MASTERARBEIT | MASTER'S THESIS

Titel | Title

Identification of the sources of DNA-reactive genotoxic substances in recycled food contact materials using in-vitro bioassays

verfasst von | submitted by

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angestrebter akademischer Grad | in partial fulfilment of the requirements for the degree of

Master of Science (MSc)

Wien | Vienna, 2025

Studienkennzahl lt. Studienblatt | Degree programme code as it appears on the student record sheet:	UA 066 659
Studienrichtung lt. Studienblatt | Degree programme as it appears on the student record sheet:	Masterstudium Lebensmittelchemie
Betreut von | Supervisor:	Univ.-Prof. Dr. Doris Marko

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Acknowledgement

I would like to express my sincere gratitude to all those who contributed to this thesis.

First and foremost, I would like to thank Univ.-Prof. Dr. Doris Marko for making it possible to conduct my Master's thesis in collaboration with the Austrian Research Institute for Chemistry and Technology (OFI), outside of the university environment, and for supporting the discussion of my results at the Institute of Food Chemistry and Toxicology.

My deepest thanks go to my external supervisor at OFI, Dr. Christian Kirchnawy. Thank you for your continuous support, for initiating contacts that allowed testing of samples outside of OFI, and for encouraging me to present my results at project meetings. I truly appreciated your persistence and guidance throughout the entire process.

I would also like to thank Dr. Elisa Mayrhofer, who helped me design the experiments and guided me through the more frustrating parts of laboratory work, especially when the bacteria didn't cooperate. Your troubleshooting skills and encouragement were invaluable.

Special thanks to the entire "Microbiology & Cell Culture" team. Thank you for all the hours we spent working side by side at the sterile hood and for your support during the countless pipetting sessions. I especially want to thank Markus Posch, MSc. for his critical input regarding the SPE setup, for indulging my creative ideas involving the muffle furnace, and for staying late on Fridays to supervise my work.

Further thanks go to my colleagues from the "Pharma & Chromatography" group. I would especially like to thank Sabine Hering, MSc. for measuring my samples and helping me interpret the data. Thank you all for the stimulating discussions and for sharing your experience when evaluating the effects of various compounds.

Last but not least, I would like to thank my family and friends. To my parents, Renate and Herbert: thank you for your unwavering support—emotionally, practically, and financially.

1 Abstract

In 2015, the European Union launched its Circular Economy Action Plan to reduce resource consumption and promote the reuse and recycling of materials, including food contact materials (FCMs) such as plastic packaging (BMK, 2022). The safe use of recycled plastics in food contact applications is tightly regulated within the EU. Regulation (EC) No 1935/2004 sets the general safety requirements, while Regulation (EU) 2022/1616 specifically governs the use of recycled plastic materials in contact with food. Currently, only recycled PET is authorised for direct food contact under strict conditions, including the use of an approved recycling process and demonstration of sufficient decontamination efficiency. For other plastics such as polyethene (PE) and polypropylene (PP), no recycling processes have yet been authorised for direct food contact.

This thesis investigates the source(s) of DNA-reactive substances detected in recycled polyolefin-based FCMs using in vitro mutagenicity testing. Substances with genotoxic potential are of particular concern, as they must be absent or below defined thresholds, such as the Threshold of Toxicological Concern (TTC) for DNA-reactive substances (0.15 µg/person/day). The Ames MPF assay was used to assess mutagenic potential. Solid-phase extraction (SPE) was applied to fractionate recycled samples and identify the physicochemical characteristics of the active components.

Positive Ames responses were consistently observed in both the flow-through and elution fractions, suggesting at least two types of mutagenic substances. Recycled materials without prior consumer use still yielded positive results, indicating that post-consumer contamination is not the main cause. Significant differences were found between printed and unprinted recycled materials, with only the printed samples showing mutagenic activity. Further investigation into printing ink components revealed that nitrocellulose (NC)-based inks, especially after thermal processing, were consistently associated with positive Ames results. Model samples using NC and azopigment solutions on LDPE foils confirmed these findings.

The results demonstrate that certain thermally degraded ink components contribute to the DNA-reactive signals observed in recycled FCMs. These findings highlight the need to consider not only the base polymer but also additives such as inks and coatings in safety assessments of recycled plastics for food contact use. Only by the integration of “design-for-recycling” can the development of a fully comprehensive possibility of a circular economy be achieved. If the recycled material should come in contact to food, it is necessary to minimize printing ink components in the input materials or find sufficient ways for deinking of the material.

Kurzzusammenfassung

Im Jahr 2015 veröffentlichte die Europäische Union ihren Aktionsplan zur Kreislaufwirtschaft, um den Ressourcenverbrauch zu senken und die Wiederverwendung sowie das Recycling von Materialien - auch von Lebensmittelkontaktmaterialien (FCM) wie Kunststoffverpackungen zu fördern (BMK, 2022). Der Einsatz von recycelten Kunststoffen im Lebensmittelkontakt unterliegt in der EU strengen rechtlichen Vorgaben. Die Verordnung (EG) Nr. 1935/2004 regelt die allgemeinen Anforderungen an die Lebensmittelsicherheit, während die Verordnung (EU) 2022/1616 speziell die Verwendung von recycelten Kunststoffen im Lebensmittelkontakt regelt. Aktuell ist ausschließlich recyceltes PET für den direkten Lebensmittelkontakt zugelassen – unter der Bedingung, dass es in einem Recyclingverfahren verarbeitet wurde, das eine ausreichende Dekontaminationsleistung nachweist. Für andere Kunststoffe wie Polyethylen (PE) und Polypropylen (PP) wurden bislang keine Verfahren genehmigt.

Ziel dieser Arbeit war es, die Quelle(n) DNA-reaktiver Substanzen in recycelten polyolefinbasierten Materialien zu identifizieren. Diese Substanzen sind aus toxikologischer Sicht besonders relevant, da sie laut TTC-Konzept (Threshold of Toxicological Concern) in einer maximalen Tagesaufnahme von 0,15 µg pro Person tolerierbar sind. Die Mutagenität wurde mit dem Ames MPF-Test überprüft. Die Proben wurden mittels Festphasenextraktion (SPE) fraktioniert, um Rückschlüsse auf die physikochemischen Eigenschaften der mutagen wirkenden Substanzgruppen zu ziehen.

In allen untersuchten Proben wurden Ames-positive Signale sowohl in der Durchfluss- als auch in der Elutionsfraktion detektiert – ein Hinweis auf mindestens zwei unterschiedliche mutagene Komponenten. Auch bei Materialien ohne Endverbraucherkontakt wurden positive Ergebnisse festgestellt, wodurch eine Kontamination durch unsachgemäße Nutzung ausgeschlossen werden konnte. Ein deutlicher Unterschied zeigte sich zwischen bedruckten und unbedruckten recycelten Materialien: Nur die bedruckten Proben wiesen eine mutagene Wirkung auf. Weitere Untersuchungen ergaben, dass insbesondere nitrozellulosebasierte Druckfarben nach thermischer Behandlung mit positiven Ames-Ergebnissen assoziiert sind. Dies konnte durch Modellproben bestätigt werden, bei denen NC- sowie Azopigment- Lösungen auf LDPE-Folie aufgebracht und thermisch behandelt wurden.

Die Ergebnisse zeigen, dass bestimmte thermisch degradierte Bestandteile von Druckfarben wesentlich zur DANN-reaktiven Aktivität in recycelten Kunststoffen beitragen können. Für die Sicherheitsbewertung von recycelten Lebensmittelkontaktmaterialien sollten daher nicht nur die Grundpolymere, sondern auch additive Komponenten wie Druckfarben berücksichtigt werden. Nur durch die Integration von „Design-for-Recycling“ kann die Entwicklung einer vollumfänglichen Möglichkeit einer Kreislaufwirtschaft geschafft werden, etwa durch die Minimierung des Einsatzes oder wirksame Deinking-Methoden.

2 Introduction

The usage of recycled plastics as food contact material is a big issue, since the conservation of resources and the avoidance of disposable products is a much-discussed point in the industry as well as in the authorities. The usage of such materials is regulated by different laws and since the regulations are very strict and the goal of a recycling rate of plastic packaging in 2030 of 55%. Since these steps need a scientific base also the research in this field is a big thing as well as different approaches of recycling and evaluation methods of the purification steps in the recycling. (European Parliament, 2024)

2.1 Regulation of Food Contact Materials on the EU Market

During the processing and shelf life of food, it comes to countless contacts with different materials including packaging but also materials of production machines or tableware and kitchenware. All of these materials are classified in the group of food contact materials (FCM). These FCMs are regulated by the Regulation (EC) No 1935/2004 on materials and articles intended to come into contact with food when placed on the European market. This regulation defines the requirements that the materials must meet to maintain food safety since through the contact a transfer of constituents from the materials into the food is possible. This may affect the safety of the food and human health as well as the quality of the food and its organoleptic properties. In general, the principle on which this regulation is based is, that the materials are inert enough to preclude the transfer of substances to the food. But since there are also food contact materials, where this behavior is a desired effect e.g. in the case of oak barrels for wine ageing, this regulation has also to consider these so-called active food contact materials. A second type of food contact material which is relatively new and builds an exception in this regulation is the group of intelligent food contact materials, these materials are designed to monitor the condition of the food. (The European Commission, 2004)

The Regulation (EC) No 1935/2004 defines 17 different groups of materials and articles which could be met through specific measures, one of these is plastics. (The European Commission, 2004)

Additionally, to Regulation (EC) No 1935/2004, the Regulation (EC) No 2023/2006 is applied to FCM. This regulation is about the good manufacturing practice for these materials and articles and the defines the quality assurance and control system as well as the adequate documentation. (The European Commission, 2006, 2011)

For plastics in food contact, the Commission Regulation (EU) No 10/2011 on plastic materials and articles intended to come into contact with food is applicable. This regulation is based on Article 5 of the Regulation (EC) No. 1935/2004. (The European Commission, 2004, 2011)

In the Commission Regulation (EU) No 10/2011 defines the requirements for manufacturing and placing on the market of materials and articles out of plastics, which are indeed to come

in contact with food, which are already in contact with food or the contact to food is foreseeable. These materials have to fulfill specific requirements which are also defined in this regulation. These requirements concern specific and overall migration limits of substances from the food contact material into the food. This regulation also defines the different food simulants which are used for the compliance testing of different foods and the testing time and temperature for different contact times and temperatures.(The European Commission, 2011)

In case of the usage of recycled plastics in contact with food, another regulation has to be applied. The Commission Regulation (EU) 2022/1616 on recycled plastic materials and articles intended to come into contact with foods is also based on Article 5 of the Regulation (EC) No. 1935/2004. In this regulation the European Commission sets new standards for the admission of technologies for the recycling of plastics intended to come into contact with foods. (The European Commission, 2022)

At the moment only mechanical PET recycling and closed loop recycling of all polymers is allowed by the term of “suitable technology”, it is also defined if it is necessary to authorize individual processes (like in case of mechanical PET recycling) or not (like in case of closed loop recycling). If new technologies should be applied, they are defined as novel technology, this means, that this technology is not established on the market yet. (The European Commission, 2022)

But it is possible to place materials and articles made of recycled plastics produced out of recycled plastics on the market for a limited time and under strict conditions. This approach ensures that the developer has the possibilities to collect data out of a large and representative number of samples to determine the suitability of the novel technology. The developer has to report the characteristics of the recycling technology as well as scientific evidence and studies showing the compliance of the material to Article 3 of Commission Regulation (EU) No. 1935/2004 and the microbiologically safety. Additionally, the efficiency of the decontamination and the migration of these contaminants to food has to be determined. (The European Commission, 2022)

2.2 The Threshold of Toxicological Concern

Since the analytical methods are getting better and better, and low concentrations of substances are possible to detect in food, the EFSA has developed a prioritisation tool for the assessment of the safety of substances of unknown toxicity.

In 2012, the first publication of the tool, called the threshold of toxicological concern, was published. This assessment is based on the structural similarities and, consequently thereof a similarity of the impact on the human body in a toxicological way.

The calculation of the TTC value is based on the distribution of NOAELs of the different Cramer structural classes. These classes are originally defined to make the expert judgment in food chemical risk assessment more transparent. The classification differs in three categories. Class 1 includes substances with a simple chemical structure and modes of metabolism exist, but a low order of oral toxicity is suggested. Normal constituents of the body are included in this class, except for hormones.

Class 2 includes substances without any structural features which would indeed a toxicity. Common components of food are included in this class.

Class 3 includes only substances that permit no strong initial presumption of safety. Only substances without any reactive functional groups are located here. But there are additional requirements when it comes to organophosphates and carbamates.

Since the variety of substances is big and not every substance is possible to identify, there are also limits set for these since a potential DNA-reactive mutagenic or carcinogenic effect is not possible to rule out.

The TTC values of the previous described classification is shown in table 1.

table 1: TTC value - classification of substances ((More et al., 2019)

Classification	**TTC value in µg/person per day**
Potential DNA-reactive mutagens and/or carcinogens	0.15
Organophosphates and Carbamates	18
Cramer Class III	90
Cramer Class II	540
Cramer Class I	1800

Since the mode of action of mutagens and carcinogens can vary between different pathways, there is a differentiation in the classification of substances in table 1. The DNA-reactive mutagens and carcinogens have a much lower limit than mutagens with another mode of action. And so, the TTC value is higher if a direct DNA-reactive mode is excluded. (EFSA 2016).

To exclude the direct DNA-reactive effect Koster et. al invented a strategy by the combination of NIAS Screening and bioassays. Since not only IAS are included in the material but also non-intentionally added substances (NIAS) have to be evaluated this assessment method is necessary to find a pragmatic and feasible approach. (Koster Sander et al., 2016)

2.2.1 *Exclusion of genotoxic effects by bioassays*

The strategy proposed by Koster et al. in 2016 is based on the analysis in carton food contact materials but has been expanded also to other food contact materials especially plastics.

The analysis is divided in 5 steps. At first a screening of the migrants that exceed the limit of cramer class III (90 µg/day) is done. In the second step the presence of highly toxic substances and substances that are excluded from the TTC concept are excluded. The third step is for the exclusion of genotoxic activity. This can be made by checking for structural alerts but a more pragmatic way is to use bioassays. It has to be noted, that a level of 0,15 µg/day has to be excluded. One important point in this step is, that the used assay has to be suitable for the testing of mixtures and not only for pure substances. In the fourth step, the results of step two and three are evaluated. So if there is a migrating substance detected which should be excluded, an identification and a substance-specific safety assessment should be performed. Migrants that exceed the 90 µg/day level have to be identified in every case. The last step is the testing of allergenic reactions. (Koster Sander et al., 2016)

In step 3 one important point is suitability of testing mixtures in the bioassay, Schilter et al. published data which show, that the Ames test is the most appropriate system, since the assay has been successfully used to text mixtures of environmental samples, medical devices and also food contact materials (Geurts et al., 2019).

2.3 Contamination of post-consumer recyclates

The recycling of packaging materials, including food packaging materials, is a goal of the circular economy action plan of the European Commission. (Commission & Communication, 2020) The sources of contamination are rising through the input material. The input material could contain non-food grade material but also other materials which disturb the recycling process. Also, typical post-consumer contamination like compounds of misuse of the packaging materials or food components which are sorbed by the FCM are in the input stream of the recycling plant. (Geueke et al., 2018)

2.3.1 *Mycotoxins*

Mycotoxins are the second metabolites produced by mould. These byproducts are a threat to the human health due to their genotoxic and cancerogenic mode of action in the human body. Due to this fact, they need to be considered as a possible contaminant of the post-consumer input material for recycling plants. Not just because of previous contamination through the food itself, but also because the collected plastics often contain residues of food, which the mould can grow on and produce mycotoxins. Before entering the recycling process, the input materials get washed with water with different detergents to clean off the food residues. It must be assumed that the biggest part of the mould and the mycotoxins are washed off. But of course, the mycotoxins can migrate into the plastics and therefore enter the recycling stream.

In Regulation (EC) No 2023/915 the maximum levels of certain contaminants in food are defined. Seven different groups regulate the limits of mycotoxins: – aflatoxins, ochratoxin a, patulin, deoxynivalenol, zearalenone, fumonisins, and T-2 and HT-2 toxins. The maximum levels are set for different types of food, e.g. groundnuts, milk, dried fruits, or cereals. There is no regulation for residues in food contact materials (Commission, 2023). Since a packaging material for food needs to be safe, contamination with mycotoxins is not acceptable.

No data is available for mycotoxin residues in post-consumer materials, neither in post-consumer recycling materials. Data is available for the degradation of different mycotoxins in food through thermal processing, which is also happening in the recycling process. The authors describe, that a reduction of mycotoxins correlated to temperature exposure exists (Kabak, 2009).

In the case of aflatoxins, the effect of heat processing was evaluated in corn, rice and wheat. In corn flour with an aflatoxin-B1 contamination of 0.05 µg/g, a reduction of 25 % was reached through incubation at 180 °C for 30 minutes (Cazzaniga et al., 2001). In the case of a sample with higher initial contamination of 0.49 µg/g, a reduction of 46 % was possible through incubation at 87 °C (Elias-Orozco et al., 2002). In rice it is shown that the reduction level highly depends on the moisture of the sample, but also on the type of aflatoxin. The reduction level is between 51 and 95 % (Castells et al., 2006).

The reduction of ochratoxin is only evaluated in wheat samples. The initial contamination level was between 0.01 – 0.05 µg/g of ochratoxin-A, and the extrusion temperature was between 116 – 200 °C. It is shown, that the reduction is the highest in the most contaminated samples treated at the highest temperature. It was possible to reduce the ochratoxin-A level up to 42 % (Scudamore et al., 2004).

The group of Fusarium-toxins is split into fumonisins, trichothecenes, and zearalenone. The reduction of these is evaluated in corn grits or flour. The corn for testing the reduction of fumonisins had an initial contamination of 5 µg/g. A reduction of up to 42 % was possible with an incubation at 160 °C. If the temperature is even higher (up to 200 °C) the reduction of fumonisins is up to 95 % (Castelo et al., 2006).

In the testing of deoxynivalenol, the corn grits were spiked with 4 µg/g, in corn flour, the contamination was at 5 µg/g (Cazzaniga et al., 2001). After the heating of the corn grits, a reduction of 53 % was reached, in the corn flour a reduction of up to 99.5 % was possible (Wolf-Hall et al., 1999).

The sample for the zearalenone was spiked with a concentration of 4.4 µg/g. The samples were heated up to 160 °C and a reduction of up to 83 % was reached (RYU et al., 1999).

Overall, it can be said, that the reduction of mycotoxins depends on the type of starting material and type of mycotoxin. Additionally, the impact of the moisture is visible – but this is not relevant in the case of packaging materials (Kabak, 2009).

