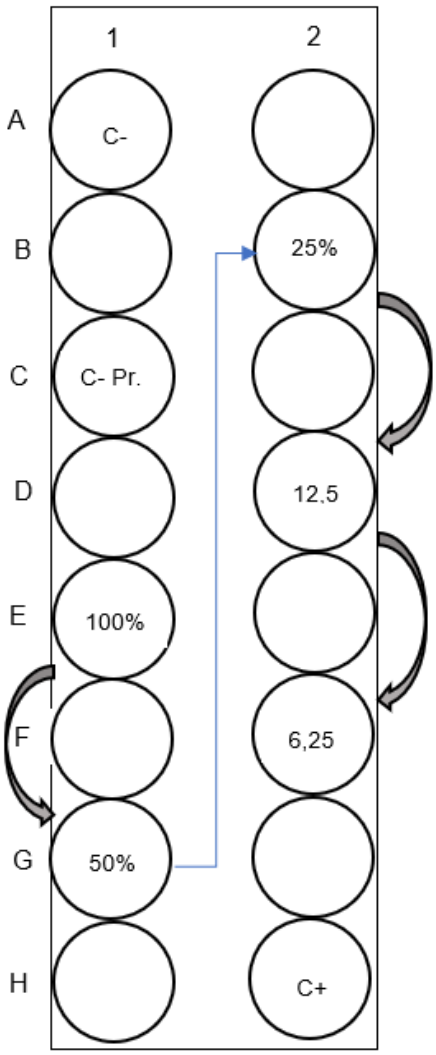

Figure 4: Dilution series made in the 96-well plate

In figure 4 the layout of the dilution series is shown. Every second well of the 96-well plate is used for the dilution series in this sequence: negative control (only DMSO, C-), process C- (extractions agent), 100% of the substance (spiked polymer sample or process C+), the dilution steps of the substance (50%, 25%, 12,5% and 6,25%) and the positive control (mutagenic substance, C+).

The dilution series of the 96-well plate is transferred to the 24-well plate. The layout of the 24-well plate is shown in figure 5.

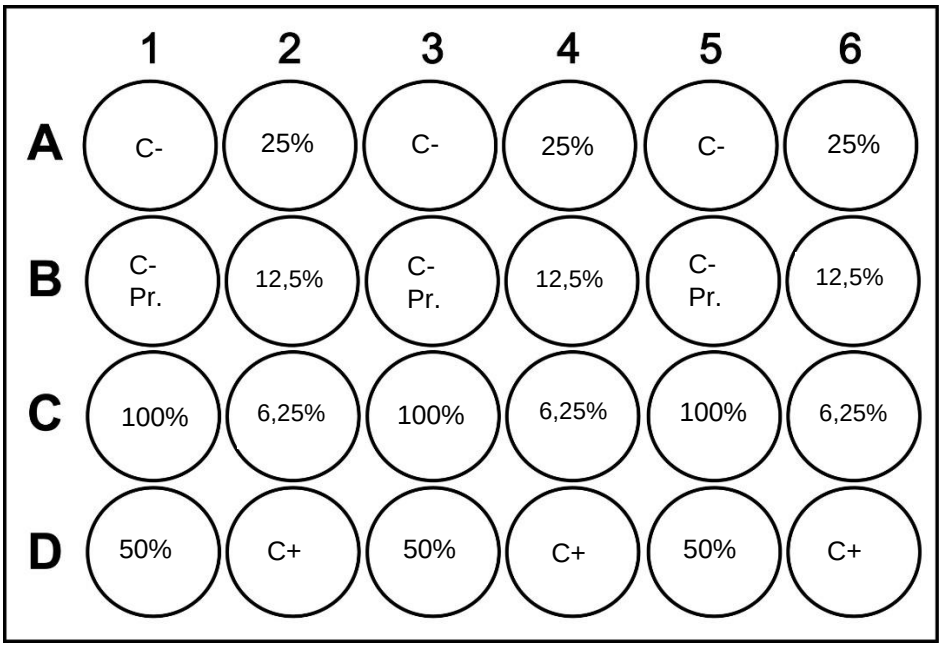

Figure 5: Plate layout of the 24-well plate

After the incubation time of 90 minutes 2,6ml of the indicator medium (violet) was added in every well of the 24-well plate. The content of the 24-well plate was transferred to three 384-well plates. The amount of 50µl of every dilution step was transferred to 48 wells of the 384-well plates. For one 24-well plate three 384-well plates were needed. This step is shown in the figure number 6.

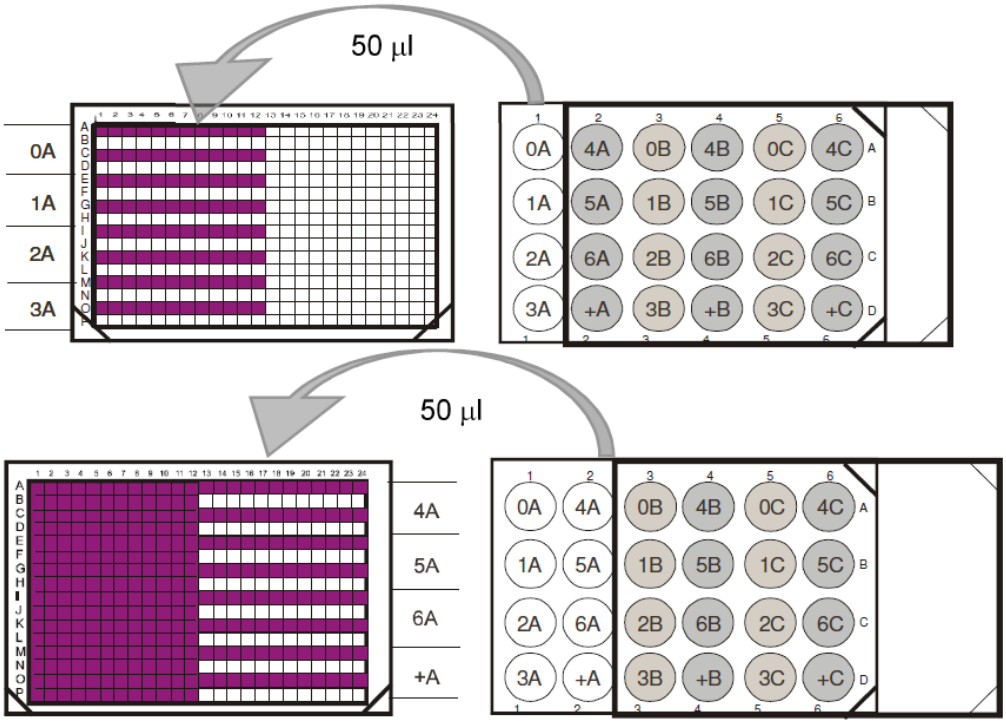

Figure 6: Transfer of the content of the 24-well plate to the 384-well plate (source: Xenometrix)

The plates were put in lockable plastic bags and incubated for 48 hours.

Concluding this chapter, figure 7 shows a summary of the procedure of the Ames MPF assay.

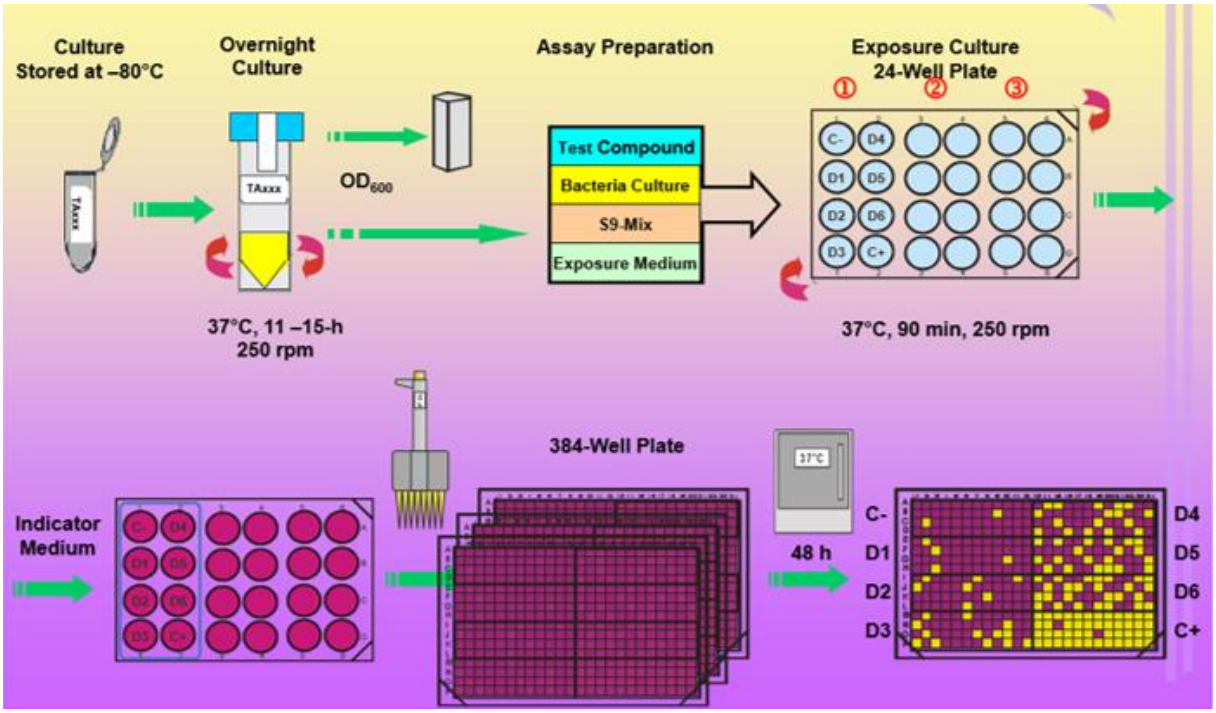

Figure 7: Summary of the procedure of the Ames MPF assay (source: Xenometrix)

3.2.3. Spiked recyclate-samples in the assay

To assess matrix effects of the recyclate-samples in the assay, the response to a known Ames-positive substance was evaluated. For this step, all polymer recyclate samples were tested in absence and presence of a positive control spike. To do this the exposure medium was spiked with the corresponding mutagenic substances in the same concentrations as described in the chapter 4.1.3. (table 15). Both plates (spiked plate and unspiked plate) were conducted in parallel for every test run.

3.3. The evaluation of the assay

After the incubation time of 48 hours the revertants of the dilution steps on the plates were counted and the negative and positive controls were criticized. Due to the change of the pH-value the indicator medium changed the colour from violet to yellow. The revertants were seen with the colour change and were dyed yellow. Most of the revertants were coloured clearly yellow but revertants which were only coloured light were counted too. Figure 8 shows a 384-well plate after the incubation time of 48 hours.

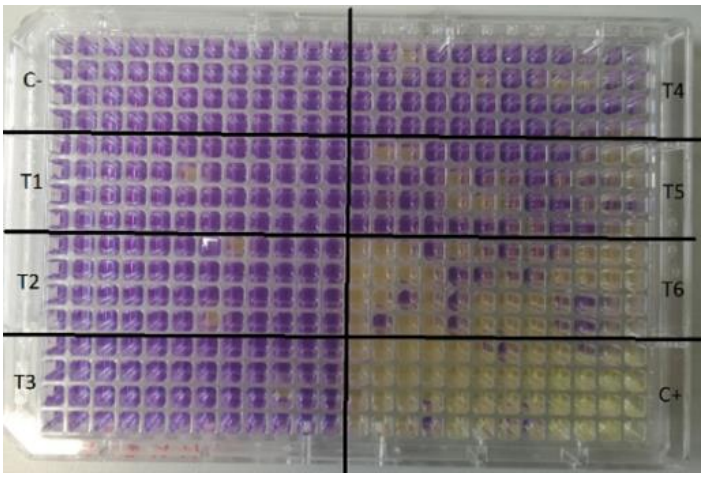

Figure 8: 384-well plate after the incubation time

The received data were registered in an Excel-datasheet which was provided from Xenometrix.

The scoring was conducted according to Xenometrix. For the determination of a positive test result the baseline (= background mutations of C-) had to be estimated. Therefore, the following formula was used:

$$Baseline = (arithmetic\ mean + SD\ of\ negative\ control)\ x\ 2$$

A baseline-value <1 was rounded up to 1 to avoid false negative results. If the test had an arithmetic mean bigger than the baseline the test result was assessed positive (according to Xenometrix). A threshold line was additionally set to avoid false positive results:

$$Positive\ threshold = baseline\ x\ 2$$

If the positive threshold was twice the baseline the result of the test was assessed positive.

The results were diagrammed in histograms in the Excel-sheet. Figure 9 shows an example of such a histogram.

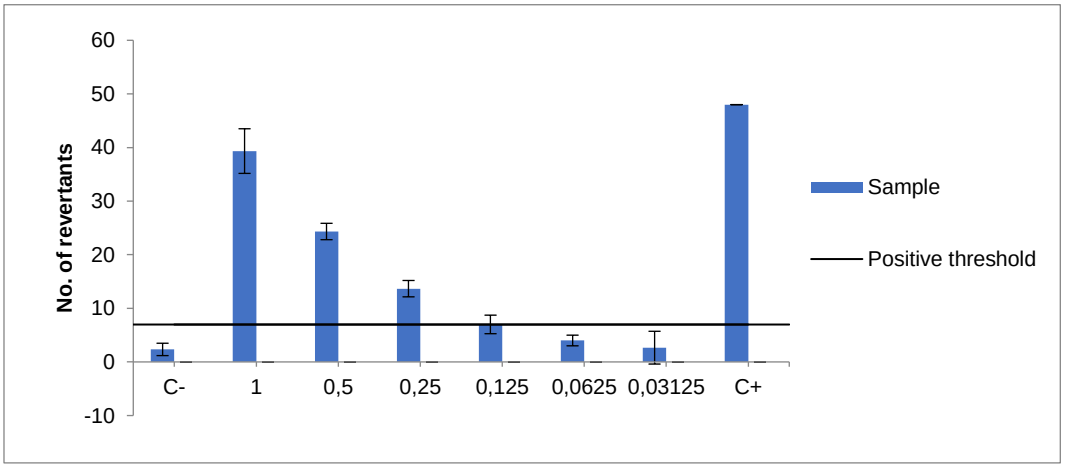

Figure 9: Example of the representation of a test result with a 1:2 dilution and a positive control (C+) on the last position

The y-axis of the histogram shows the amount of the revertants (arithmetic mean of three 384-well-plates of one test run) and the x-axis shows the negative control and the dilution steps and if necessary, also the positive control. In this figure there is a negative control on the first position of the x-axis. On the second position of the x-axis there are 100% of the sample. The next five steps are the dilution steps of the sample (1:2 dilution) and on the last position there is the positive control.

If the values of the revertants were above the positive threshold, the sample was considered as positive in the assay.

4. Results

At first the results of the tests for the biological substance recovery are presented and finally the results of the tests with the recyclate-samples are shown. The results are listed according to the different recyclate sample-types. As already mentioned, the PS- and PET-recyclate samples were migrated with ethanol as extracting agent and the other polymer recyclate types (HDPE, LDPE, PP) were migrated with dichlormethane as extracting agent. All samples were prepared under the same conditions described in the chapter 4.1.1., 4.1.2. and 4.1.3. The bacteria strain TA98 was tested with 2-NF and 2-AA/2 and the bacteria strain TA100 was tested with 2-AA/1 and 4-NQO.

4.1. Tests for the biological substance recovery

For these tests the final concentration of the mutagenic substances in the samples and the concentration of the positive controls C+ in the assay were equal:

2-NF: 50µg/ml

2-AA/2: 25µg/ml

2AA/1: 62,5µg/ml

4-NQO: 2,5µg/ml

The blue bars in the histograms show the spiked polymer samples (50ml extracting agent + 10mg polymer + mutagenic substance) and the green bars show the process C+ samples (50ml extracting agent + mutagenic substance). In these assays a 1:2 dilution was made.

4.1.1. Sample-type: HDPE01

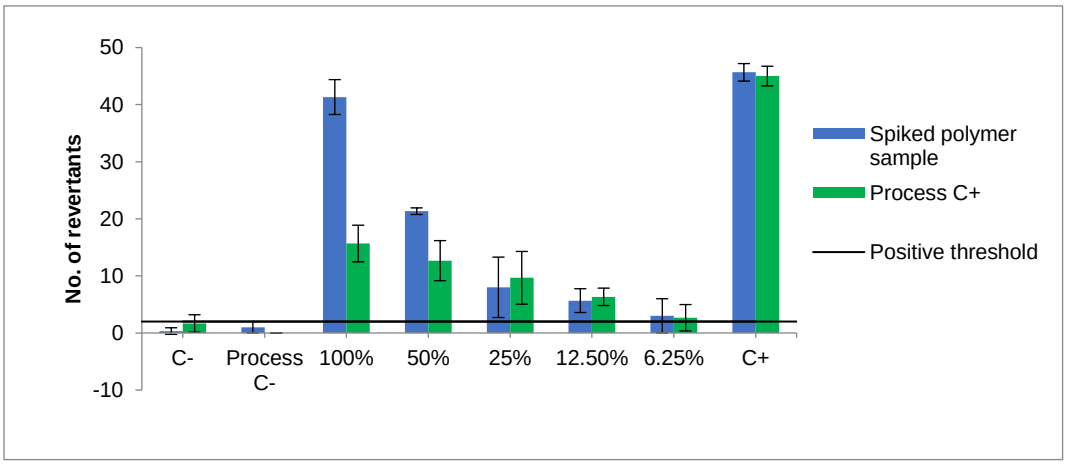

Figure 10: Test for the substance recovery with HDPE01: TA98 and 2-NF -S9

In this figure the very low response of the top concentration of the process C+ sample is noticeable while the top concentration of the spiked polymer sample is high. This means that the substance 2-NF works well with the polymer sample HDPE but it appears that parts of the process C+ sample was lost during sample preparation. The biological substance recovery of the spiked polymer sample is 91% and of the process C+ is 35%.

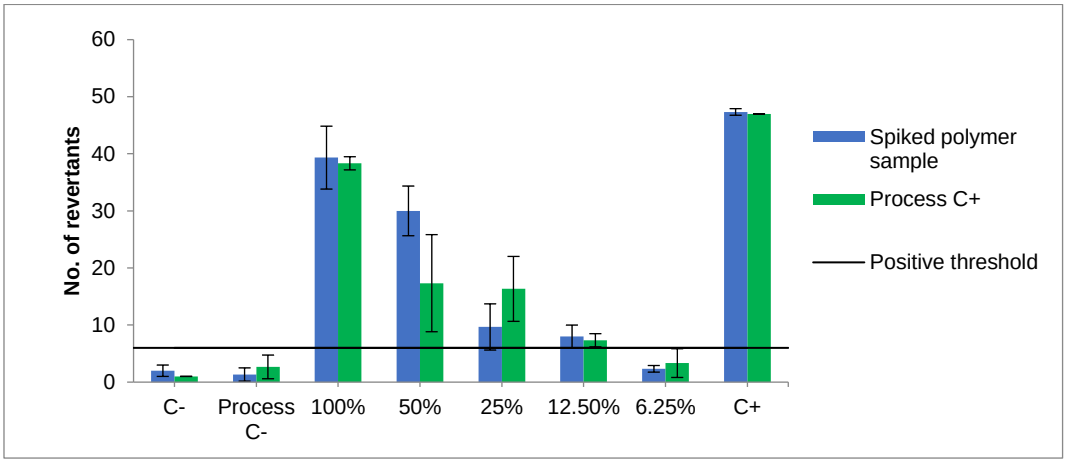

Figure 11: Test for the substance recovery with HDPE01: TA98 and 2-AA/2 +S9

In figure 11 the spiked polymer sample and the process C+ both show high revertant numbers by almost 40 revertants. The maximum possible number of revertants is 48. The positive controls in the assay show the highest revertant numbers with 48 revertants. The biological substance recovery of the spiked polymer sample is 83% and of the process C+ 82%.

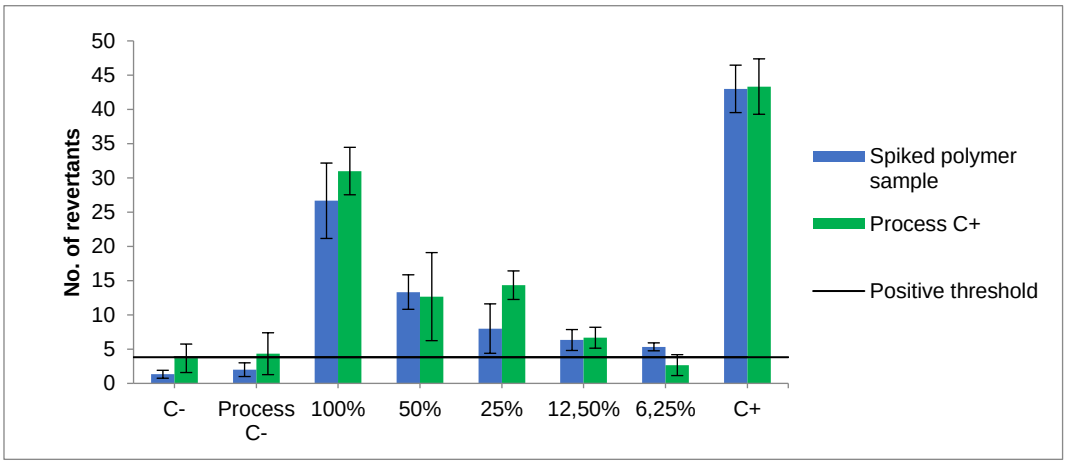

Figure 12: Test for the substance recovery with HDPE01: TA100 and 2-AA/1 +S9

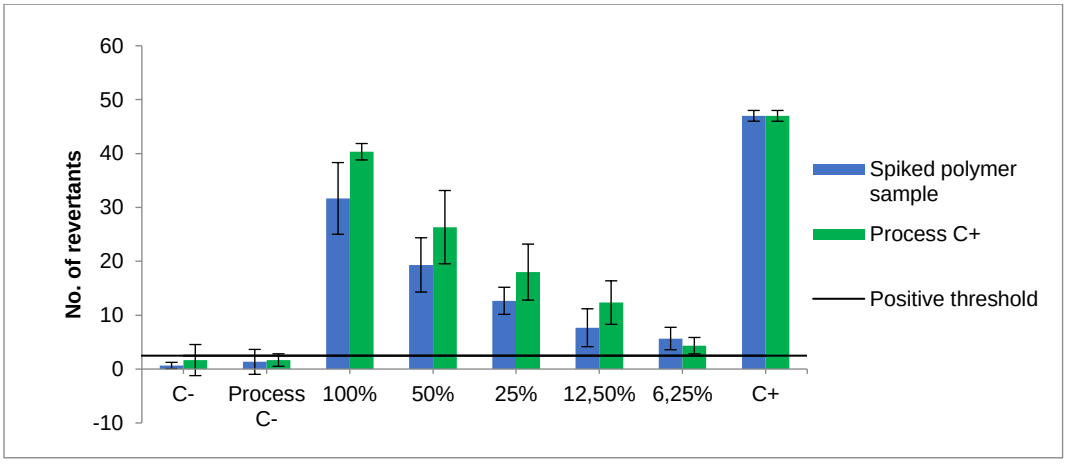

Figure 13: Test for the substance recovery with HDPE01: TA100 and 4-NQO -S9

In figure 12 and 13 the spiked samples and the C+ process samples show lower revertant numbers but almost between 25 and 40 revertants. The positive controls in the assay show

high revertant numbers (almost 48 revertants). The biological substance recovery of 2-AA/1 (figure 12) is 62% for the spiked polymer sample and 72% for the process C+ sample. The biological recovery of 4-NQO (figure 13) is 67% for the spiked polymer sample and 86% for the process C+ sample. The substance recoveries are above 60%. This means that the substances 2-AA and 4-NQO work with the polymer HDPE in the assay.

4.1.2. Sample-type: PP02

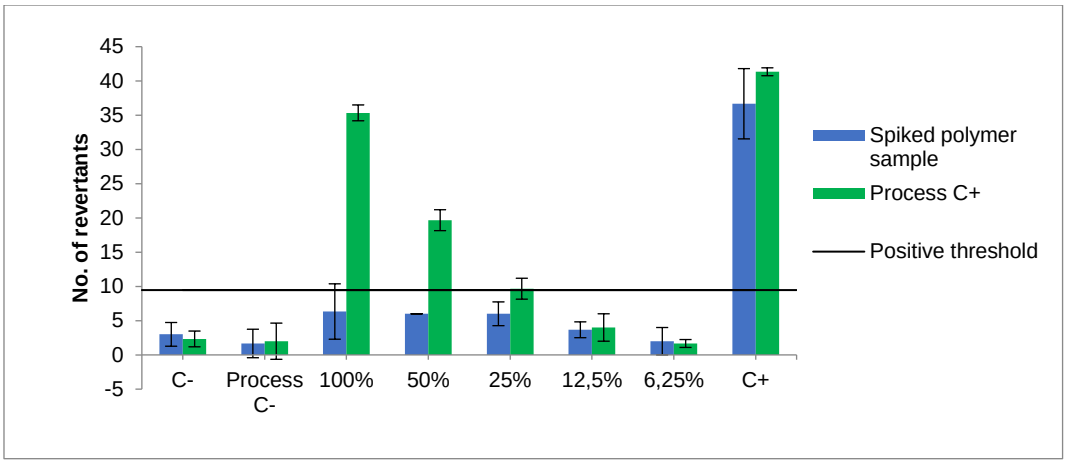

Figure 14: Test for the substance recovery with PP02: TA98 and 2-NF -S9

In this figure the top concentration of the spiked polymer sample is very low while the top concentration of the process C+ is high. It is possible that the substance 2-NF interacted with the polymer sample and consequently the genotoxicity of the substance is reduced. The dilution series of the process C+ can be seen very clearly and looks satisfactory. The biological substance recovery of the spiked polymer sample is 17% and of the process C+ 85%.

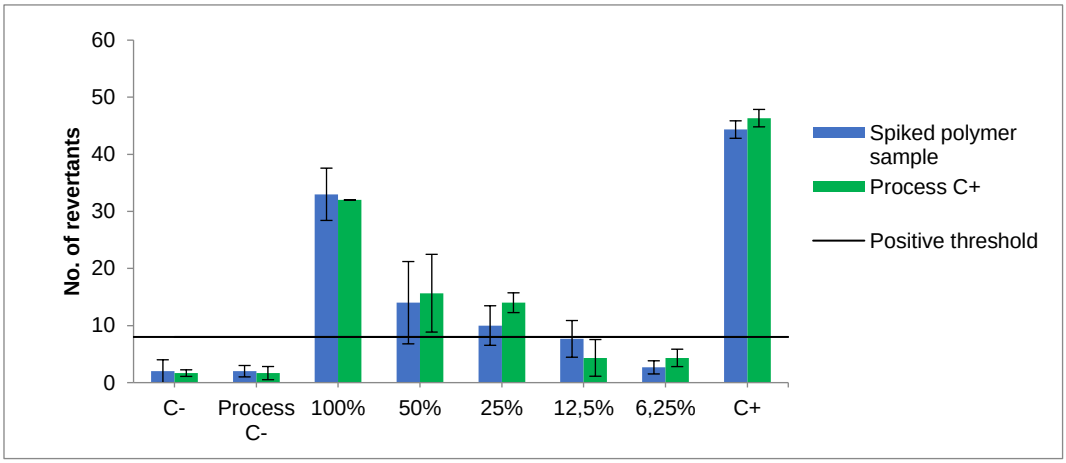

Figure 15: Test for the substance recovery with PP02: TA98 and 2-AA/2 +S9

In figure 15 the spiked sample and the C+ process sample show lower revertant numbers but certainly around 30 revertants. The biological substance recovery of the spiked polymer sample is 74% and of the process C+ 69%. The substance recoveries of both, spiked polymer sample and process C+ are above 60% so it can be said that the substance 2-AA works well with the polymer PP in the assay.