2.3.2 *Scalping*

If it comes to packaged food, there is always an interaction between the packaging materials and the foodstuff itself. On the one side, there is the migration of substances into the foodstuff from the polymer. These substances are for example monomers or additives which show a major problem for safety and off-flavours. On the other side, there is an adsorption process happening from the foodstuff to the polymer, which is called scalping. This affects aroma compounds, fats, organic acids, or pigments which leads to a loss of aroma in the foodstuff and damage to the packaging material. (Adams et al., 2019)

The intensity of the scalping process highly depends on the intrinsic and extrinsic factors to which the foodstuff is exposed during its shelf life.

One of the most important extrinsic factors is storage time and temperature. In LDPE and OPP are available data for different storage conditions. In these cases, a study with 10 different flavour compounds in an aqueous phase was done. The selection of the flavour compounds tries to cover differences in functional groups and polarity since this also influences the scalping effect. The storage time was between 1 hour and 14 days and the storage temperature was at 4 °C, 20 °C, or 40 °C. It is shown that the rate and quantity of scalping are

directly influenced by the type of polymer. In the direct comparison between polyolefins like LDPE and PP and other polymers like PC or PET is noticeable, that the total amount of flavour compounds is in polyolefins much higher than in the comparison group. But it is also shown, that the temperature has not that big impact on the scalping as assumed. (Van Willige et al., 2002)

In Europe the usage of post-consumer recycled PET in food contact is already possible but the recycling plants have to show the efficiency of the cleaning process. To evaluate the input material of these plants, studies about the input material and the contaminations of this are made.

In a study Roland Franz and Frank Welle showed the amount of non-food PET in PET recycling streams and the contamination level in the rPET produced of these charges. First, it is not fully possible to differentiate between beverage container and non-food containers in the flakes for the input material. In general, the recyclers buy prewashed flakes for their super-clean recycling process. From market data, it is possible to say, that the recycling feed stream of bottle-to-bottle PET recycling includes up to 5 % non-food containers, but the actual percentage will vary depending on the collection system.

The contamination level of these mixed non-food ranges from the sub-mg/kg concentration up to 30 mg/kg, if very volatile substances are excluded since data show, that these substances are highly effective cleaned in the super-clean process. Also, nearly all identical substances could be assigned to Cramer classes I, II or III. (Franz & Welle, 2020)

2.3.3 *Printing Inks*

Packaging does not just have the purpose to protect the packaged foodstuff from extrinsic factors. It also has an informative marketing function. These functions can only be fulfilled with a printed surface or a printed banderole. There are research projects to implement deinking steps to the recycling processes, but for now, this is not the state of the art. But since these compounds are not regularly removed before the recycling process, they also must be considered in the assessment of possible contaminants of the input material (Skoda, 2022).

The composition of printing inks always depends on the printing process in which they should be used, but in general, they consist of three parts. At first the colourants – this part is represented by organic or inorganic pigments. The second part is the vehicle, which makes the printing process possible. This vehicle can be a binder (like polymer or resin) or a solvent. The last part are additives. This part is represented by a big and heterogeneous group. In this group can be used antifoam agents, antistatic agents, biocides, inhibitors, stabilizers, and many more (Josef Sutter et al., 2011).

Printing ink for the application of food contact materials has to be designed according to the EuPIA “Guideline on Printing Inks applied to Food Contact Materials” (EuPIA, 2020).

2.3.3.1 Colourants

Colourants are the colouring part of the printing ink. There are different types of pigments used to create a certain colour. It is possible to use organic pigments like azo pigments, or inorganic pigments like titanium dioxide for a white colour or carbon black for a black colour, for metallic effect are powdered aluminium or brass used (EuPIA, 2020).

Inorganic pigments or pigments for metallic effect are often pigments including transition metals. These metals have catalytic properties which can lead to undesirable and uncontrollable reactions in the recycling process since the input material is not that clearly defined (Winkler, 2014).

Azo pigments are a common type of pigment that is used in printing inks. The common structure of these pigments is the azo group. This group also shows a reactive centrum of the molecule. Azo pigments are known as a source of contamination of primary aromatic amines (PAAs) because of raw material residues in the final product. Additionally since azo pigments are not thermal stable substances, during the recycling process, different degradation products occur including benzene, PAAs like aniline or diaminobenzene (Nguyen & Saleh, 2020).

In the testing of the thermal degradation of ten different azo pigments, it is shown that the highest occurrence of degradation products is between 200 °C and 300 °C (Nguyen & Saleh, 2020). Since this is also the temperature used in the recycling process, azo pigments are likely to build degradation products also in this process.

2.3.3.2 Vehicle

As the vehicle of the printing inks are different substances used depending on the printing technique which is used. Nitrocellulose is a binder which finds a broad application but also other resins like maleic acid or synthetic resins like polyvinyl butyral, polyamide or polyurethane are often used. These binders are mostly used combined with solvents like alcohols or esters to enhance the workability of the printing inks, additionally, additives like plasticisers, slip additives or adhesion promotors are used (EuPIA, 2020).

But there are also water-based systems which use styrene-acrylic co-polymers, acrylic co-polymers or maleic resins with solvents like water, isopropanol, glycol ether or propylene glycol. This system needs also additives for example amines, biocides, defoamers, wetting agents, waxes or slip agents (EuPIA, 2020).

For nitrocellulose is data available, that shows the thermal degradation in a thermographic analysis. It is shown, that the thermal degradation differs between the nitrogen content of the nitrocellulose, with a lowering of the nitrogen content a rise of the degradation temperature occurs but the peak of degradation is in all cases between 200 °C and 205 °C (Pourmortazavi et al., 2009). It is not shown which degradation products are built in the heating but in 2019 a new source of contamination of pharmaceuticals was reported to the authorities. It was possible to identify the building of *N*-nitrosodimethylamine and *N*-nitrosodiethylamine during the heat-sealing of the blister packaging. The foil which was used in this process was printed with amine-containing printing ink, during the heating, a reaction with the nitrocellulose occurred and the *N*-nitrosamines were built (EMA, 2020).

2.4 Analytical Techniques – State-Of-The-Art

In the analysis of FCM, a distinction must be made between IAS and NIAS. In case of IAS, there is a Union list of authorized substances defined in the EU Regulation 10/2011, for these substances a generic specific migration limit of 60 mg/kg food is applied, for some substances a specific migration limit is defined.

But not only IAS can migrate from the FCM into the food, also NIAS may migrate. This is possible through impurities or degradation products of IAS but also if it comes to recycling through post-consumer contamination and degradation products through the recycling process. These NIAS are not conducted by the Union list but in Art. 19 of EU Regulation 10/2011. The NIAS have to be assessed according international recognized scientific principles of risk assessment. A specific procedure is not defined in the EU Regulation 10/2011 (Nerín et al., 2022).

To categorize NIAS there are two different approaches, on the one side by origin and on the other side by category. If you look at them after origin, the three main categories are side products, breakdown products or contaminations. But it is also possible to divide them after category, in this case, there are four different situations. First one is identified and assessed NIAS, the second one are the identified but not assessed one, the third one are detected but not identified one and the fourth one are the not detected one (Geuke, 2018).

For the screening of NIAS in general, there is not just one perfect technique which can be applied. It is necessary to combine different since the characteristics of the FCM and migrants can differ a lot. For the analysis of volatile and semivolatile substances different GC-MS techniques may be applied. For the analysis of non-volatile and semivolatile substances, LC-MS methods are used. For the risk assessment, the individual concentrations of individual NIAS need to be known. Since standards are often missing, the quantification is made by a comparison to one or several internal standards.

Typical NIAS compounds from the category side products are PAA as a reaction product from isocyanate residuals in PU adhesives, oligomers from polystyrene (Geuke, 2018).

If it comes to the analytical techniques, always the strengths and weaknesses of the methods need to be considered. Most of the analysis and advances of NIAS are made with GC-MS and HPLC-MS, in many cases a highly sensitive method is necessary and so a high-resolution mass spectrometry is needed. But also, enough knowledge of the food simulant used and the migrate is necessary for a reliable analysis of the samples. (Nerín et al., 2022)

For some samples, especially with a larger number of substances, a combination of different techniques is necessary. So, the BfR recommends to consider a pre-separation and

concentration of substances by SPE or a multidimensional chromatographic technique (GCxGC, 2D HPLC or HPLC-GC) to improve separation. (BfR, 2011)

If it comes to the results and the interpretation of the results, the results are always separated in known and unknown substances. For the known substances, the risk assessment is based on the toxicological data of the actual compound. For the unknown substances, the risk assessment is carried out according the TTC concept and often bioassays are useful to exclude a direct-DNA reactive effect. (Koster Sander et al., 2016)

2.5 The Ames Test

The Ames test, also called bacterial reverse mutation assay, was invented in 1971 by Bruce Ames and is an assay to detect direct DNA reactive genotoxic effects of substances. The basic principle of the test is to expose the bacteria to the compound which should be tested and count the bacteria after an incubation of the petri dishes. The name “bacterial reverse mutation assay” is caused by the principle of back mutation in the mutation of the histidine operon which is responsible for the growth after the incubation since the growth medium of the bacteria is low in histidine (Ames, Lee, et al., 1973).

For the Ames test commonly, tester strains of *Salmonella Typhimurium* are used which have modification of the DNA to make these more sensitive to mutagenic substances. They have different modifications, the first and most important one is a mutation in the synthesis of histidine. The type of mutation depends on the used strain, the TA98 has a frameshift mutation through a single base-pair deletion in the *hisD3052*, the TA100 has a base-pair substitution on the *hisG46* which alters the first enzyme of the histidine biosynthesis. Both mutations lead to a histidine-auxotroph mutant of the *Salmonella Typhimurium* (Mortelmans & Zeiger, 2000).

Additionally, to the mutation on the histidine-operon, the bacteria have mutation in the *uvrB* gene, which lead to an elimination of the repair mechanism of the DNA, a mutation on the *rfa*-gene leads to a defective lipopolysaccharide layer. This mutation is essential to gain a better diffusion of the testing substance into the bacteria. The tester strains also contain a pKM101 R-factor plasmid which is responsible for an enhanced chemical and UV-induced mutagenesis and an ampicillin resistance (Mortelmans & Zeiger, 2000).

Since the metabolic system differs between prokaryotic and eukaryotic organisms which have an impact on the effects in the organism, it is necessary to include a system of metabolic activation to the testing. This is made through the usage of the 9000xg supernatant of a rat liver extract which is rich in enzymes out of the cytochrome P450 group (Ames, Durston, et al., 1973).

In the meanwhile, many variations of the classical Ames test in petri dishes are implemented and also accepted in the scientific community and authorities. Beside the classical testing in a

plate incorporation method, the preincubation method, the fluctuation method and the suspension method are described (OECD, 2020).

The testing of substances in the Ames test is used in the development of pharmaceuticals and medical products as well as in the testing of water and wastewater. So it is seen, that the Ames test is implemented in very different areas (ISO/TC 147/SC 5, 2012).

Schilter et al. summed the studies in the field of food contact materials up and started the need of a simple and fast variant of the Ames test (Schilter et al., 2019). So Rainer et al. compared different Ames variations to find a variation with a low limit of detection to comply with the requirements for the testing of food contact materials (Rainer et al., 2018). In another study already food contact material migrates were tested in the Ames MPF and showed, that the Ames MPF is a proper method to do safety assessment of food contact material (Rainer et al., 2019).

In the risk assessment of food contact materials, the current approach is based on the EU Regulation 10/2011. In this regulation the minimum requirement for the analytical limit of detection is 0.01 mg/kg for unknown substances migrate into food. To confirm the capability of the Ames test, Rainer et al. evaluated the limit of detection. For the comparison of the LECs with the detection limit to be achieved, a conversion of the 0.01 mg/ kg in mg/ L needs to be done. So the 0.01 mg/ kg are multiplied by a global concentration factor of 40, this factor is achievable in the sample preparation. To get the results of the Ames data in an adequate format, mg/ L, the results need to be divided by 2.7 since the amount of top agar which is used in a plate assay is 2.7 mL. (Rainer et al., 2018)

In the comparison of the LECs of the standard substances from the validation list for in vitro genotoxicity tests 50% of the samples (n=16) meet the limit of detection of 0.4 mg/ L.

In a study about impurities in pharmaceutical products more than 80 % of the 454 compounds are below the limit of detection of 0.4 mg/ L (Tarca et al., 2013).

The number of relevant substances, which are identified and genotoxic is rather small in the field of food contact materials. Only 10 % of the substances (n=40) meet the limit of detection of 0.4 mg/ L. And this study just takes pure substances in account, matrix effects are not considered.

2.6 State-of-the-Art and Future Challenges

In 2017 Ksenia Groh published a detailed review of the current data on the *in vitro* toxicity testing of food contact materials. There are not just publications in the field of genotoxicity testing also the cytotoxicity and endocrine activity have been a research field in the past.

The first publications and research were done in the 1990ies, in this time the focus was on the comparison of PET-bottled and glass-bottled water. The results of the different samples matched and positive effects just occurred after a long-term storage of one month and after storage in daylight (De Fusco et al., 1990). The other test of PET-samples showed continuously negative results in the Ames test. Also, the tested PLA showed negative results in the Ames test. The only FCM tested as virgin and recycled material has been paper and paperboard. In 2002 Binderup et al. did tests of water and ethanol extracts and got weak positive results in the Ames test but it was not possible to confirm the effects by a higher concentration of the sample (Binderup et al., 2002).

Till 2017 the focus in the recycling of material in contact with food was in the paper and paperboard field.

But since PET got an EFSA- and FDA-approval for selected recycling streams and recycling plants and the increasing demands for a circular economy and recycling without downcycling. The focus is more and more in the recycling of other plastics like PE or PP and getting approval for recycling plants for these plastics.

So far, only one study is published on *in-vitro* bioassay results of recycled polyolefins and polystyrene, which are not yet authorized for food contact. Mayrhofer et al published data about the testing of 119 plastics recyclate samples in the Ames test. It is shown, that none of the PET samples showed a DNA-reactive activity, but the HDPE and LDPE and also PP samples had a high number of positive results, especially in the output fraction of the recycling (Mayrhofer et al., 2023). Some of the samples also got through a chromatographic analysis but not all substances were possible to identify. But a small number of the identified also show a genotoxic alert in the toxicological classification (Rung et al., 2023).

Since the identification of the source of the substances and also the substances itself which show the DNA-reactive activity in the Ames test was not possible by know, the aim of this thesis is to collect more information about the physiochemical properties and test the impact of different constituents of the final packaging material.

3 Material and Methods

3.1 Chemicals

In table 2 all chemicals which were used in this work are listed.

table 2: list of required chemicals, abbreviations, CAS numbers and suppliers

Chemical name	Abbr. / Formula	CAS number	Supplier
2-Aminoanthracene, 96 %	2-AA	613-13-8	Sigma Aldrich
2-Nitrofluorene, 98 %	2-NF	607-57-8	Sigma Aldrich
4-Nitroquinoline N-oxide, ≥98 %	4-NQO	56-57-5	Sigma Aldrich
Acetic acid, 100 %	HAc	64-19-7	VWR
Acetone, ≥99 %	-	67-64-1	VWR
Ampicillin sodium salt	Amp	69-52-3	Sigma Aldrich
Bromocresol purple sodium salt, 90 %	Bromocresol purple Na salt	62625-30-3	Sigma Aldrich
Citric acid monohydrate, ≥99 %	-	5949-29-1	Sigma Aldrich
Collodion-solution, 4%	NC	9004-70-0	Sigma Aldrich
D-(+)-Biotin, ≥99 %	Bio	58-85-5	VWR
D-glucose 6-phosphate sodium salt solution	G-6-P	1184-16-3	Sigma Aldrich
D-Glucose, anhydrous	-	50-99-7	VWR
4,4'-Diaminodiphenylmethane, ≥97 %	MDA	101-77-9	Sigma Aldrich
Dichloromethane	DCM	75-09-2	Supelco
2,4-Dimethylaniline ≥99%	DMA	95-68-1	Sigma Aldrich
Dimethyl sulfoxide	DMSO	67-68-5	Merck
Dipotassium hydrogen phosphate	K_2HPO_4	7758-11-4	Merck
Ethanol, ≥99.9 %, LiChrosolv®	EtOH	64-17-5	Merck
L-Histidine, ≥99 %	His	71-00-1	Sigma Aldrich
Magnesium chloride hexahydrate, 99.0-102.0 %	$MgCl_2 \cdot 6H_2O$	7791-18-6	Sigma Aldrich
Magnesium sulphate heptahydrate	$MgSO_4 \cdot 7H_2O$	10034-99-8	Merck
Methanol, >99.8 %	MeOH	67-56-1	J.T.Baker
Nicotinamide adenine dinucleotide phosphate sodium salt, >97 %	NADP	69899-85-8	AppliChem

table 2 continued: list of required chemicals, abbreviations, CAS numbers and suppliers

Chemical name	Abbr. / Formula	CAS number	Supplier
Nitrogen-gas, 99,999 %	-	7727-37-9	Messer Austria GmbH
Nutrient broth No. 2	Growth medium	-	Oxoid
o-Anisidine, ≥99 %		90-04-0	Sigma Aldrich
Phosphoric acid, 85 %	H_3PO_4	7664-38-2	Supelco
Potassium chloride, 99.5-101.0 %	KCl	7447-40-7	VWR
Rat liver S9, phenobarbital/β-naphthoflavone induced, lyophilized	S9	-	Xenometrix
Sodium ammonium hydrogen phosphate tetrahydrate	$NaNH_4PO_4 \cdot 4H_2O$	7783-13-3	Merck
Sodium dihydrogen phosphate dihydrate, ≥99.0 %	$NaH_2PO_4 \cdot 2H_2O$	13472-35-0	VWR
Sunset Yellow FCF, dye content 90%	Azo	2783-94-0	Sigma Aldrich
Titanium (IV) oxide, 99-100.5%	TiO_2	13463-67-7	VWR

3.2 Consumables

In table 3 the necessary consumables are listed.