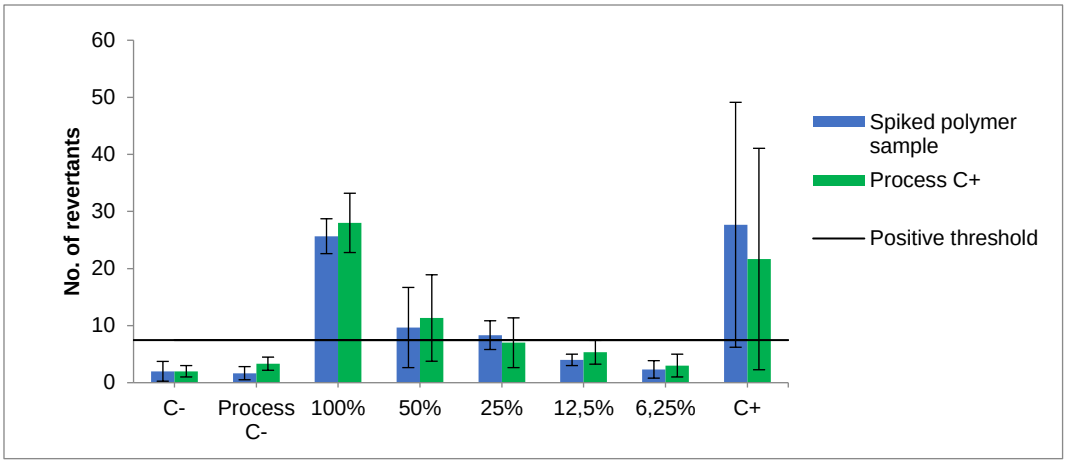

Figure 16: Test for the substance recovery with PP02: TA100 and 2-AA/1 +S9

In figure 16 the response of the positive control is very low in comparison to the other assays. Perhaps the substance got instable due to the Freeze – Thaw cycles by the usage, thus the mutagenicity of the substance was reduced. Consequently, the response of the polymer sample and C+ process sample is also low. Due to the very low revertant numbers of the positive controls in the assay, the biological substance recoveries are very high at 93% for the spiked polymer sample and 129% for the C+ process.

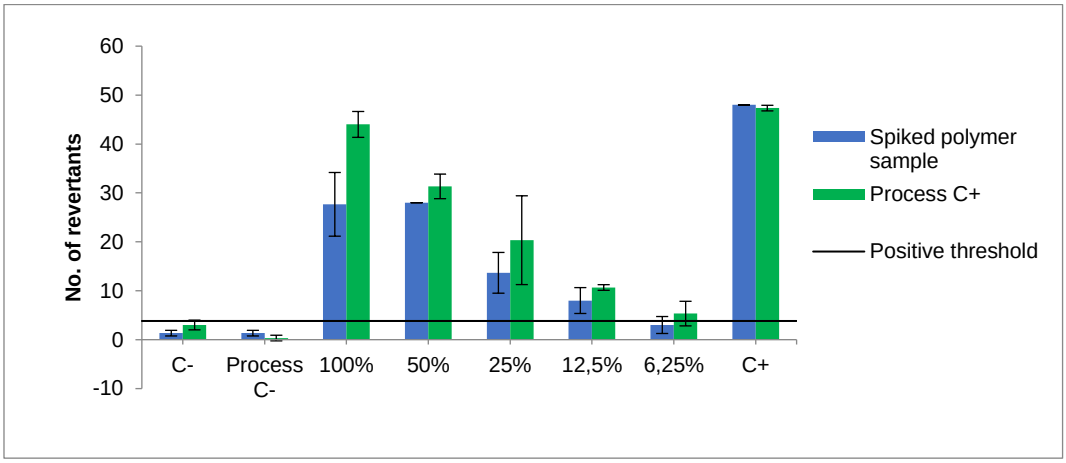

Figure 17: Test for the substance recovery with PP02: TA100 and 4-NQO -S9

In this figure the process C+ shows good revertant numbers around 40 revertants. But compared to the positive controls (C+) in the assay the top concentration of the spiked polymer sample is very low. Assuming that 4-NQO normally shows a very good response in the assay, it is possible that 4-NQO interacted with the polymer sample and this has an effect on genotoxicity or that some substance was lost due to errors in sample preparation steps. The dilution series can be seen very clearly and appears satisfactory, 4-NQO is just a bit too highly dosed for an exact dilution series. The biological substance recovery of the spiked polymer sample is low with 58%. The biological substance recovery of the process C+ is very high with 93%.

4.1.3. Sample-type: LDPE02

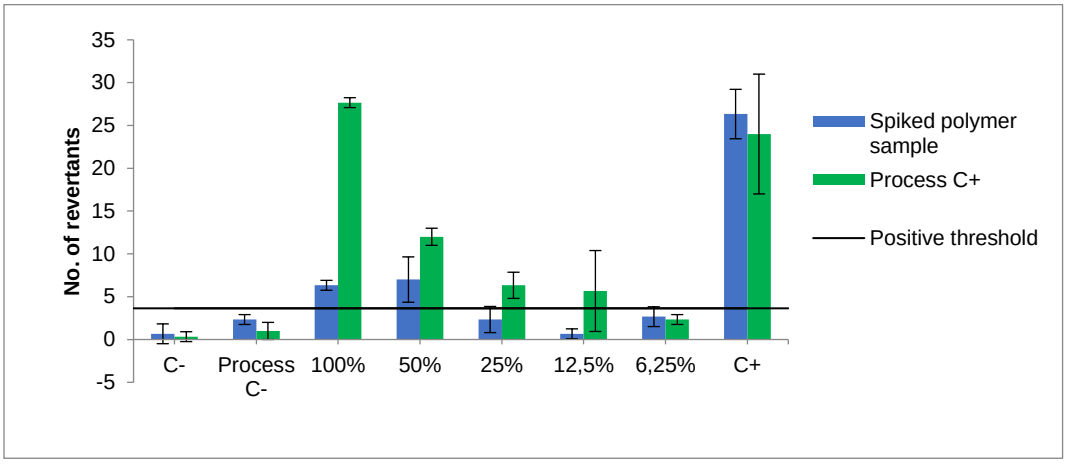

Figure 18: Test for the substance recovery with LDPE02: TA98 and 2-NF -S9

In this figure the very low response of the spiked polymer sample is noticeable while the top concentration of the process C+ is high. The positive controls in the assay show a bit lower revertants than usual (almost between 20 and 30 revertants). It appears that 2-NF reacted with the sample and consequently lost activity. The biological substance recovery of the spiked polymer sample is very low with 24%. Due to the lower response of the positive control in the assay the biological substance recovery of the process C+ is 115%.

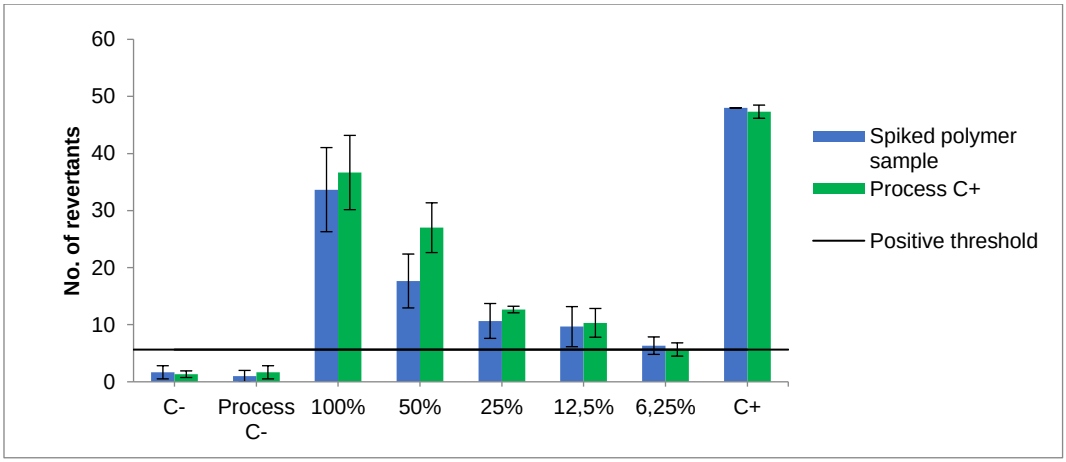

Figure 19: Test for the substance recovery with LDPE02: TA98 and 2-AA/2 +S9

In figure 19 the spiked sample and the C+ process sample show lower revertant numbers but certainly between 30 and 40 revertants. The biological substance recovery of the spiked polymer sample is 70% and of the process C+ 78%. The substance recoveries of both, spiked polymer sample and process C+ are above 60%, so it can be said that the substance 2-AA works well with the polymer sample LDPE in the assay.

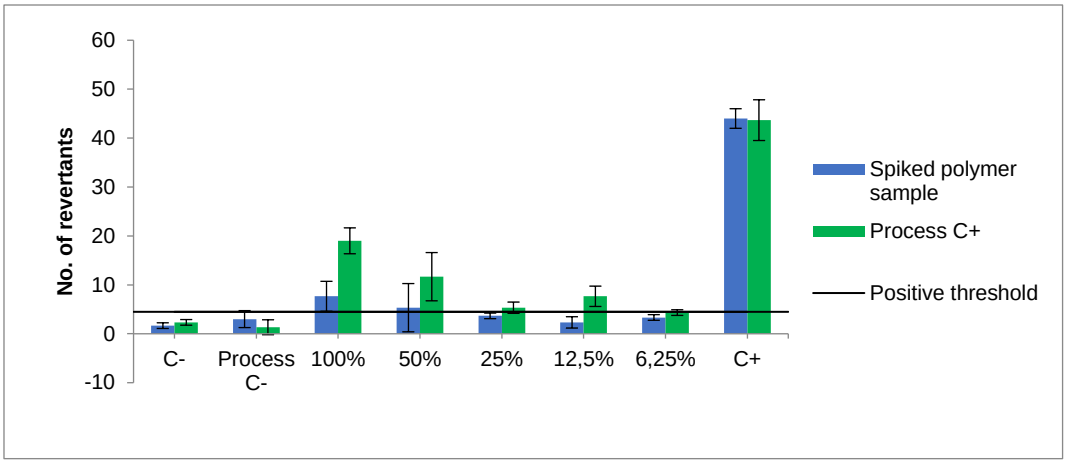

Figure 20: Test for the substance recovery with LDPE02: TA100 and 2-AA/1 +S9

In this figure the top concentration of both, spiked polymer sample and C+ process is very low. The biological substance recovery of the spiked polymer sample is 20% and of the process C+ is 40%. It is likely that substances were lost here due to errors made during sample preparation.

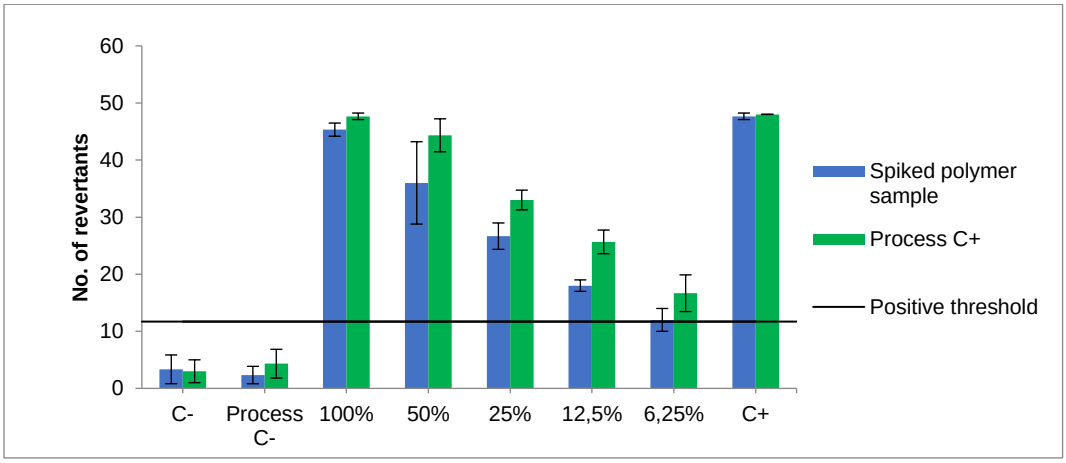

Figure 21: Test for the substance recovery with LDPE02: TA100 and 4-NQO -S9

In figure 21 the response of 4-NQO in both, the spiked polymer sample and process C+ sample is very good. The steps of the dilution series are clearly visible and the results are satisfactory, 4-NQO is just a bit too highly dosed for an exact dilution series. The biological substance recoveries in this test are very high with 95% for the spiked polymer sample and 99% for the process C+.

4.1.4. Sample-type: PS03

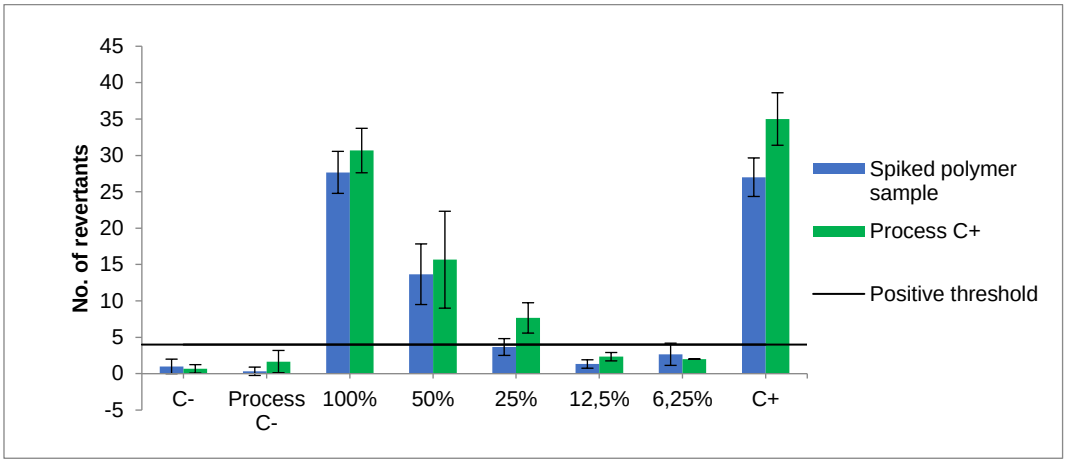

Figure 22: Test for the substance recovery with PS03: TA98 and 2-NF -S9

In figure 22 the spiked sample and the C+ process sample show lower revertant numbers but certainly between 25 and 35 revertants. The positive controls in the assay also show lower responses than usual. Due to the lower responses of the positive controls the biological substance recoveries are high in this test at 102% for the spiked polymer sample and 88% for the process C+. Perhaps the substance 2-NF got instable due to the Freeze – Thaw cycles by the usage, thus the mutagenicity of the substance was reduced in this test. The steps of both dilution series are clearly visible.

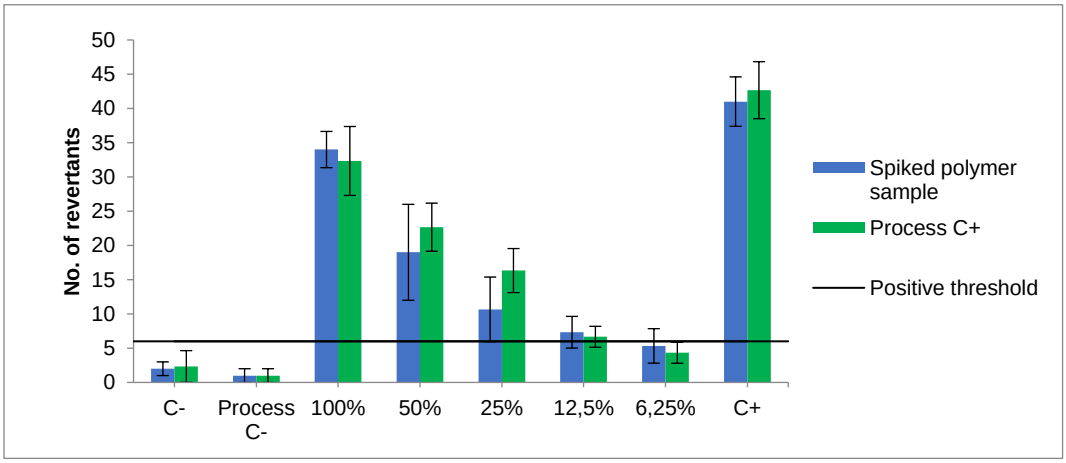

Figure 23: Test for the substance recovery with PS03: TA98 and 2-AA/2 +S9

In figure 23 the spiked sample and the C+ process sample show lower revertant numbers but certainly between 25 and 35 revertants. The positive controls in the assay also show fewer responses than usual (around 40 revertants). The biological substance recovery of the spiked polymer sample is 83% and of the process C+ is 76%. The steps of the dilution series are clearly visible and appear satisfactory, 2-AA is just a bit too highly dosed for an exact dilution series.

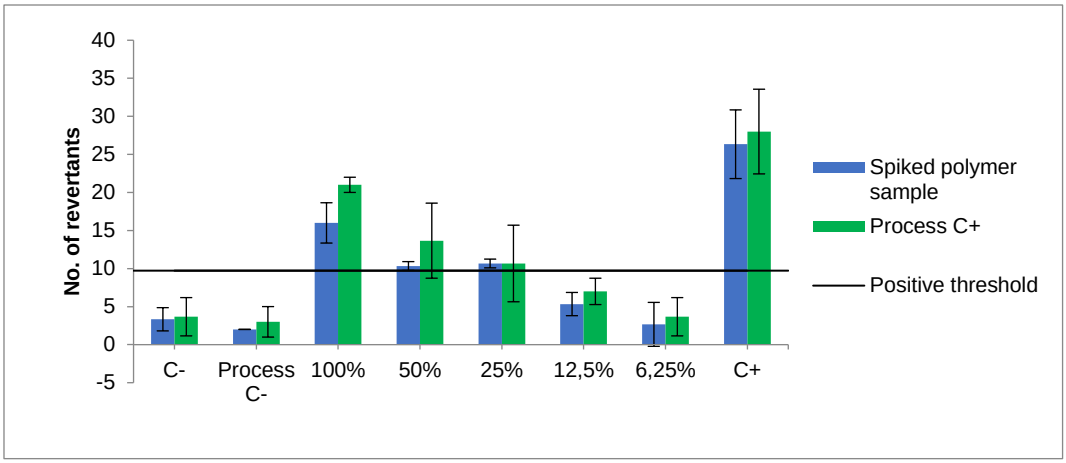

Figure 24: Test for the substance recovery with PS03: TA100 and 2-AA/1 +S9

In the test in figure 24 it seems as if the substance 2-AA does not work well in the assay because the positive controls are also lower than usual (under 30 revertants). The top concentrations of the polymer sample and process C+ show also very low revertant numbers between 15 and 25 revertants. Perhaps the substance 2-AA got instable due to the Freeze – Thaw cycles by the usage, thus the mutagenicity of the substance was reduced in this test. It is also likely that parts of the substance were lost during the sample preparations steps. The biological substance recovery of the spiked polymer sample is 61% and of the process C+ 75%.

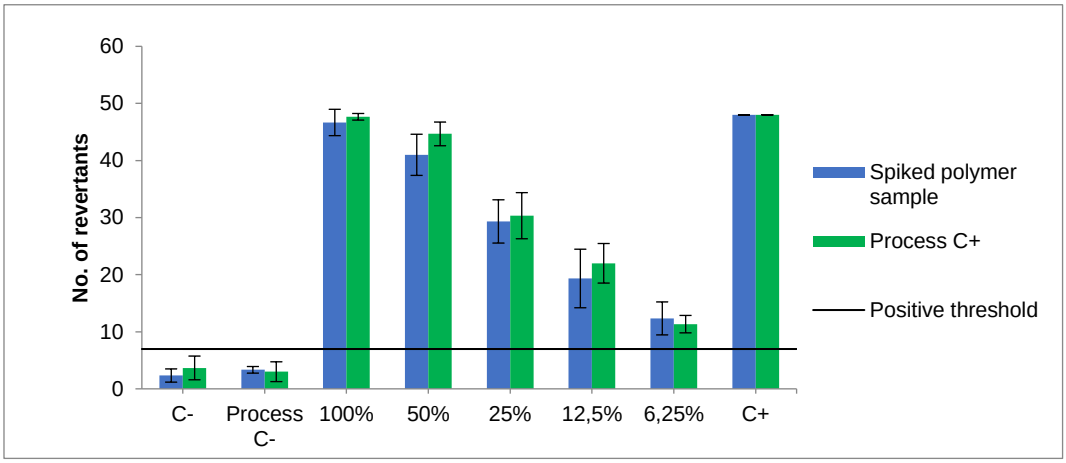

Figure 25: Test for the substance recovery with PS03: TA100 and 4-NQO -S9

In this figure the substance 4-NQO shows very good responses with the polymer sample and also with the process C+ sample. The steps of the dilution series are clearly visible and look satisfactory, 4-NQO is just a bit too highly dosed for an exact dilution series. The biological substance recoveries of both, the spiked polymer sample and the process C+ are very high at 97% for the sample and 99% for the process C+.

4.1.5. Sample-type: PET03

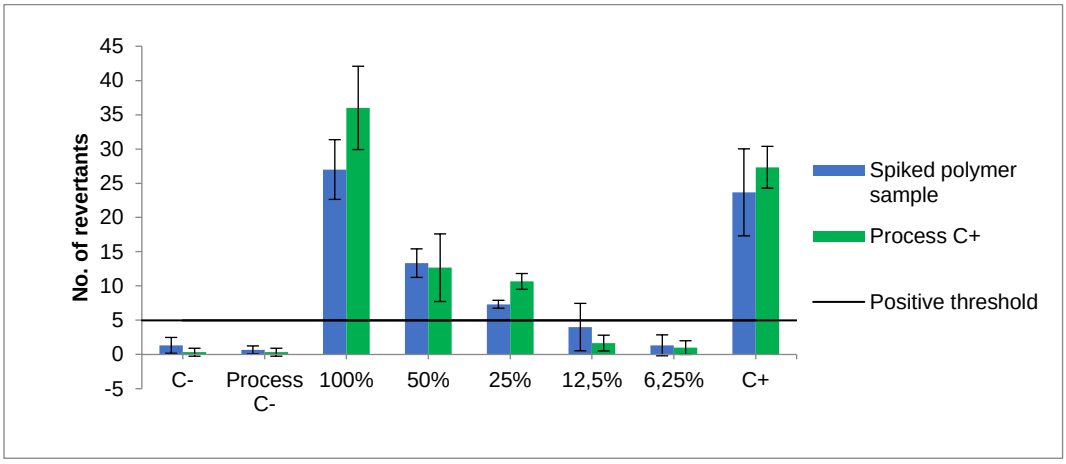

Figure 26: Test for the substance recovery with PET03: TA98 and 2-NF -S9

In this figure it is noticeable that the positive controls in the assay show very low revertant numbers between 25 and 30 revertants. The polymer sample and the process C+ sample show revertant numbers between 25 and 40 revertants. Perhaps 2-NF got instable due to the Freeze – Thaw cycles by the usage, thus the mutagenicity of the substance was generally reduced in this experiment. Furthermore it is possible that 2-NF interacted with the polymer sample during the sample preparation steps and because of this the response of the polymer sample is lower than the response of the C+ process. Due to the lower responses of the positive controls in the assay the biological substance recoveries are very high at 128% for the spiked polymer sample and 132% for the process C+.

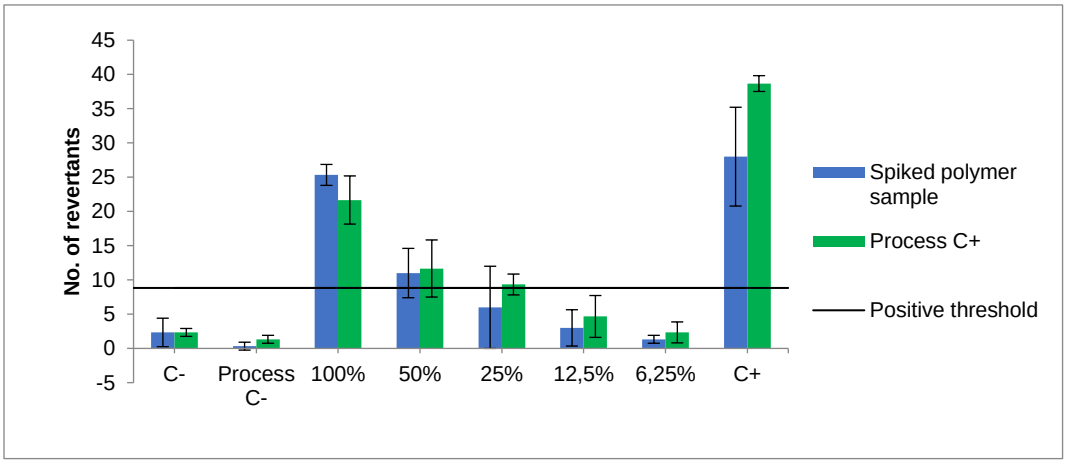

Figure 27: Test for the substance recovery with PET03: TA98 and 2-AA/2 +S9

In figure 27 it is noticeable that one positive control works well in the assay, while the other positive control shows fewer revertant numbers. It is likely that there was an error while pipetting the positive controls. The revertant numbers of the polymer sample and the process C+ are both low between 20 and 30 revertants. It is possible that errors during sample preparation may have resulted in loss of substance. The biological substance recovery of the spiked polymer sample is very high with 91%. This is because of the low response of the positive

control in the assay. The biological substance recovery of the process C+ is very low with 56%.

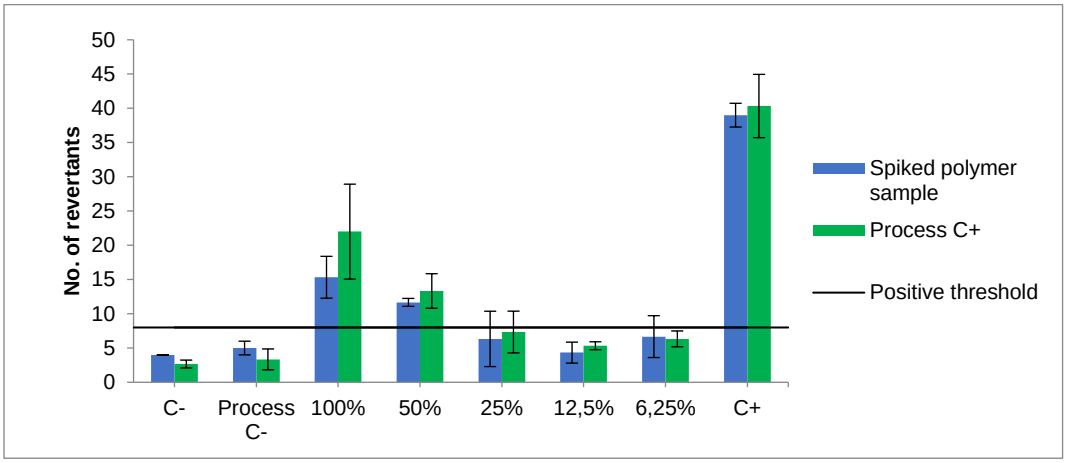

Figure 28: Test for the substance recovery with PET03: TA100 and 2-AA/1 +S9

In comparison to figure 28, the positive controls in figure 34 with the same pure substance show good revertant numbers over 40 revertants. The revertant numbers of the polymer sample and the process C+ are both low between 15 and 25 revertants. It is possible that errors during sample preparation may have resulted in loss of substance. As a result, the biological substance recoveries are very low at 39% for the spiked polymer sample and 35% for the process C+.

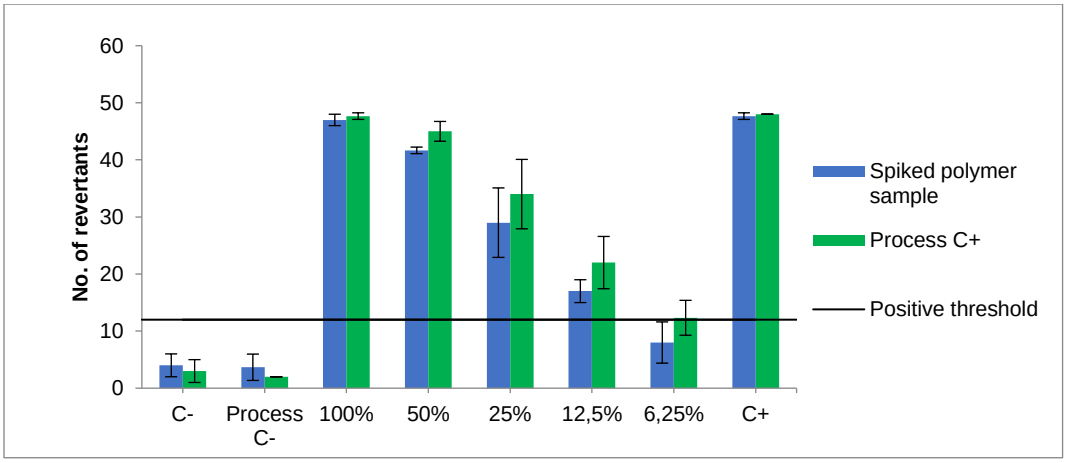

Figure 29: Test for the substance recovery with PET03: TA100 and 4-NQO -S9

In this experiment the responses of the polymer sample and C+ process sample are very high (almost 48 revertants). The dilution series appears satisfactory, 4-NQO is just a bit too highly dosed for an exact dilution series. The biological substance recoveries are very high at 99% for both, the spiked polymer sample and the process C+.