table 3: list of consumables and their suppliers

Product name	Supplier
24-well microtiter plates incl. lid, PS, sterile	VWR
384-well microtiter plates incl. lid, PS, sterile	VWR
96-well microtiter plates incl. lid, PS, sterile	VWR (Thermo Fisher)
Bottle-top sterile filter, PES, 0.2 µm, 1,000 mL	VWR
Bottle-top sterile filter, PES, 0.2 µm, 250 mL	VWR
Centrifuge tubes, 15 mL, sterile	VWR
Centrifuge tubes, 50 mL, sterile	VWR
Cuvette, PS, semi-micro	VWR

table 3 continued: list of consumables and their suppliers

Product name	Supplier
Disposable graduated pipettes, 10 mL, sterile	VWR
Disposable graduated pipettes, 25 mL, sterile	VWR
Female luer coupler	Supelco
Glass bottles “Schott”, 1 L	VWR
Glass bottles “Schott”, 100 mL	VWR
Glass bottles “Schott”, 2 L	VWR
Glass bottles “Schott”, 250 mL	VWR
Glass bottles “Schott”, 500 mL	VWR
Glass crimp vial, 2 mL	Merz Brothers
Glass Erlenmeyer flask, 100 mL	VWR
Glass Erlenmeyer flask, 50 mL	VWR
Glass funnel, 100 mm	VWR
Glass funnel, 150 mm	VWR
Glass powder funnel	VWR
Glass vials, 2 mL, with PTFE-coated lid	Merz Brothers
Glass vials, 20 mL, clear, with PTFE-coated lid	Merz Brothers
Glass vials, 40 mL, clear, with PTFE-coated lid	Merz Brothers
Graduated glass pipette, 10 mL	VWR
Graduated glass pipette, 20 mL	VWR
Graduated glass pipette, 25 mL	VWR
Graduated glass tube for rack R-6 Analyst	BÜCHI
Male luer coupler	Supelco
Measuring cylinder, 1,000 mL	VWR (Brand)
Measuring cylinder, 100 mL	VWR (Brand)
Measuring cylinder, 250 mL	VWR (Brand)

table 3 continued: list of consumables and their suppliers

Product name	Supplier
Measuring cylinder, 500 mL	VWR (Brand)
Microcentrifuge tubes, 1.5 mL + 2 mL, PCR clean	Eppendorf
Petri dishes	VWR
Pipette tips with filter, 1000 µL, sterile	Th. Geyer
Pipette tips with filter, 1250 µL, sterile	Th. Geyer
Pipette tips with filter, 10 µL, sterile	Th. Geyer
Pipette tips with filter, 20 µL, sterile	Th. Geyer
Pipette tips with filter, 200 µL, sterile	Th. Geyer
Pipette tips without filter, 1250 µL	Eppendorf
Pipette tips without filter, 1200 µL	Sartorius
Pipette tips without filter, 200 µL	VWR
Positive displacement pipette tips “Combitips®”, 0.1 mL, Eppendorf quality	Eppendorf
Positive displacement pipette tips “Combitips®”, 10 mL, Eppendorf quality	Eppendorf
Positive displacement pipette tips “Combitips®”, 50 mL, Biopur®	Eppendorf
PTFE-coated lid for glass bottles	VWR
Reagent reservoirs, 100 mL, sterile	VWR
Round flask, 29/32 NS, 250 mL	VWR (Lenz-Laborglas)
SPE column “Isolute® C18”, 6cc, 500 mg	Biotage
SPE column “Oasis® HLB”, 6 cc, 200 mg	Waters
SPE reservoir, 6 cc	Supelco
SPE tube adapter	Supelco
Syringe sterile filter, cellulose acetate, 0.2 µm	VWR
Syringe, 20 mL + 50 mL, sterile	VWR / Henke-Sass Wolf
Visiprep™ SPE manifold solvent guide needles	Supelco

3.3 Devices

In table 4 all devices are listed.

table 4: list of devices and their suppliers

Device name	Supplier
Analytical balance “A 2005”	Sartorius
Analytical balance “Entris 224i-1S”	Sartorius
Analytical balance “XS205”	Mettler Toledo
Balance “KB2400-2N”	Kern
Cell density meter “Ultrospec® 10”	Biochrom
Electronic multichannel pipette “Xplorer®”, 8-channel, 50-1200 µL	Eppendorf
Electronic multichannel pipette “Picus®NxT”, 8-channel, 50-1200 µL	Sartorius
Electronic pipetting aid "Powerpette Pro"	VWR
Electronic positive displacement pipette "Multipette® E3x"	Eppendorf
Freezer, -20°C	Liebherr
Incubator “3501”	Rumed
Incubator “BK 3108”	Ehret
Incubator “StabiliTherm EB 118-2”	Thermo Fisher
Laminar flow workbench "Biosafe 7-130"	Ehret
Laminar flow workbench “HS 12/2”	Heraeus
Laminar flow workbench “Hera Safe HSP09”	Heraeus
Manual multichannel pipette “Pipetman®”, 8-channel, 2-20 µL	Gilson
Muffle furnance, Nabertherm L9/11/C6	Nabertherm
Parallel evaporator “Syncore® Analyst”	BÜCHI
Platform, 230 V, 50/60 Hz	BÜCHI
Condenser, standard	BÜCHI
Round receiving flask, KS24/20, P+G coated (2 L)	BÜCHI
Crystal rack “R-6 Analyst” incl. vacuum cover	BÜCHI

table 4 continued: list of devices and their suppliers

Device name	Supplier
Interface “I-300 Pro”	BÜCHI
Vacuum pump “V-300”	BÜCHI
Recirculating chiller “F-314”	BÜCHI
pH Meter “VWR PH 110”	VWR
Piston pipette “Pipetman®”, 1-10 µL	Gilson
Piston pipette “Pipetman®”, 200-1000 µL	Gilson
Piston pipette “Pipetman®”, 20-200 µL	Gilson
Refrigerator, 4°C	Liebherr/ NordCap
Shaking incubator “3032”, amplitude 25 mm	GFL
SPE Vacuum Manifold “Visiprep™”	Supelco
Steam steriliser “Varioklav®”	HP Labortechnik
Ultra-low freezer, -80°C	Telstar Technologies
Ultra-pure water preparation system “Reference A+”	MilliQ
Ultrasonic bath “Sonorex Super RK 514”	Bandelin
Vacuum pump “MZ 2 NT”	Vacuubrand
Vacuum pump “MZ 2”	Vacuubrand
Vortex “VV 3”	VWR
Water bath “1008”	GFL

3.4 Strains

All Ames tester strains, necessary for the testing, are listed in table 5.

table 5: list of Ames tester strains and their supplier

Name	Abbr.	Supplier
Ames tester strain TA98	TA98	Xenometrix
Ames tester strain TA100	TA100	Xenometrix

3.5 Sample Preparation

In the experiments, different sample types were used. Depending on the sample a different sample preparation was necessary.

For testing pure substances, the substance needed to be dissolved in DMSO to reach a concentration of 50 mg/ mL. These samples were directly tested in the Ames test, no migration and/or concentration was necessary.

In some experiments, specific test items were made. These samples were prepared for a systematic analysis of different printing ink components. A combination of pigment and/or binder was suspended in ethanol (see table 6) and the mixtures were applied to 1 m² of LDPE mono foil. This foil was also used as a negative control sample. The prepared samples were either used directly for migration (no treatment) or incubated at high temperatures beforehand to mimic a recycling process. The heat treatment at 160 °C was done in a hydraulic press, the heat treatment at 230 °C was done in a muffle furnace in nitrogen atmosphere. All of the prepared samples (non-treated and treated) need a migration to test in the Ames test. As well as the sent samples from project members, mostly input and output materials of a recycling plant. The migration is described in 3.6.

table 6: list of different samples and treatment conditions, TiO_2 = Titanium Dioxide, Azo = Sunset Yellow FCF, NC = Collodion solution

Sample name	Pigment [g/m²]		Binder [mL/m²]		Solvent [mL/m²]		Treatment
TiO_2 untreated	TiO_2	3.6	none		EtOH	20	none
TiO_2 160 °C	TiO_2	3.6	none		EtOH	20	20 min; 160 °C
TiO_2 230 °C	TiO_2	3.6	none		EtOH	20	20 min; 230 °C
TiO_2 + NC untreated	TiO_2	3.6	NC	15	EtOH	5	none
TiO_2 + NC 160 °C	TiO_2	3.6	NC	15	EtOH	5	20 min; 160 °C
TiO_2 + NC 230 °C	TiO_2	3.6	NC	15	EtOH	5	20 min; 230 °C
Azo untreated	Azo	3.6	none		EtOH	20	none
Azo 160 °C	Azo	3.6	none		EtOH	20	20 min; 160 °C
Azo 230 °C	Azo	3.6	none		EtOH	20	20 min; 230 °C
Azo + NC untreated	Azo	3.6	NC	15	EtOH	5	none
Azo + NC 160 °C	Azo	3.6	NC	15	EtOH	5	20 min; 160 °C
Azo + NC 230 °C	Azo	3.6	NC	15	EtOH	5	20 min; 230 °C

3.6 Migration

The migration of the samples was done based on Regulation No. 10/2011 on plastic materials and articles intended to come into contact with food. To cover worst-case conditions, the migration was always done with 95 % ethanol for 10 days at 60 °C. After the migration, the solvent was transferred to a fresh glass bottle and stored at 4 °C until concentration.

The migration was done in Schott glass bottles or glass vials, depending on the final volume of the solvent. To prevent migration from the PE-lid, a PTFE seal was used to cover the lid. Additionally, negative controls without any sample materials were prepared to check contaminations through glass ware, PTFE seals or solvents.

In order to achieve comparable results between food contact materials (e.q. foils) and granules a mass to volume ratio of 0.3 g polymer per mL solvent was applied for all experiments.

3.7 Concentration

The concentration of the migrates was done in a parallel evaporation instrument, the Syncore® Analyst R-6 from Büchi. This instrument concentrates six samples at the same time with a volume of up to 300 mL per sample. The glasses of the Syncore® Analyst R-6 have an appendix on the bottom with a volume of 1 mL, which is cooled to prevent evaporation to dryness. To change the solvent from 95 % ethanol to bioassay-compatible DMSO, it is necessary to prevent the cooling of the appendix with a bypass system after the addition of DMSO. The whole set-up of the Syncore® Analyst is shown in figure 1.

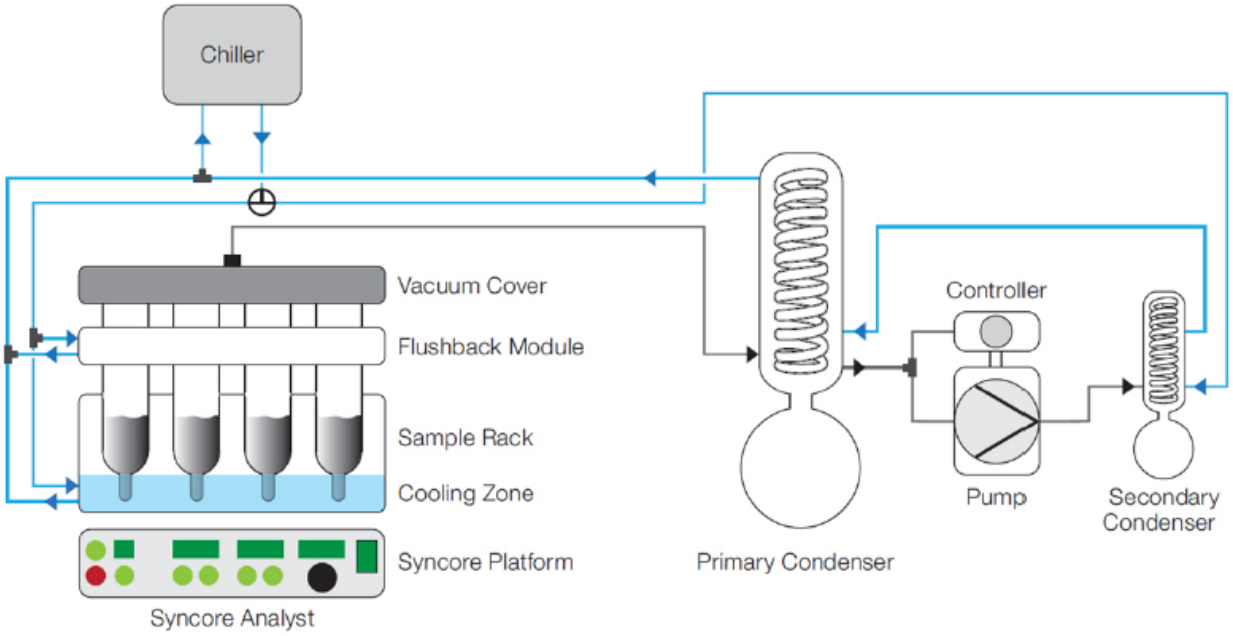


figure 1: schematic set-up of a Syncore® Analyst system including a vacuum pump and a chiller. Blue lines represent the cooling water loop (Büchi, 2012).

For the concentration the glasses of the Syncore® Analyst R-6 were prewashed with 95 % ethanol. From the migrates a 1 mL aliquot was taken in a 2 mL glass vial with a crimp lid. The glasses were put in the sample rack and the vacuum cover was closed. The heating of the platform was set to 60 °C and the temperature of the vacuum cover was set to 55 °C. The rotation of the sample rack was set at 300 rpm. For concentration a vacuum gradient was applied. The vacuum profile is shown in table 7. During evaporation the bypass was opened to ensure that cooling water reached to appendix. The temperature of the cooling water was set to 5 °C.

table 7: pressures used for the evaporation of migrates

	Pressure [mbar]	**Time [min]**		**Pressure [mbar]**	**Time [min]**
Step 1	800	5	Step 7	90 - 70	2
Step 2	400	3	Step 8	70	8
Step 3	400 - 150	2	Step 9	120	5
Step 4	150	2	Step 10	130	5
Step 5	150 - 90	2	Step 11	130 - 1000	10
Step 6	90	2			

After the sample volume was reduced to approximately 1 mL DMSO was added to the glasses and the bypass was closed to stop the appendix cooling and to allow solvent exchange. The volume of DMSO added to the sample depended on the previous volume of non-concentrated solvent, as a concentration factor of 100 was anticipated by the concentration.

This solvent exchange was done at the same temperatures and rotation settings as before, but at a pressure of 50 mbar for 30 to 180 minutes, depending on the intended residual volume. After reaching the volume the DMSO extracts were transferred to 2 mL glass vials closed with a screw cap and stored at -20 °C until testing.

3.8 Fractionation

The fractionation of sample migrates was done with Biotage ISOLUTE C18 or Oasis HLB 6 cc Vac columns on a Visiprep Vacuum Manifold (24 port, Supelco).

Before starting the fractionation, the sorbents were equilibrated. For that, the vacuum was set to 70 mbar and the solvents listed in table 8 were consecutively drawn through the columns.

table 8: solvents used for the equilibration of SPE-cartridges for sample fractionation

Volume [mL]	Solvent	Composition
2 x 5 mL	Dichloromethane: Ethanol	8:2
2 x 5 mL	Methanol	100 %
2 x 5 mL	Ultrapure water	-

After column equilibration, the samples were loaded and fractionated. All elution and flow-through fractions were collected separately in 20 mL glass vials.

As the samples must not exceed an ethanolic content of 10 % when loaded to the sorbent materials, 95 % ethanol migrate were first concentrated by parallel evaporation using the concentration protocol in 3.7 to reduce the ethanol volume to 2 mL. Then, the concentrated ethanol migrates were filled up with ultrapure water to reach an ethanol concentration of 10 %. Further, 0.4 % of a NaH_2PO_4 buffer was added. The samples were loaded to the column in 2 mL steps which was followed by a washing step with 2x 5 mL ultrapure water containing 0.4 % NaH_2PO_4-buffer. After loading the cartridges were dried under a gentle stream of air for 2 hours.

The fractionation was done in reverse-flush mode, i.e. the columns were inverted before elution. For that, an adapter was put on the open end of the cartridge to close it and connect this end directly to the manifold. On the tip of the cartridge another adapter was placed to install a reservoir tube which serves as a container to apply the elution buffer. Depending on the experimental design different elution buffers were used. Each elution buffer was applied 4 times using 2 mL for each of these steps. The different solvents are shown in table 9.

The 100 % methanol fractions were concentrated by evaporation by air blow-down. The other fractions were concentrated using the Syncore® Analyst R-6. In both cases this step was combined with a solvent exchange to DMSO using a final DMSO volume of 400 µL per fraction.

table 9: list of different fractionation experiments and corresponding elution buffers

Fractionation No.	Column		Solvent	Composition
Fractionation 1	Oasis HLB, 200 mg, 6cc	Buffer 1	Methanol (in water)	50 %
		Buffer 2	Methanol	100 %
		Buffer 3	Methanol/Dichlormethane	3/1
		Buffer 4	Methanol/Dichlormethane	1/3
		Buffer 5	Dichlormethane	100 %
Fractionation 2	Oasis HLB, 200 mg, 6cc	Buffer 1	Methanol (in water)	12.50 %
		Buffer 2	Methanol	25 %
		Buffer 3	Methanol (in water)	37.50 %
		Buffer 4	Methanol (in water)	50 %
		Buffer 5	Methanol (in water)	62.50 %
		Buffer 6	Methanol	75 %
		Buffer 7	Methanol (in water)	87.50 %
		Buffer 8	Methanol	100 %
Fractionation 3	Biotage Isolute C18 500 mg, 6cc	Buffer 1	ultrapure water	100 %
		Buffer 2	Methanol	100 %
Fractionation 4	Biotage Isolute C18 500 mg, 6cc	Buffer 1	ultrapure water	100 %
		Buffer 2	Methanol	100 %
Fractionation 5	Biotage Isolute C18 500 mg, 6cc	Buffer 1	ultrapure water	100 %
		Buffer 2	Methanol	100 %

3.9 Miniaturized Ames Test

The media used for Ames testing in the miniaturized format were prepared following the international standard ISO 11350:2012 “Water quality – determination of the genotoxicity of water and wastewater – Salmonella/microsome fluctuation test (Ames fluctuation test)”. The testing procedure followed the protocol provided by Xenometrix AG.

3.9.1 *Growth medium*

To grow the bacteria Nutrient Broth No. 2 from Oxoid was used. The medium was prepared according to supplier’s instructions. 10 g of the powder was placed in a Schott bottle and 400 mL ultrapure water was added. The bottle was autoclaved for 20 min at 121 °C. After cooling to ambient temperature, the medium was aliquoted to sterile 50 mL PE tubes under sterile conditions. After labelling the tubes were stored at 4 °C.

3.9.2 *Exposure Medium*

To prepare the exposure medium 900 mL ultrapure water was filled into a Schott bottle. The substances from table 10 were added and dissolved, one at a time. Then, the solution was filled up to 1000 mL with water and the pH was checked using a pH test strip. A pH of 7.0 ± 0.2 was acceptable. After checking the pH, the solution was sterilized by sterile filtration using a 0.2 µm bottle top filter. The sterile medium was labelled and stored at 4 °C for up to two months.

Before testing the exposure medium was supplemented with biotin (0.5 mM) and histidine (6.4 mM). After adding the amino acids the medium is stable for 2 weeks at 4 °C.

table 10: components for the preparation of the exposure medium for Ames MPF

Substance	**Concentration [g/L]**
Magnesium sulphate heptahydrate	0.2
Citric acid monohydrate	2.0
Dipotassium hydrogen phosphate	10.0
Sodium ammonium hydrogen phosphate tetrahydrate	3.5
D-Glucose	4.0

3.9.3 *Reverse Indicator Medium*

For the reverse indicator medium three solutions were prepared separately. Solution 1 and the indicator solution were combined before autoclaving to obtain solution A, and solution B was autoclaved separately.

For Solution 1 a Schott bottle was filled with 900 mL ultrapure water before adding the salts listed in table 11 in the section Solution 1. The salts were added and dissolved consecutively. The volume was filled up to 1 L with ultrapure water. For the indicator solution bromocresol-purple sodium salt was dissolved in 30 mL ultrapure water in a PE vial. Then the solution was added to solution 1. After mixing both solutions the total volume was equally split to two Schott bottles for autoclaving.