4.1.6. Determination of the biological substance recovery

To determine the biological substance recovery the revertant amount of the positive controls (C+) in the assay was compared with the revertant amount of the top concentrations of the

spiked polymer samples and the process C+ samples. The results are listed in the following table number 16.

	2-AA/1	2-AA/2	2-NF	4-NQO
HDPE: spiked polymer sample	62%	83%	91%	67%
HDPE: C+ process	72%	82%	35%	86%
LDPE: spiked polymer sample	20%	70%	24%	95%
LDPE: C+ process	40%	78%	115%	99%
PP: spiked polymer sample	93%	74%	17%	58%
PP: C+ process	129%	69%	85%	93%
PS: spiked polymer sample	61%	83%	102%	97%
PS: C+ process	75%	76%	88%	99%
PET: spiked poylmer sample	39%	91%	128%	99%
PET: C+ process	35%	56%	132%	99%

Table 16: Results of the biological substance recovery with all polymer recyclate samples

4.2. Tests with the recyclate-samples

These tests were performed to exclude that the used standard samples have cytotoxic activities. If the spike recovery falls below 60%, cytotoxicity or growth inhibiting effects are assumed.

For this purpose a spiked and an unspiked plate were conducted in parallel. For the spiked plate, the exposure medium of the final bacteria mix was spiked with the mutagenic substances so that the final concentrations in the assay were: 2-NF: 2µg/ml

2-AA/2: 2,5µg/ml

2AA/1: 2,5µg/ml

4-NQO: 0,1µg/ml

The positive control substances which were used in the assay had the concentrations: 50µg/ml for 2-NF, 25µg/ml for 2-AA/2, 62,5µg/ml for 2-AA/1 and 2,5µg/ml for 4-NQO. For the first screening 100% of the samples were tested undilutedly. If a sample was tested positive it would be necessary to make a 1:2 dilution in a further assay with the positively tested sample.

The blue bars show the recyclate samples and the green bars show the spiked plate which was conducted parallel to the unspiked plate.

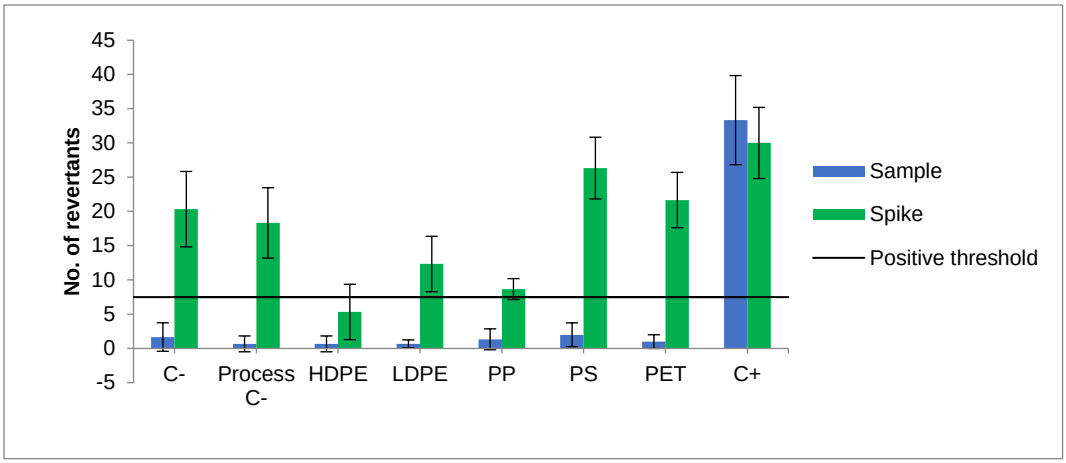

Figure 30: Test with recyclate-samples: TA98 and 2-NF -S9

This test was conducted several times, but the spiked plate showed low responses in every test run. It seems as if the pure substance 2-NF and the polymer samples do not work together in the assay.

The next three figures (31, 32, 33) also showed the recyclate-samples with the substance 2-NF.

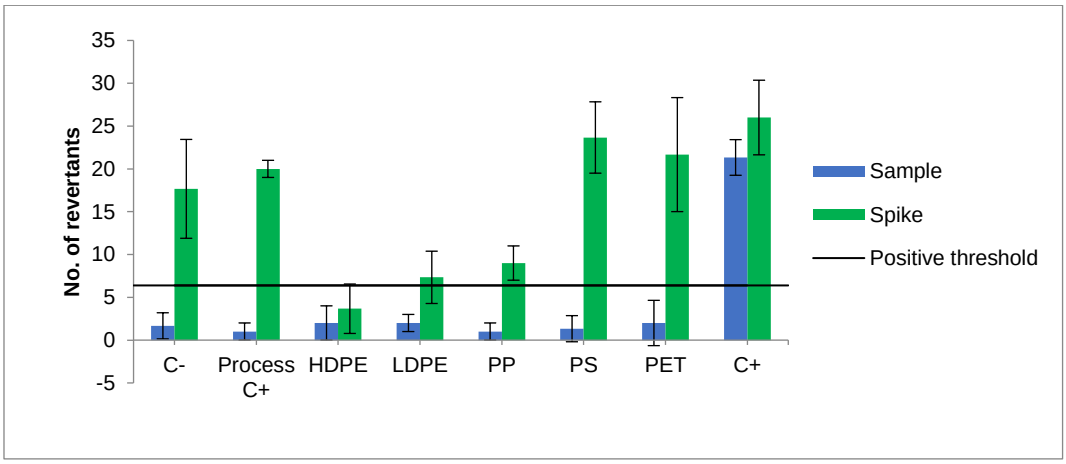

Figure 31: Test with recyclate-samples: TA98 and 2-NF -S9: first repeat

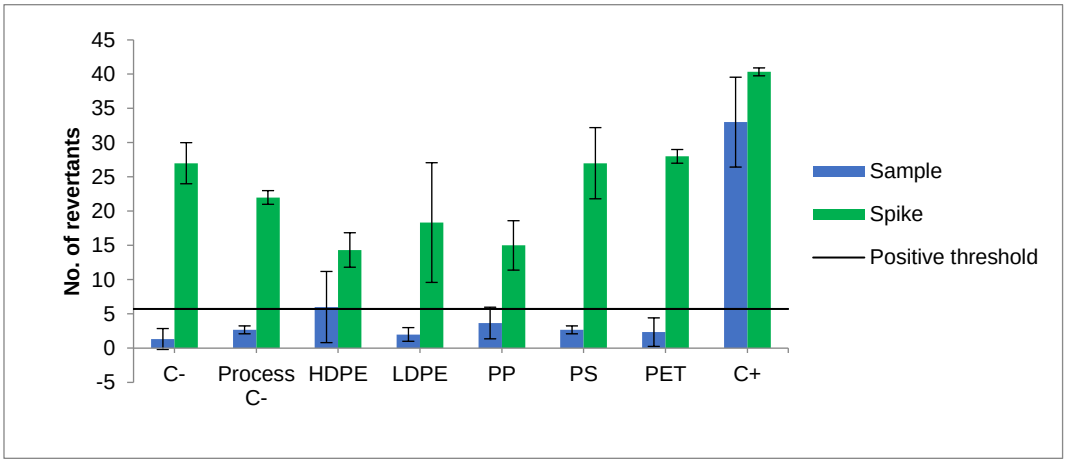

Figure 32: Test with the recyclate-samples: TA98 and 2-NF: second repeat

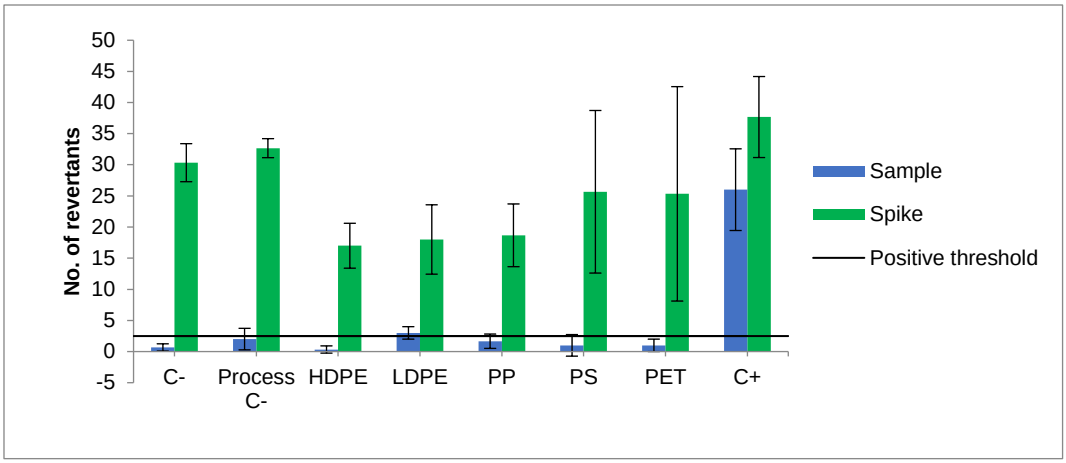

Figure 33: Test with recyclate-samples: TA98 and 2-NF -S9: third repeat

As the pictures show, the substance 2-NF caused problems in connection with the plastic polymer recyclate samples. The positive controls in the assay also show lower revertant numbers than usual (between 20 and 35 revertants).

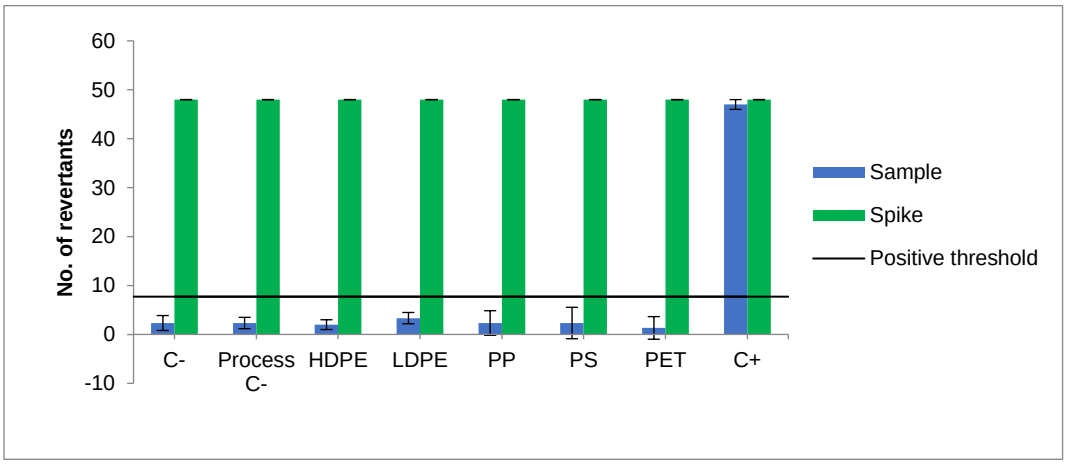

Figure 34: Test with recyclate-samples: TA98 and 2-AA/2 +S9

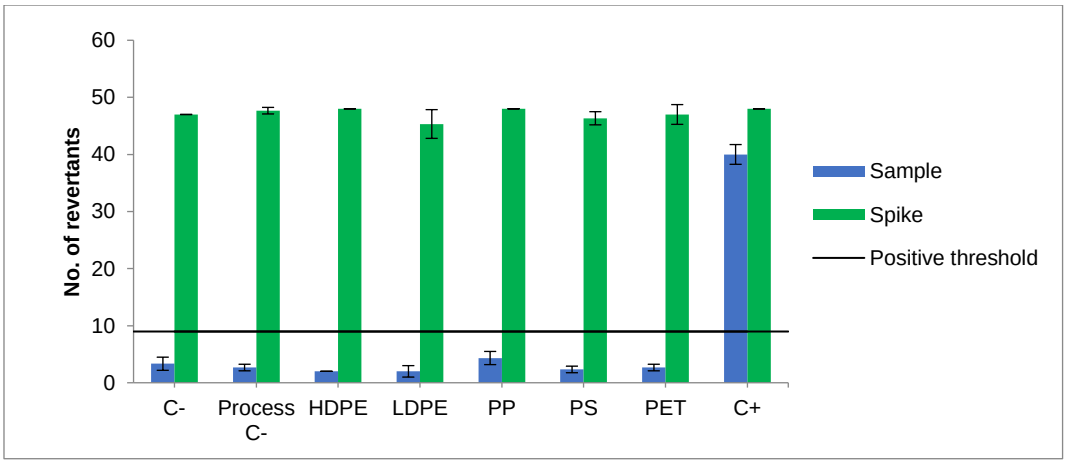

Figure 35: Test with recyclate-samples: TA100 and 2-AA/1 +S9

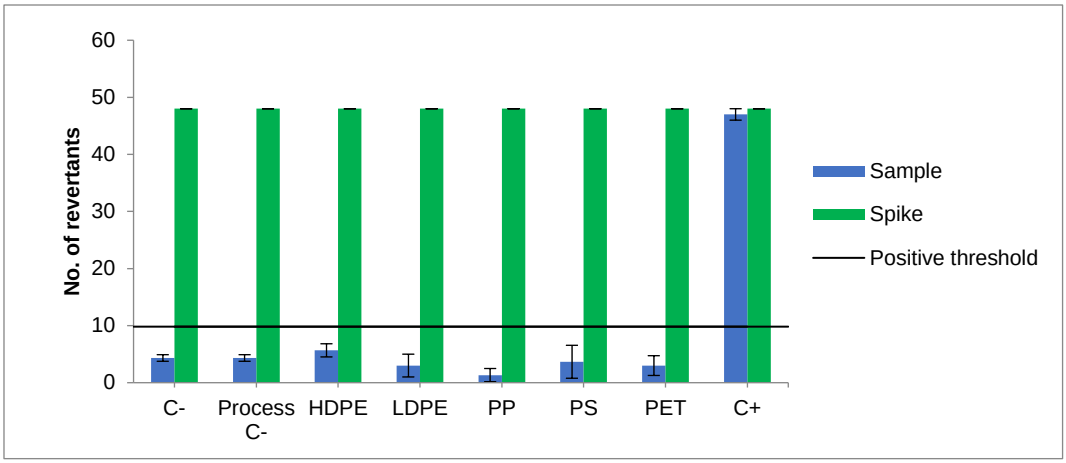

Figure 36: Test with recyclate-samples: TA100 and 4-NQO -S9

The experiments with the other pure substances 2-AA and 4-NQO in figure 34, 35 and 36 worked well. The spiked plates always show a very high response in the assay with almost 48 revertants in every test run. The plastic polymer recyclate-samples clearly show negative results.

4.3. Further Experiments

4.3.1. Biological substance recovery with ethanol as extracting agent under standard migration conditions

These experiments were made to see if the mutagenic substances can be detected after the standard migration conditions with long and high impact of heat according to the law EU 10/2011. This migration conditions are the harshest migrations conditions. For the migration with ethanol as extracting agent, 300ml of 95%-ethanol was prepared in big schott bottles with the mutagenic pure substances 2-NF, 2-AA/1, 2-AA/2 and 4-NQO. The migration time was 10 days by 60°C in the drying oven. A solvent blank, only 300ml ethanol was run with the process positive controls. The final concentrations of the substances in the assay were equal to the other experiments: 50µg/ml of 2-NF, 25µg/ml of 2-AA/2, 62,5µg/ml of 2-AA/1 and 2,5µg/ml of 4-NQO. The C+ controls in the assay had the same concentrations. The substances 2-AA/1 and 4-NQO were tested with the tester strain TA100 and the substances 2-AA/2 and 2-NF were tested with the tester strain TA98. 2-AA was tested with the S9-mix. The results are shown in figure 37.

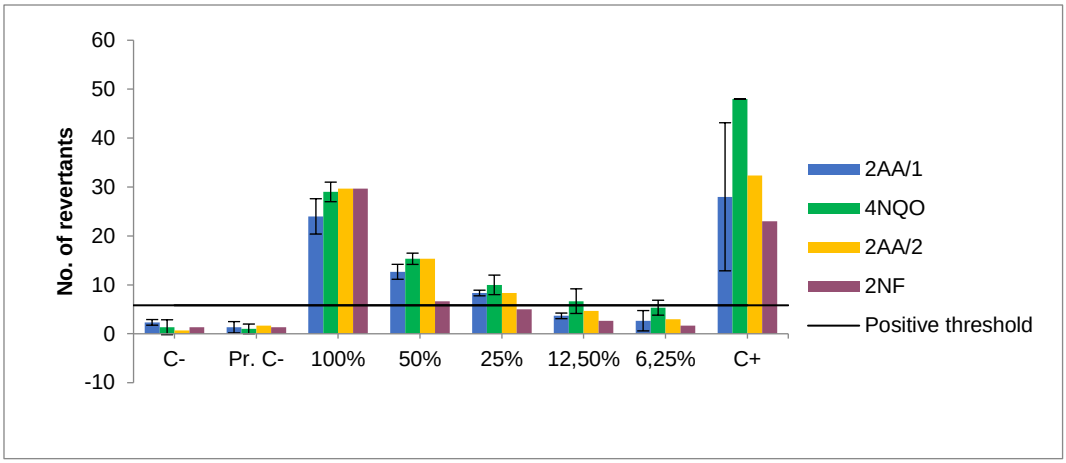

Figure 37: Biological substance recovery with ethanol as extracting agent

The blue bars show the test results of the substance 2-AA/1. The greens bars show the results of the substance 4-NQO. The orange bars show the results of 2-AA/2 and the violet bars show the results of 2-NF. The C+ controls of 2-AA/1, 2-AA/2 and 2-NF show fewer revertant numbers compared to the C+ control of 4-NQO.

The 100% of the process C+ of 4-NQO shows fewer revertant numbers in comparison to the C+ control in the assay. The substance 4-NQO normally has high mutagenicity and works well in the assay so it seems as if the substance was lost during the migration process or/and the sample preparation steps.

The 100% of the other pure substances show high revertant numbers in comparison to their C+ controls in the assay. Perhaps 2-AA and 2-NF got instable due to the defrosting and frosting by the usage, thus the mutagenicity of the substance was generally reduced in this experiment.

So it can be said that all four substances can be detected after the standard migration conditions. It seems as if the long impact of heat does not influence the mutagenicity of the pure substances because the results are similar to the results with the polymer recyclate samples which were extracted with dichlormethane under short heat impact with lower temperatures.

4.3.2. rPET-samples

For these experiments six rPET-samples were received from our partner-institute OFI. The used mutagenic spike substances were 2-AA in two different concentrations, 2-NF and 4-NQO. 2-AA was tested with the bacteria tester strains TA98 and TA100 and the S9-mix as metabolic activator system. 2-NF was tested with TA98 and 4-NQO was tested with TA100. Equal to the tests with the spiked recyclate-samples, a spiked and an unspiked plate were conducted in parallel. For the spiked plate, the exposure medium of the final bacteria mix was spiked with the mutagenic substances so that the final concentrations in the assay were: 2µg/ml for 2-NF, 2,5µg/ml for 2-AA/1, 1µg/ml for 2-AA/2 and 0,1µg/ml for 4-NQO. The posi-

tive control substances which were used in the assay had the concentrations: 50µg/ml for 2-NF, 25µg/ml for 2-AA/2, 62,5µg/ml for 2-AA/1 and 2,5µg/ml for 4-NQO. For the first screening 100% of the samples were tested undilutedly.

The results are shown in the next four figures. The blue bars show the rPET-samples and the green bars show the spiked plate which was conducted in parallel to the unspiked plate. The numbers 1 to 6 on the horizontal axis are the 6 different rPET-samples.

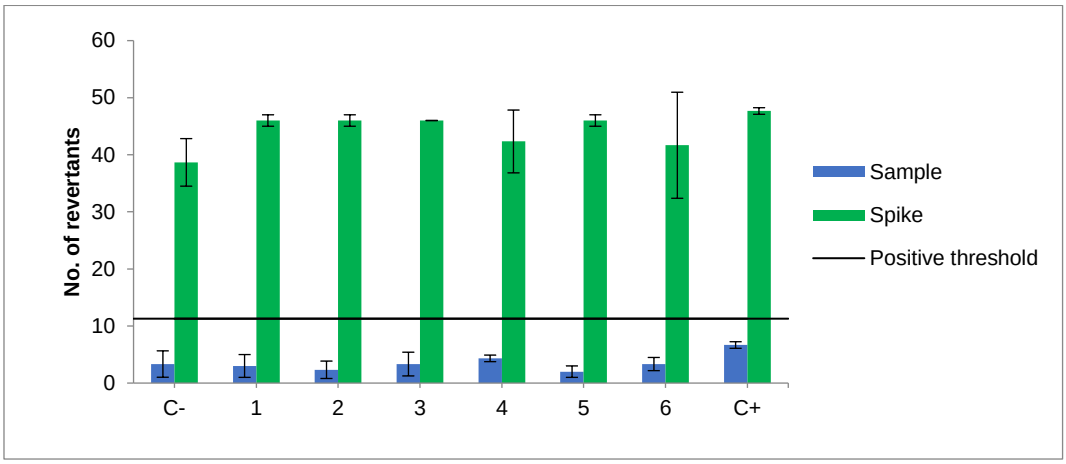

Figure 38: Test with rPET-samples: TA100 and 2-AA/1 +S9

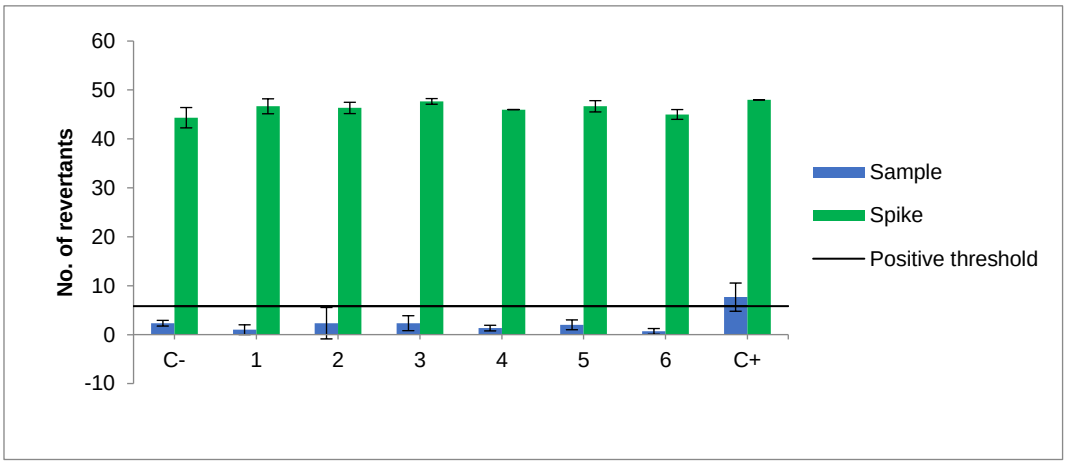

Figure 39: Test with rPET-samples: TA98 and 2-AA/2 +S9

In the tests with the substance 2-AA (figure 38 and 39) the positive control in the assay shows very fewer revertants. The spiked plate clearly shows high revertant numbers. It is likely that there was an errow while pipetting the positive controls.

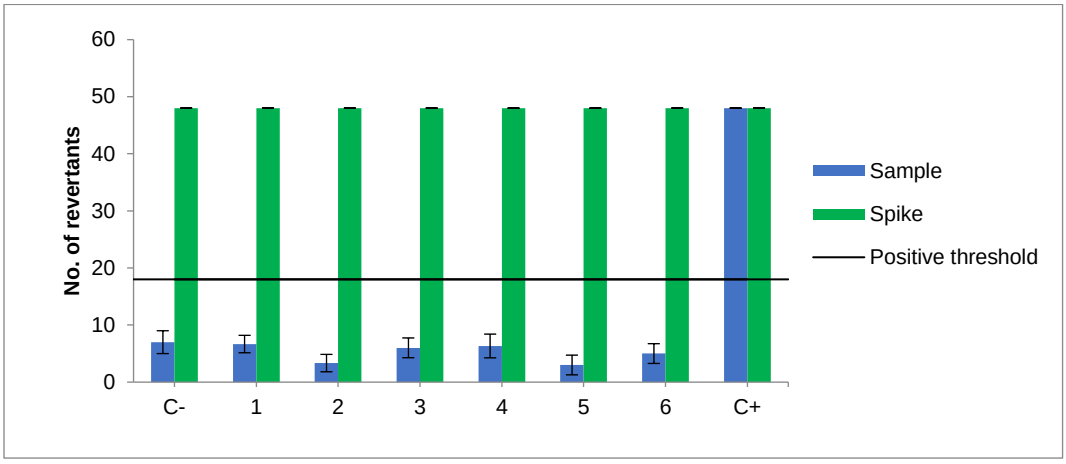

Figure 40: Test with rPET-samples: TA100 and 4-NQO -S9

In figure 40 the high background is noticeable which correlates with false positive results.

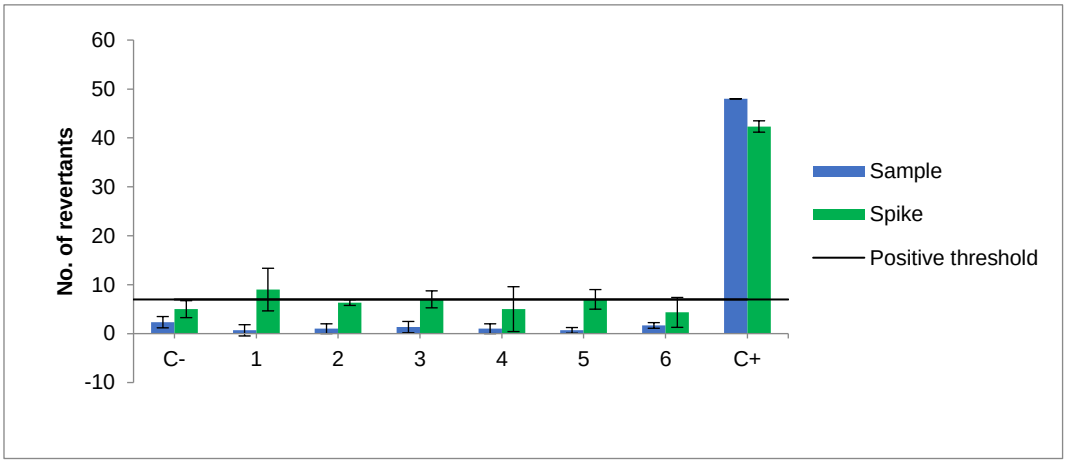

Figure 41: Test with rPET-samples: TA98 and 2-NF -S9

As already seen in the experiments with the spiked recyclate-samples, 2-NF does not operate well with plastic samples in the assay. This effect also exists with the rPET-samples in figure 41. It seems as if the samples interact with the pure substance 2-NF and consequently reduce their mutagenic effect in the assay.

In these experiments the rPET-samples also clearly show negative results.

4.3.3. Different growth-times of the tester strains

For these experiments different growth-times of the bacteria tester strains were researched. The tester strains TA98 and TA100 were tested after 18 hours growth-time, 12 hours growth-time and overnight culture rejuvenation. Therefore 10µl of the frozen bacteria stock was added to 3ml nutrient broth No. 2 and 3µl ampicillin. The bacteria culture was incubated in the Orbital shaker by 250 rpm once for 18 hours and once for 12 hours. Then the bacteria culture was directly taken for the AMES test. An older bacteria culture which was stored between one and two weeks at 4°C was taken for the test with the overnight culture rejuvenation. 200-400µl of the bacteria culture were mixed with 3ml nutrient broth No. 2 and 3µl ampicillin and

incubated in the Orbital shaker by 250rpm for a few hours till the OD was >2. The mutagenic substances 4-NQO, 2-NF, MMS and ENU were used. The top concentrations of the substances were 1µg/ml for 4-NQO, 2500µg/ml for MMS and 5000µg/ml for ENU. In the assay a 1:2 dilution was made.

The results are shown below. The blue bars show the number of revertants after 18 hours-growth-time, the green bars after 12 hours-growth-time and the yellow bars show the overnight culture rejuvenation.