For solution B a Schott bottle was filled with 700 mL ultrapure water. 4.0 g D-glucose was added. After dissolving the volume was filled up to 800 mL which was then split into two Schott bottles. All solutions were autoclaved for 20 min at 121 °C.

After the solutions had cooled down to room temperature, one bottle of solution B was added to one bottle of solution A and 20 mL biotin (0.5 mM) under sterile conditions.

table 11: components for the preparation of the different solutions for the reverse indicator medium for Ames MPF

		Substance	**Mass [g]**	**Volume [mL]**
Solution A	Solution 1	Magnesium chloride hexahydrate	0.2	1000
		Citric acid monohydrate	2.0	
		Dipotassium hydrogenphosphate	10.0	
		Sodium ammonium hydrogenphosphate tetrahydrate	3.5	
	Indicator solution	Bromocresol-purple, sodium salt	0.051	30
Solution B		D-Glucose	4.0	800

3.9.4 *Spikes*

To provide positive controls for the different Ames testing conditions, 4 different spike solutions were prepared. Their concentrations and the corresponding testing conditions are given in table 12. The solutions were prepared using DMSO.

table 12: substances used as positive controls and their concentrations in the different testing conditions in Ames MPF

Testing condition	Substance	Concentration [mg/mL]
TA 98 -S9	2-Nitrofluorene	2.8
TA 98 +S9	2-Aminoanthracene	0.7
TA100 -S9	4-Nitroquinoline N-oxide	0.14
TA100 +S9	2-Aminoanthracene	1.75

The spike solutions were used directly to prepare spiked exposure medium. If they were applied to 24-well plates as positive controls instead, an additional dilution step (3.6 µL spike stock + 196.4 µL DMSO) was needed.

3.9.5 *S9-Mix*

For tests with metabolic activation buffer solutions for the S9-fraction were prepared. Stock solutions were prepared by dissolving the reagents listed in table 13 in ultrapure water, followed by filtration through a sterile filter (0.2 µM). The stocks were stored at 4°C (salt solutions) or -20 °C (G-6-P, NADP). Before testing an S9 mixture was prepared freshly by mixing the stocks and S9 fraction in the volumes given in table 13.

table 13: substances and volumes used for the preparation of the S9-mixture

Substance	Stock concentration [M]	Volume per 24-well plate [µL]
Potassium chloride	1	34.7
Magnesium chloride hexahydrate	0.25	33.6
Sodium dihydrogen phosphate	0.2	535.5
Glucose-6-phosphate	0.2	26.3
Nicotinamide adenine dinucleotide phosphate disodium salt	0.04	105
S9 fraction		315
	Total:	**1050**

3.9.6 *Preparation of aliquots of the cryogenic culture*

Cryogenic cultures, purchased from Xenometrix, were reconstituted and used to prepare aliquots. For that, a cryogenic culture, retrieved from the -80 °C freezer, was thawed for 5 minutes at room temperature, before 200 µL growth medium and 30 µL glycerol was added. After resuspension by pipetting up and down and vortexing, the bacteria were distributed in 10 µL aliquots to microcentrifuge tubes. These tubes were labelled and stored at -80 °C.

3.9.7 *Procedure*

The Ames testing procedure extends over a period of 4-5 days and can be divided into 1) preparation of the overnight culture, 2) exposure and incubation and 3) counting of the revertant wells.

3.9.7.1 Day 1 – Preparation of the Overnight Culture

To cultivate the bacteria 10 mL growth medium was transferred to a 50 mL PE tube and 10 µL of an ampicillin solution [50 mg/mL] as well as 10 µL of the bacterial cryo-culture was added. The PE tube was labelled with the respective strain and incubated overnight in the orbital shaker at 37 °C and 150 rpm at an angle of about 45 °. As a sterility control a PE tube containing only growth medium was used.

Bacterial growth was followed by measuring the optical density. If an OD_{600} of 2-2.5 was reached, the bacteria were retrieved from the incubator to continue with the exposure to test substances/samples.

3.9.7.2 Day 2 – Exposure to the test substances

The miniaturized Ames test was carried out in 24-well format. On each plate, a negative control (C-), and if not tested with spiked medium, also a positive control (C+) was applied (spiked negative controls served as positive control if a parallel spike test was performed). As each sample or control was applied in triplicates, this leads to a test capacity of 6 or 7 different samples or sample dilutions on each 24-well plate. In each well e10 µL DMSO (negative control), diluted spike (positive control) or testing compound was applied.

Further, an exposure mix was prepared, with slightly different composition depending on the testing condition. The different mixes are described in table 14. To tests for inhibitory effects induced by sample treatment, a spiking test was run in parallel by adding 5 µL of spike solution to the exposure medium. 240 µL of the exposure mix was added to each well of the 24-well plate. The plates were incubated for 90 minutes at 37 °C and 250 rpm (2.5 cm amplitude).

table 14: composition of exposure mix for one 24-well plate for different testing conditions

	TA98 -S9	TA98 +S9	TA100 -S9	TA100 +S9
Exposure medium	6.3 mL	5.25 mL	6.65 mL	5.6 mL
S9-Mix	-	1.05 mL	-	1.05 mL
Bacterial suspension	0.7 mL	0.7 mL	0.35 mL	0.35 mL

After incubation 2.6 mL of reverse indicator medium was added to each well. With an 8-channel pipette, the mixture was transferred to 384-well plates, distributing 50 µL in each well. The sample application scheme for 24- and 384-well plates is shown in figure 2. Each 24-well plate results in three 384 -well plates, where one section of 48 wells corresponds to one original well on the 24-well plate (i.e. one measurement). The plates were placed in an incubation bag (to prevent evaporation) and incubated for 48 – 72 hours at 37 °C.

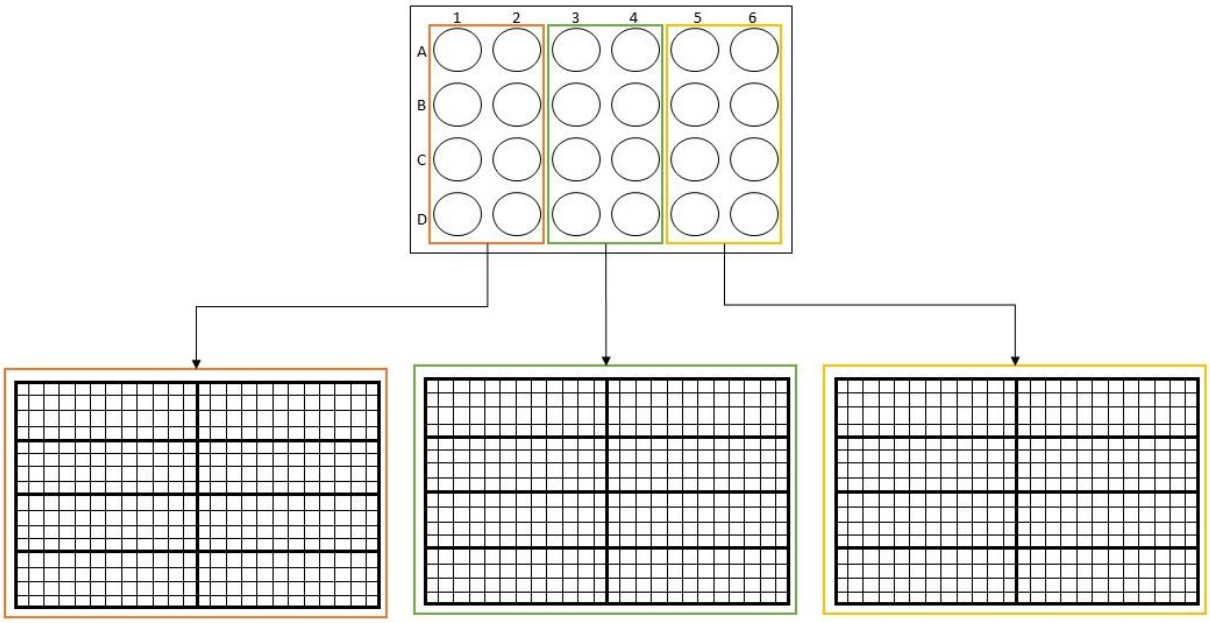


figure 2: scheme for the transfer of the samples from a 24-well plate to three 384-well plates

3.9.7.3 Day 4/5 – Plate counting

To evaluate the testing results, the number of positive wells in each 48-well section was counted. Each well which is yellow or differs from the original purple color of the indicator medium was counted as a positive well.

Samples were scored by comparing their response to the level of background mutations in the negative control. For that a baseline was calculated based on the revertant wells of the negative control according to the formula in equation 1. If the baseline was ≤1, it was set to 1.

A sample scored a positive result, if the mean revertant count of the sample was ≥2-fold higher than the baseline.

equation 1: formula to calculate the baseline; m= mean, sd= standard deviation

$$baseline = m + sd$$

To evaluate the inhibitory activity of the samples, the revertant wells of the spiked samples and the spiked negative control were scored. The spike recovery was calculated by dividing the arithmetic mean of the spiked sample revertants by the arithmetic mean of the spiked negative control revertants. A threshold of 60 % spike recovery was defined. If the sample showed ≤60 % spike recovery, the sample was considered inhibitory in the Ames test.

3.10 Agar-plate based Ames Test

3.10.1 *Media*

To test samples in the agar-plate-based format of the Ames test the media and solutions prepared for the miniaturized Ames test were used. Only the reverse indicator medium was replaced by a solid medium which does not contain indicator solution, but Agar agar instead. The composition of this medium is shown in table 15. The compounds of solution A were dissolved in 600 mL ultrapure water and the D-glucose of solution B was dissolved in 200 mL ultrapure water. Both solutions were autoclaved for 15 minutes at 120 °C. After cooling to 60 °C the solutions were combined and stored at 60 °C to keep the medium liquid. Directly before use the medium was tempered to 45 °C and 17 mL biotin solution (0.5 mM) was added.

table 15: composition of the solid medium for the Ames test in Petri dishes

	Compound	**[g]**
Solution A	Magnesium sulfate heptahydrate	0.18
	Citric acid monohydrate	1.7
	Dipotassium hydrogenorthophosphate	8.7
	Sodium ammonium hydrogen phosphate tetrahydrate	3.0
	Agar agar	12
Solution B	D-glucose	3.5

3.10.2 *Procedure*

To produce comparable results to the miniaturized Ames test, the plate-based assay was done the same way as the miniaturized Ames test up to the exposure step in 24-well plates (see 3.9.6 Preparation of aliquots of the cryogenic culture

Cryogenic cultures, purchased from Xenometrix, were reconstituted and used to prepare aliquots. For that, a cryogenic culture, retrieved from the -80 °C freezer, was thawed for 5 minutes at room temperature, before 200 µL growth medium and 30 µL glycerol was added. After resuspension by pipetting up and down and vortexing, the bacteria were distributed in 10 µL aliquots to microcentrifuge tubes. These tubes were labelled and stored at -80 °C.

Procedure). After the exposure the procedure differed from the miniaturized Ames test.

250 µL exposure mix (in each well of the 24-well plate) was transferred in a Petri dish and covered with about 20-30 mL of the solid Ames medium. After hardening the plates were incubated at 37 °C for 48 – 72 hours. Afterwards, the revertant colonies on each agar plate were counted. The evaluation was identical to the miniaturized Ames test.

4 Results and Discussion

This thesis is one part of the results of the project “Polycycle” of the OFI. Some of the experiments base on previous results and the provided sample material was mostly provided by industry partners of the project. Some of the results are already published by Mayrhofer et al. In this publication the DNA-reactive effects in many recycled polyolefin samples are shown but the source of the substances and the identification of the substances was not possible yet (Mayrhofer et al., 2023).

The results of this thesis can be divided into two main parts.

In the first part, the experiments were done with a post-consumer recycling material which already showed really high mutagenic activity. To prove the results of the tests and to identify the physiochemical properties of the mutagenic substance(s), different experiments were carried out.

The tests of 4.1, 0 and 4.3 are carried out with extracts of a postconsumer recycling LDPE material shown in the right part of figure 3. In the left part of the figure the input material of the recycling stream is shown.

figure 3: sample material of the fractionation; input (left) and output (right) of the recycling stream.

The focus of the second part is on the discovery of the source of the mutagenic signal. This was done by the screening of own samples and well-defined samples from the industry. The focus in this part is on the impact of different types of printing ink and the influence of changes in process parameters.

4.1 General experiments for testing the right test set-up

To narrow the scope of the experiments, the mutagenicity testing was limited to strain TA98 with metabolic activation (S9) and the Ames MPF assay. This methodological decision was supported by the results of the preliminary experiments described in Sections 4.1.1 and 4.1.2. Due to the variability in detection limits, the focus was initially placed on samples showing the strongest mutagenic responses.

4.1.1 *Comparison in different testing conditions*

Initially, the sample was tested under all four standard conditions in the Ames MPF assay: TA98 and TA100, each with and without metabolic activation (S9). figure 4 illustrates the lowest effective concentration (LEC) for each condition. The LEC is defined as the highest dilution factor at which a positive mutagenic response was still observed.

No DNA-reactive activity was detected in TA98 without metabolic activation. In contrast, TA98 with metabolic activation showed positive responses up to a 1:100 dilution for migrate 1 and up to a 1:316.2 dilution for migrate 2. For TA100 without metabolic activation, mutagenic activity was observed in the undiluted sample of migrate 1 and at a 1:3.162 dilution in migrate 2. In TA100 with metabolic activation, positive responses were seen up to a 1:10 dilution for both migrates.

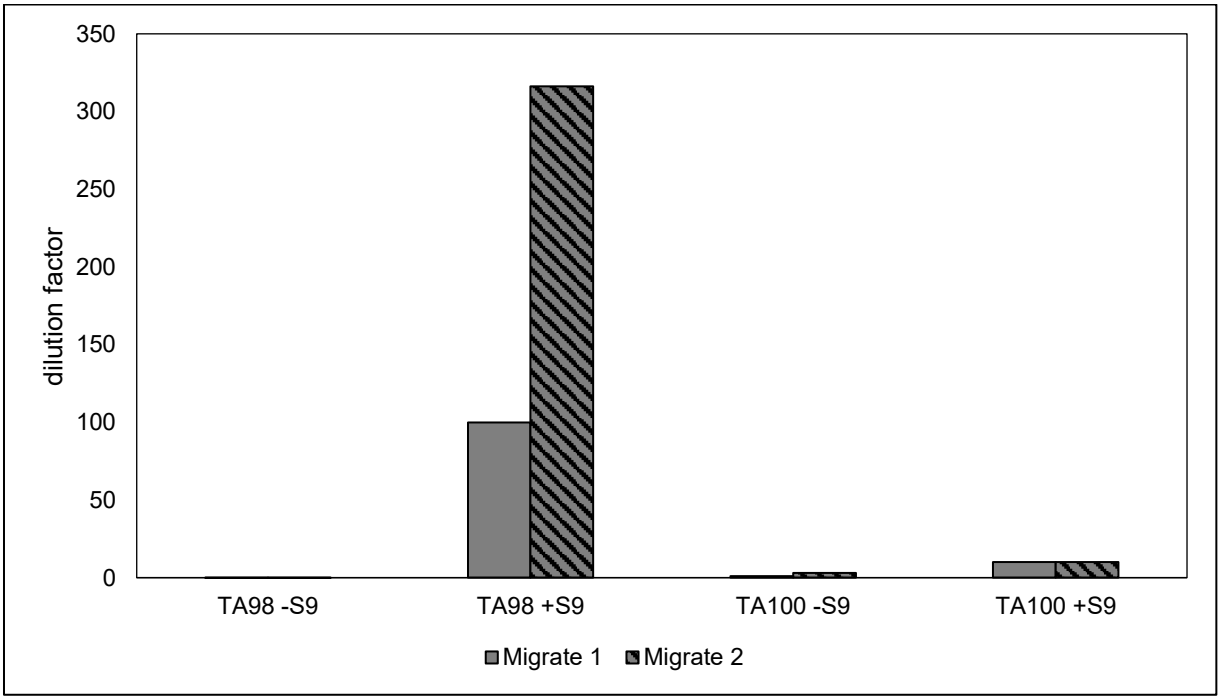


figure 4: lowest effective concentration of the DNA-reactive activity tested in Ames MPF TA98 or TA100 with our without metabolic actiabtion (S9) of the extract of a recycled plastics sample

The results showed that the strongest DNA-reactive signal was observed in the TA98 strain with metabolic activation. Based on this finding, all further experiments were conducted under these conditions. TA98 is commonly used to detect substances that induce frameshift

mutations. The requirement of metabolic activation indicates that the parent compound itself is not directly DNA-reactive, but its metabolites are. This is a plausible finding, as directly DNA-reactive substances are often highly reactive and are likely degraded during the recycling process. In contrast, substances that require metabolic activation tend to be more stable and may persist through recycling. An example of such compounds is polycyclic aromatic hydrocarbons (PAHs), which exhibit high genotoxic potential after bioactivation.

4.1.2 *Comparison of different Ames test variants*

To exclude the possibility that the observed effects were caused by biochemical interference or pH changes in the absence of bacterial growth, a comparison between the miniaturized Ames test (Ames MPF) and the traditional plate-based Ames test was conducted. As these two methods rely on different endpoints - counting of revertant colonies on agar plates versus detection of reverted wells via colorimetric readout - the likelihood of false-positive results due to non-mutagenic interference is minimized.

The sample was tested in a serial dilution, and the results from both assay formats are presented in figure 5. Comparable signal intensities were observed in both systems, supporting the suitability of the Ames MPF format for testing recycled material extracts.

A clear dose-response relationship was observed in both assays, particularly at higher concentrations. However, the dose-response curve exhibited a flattening or decline at the highest concentrations, which can be attributed to inhibitory effects of certain sample components. These inhibitory effects may mask the DNA-reactive signal at high doses.