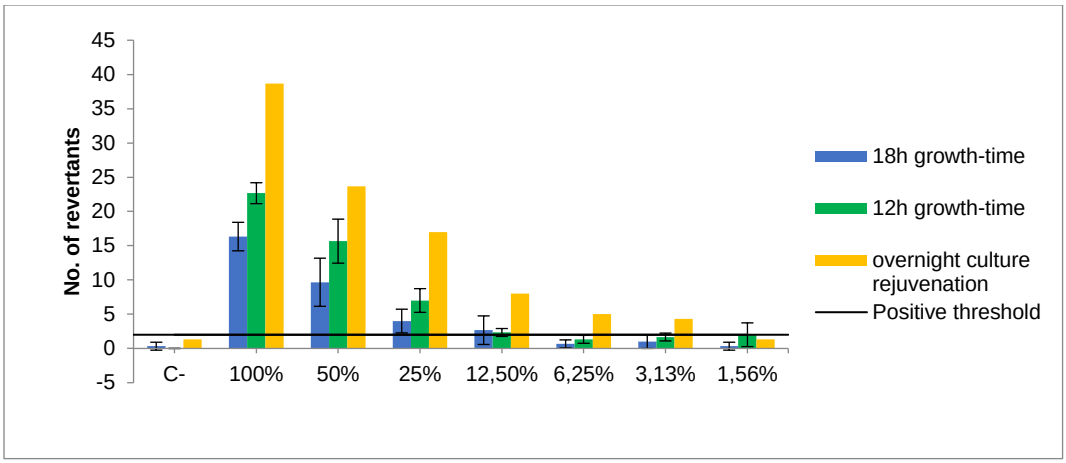

Figure 42: Different growth-times: TA98 and 4-NQO

The overnight culture rejuvenation of TA98 with 4-NQO shows the best results.

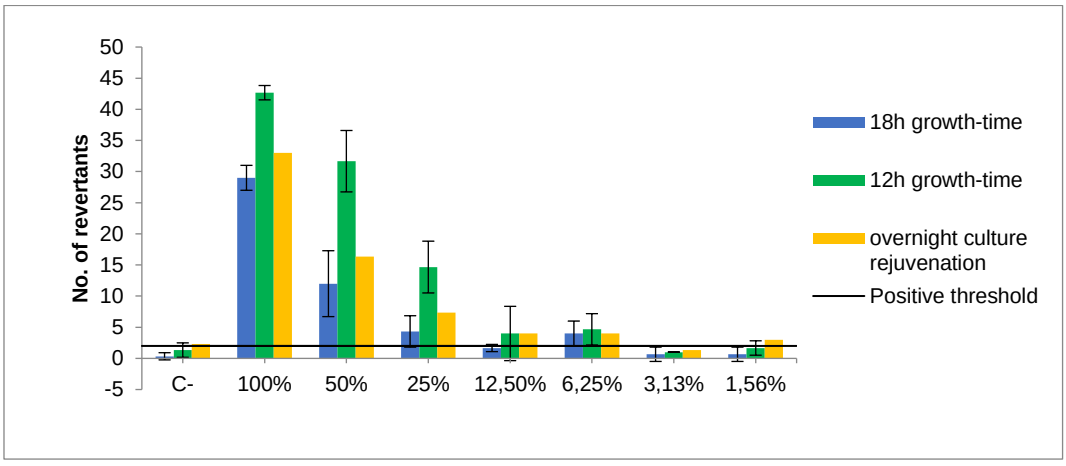

Figure 43: Different growth-times: TA98 and 2-NF

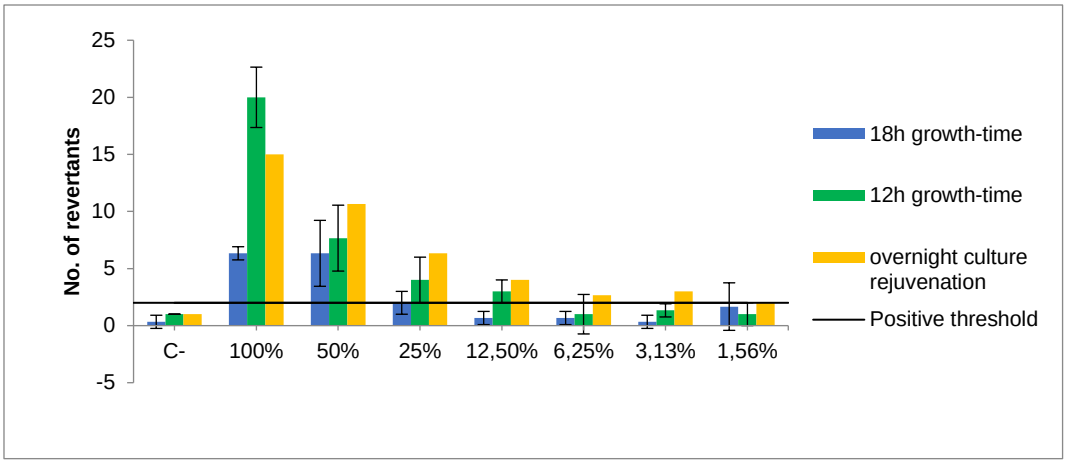

Figure 44: Different growth-times: TA98 and ENU

In the experiments with TA98 and the substances 2-NF (figure 43) and ENU (figure 44) the best results were produced after the 12 hours-incubation of the tester strains.

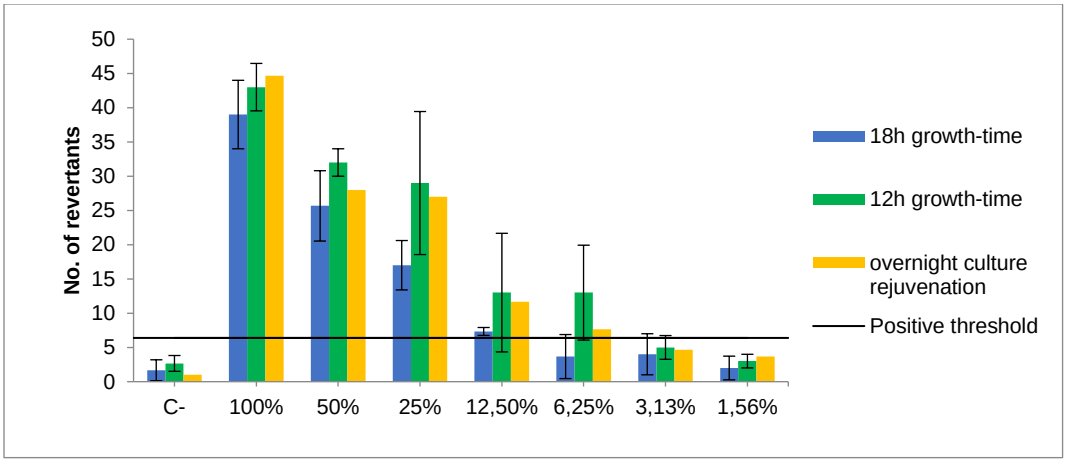

Figure 45: Different growth-times: TA100 and 4-NQO

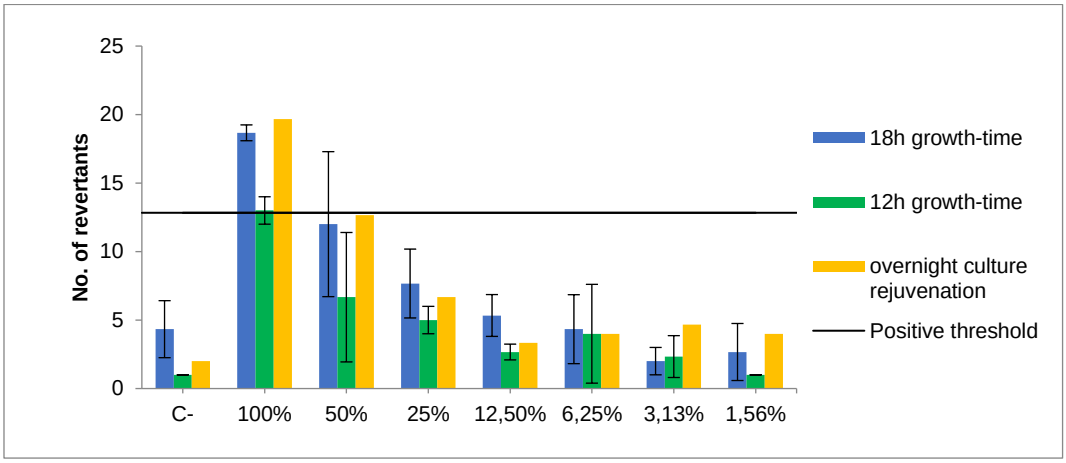

Figure 46: Different growth-times: TA100 and MMS

In the tests with TA100 and the pure substances 4-NQO (figure 45) and MMS (figure 46) the best results were produced after the overnight culture rejuvenation. The very high background is noticeable which is related to false negative results.

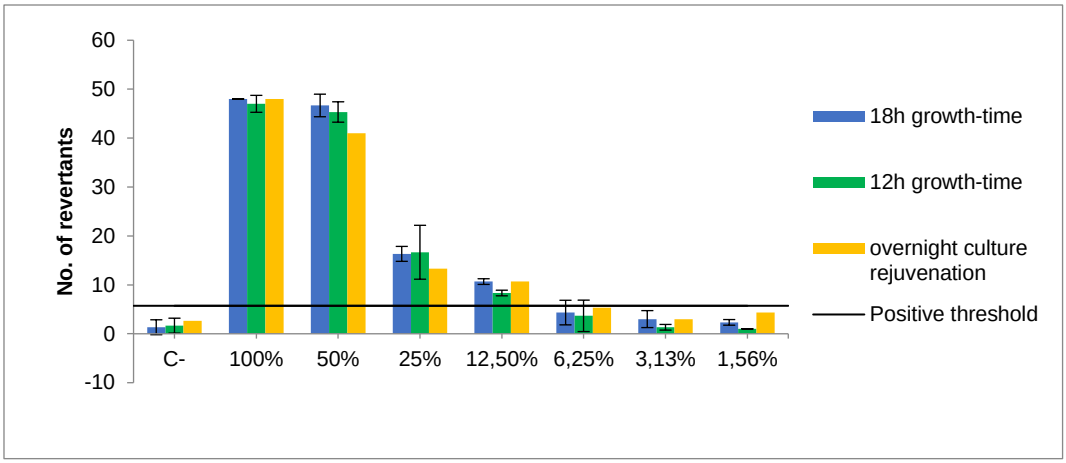

Figure 47: Different growth-times: TA100 and ENU

The substance ENU shows similar results with the tester strain TA100 after 18 hours-incubation, 12 hours-incubation and overnight culture rejuvenation.

In summary it can be said that the 12 hours-incubation and culture rejuvenation produce the best results in the assay. But in general results also depend on the mutagenicity of the pure substances and on the bacteria tester strains.

4.3.4. Tests with bacteria tester strains TA97, TA1535 and TA1537

These experiments were performed to see if the bacteria tester strains TA97, TA1535 and TA1537 work in our Ames MPF format to be able to do further experiments with these bacteria in the future. The tester strains TA97, TA1535 and TA1537 were tested with BaP, 2-AF, 2-AA, 9-AA, ENU, MMS and 4-NQO. The top concentrations of all mutagenic substances were 5000µg/ml. In the experiments with the pure substances BaP and 2-AF the S9-mix was added as a metabolic activator system. The dilution series was made with a √10 dilution.

The best results of this experiment series are shown below.

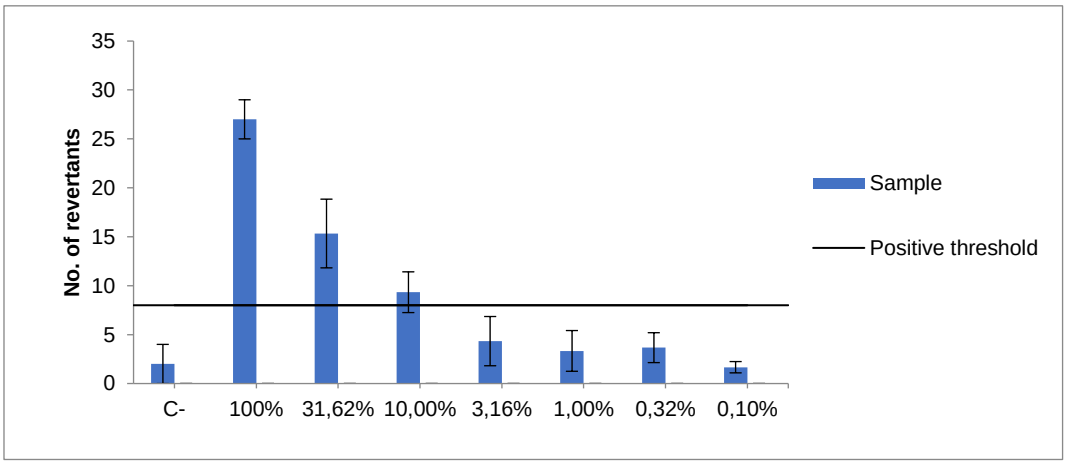

Figure 48: TA97 and MMS

The dilution series with MMS and TA97 works well in the assay. Only the top concentration shows lower revertant numbers for about 25 revertants.

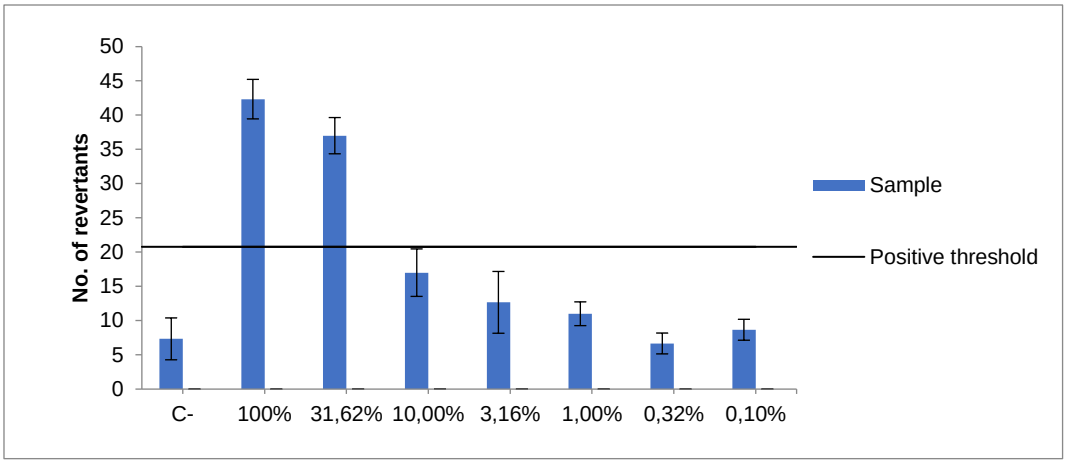

Figure 49: TA1537 and MMS

In the experiment with MMS and the bacteria tester strain TA1537 the background is very high. The dilution series works but the rate of spontaneous mutations is high.

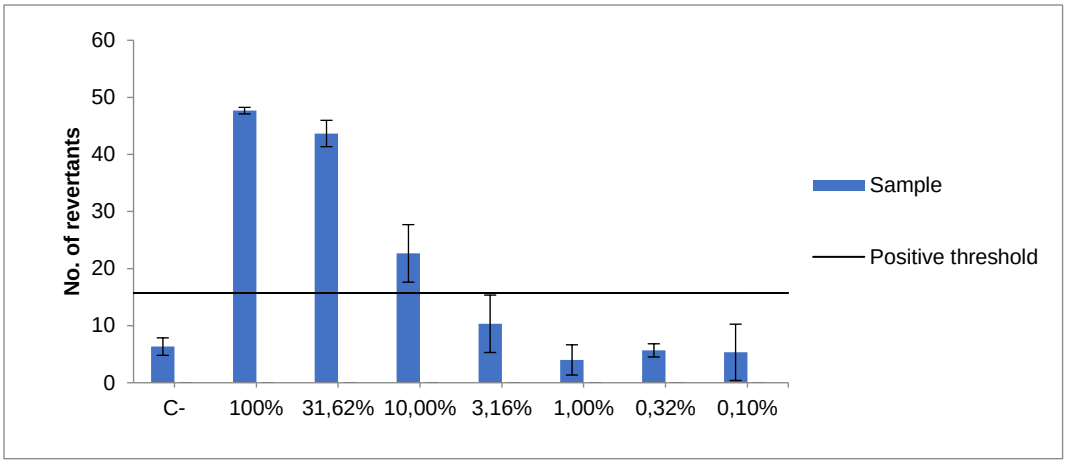

Figure 50: TA97 and 2-AA

In the experiment with 2-AA and the bacteria tester strain TA97 the background is also very high and the dilution series does not work so well in the assay. The response of the pure substance is very high about 48 revertants.

It is necessary to further test and optimize these bacteria tester strains to achieve better results in the assay.

5. Discussion

5.1. Migration process and sample preparation

According to the EU regulation 10/2011 on plastic materials and articles intended to come into contact with food and to the DIN norm EN 1186 food simulants for migration studies should be chosen corresponding to the properties of the packed foodstuff. Both documents include lists of food simulants assigned to certain food categories. Additionally, standard EN 1186-1 allows the use of 95% ethanol or iso-octane as a substitute testing if migration testing is technically not feasible. So there are many options to do migration studies. But there are no explicit guidelines relating to the migration process, the food simulants and the sample preparation so it is difficult to work under the same conditions for all packaging materials. The comparison of food simulants with packaged foodstuff is questionable. Food simulants can not exactly replicate the texture of real foodstuff. Samples which were tested positively in the Ames MPF assay should be confirmed by migration studies in more relevant food simulants.

The sample preparation process of FCM migrates is very long-lasting and extensive. For the pre-concentration with the rotary evaporation the available equipment allowed only to prepare one sample at a time and the solvent change (evaporation under vacuum) can last a few days. Due to the many preparation steps there are many sources of error which can lead to substance losses in the migrate samples.

The sample preparation is prone to substance losses which depends on the volatility and the physio-chemical properties of the mutagenic substances. It appears that the substance 4-NQO can be retained without substance loss and the substances 2-NF and 2-AA are expected to be lost. The test runs with 2-NF and 2-AA show in general different results relating to the biological substance recovery while the test runs with 4-NQO show a good biological substance recovery. The results of the test show that 2-NF does not work ideally in the assay and during the sample preparation process it seems as if the substance easily gets lost perhaps because of the hydrophobic character of 2-NF. According to Adahchour et al. (2010) only 7% of the potential genotoxic substances are highly volatile. The loss of volatile substances during the preparation process seems to be acceptable. Although, improved recoveries for relatively volatile substances are desirable to solve this problem. These may be achieved by alternative and more selective evaporation methods. Furthermore, Rainer et al. reported 2018 that only 10% of genotoxic substances of packaging migrates could be detected by the Ames MPF assay[123].

[123] (Bernhard Rainer E. P.-K., 2018)

5.2. Tests for the biological substance recovery

It is possible that the polymer recyclate samples interact with the mutagenic substances and thus the substances can lose their genotoxic effect. This can be seen in figure 18 with LDPE and 2-NF. The top concentration of the spiked polymer sample is very low while the top concentration of the process C+ is high. It appears that LDPE interacts with 2-NF and thus 2-NF loses its mutagenicity and shows a very low response in the assay. This effect can also be seen in figure 14 with PP and 2-NF. Due to the molecular structure of 2-NF it is probable that this substance interacts with LDPE and PP and thus the genotoxic effect of 2-NF is reduced. Compared to the test results with the other pure substances, it seems as if 2-NF generally shows less response in the assay. In the tests with 2-NF the positive controls in the assay often show lower revertant numbers than the other mutagenic substances. However it is noticeable that in figure 10 with HDPE and 2-NF the substance recovery of the spiked sample polymer is high with 91% and the substance recovery of the process C+ is only 35%. This means that 2-NF works with the recyclate-sample HDPE, but it appears that parts of 2-NF were lost during sample preparation of the process C+.

In comparison to figure 20 with LDPE and 2-AA the top concentration of both, the spiked polymer sample and the process C+ is very low. In this case it is possible that parts of the mutagenic substance were lost because of errors during the sample preparation or that 2-AA got instable because of the Freeze-Thaws cycles and consequently lost genotoxic activity. This can also be seen in figure 28 with PET and 2-AA: the substance recovery of the polymer sample and of the process C+ are also low (39% and 35%).

In some cases, the positive control substance (C+) in the assay did not work well and due to this the substance recovery seems to be good, although the amount of the revertant colonies in the assay is low. This can be seen in figure 16 with PP and 2-AA/1, in figure 22 with PS and 2-NF and in figure 26 with PET and 2-NF. In this case, the calculated substance recovery in the test was not achieved.

In figure 24 with PS and 2-AA/1 and in figure 27 with PET and 2-AA/2 the positive controls in the assays show very low responses. Furthermore the top concentrations of the spiked polymer samples and of the process C+ also show very low responses. In this case it is likely that the mutagenic substance 2-AA got instable due to the Freeze-Thaws cycles and so lost their mutagenicity.

In the tests with the recyclate-sample HDPE with 2-AA/1 (figure 12) and 4-NQO (figure 13) it seems that parts of the substances were lost during the sample preparation because the substance recoveries are between 62% and 86%. The positive controls in the assay show very high revertant numbers. This can also be seen with the substance 2-AA/2 and the recy-

clate-samples PP (figure 15), LDPE (figure 19) and PS (figure 23). The biological substance recoveries are between 69% and 83%.

The substance 2-AA only achieved very good results in one test with the recyclate-sample HDPE (figure 11). It seems as if 2-AA was very susceptible during sample preparation and also to the freeze-thaws cycle.

The substance 4-NQO shows the best substance recovery rates with almost 100% in almost all performed tests. This can be seen in figure 21 with the recyclate-sample LDPE, in figure 25 with PS and in figure 29 with PET. The dilution series with 4-NQO also looks satisfactory and it seems as if the substance worked well in the assay with the recyclate-samples. In only one test the substance recovery of 4-NQO in the spiked polymer sample is very low in comparison to the process C+. This is shown in figure 17 with the recyclate-sample PP. The substance recovery of the process C+ is very high at 93% and of the spike polymer sample only at 58%. It appears that 4-NQO interacts with the polymer PP and this affects the mutagenicity of the substance in the assay.

The results of the biological substance recovery seem to be very different depending on the mutagenic substances, bacteria tester strains, sample materials and so on. The LOD-values which are achieved in migrates depend on the pre-concentration steps and on dilutions up to the exposure step during the test procedure. This shows the importance of minimising substance losses during the sample preparation to achieve low LOD-values in the assay[124].

5.3. Tests with recyclate-samples

The plastic polymer recyclate samples clearly show negative results in the assay. The spiked plates ran parallel to the unspiked plates to assess matrix effects of the recyclate samples in the assay. If the spiked sample showed a spike recovery (amount of revertants) of less than 60% in comparison to the spiked solvent control it was considered as “inhibitory effect” in the assay. This can be seen in figure 30, 31, 32 and 33 with the mutagenic substance 2-NF. The experiment with the substance 2-NF was repeated several times but never showed better results. As a comparison to the test results with the other substances (figures 34, 35, 36) the responses of the spiked plates were constantly high (almost 100%). It seems as if the polymer recyclate samples interact with 2-NF in the assay and reduce their mutagenicity. The samples PET and PS work better with 2-NF, but the other samples HDPE, LDPE and PP show very low revertant numbers. The polymer samples HDPE, LDPE and PP showed the lowest spike recovery. In this case a dilution series for HDPE, LDPE and PP should be prepared to detect potential genotoxic effects which can be masked by cytotoxicity in the assay.

[124] (Bernhard Rainer E. M.-K., 2019)

5.4. Tests for the biological substance recovery under standard migration conditions

The substances 2-AA, 4-NQO and 2-NF can be detected after the harshest migration process of 60°C for 10 days. It must be said that the positive controls of 2-AA and 2-NF show low revertant numbers in the assay (between 20 and 35 revertants). Only the positive control of 4-NQO show high response with 48 revertants. This may indicate that 2-AA and 2-NF have become unstable due to the Freeze-Thaw cycles and therefore do not show the usual response in the assay. This would also explain the lower revertant numbers of the process C+ with 2-AA and 2-NF. The process C+ of 4-NQO also shows lower revertant numbers. It is possible that some substance was lost due to errors during the sample preparation because 4-NQO usually shows high recovery rates. The results are similar to the results with the plastic polymer recyclate-samples which were prepared under weaker migration conditions (40°C for 24h). Therefore it can be assumed that genotoxic activity can be detected after the long and high impact of heat during the migration process. But due to the low revertant numbers of the positive controls in the assay, this experiment should be repeated to ensure that the substances can show the usual genotoxic effect.

5.5. rPET-samples

The rPET-samples of these experiments clearly show negative results in the assay. Just like the experiments with the recyclate-samples the spiked plates ran parallel to the unspiked plates to assess matrix effects of the samples in the assay. As already mentioned in the previous tests, there were also problems with 2-NF and the polymer recyclate-samples here. It seems as if the pure substance 2-NF does not work well with polymer samples in the assay. This can be seen in figure 41. The spiked samples showed a very low spike recovery well below 60%. In this case a dilution series for the rPET-samples should be prepared to detect potential genotoxic effects which can be masked by cytotoxicity in the assay.

5.6. Different growth-times of the tester strains

In figure 42 with the substance 4-NQO the overnight culture rejuvenation of TA98 shows the highest response of the top concentration with almost 48 revertants. The 12 hours and 18 hours growth-times of the bacteria culture show low revertant numbers under 25 revertants. In figure 43 and 44 the bacteria TA98 show the highest revertant numbers after 12 hours growth-time but it must be mentioned that with the substance ENU (figure 44) the number of revertants is generally much lower in the assay (only ~ 20 revertants at top concentration). In figure 45 and 46 the highest responses are produced after the overnight culture rejuvenation of the bacteria tester strains TA100. However the revertant number in the test with the substance MMS (figure 46) is also much lower in the assay than usual. In figure 47 all three different growth-times of the bacteria TA100 show the same results with high revertant num-

bers between 40 and 48 revertants. In conclusion, the substance ENU with TA98 and MMS with TA100 did not work so well in the assay. The 12-hours growth-time and the culture rejuvenation produce the best results but in general the results also depend on the mutagenicity of the pure substances and the bacteria tester strains and how they act together in the assay.

5.7. Ames MPF assay

The Ames assay is a fast, simple and cost-efficient screening method for detecting FCM in migrates. It is a good alternative to reduce *in-vivo* bioassays and animal testing. But for some tests the liver-enzyme S9 is needed which is extracted from rats. This rat-liver-extract is expensive, and many rats must die because of the tests. Depending on the variant of testing method the amount of the bacteria tester strains changes and the usage of the materials and amount of work are different. To test FCM migrates by the Ames MPF assay relatively large volumes of sample migrates are needed because several test runs under various conditions are necessary to determine the full mutagenic potential of the test samples.

As a comparison to the original Ames test the Ames MPF assay only requires a little amount of the samples (10µl for top concentration). The standard petri dish-based Ames test needs a sample volume of at least 50 µL for top concentration. Due to the difficult and extensive production and preparation of the migrate samples this is an important item.

Furthermore the procedure and the scoring of the Ames MPF assay is easier and faster. The guideline is only the threshold which is twice the baseline (=background mutations of C-). However different bacteria tester strains have various spontaneous mutations. TA98 for example has a lower rate of spontaneous mutations and TA100 has a higher rate of spontaneous mutations. These variabilities of spontaneous mutations are not included. A higher rate of spontaneous mutations makes the assay very difficult or even impossible to evaluate. The use of a minimum baseline adapted for every tester strain could be the solution for this problem. Another disadvantage of the Ames MPF testing protocol is the limited dynamic range because there are only 48 wells for the scoring of each datapoint. In comparison to the standard petri dish the maximum number of revertant colonies is much higher and theoretically unlimited. The LOD- and LEC-values have to be improved in the future to also detect very little concentrations of mutagenic substances. This is important to make sure that the recyclable packaging materials are substantially safe for the customer’s use.

6. Conclusion

In this master's thesis the plastic polymer recyclate samples explicitely show negative results in the Ames MPF assay and thus there are not any genotoxic substances found in the migrates. For the project "Polycycle" this means that it seems possible to recycle the different types of plastic without health concerns to humans. However it must be mentioned that only a few samples were tested for this master's thesis and therefore the sample size is not representative. In the future many more recyclate-samples will have to be tested in order to be able to make reliable statements. It is also necessary to perform several tests with these polymer recyclate-samples under different migration conditions because the sample preparation process is prone to human and technical mistakes and consequently to substance losses. In the future it is necessary to determine explicit guidelines for the sample preparation process and to find alternative and more selective methods.

As a conclusion I am of the opinion that the presented results are a step forward to more safety for human's health and a contribution to the recovery of the environment as they show that recycled plastic packaging materials can be reused for foodstuff.

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