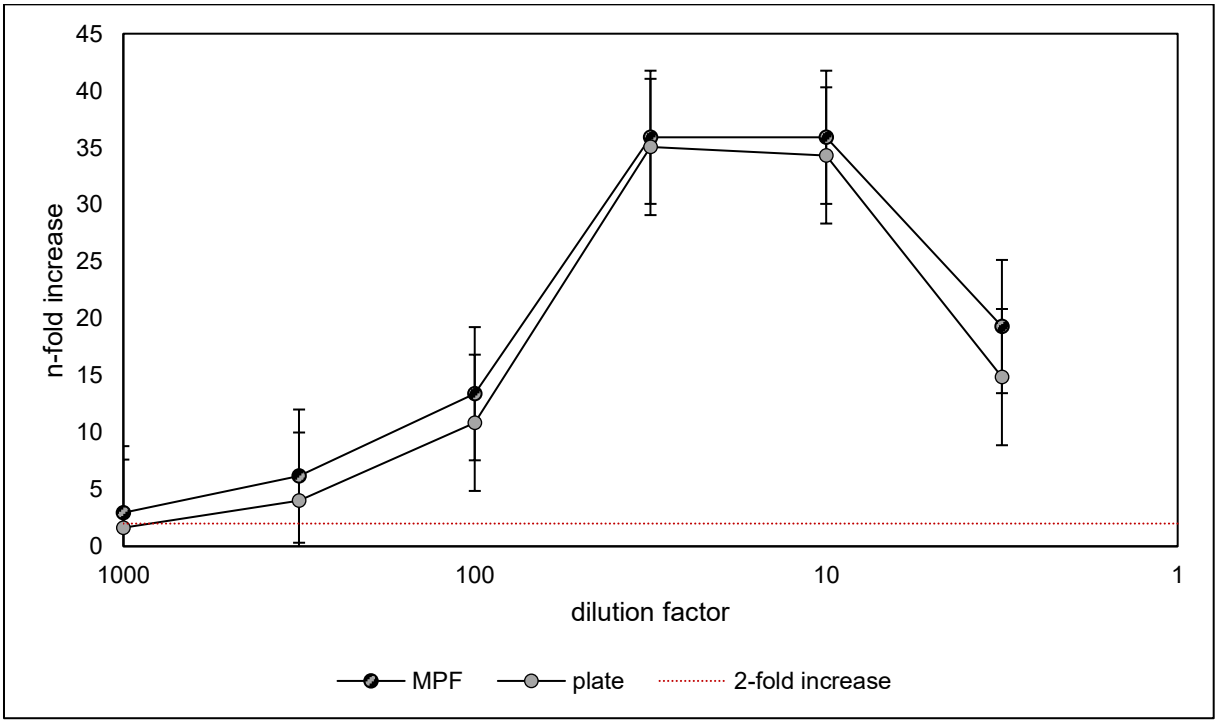


figure 5: comparison of the DNA-reactive activity of a recycled plastics sample in a semi-logarithmic serial dilution shown in the Ames MPF and plate-Ames in TA98 + S9

4.2 Migration Kinetics

This experiment compares the influence of different migration durations and solvents on the extractability of substances from the sample. Migration times ranged from 0 hours—defined as the immediate exposure of the material to the solvent—to 10 days at 60 °C. Two solvents were used: distilled water and 95% ethanol. To assess the total migration, the dry residue of each extract was determined via rotary evaporation, following the procedure outlined in DIN EN 1186-3:2022-10.

figure 6 presents the results of the experiment. The bars represent the percentage of total dry residue, normalized to the ethanol (95%) extract after 10 days, which is set as 100%. The superimposed dots indicate the lowest effective concentration (LEC) of the migrates in the Ames MPF assay.

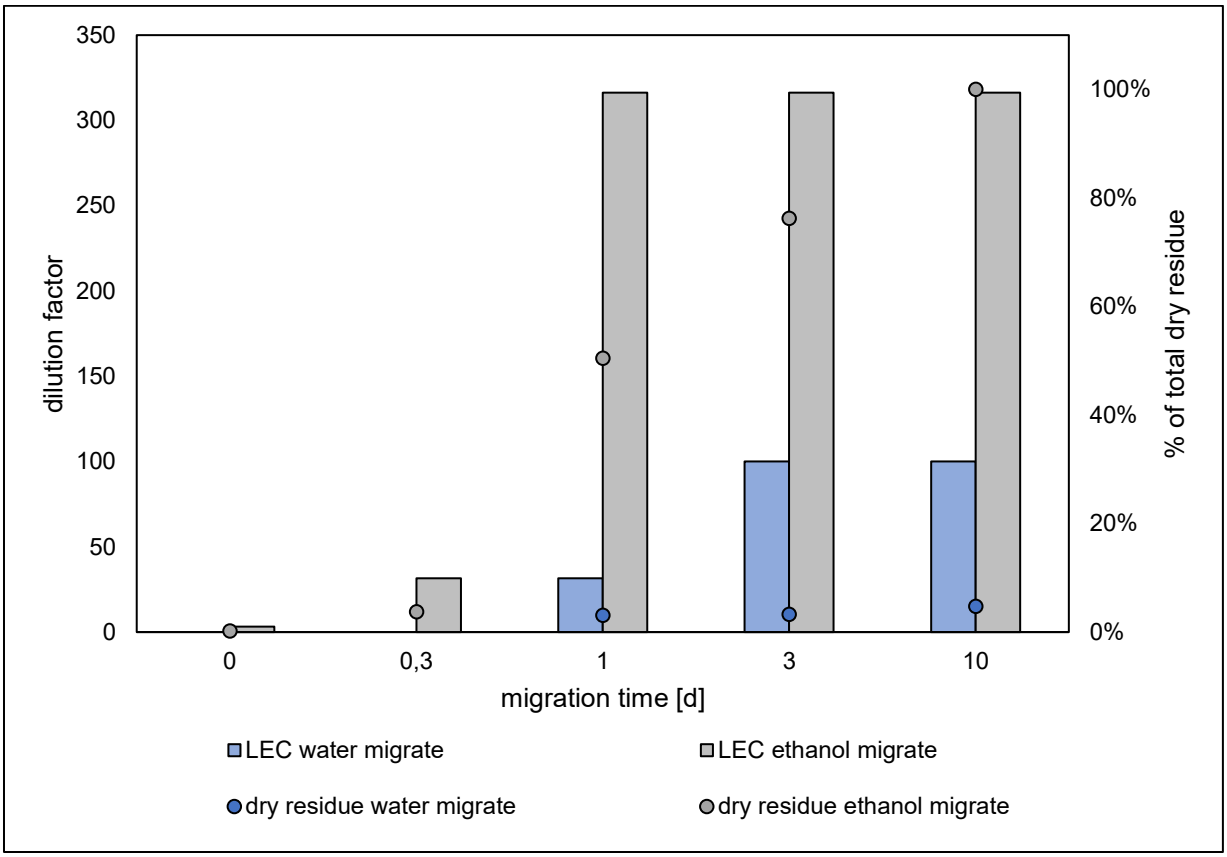


figure 6: comparison of the dry residue and the lowest effective concentration (LEC) in TA98+SA9 of a recycled plastics sample of one tested recycled material sample in different migration time (spilling, 8 hours, 1,3 and 10 days) and solvent (distilled water and 95% ethanol)

The results show that the amount of dry residue differs significantly depending on the solvent used, but also that migration time influences the total migration. The dry residues of the water-based migrates are considerably lower than those of the ethanol-based ones.

Regarding genotoxicity, the lowest effective concentrations (LECs) demonstrate that the ethanol migrates exhibit significantly higher mutagenic activity than the water migrates.

Notably, the LEC for the ethanol migrate reaches a dilution factor of 316.2 after just one day of migration at 60 °C, and this value remains constant over the entire migration period. In contrast, the water migrates do not reach this level of DNA-reactive signal under the same conditions.

These differences can be explained by the chemical properties of the polymer and the nature of the interaction between the simulant and the material. Polyethylene consists of apolar hydrocarbon chains, making it more susceptible to interaction with organic, apolar solvents such as ethanol than with polar solvents like water. Ethanol can partially swell or soften the polymer surface, thereby increasing the effective surface area available for migration—a key factor in migration kinetics (Sperling, 2005).

4.3 Fractionation experiments

As the results of the nontarget screening showed, a big number of different substances is present in the extracts of the samples. To lower the variety of substances a solid phase extraction with a fractionation is done. The aim of the experiment is to separate the different substances by their chemical properties which can also help in the identification of the substances or their groups.

4.3.1 *Fractionation 1*

The first fractionation was done on an Oasis HLB column, this column is a universal column and there are interactions of hydrophile and lipophile compounds with the solid phase. All the fractions had been tested to see the direct DNA-reactive activity of each of them.

In figure 7**Fehler! Verweisquelle konnte nicht gefunden werden.** the results of the Ames test of the different fractions were shown. It was shown that the flow through of the loading-step still contains some mutagenic substances, the washing water shows a mutagenic signal only in the undiluted sample. The first elution-fractions (50% MeOH, 100% MeOH) showed a mutagenic signal up to the dilution with 0,1% sample but the 100% MeOH fraction also includes some inhibitory substances. In the fraction of mixture from dichloromethane and MeOH the mutagenic effect was also present till the 3,16% sample dilution step. So, this showed, that the DNA-reactive signal was lower than in the fractions before. The fraction with pure dichloromethane showed only in the undiluted sample a mutagenic activity.

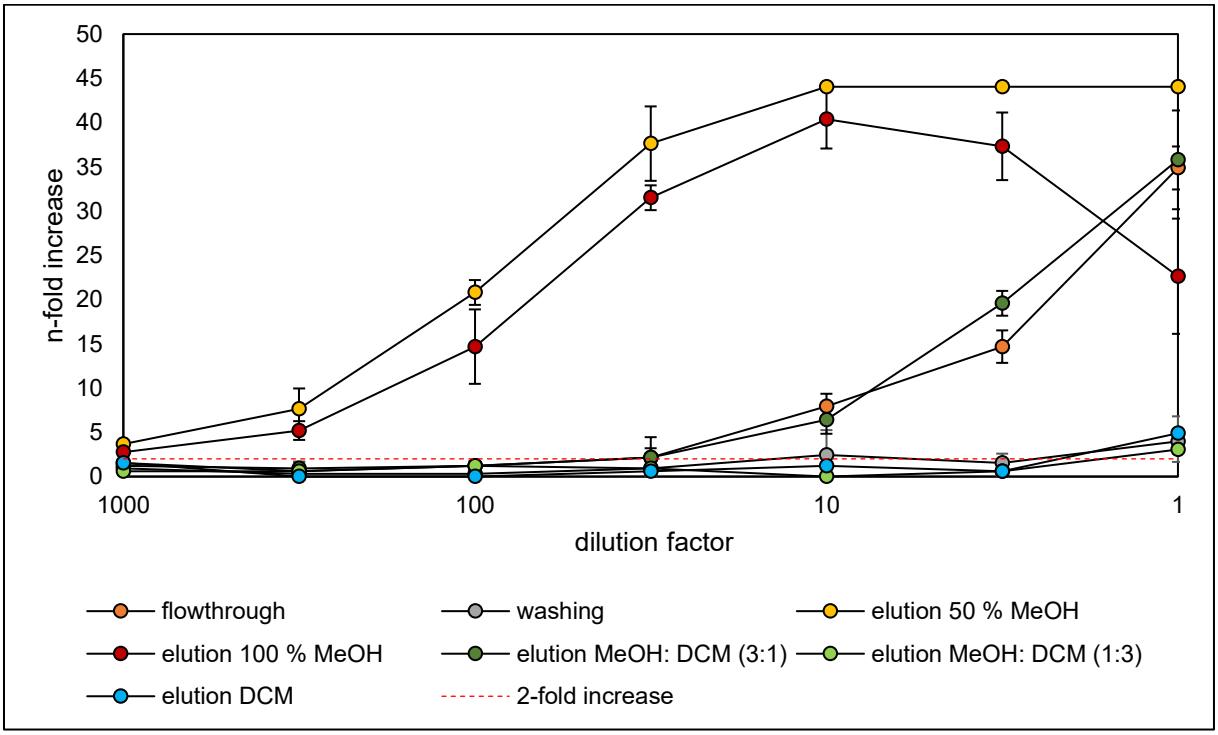


figure 7: Results of the fractionation of a recycled sample on a HLB column (Oasis): the DNA-reactive signal in Ames MPF TA98+S9 of each fraction is shown in a semi-logarithmic serial dilution.

The DNA-reactive effect of the flow through can have different reasons, on the one side it is possible, that the column is completely loaded and no free binding spots are available. It is also possible that the substances which resume in the flow through have other chemical properties and do not bind on the column. This would be for example small molecules with a high polarity.

The shrinking of the DNA-reactive effect in the elution steps does not have to be associated with the change in the composition of the solvent. It can also be assumed, that the substance is washing out over the time. At it is shown that the substance does not bind that strong to the solid phase since the elution with 50% MeOH already shows a high DNA-reactive activity.

4.3.2 *Fractionation 2*

In the second fractionation the aim was to improve the elution steps since the elution steps of the first fractionation showed high mutagenic activity or just low and no in-betweens. The fractionation was done on a Biotage HLB-column again.

In figure 8, the results of the fractionation are shown. In the first four fractions (12,5%, 25%, 37,5% and 50% MeOH) the undiluted sample always reaches the 40-fold increase to the baseline and the LEC is in the area of under 1% sample in the dilution. The fractions of 62,5% and 75% MeOH show a slope of the mutagenic signal, which indicates an inhibitory signal in the fraction, in these fractions also a LEC of 1% sample is reached. In the last two fractions (87,5% and 100% MeOH) the mutagenic signal in the undiluted sample is about 20fold increase above the baseline and the LEC reaches to 1% or 10% sample.

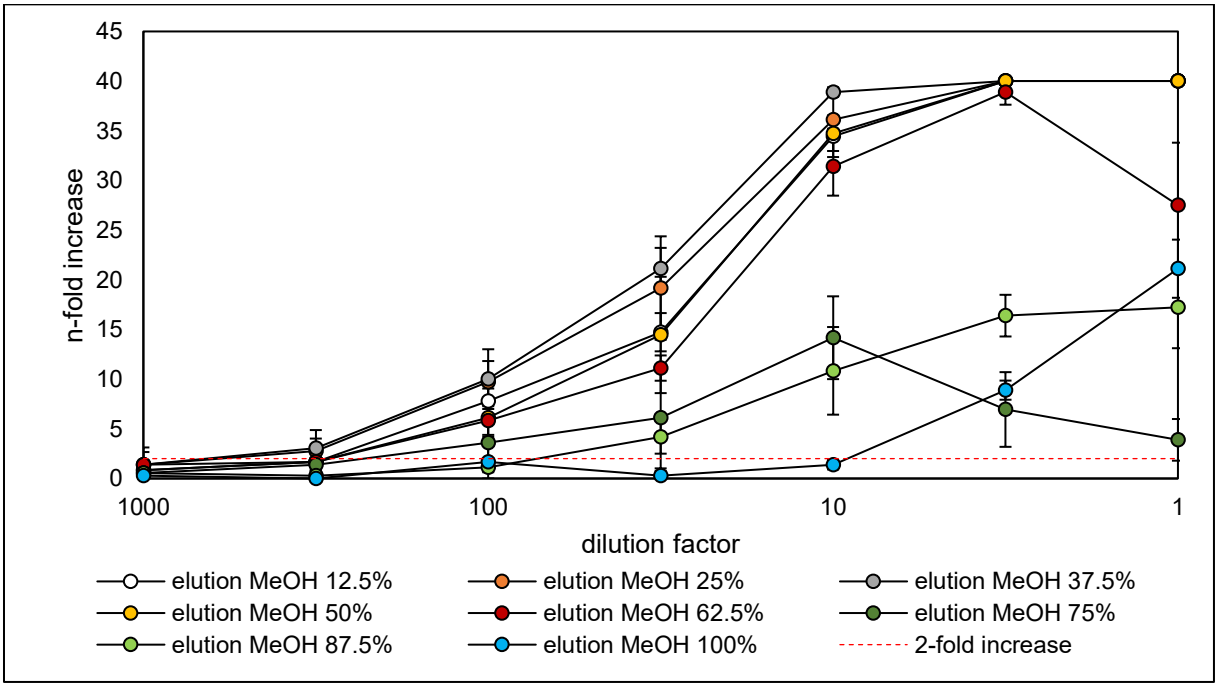


figure 8: Results of the fractionation of a recycled sample on a HLB column (Oasis): the DNA-reactive activity in Ames MPF TA98+S9 of each elution fraction is shown in a semi-logarithmic serial dilution.

4.3.3 *Fractionation 3*

In the next step the fractionation was repeated with a Isolute C18 column (Biotage). This column is not as broadly applicable as the HLB column, which makes the properties of the molecules differentiated even better.

In figure 9 the results of the Ames test are shown in a serial dilution. It is shown that all of the fractions have a DNA-reactive signal. The flowthrough fraction of the loading has a LEC of 31, the washing step a LEC of 3,1 and the elution a LEC of 316,2. So these results confirm the assumption, that there must be different substance groups with different physiochemical properties in the recycling sample.

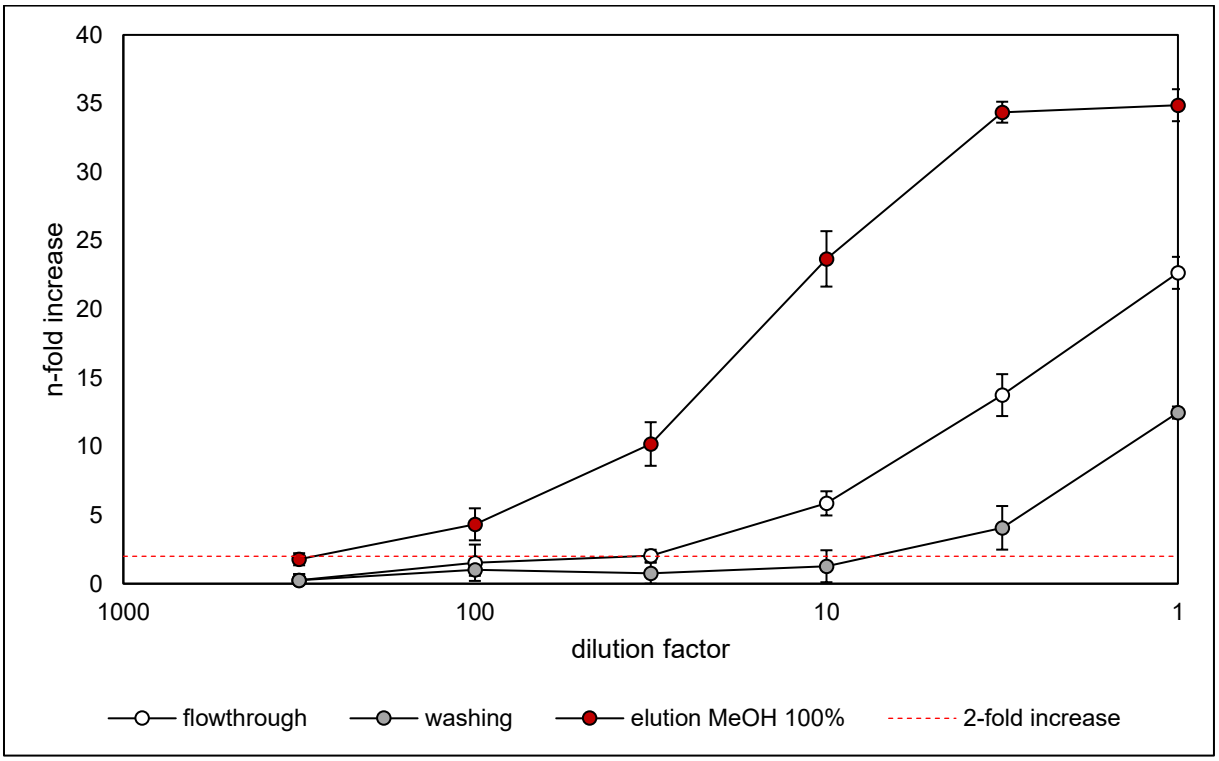


figure 9: Results of the fractionation of a recycled sample on an Isolute C18-column (Biotage): the DNA-reactive activity in Ames MPF TA98+S9 of each fraction is shown in a semi-logarithmic serial dilution.

If the results shown in figure 7 (HLB column) and the results shown in figure 9 (C18 column) are compared, it becomes evident that a signal is observed in the flow-through fraction in both cases. The LEC of this fraction is approximately 31.6. However, if the results of the washing step are compared, the sample on the C18 column shows a DNA-reactive activity up to a 3.16-fold dilution. This indicates the presence of substances with a really low interaction to the stationary phase. A comparison of the elution fraction based on the LEC is not sufficient in this case, since the elution on the HLB-column was done according to a different protocol (more steps) than that used for the C18 column.

4.3.4 *Fractionation 4*

To identify if the high number of substances is responsible for the signal in the flow through (due to an overload of the column) or if there are substances with different physiochemical properties a fractionation of the actual fraction was done. So, the loading of the original sample was fractionated on another column and the elution-fraction of the sample was also fractionated another time, as shown in the upper part of figure 10. The results of the experiment are shown in the lower part of figure 10, it is shown, that the main signal of the previous loading or elution fraction remain in the same fraction in the second fractionation. Since the experiment was performed on an C18 column it is possible to say, there are at least two substance-groups in the sample with different physiochemical properties.

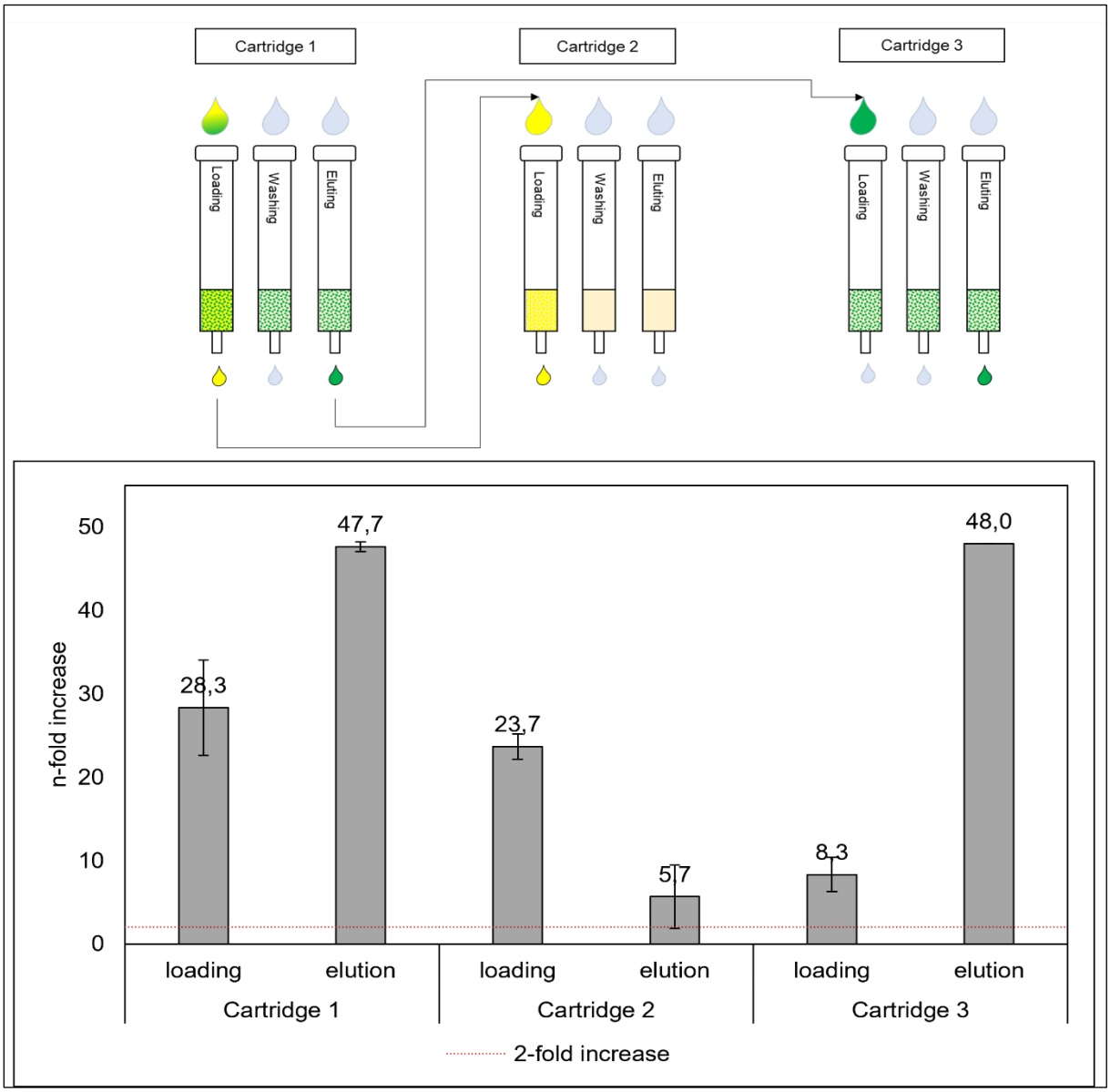


figure 10: Results of the fractionation of fractions of a recycled sample on an Isolute C18-column (Biotage): the DNA-reactive signal in Ames MPF TA98+S9 is shown in the undiluted sample

4.3.5 *Fractionation 5*

The next step was to see the impact of the pH on the substances in the sample. The sample was loaded on a C18-column with a pH 3, unbuffered, pH 7 and pH 11.3. In figure 11 the results of this experiment are shown. The n-fold increase of the Ames-Test results as well as the spike recovery as an indicator for the inhibitory signal of the fraction are shown. Since the n-fold increase is limited by 48 cause of the test setup, the results with the most interesting information are the values of the flow through-fraction. It is shown that the direct-DNA reactive signal is decreasing with increasing pH-value. At a pH 3 the n-fold increase of the flow through is about 19, compared to the pH 7 and pH 11.3, where the value is at about 6. It is shown, that a part of the effect might be caused by substances which cannot bind, regardless the pH-value, on the column.

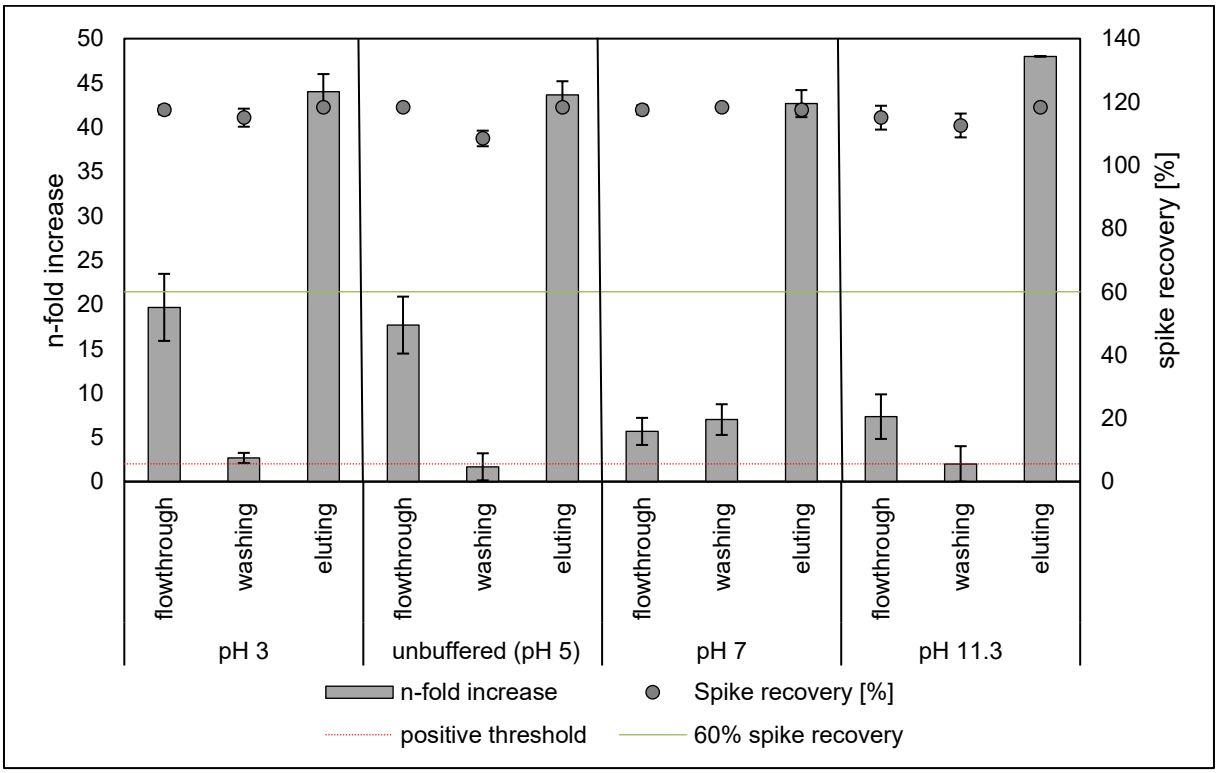


figure 11: Results of testing the DNA-reactive activity in Ames MPF TA98+S9 of the DMSO-concentrates of the flow through, washing step and elution fraction of a recycling sample in pH 3, unbuffered (pH 5), pH 7 and pH 11.3.

According to the literature, the observed pH-dependent changes in the flow-through and the corresponding n-fold increase can be attributed to the presence of substance groups such as primary aromatic amines (PAAs), which undergo charge alterations depending on the pH. At a pH of 3, PAAs are predominantly protonated and thus carry a positive charge, resulting in poor retention on the C18 column. As the pH increases, deprotonation occurs, leading to a reduced positive charge and consequently stronger interactions with the hydrophobic stationary phase of the C18 column.

The results of the fractionation highlight the complex composition of the recycling samples, which contain a diverse mixture of chemically different mutagenic substances. The use of solid-phase extraction on different columns (HLB and C18) and varying elution and pH conditions enabled the separation and partial characterization of these substances based on their physicochemical properties.

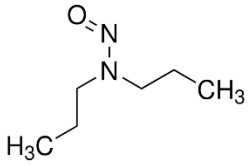
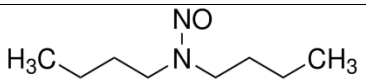
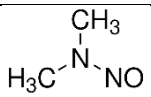
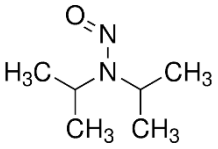
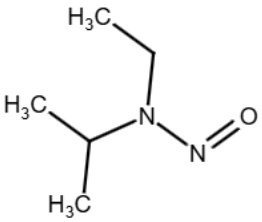
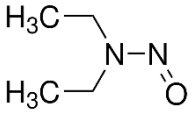
The presence of mutagenic activity in both the flow-through and multiple elution fractions indicates that a simple extraction or purification step is insufficient to isolate or remove all mutagenic compounds. Some substances bind weakly or not at all due to polarity or molecular size, while others show stronger retention.

The pH-dependent binding behavior, especially of primary aromatic amines, demonstrates that changes in molecular charge significantly influence interactions with the stationary phase and, consequently, separation efficiency. Protonation at low pH reduces binding affinity, leading to higher mutagenicity in the flow-through fraction, while deprotonation at higher pH promotes stronger binding.

4.4 Analysis of N-Nitrosamines

In the GC-MS Screening the fractions of a fractionation of the recycled plastic sample were tested on the substances in table 16.

table 16: compounds of the N-nitrosamines Screening with CAS number, structure, LOD (limit of detection) and LOQ (limit of quantification)

Compound	Structure	CAS Nr.	LOD [ng]	LOQ [ng]
NDPA		621-64-7	2.25	8.79
NDBA		924-16-3	2.53	8.84
NDMA		62-75-9	1.57	5.58
NDiPA		601-77-4	2.7	9.41
NEiPA		16339-04-1	2.36	8.26
NDEA		55-18-5	2.47	8.62

In table 17 the results of the screening are shown. It is shown, that only NDPA and NDBA are present in the sample and the fractionation of the sample works pretty well, since in the flowthrough sample no substances are detectable. This shows, that all of the *N*-nitrosamines are captured by the column.

*table 17: results of the N-Nitrosamine screening of the sample and their fractions, * marked values are out of the calibration but measured*

	NDPA	**NDBA**	**NDMA**	**NDiPA**	**NEiPA**	**NDEA**
unfractionated	399.146*	144.858*	n.d.	n.d.	n.d.	n.d.
flowthrough	n.d.	n.d.	n.d.	<LOD	n.d.	n.d.
Elution 0.3	317.447*	129.839*	n.d.	<LOD	n.d.	n.d.
Elution 3	987.095*	918.475*	n.d.	<LOD	n.d.	n.d.

The detected substances of this GC-MS Screening were also tested in the Ames-MPF – NDPA and NDBA. In in silico analysis via VEGA NOVA all of the N-Nitrosamines tend to act in form of point mutations, so the TA100 is the strain which would be positive in this case.

In the testing of the substances in TA98 + S9 no direct DNA-reactive effects could be seen.

These results suggest that the N-Nitrosamine are not responsible for the direct-DNA reactive effect of the samples, especially not to this intensity.

4.5 Systemical Screening of different sample types of input and output material

Since the analysis of the different fractions and the chemical analysis of these did not led to any results regarding the origin of the direct-DNA-reactive substances, a systematical screening of different fractions of the recycling process and different input materials was done. In this testing, three different pairs of input and output material of a recycling plant were tested.

The samples are migrated for 3 days at 60°C with 95% ethanol. This extract was fractioned on a HLB column as described in 3.8.

On the one side, the concentration of the primary aromatic amines was analysed by Ciboris group and on the other side the DMSO-Extracts were tested in the Ames test. The three substances with the highest concentration in the analytic were also tested as pure substances and as a mixture to see the combinatory effect of these in the Ames test.

4.5.1 *Comparison of the DNA-reactive activity*

Three different sample pairs of output and input material were tested in the Ames test. Sample pair 1 are also the output sample which were used in the fractionation experiments and pictured in figure 3. Since the recycling process always includes cleaning processes the assumption of the results for this experiment was, that the mutagenic signal after the recycling step is much lower than before.

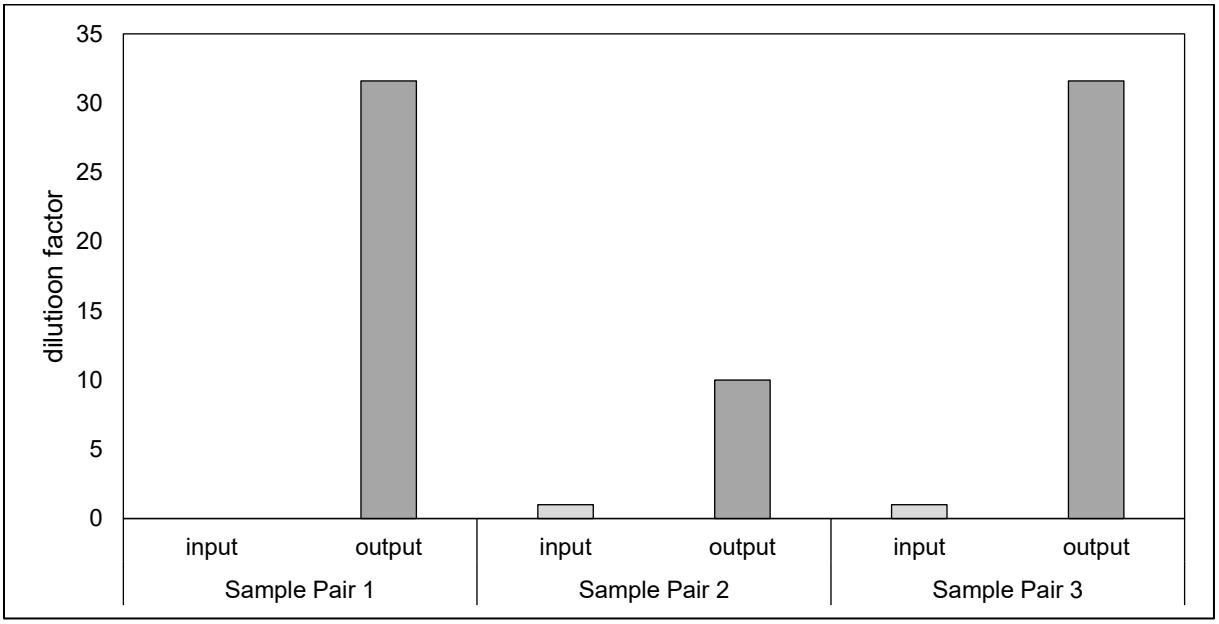


figure 12: Lowest effective concentration of testing the DNA-reactive activity in Ames MPF TA98+S9 of extracts of the input and output material samples from three different recycling material sample pairs

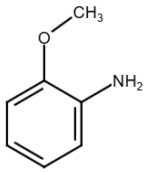
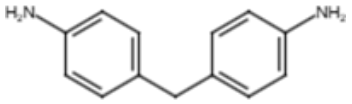
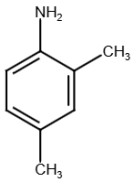
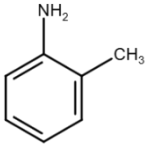
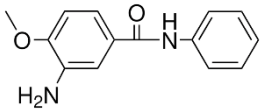
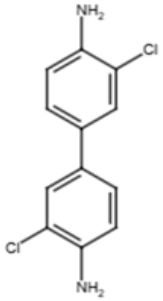
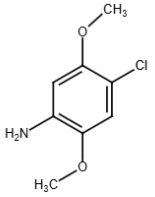
In figure 12 the LEC of the different samples are shown. It is shown, that the mutagenic activity is always much higher in the output-fractions of the sample pairs. In this experiment it was shown, that the mutagenic activity of the material increases significantly during the recycling

process but finding the source is not possible with this samples since the input material are fractions of post-consumer materials and the chain of use is not traceable.

4.5.2 *Analysis of Primary Aromatic Amines*

Since the input material consists of different printed and non-printed packaging materials an analysis of primary aromatic amines was done, since these are a typical degradation product of azo-pigments. The relevant substances are listed in article 43 in REACH.

table 18: substances of PAA-Screening by Ciboris

Compound	CAS-Number	Structure
o-Anisidine	90-04-0	
4,4'-Diaminodiphenylmethane	101-77-9	
2,4-Dimethylaniline	95-68-1	
o-Toluidine	95-53-4	
3-Amino-4-Methoxybenzanilide	120-35-4	
3-3-Dichlorobenzidine	91-94-1	
4-Chloro-2,5-Dimethoxyaniline	6358-64-1	

The results of the analysis are shown in figure 13. The plotted data is the factor of the concentration in the output material to the concentration of the input material. So, it is shown, that there is no substance listed, which is reduced during the recycling process.

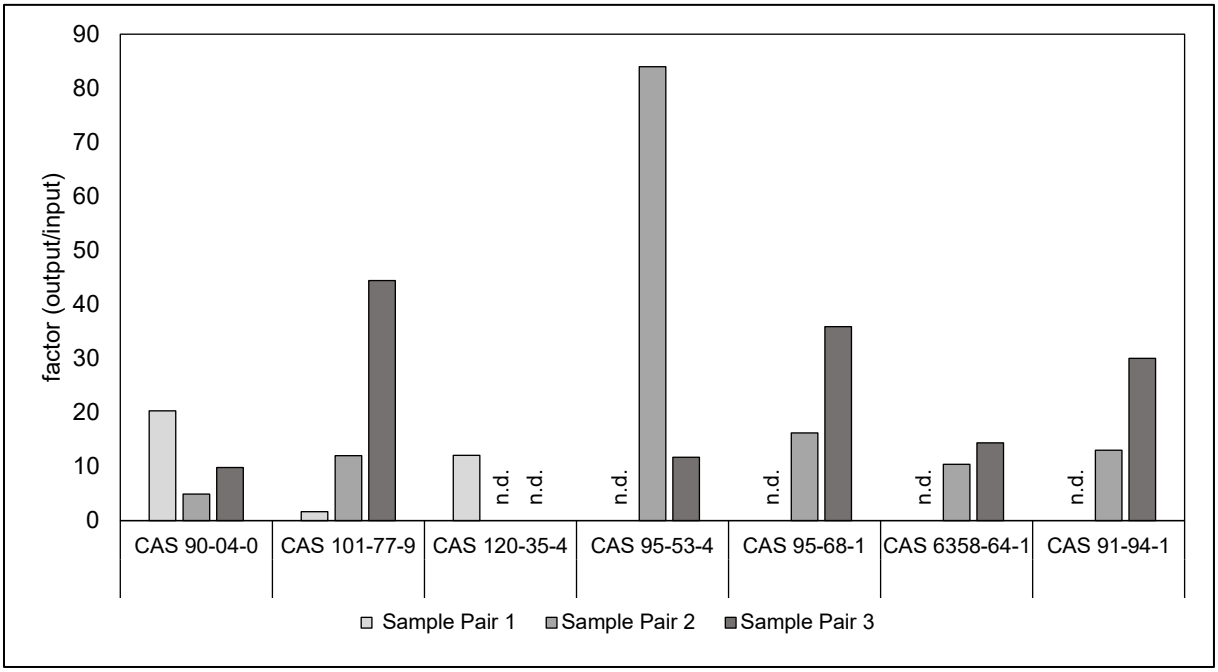


figure 13: Results of the PAA analysis shown in factor of output-concentration to input-concentration, n.d.= not detected

In table 19 the concentrations in the output fractions are listed. Since the data of DNA-reactive activity of pure substances in the Ames test from the literature are often very difficult to compare, due to different testing conditions, the substances with the highest concentration are tested in the TA98 + S9 pure but also as a mixture to check the combinatory effects.

Based on these values the three substances which are analyzed as pure substance and 1:1:1 mixture was selected. The chosen compounds are marked in a red frame.

table 19: concentration of PAAs in the output fractions. Red framed substances are further tested in Ames test. LOQ = 10 ppb, n.d.= not detected

Compound	**CAS-Number**	**Output 1**	**Output 2**	**Output 3**
o-Anisidine	CAS 90-04-0	264	49	98
4,4'-Diaminodiphenylmethane	CAS 101-77-9	150	12	444
3-Amino-4-Methoxybenzanilide	CAS 120-35-4	121	< LOQ	< LOQ
o-Toluidine	CAS 95-53-4	n.d.	84	117
2,4-Dimethylaniline	CAS 95-68-1	< LOQ	162	359
4-Chloro-2,5-Dimethoxyaniline	CAS 6358-64-1	< LOQ	104	144
3-3-Dichlorobenzidine	CAS 91-94-1	n.d.	13	30

In figure 14 the results of the testing of the pure substances are shown. The starting concentration was 1000 ppb and the serial dilution was done by a factor of 2. As marked, the sample of 4,4'-Diaminodiphenylmethane and 2,4-Dimethylaniline showed inhibitory effects in the initial concentration but in the diluted samples no inhibitory effect was seen. If you compare the measured concentrations in the samples and the results in the Ames test, it is shown, that the occurrence of these PAAs cannot be responsible for the effect seen in the samples shown in 4.5.1. Since it is known, that the effect of single substances cannot just be added to each other also the 1:1:1 mixture of these three substances was tested in the Ames test to see if there are any combinatory effects. The results of the serial dilution are shown in figure 15. The sample with 316,2 ppb of each substance showed a high inhibitory effect but already in the first dilution step to 100 ppb the spike recovery was at 100% and so no inhibition was seen. A DNA-reactive effect is just seen in the dilution step of 31,62 ppb. In this dilution, the n-fold increase was at 3.9.

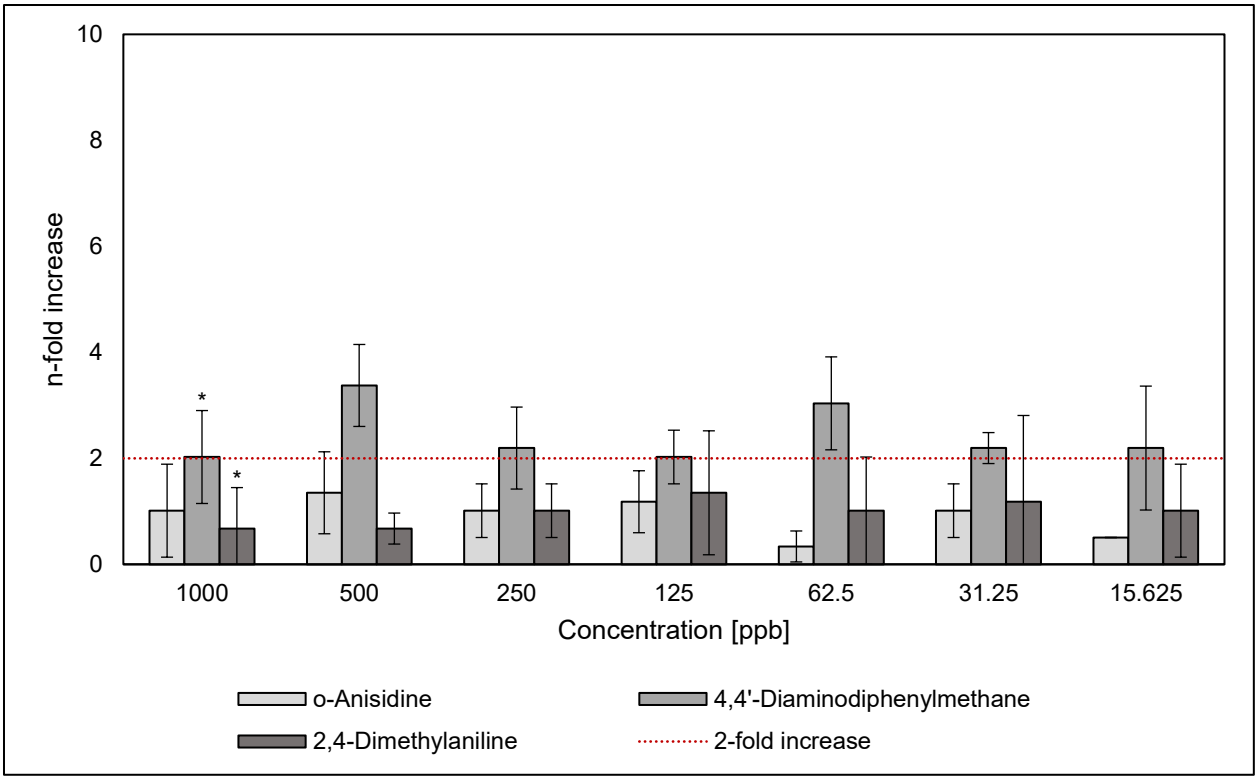


*figure 14: Results of testing DNA-reactive activity in Ames MPF TA98+S9 of o-Anisidine, 4,4'-Diaminodiphenyl-methane and 2,4-Dimethylaniline. the *-marked columns showed an inhibitory signal in the test.*

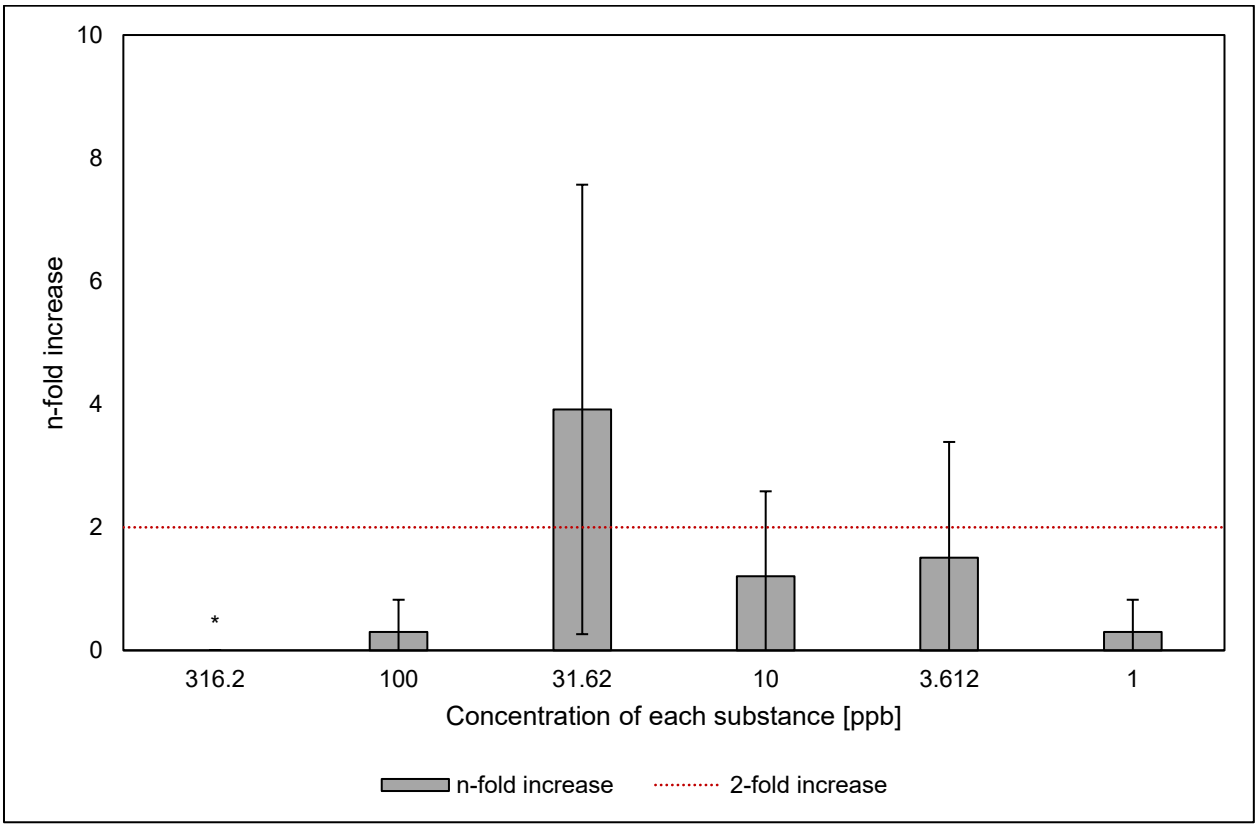


*figure 15: Results of testing DNA-reactive activity in Ames MPF TA98+S9 of a 1:1:1 mixture o-Anisidine, 4,4'-Diaminodiphenylmethane and 2,4-Dimethylaniline. the *-marked columns showed an inhibitory signal in the test*

4.6 Testing of different printed materials and printing ink components

In the project the industry partner provided different sample types of very well-defined materials. These samples were tested to exclude the impact of the consumer handling and so the risk of contamination through scalping or mold induced mycotoxins.

On the one side samples of a recycled unprinted but metallized PE-foil and the same foil but printed with a PU-based blue ink as a virgin and recycled sample were provided.

To exclude also the impact of the blue-pigment other samples with a white print were provided. These printing ink typically contains TiO_2 as a white pigment. The provided samples were printed with nitrocellulose-, polyurethane- and polyvinyl butyral- based inks to test the impact of the binder.

4.6.1 *Blue printed samples – comparison of virgin and recycled material*

In this experiment, a metallized polyethylene (PE) foil was analyzed using the Ames test. The virgin sample consisted of a blue-printed, metallized PE foil with a polyurethane (PU)-based printing ink. The recycled sample was derived from this virgin material following a mechanical recycling process. The results of the Ames test are presented in figure 16. While the virgin material exhibited no detectable mutagenic activity, the recycled sample showed a positive response, with a lowest effective concentration (LEC) below 3.125% (corresponding to a 1:32 dilution).

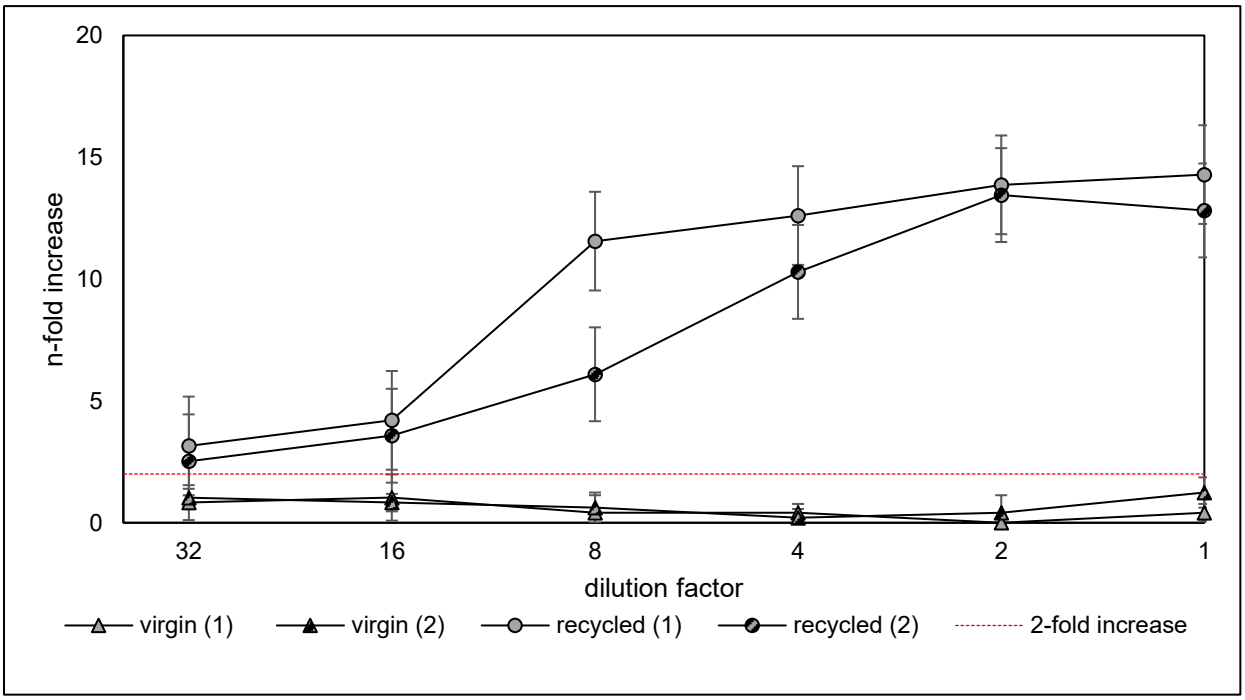


figure 16: Comparison of the results of testing DNA-reactive activity in Ames MPF TA98+S9 of extracts of virgin and the corresponding recycled material in a semi-log serial dilution and duplicate testing

Since both of the previously tested samples contained printing ink components, an additional recycled sample of unprinted but metallized PE foil was included to assess the potential contribution of the printing ink to the observed effects. The summarized results of the undiluted samples, along with a comparison between the printed and unprinted recycled foils, are presented in figure 17.

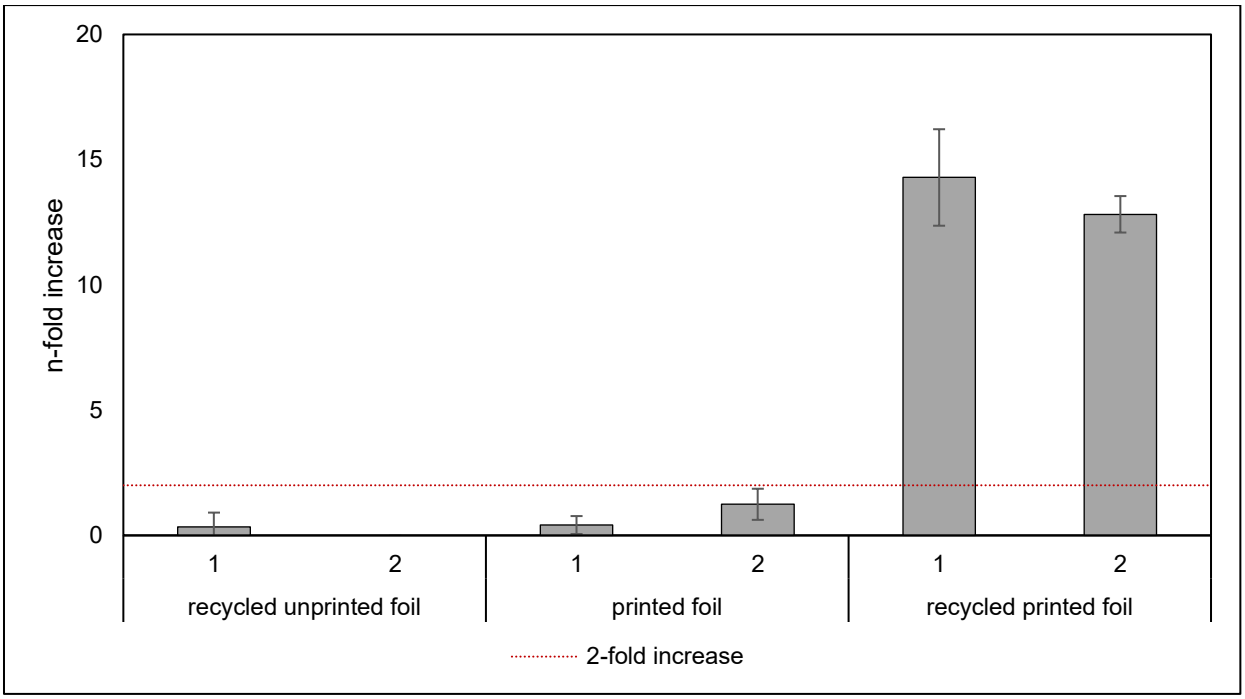


figure 17: Results of testing the DNA-reactive activity in Ames MPF TA98+S9 of extracts of the recyced unprinted foil and the virgin and recycled printed foil

The recycled, unprinted foil did not exhibit any direct DNA-reactive activity in the undiluted sample. This indicates that the components of the printing ink contribute to the mutagenic activity observed in the recycled printed material.

Since the printing ink can include many different components, as described in 2.3.3, the next steps are to test different of these components after a heating process.

4.6.2 *Testing of different binder in printing ink after the recycling process*

To evaluate the influence of different binders in the absence of organic pigments, white-printed samples were used in this experiment. White printing inks typically contain titanium dioxide (TiO_2) as the pigment. The tested binders included nitrocellulose (NC), polyurethane (PU), and polyvinyl butyral (PVB). As shown in figure 18, the samples containing PU and PVB exhibited no significant mutagenic activity, with results remaining below the 2-fold increase threshold. In contrast, the sample printed with nitrocellulose showed pronounced mutagenic activity, with a positive signal detectable down to a 3.125% sample concentration.

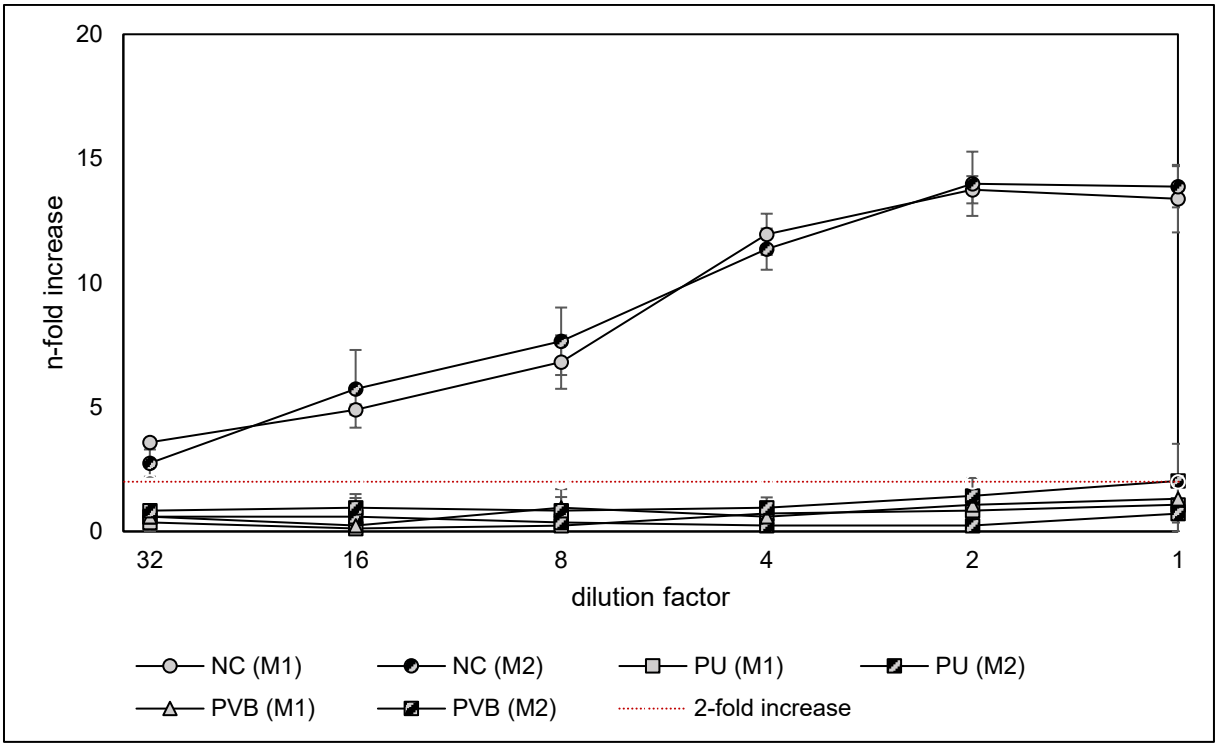


figure 18: Results of testing the DNA-reactive activity in Ames MPF TA98+S9 of extracts of different binder-types after heating at 230 °C. The results are shown in a serial dilution (1:2) in duplicate test. NC= Nitrocellulose, PU= Polyurethane, PVB= Polyvinyl butyral

These findings indicate that the thermal degradation of nitrocellulose alone, without the presence of organic pigments, can lead to the formation of mutagenic compounds. However, as discussed in section 4.6.1, positive signals were also observed with other binder systems, suggesting that nitrocellulose degradation products are not the sole contributors to the mutagenicity observed in post-consumer recyclates. To further investigate the potential contribution of other ink components, such as stabilizers, additional tests were conducted using formulations containing only nitrocellulose, as well as nitrocellulose combined with titanium dioxide.

4.6.3 *Testing the impact of TiO_2*

In the next step, sample materials were prepared using LDPE foil coated with a nitrocellulose solution, with and without the addition of titanium dioxide (TiO_2). These samples were subjected to two different thermal treatments: heating in a hydraulic press at 160 °C and heating in a muffle oven at 230 °C under a nitrogen atmosphere to simulate the oxygen-free conditions present in industrial recycling processes. The results, presented in figure 19, show the n-fold increase values for the various samples.

The first section of figure 19 includes the unheated control samples, which were tested to determine whether the raw materials exhibited any inherent mutagenic activity. Since the n-fold increase remained below the positive threshold of 2, it can be concluded that the starting materials themselves do not induce direct DNA-reactive effects.

When comparing the two thermal treatments, a clear increase in mutagenic activity is observed in the samples heated at 230 °C compared to those heated at 160 °C. Furthermore, the presence of TiO_2 appears to enhance the mutagenic response in both temperature conditions. The samples containing a mixture of nitrocellulose and TiO_2 exhibited higher n-fold increases than those with nitrocellulose alone. These findings suggest that both higher processing temperatures and the presence of TiO_2[1] can contribute to the formation of mutagenic degradation products.

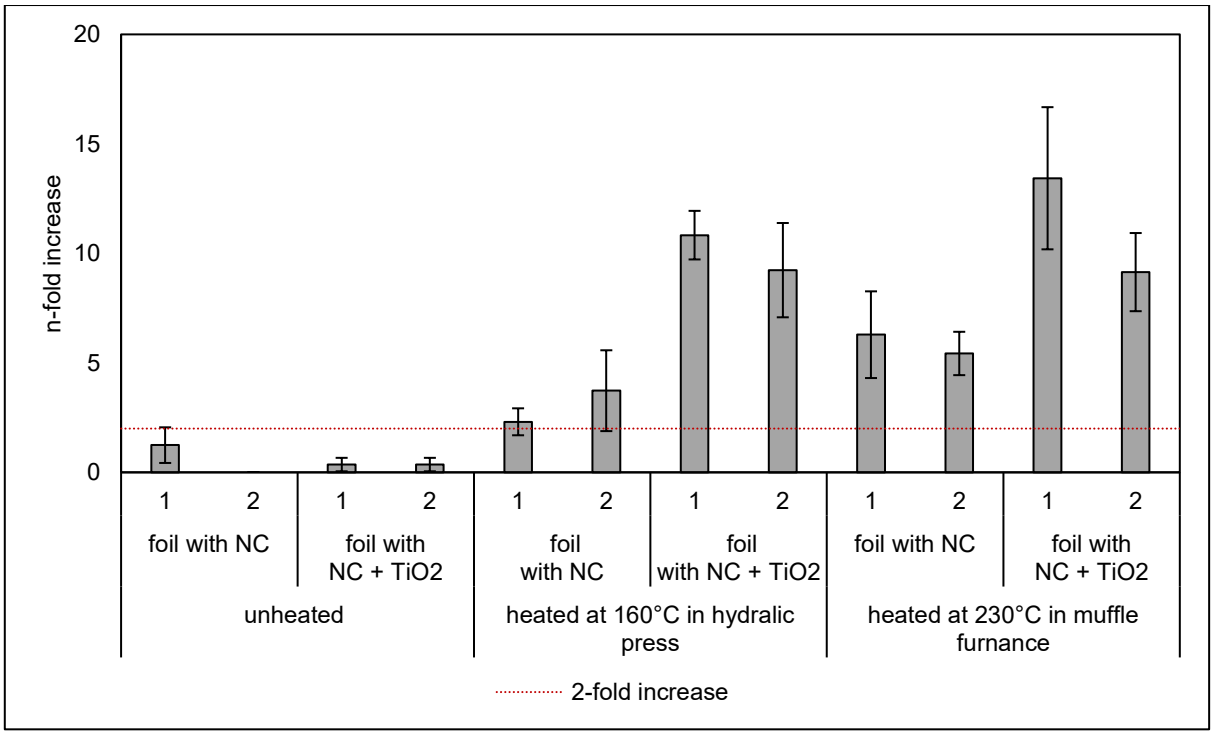


figure 19: Results of testing the DNA-reactive activity in Ames MPF TA98+S9 of extracts of the LDPE foil with nitrocellulose solution (NC) and NC and titanium dioxide (NC + TiO_2) unheated and heated at 160°C or 230°C

[1] Regarding the catalytic effect of TiO_2 in printing inks, some project partners out of the printing ink industry mentioned, that special coated TiO_2 usually is used in printing ink to inhibit this effect.

These findings suggest that the observed DNA-reactive activity is caused by thermal degradation products of nitrocellulose. It is well known that nitrocellulose can generate directly DNA-reactive compounds such as N-nitrosamines (EMA, 2020). However, N-nitrosamines typically exhibit their genotoxic activity in strain TA100, as they primarily induce base-pair substitutions rather than frameshift mutations, which are detected by strain TA98 (Thomas et al., 2024).

4.6.4 *Testing of the impact of Azopigments*

After including the binder in 4.6.3, in the next step also an azopigment was included in the testing. For this experiment the samples were prepared using the LDPE foil and a solution with azopigment (sunset yellow FCF) with and without nitrocellulose to see if the azopigment itself already shows DNA-reactive effects before and after heating in the muffle furnace at 230 °C for 20 minutes.

In figure 20 the DNA-reactive effects of the different samples are shown. The results show that the azo pigment itself shows no effects in the TA98 + S9 before heating the samples either with or without adding NC to the sample.

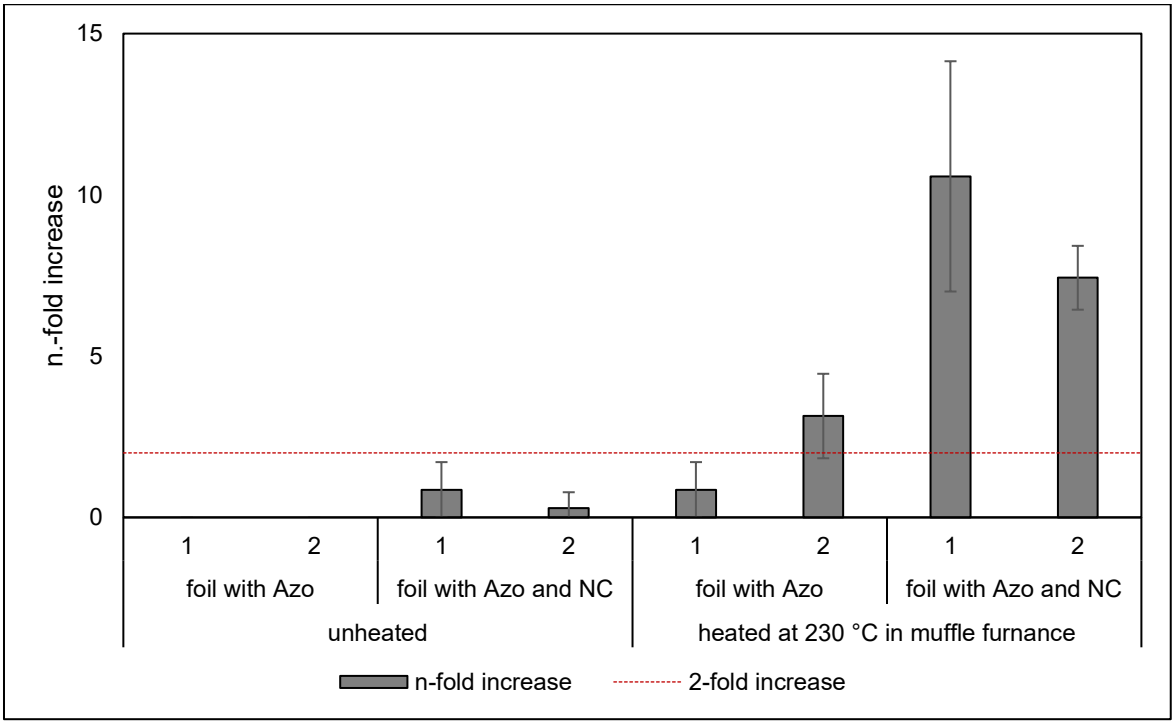


figure 20: Results of testing the DNA-reactive activity in Ames MPF TA98+S9 of extracts of the LDPE foil with azopigment (sunset yellow FCF, Abbr: Azo) and nitrocellulose solution and azopigment (NC + Azo) unheated and heated at 230°C

If it comes to heating the foil and azopigment without adding NC the results are equivocal since one of the double determinations is negative and the other positive (greater than the 2-fold increase barrier). But the samples including azo-pigment and nitrocellulose bring clearly positive results in the TA98+ S9. If the n-fold increase of the samples including azo pigments

and TiO_2 are compared, it is shown that the values are in the same height so it can be not said if the effect is only by the nitrocellulose or also the azopigment shows maybe combinatory effects after heating together with nitrocellulose.

So if sunset yellow FCF is under high heat, the structure can decompose into two primary aromatic amines – 4-aminobenzenesulfonic acid (CAS 121-57-3) and 1-amino-2-naphthol-6-sulfonic acid (CAS 5639-34-9), the structures to these decomposition are shown in figure 21. For these two substances, no Ames-data is available in the literature.

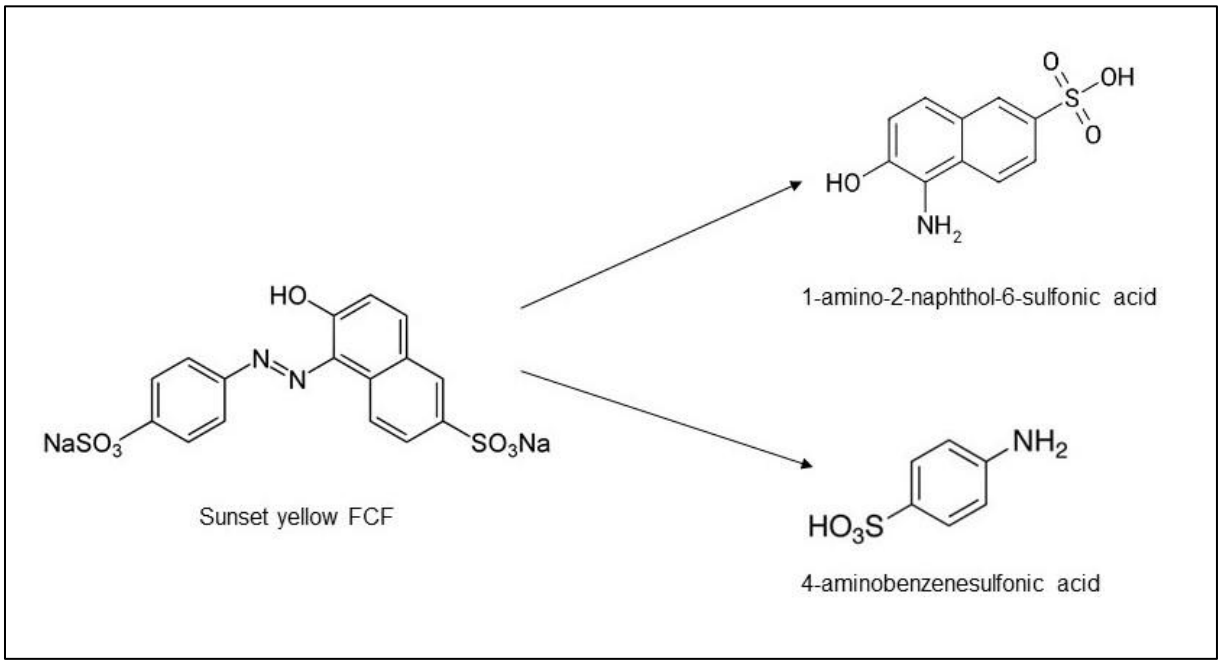


figure 21: decomposition of Sunset yellow FCF into its decomposition products 4-aminobenzenesulfonic acid and 1-amino-2-naphthol-6-sulfonic acid

In general, azopigments are known for building direct-DNA reactive compounds in their production (based on the raw material and an incomplete reaction) and if a decomposition takes place since the structure of these is based on azo-groups which can be cracked to primary aromatic amines. This decomposition is possible through a reductive environment like the human gastro-intestinal tract or even if the pigments are exposed to high heat like in the recycling process. In the REACH-regulation the list of PAA which needs to be analyzed if a azopigment is used is published (The European Parliament, 2006). But since the occurring PAA is strongly influenced by the structure of the azopigment, this list is not covering all possible substances especially if the statement of the BfR from 2017, that a safety assessment of printing ink for food contact materials is due to a lack of toxicological information about the used substances is not possible (BfR, 2017).

5 Conclusion

This thesis contributes to the ongoing efforts to evaluate the safety of recycled polyolefin-based food contact materials. The results demonstrate that DNA-reactive substances persist in recycled materials even without consumer contamination and are primarily linked to the presence and degradation of printing inks.

Through solid-phase extraction (SPE) fractionation, further insights into the physicochemical properties of these mutagenic substances were gained. One key finding is that no single compound or substance group accounts for the observed direct DNA-reactive activity in the Ames test. Instead, multiple active fractions suggest the presence of several classes of direct-acting mutagens.

Chromatographic analysis revealed N-nitrosamines and primary aromatic amines (PAAs). However, Ames tests on selected individual substances, chosen for their high concentrations, did not fully explain the mutagenicity of the total sample extracts. Since PAAs are known degradation products of azo pigments, further investigation focused on printing ink components.

Tests on defined printed and recycled materials showed positive results for samples printed with PU-based blue ink and NC-based white ink. As colored inks are typically more complex than white inks (which often contain titanium dioxide), initial simulations used simplified white-ink formulations, which also showed positive Ames test results after heating. Titanium dioxide was shown to have a catalytic effect, particularly at lower temperatures (160 °C).

Azopigment (Sunset Yellow FCF) testing alone showed no mutagenicity when only heated, but when combined with NC, positive results emerged—comparable to the white-ink samples.

This thesis marks an important step toward identifying the sources of DNA-reactive substances in recycled polyolefins. Certain printing ink components were identified through analysis of real and model samples, though the exact mutagens remain unknown.

Further research should focus on defined sample systems and integrate mutagenicity testing with advanced chromatographic and spectrometric methods for precise substance identification and risk assessment.

Ultimately, identifying and removing the responsible compounds could enable safer recycling processes and significantly enhance the safety of food packaging made from recycled plastics. This would also support the realization of a truly circular economy for polyolefin-based materials.

6 Literature

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