SafeCycle – Guidance Document

CORNET Project: Analysis of recycling processes and recyclates and their applicability for various packaging scenarios

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1 Motivation and aim of the SafeCycle project

DNA-reactive mutagens and/or carcinogens have at least 120-fold lower thresholds than any other substance group in the EFSA's safety assessment of recycling processes (EFSA, 2019, EFSA, 2024). Therefore, currently the minimum decontamination efficiency of a recycling process currently depends directly on the worst-case assumptions of EFSA on the presence of mutagenic (direct DNA-reactive) contaminants. So far, EFSA assumed that 100% of all contaminants are DNA-reactive, as there was insufficient scientific data on potential DNA-reactive contaminants in recycled plastic. This approach seems very conservative, given that DNA-reactive mutagens are banned for practically all applications. Therefore, in 2020, Fraunhofer IVV, OFI and FH Campus Vienna embarked on a comprehensive scientific study as part of the PolyCycle project, investigating the presence of DNA-reactive mutagens in recycled plastics. DNA-reactive mutagenic substances represent a broad range of different chemicals and there is no single method that can reliably detect all potential DNA-reactive mutagens. Therefore, in the project PolyCycle chemical analysis with non-target gas chromatography coupled with mass spectrometry (GC-MS) was combined with biological testing using the Ames test, that specifically detects mutagenic contaminants, including currently unknown substances. For PET the results demonstrated that there are no indications for contaminations with DNA-reactive mutagens. However, for polyolefins (PE, PP) and polystyrene (PS) samples, which are not yet authorized for food contact, the study confirmed EFSA's assumption that recycled plastics can be contaminated with DNA-reactive mutagens. The *in vitro* bioassay results revealed that many samples were systematically contaminated with DNA-reactive mutagens, whereas others did not exhibit any cause for concern. Those mutagenic contaminants could not be detected by standard GC-MS screenings (Mayrhofer et al., 2023).

The SafeCycle project aimed to identify the risk factors leading to contamination with DNA-reactive substances, and to provide recommendations on how to avoid critical contamination. This shall enable a future application of recycled polyolefins and polystyrene in sensitive applications such as food contact. For this purpose, the analytical methodology was extended by non-target-analytics with high performance liquid chromatography coupled with high resolution mass spectrometry (HPLC-HRMS) to be able to detect a broad range of substances including also non-volatiles, that would be overlooked by standard GC-MS screenings. A systematic testing process was carried out on defined research samples to evaluate the influence of different critical parameters (e.g. filling, content, additives, printing, adhesives, decontamination, temperature, etc.).

This document addresses contact-sensitive packaging, and its methods comply with hazard identification rather than risk assessment. It is important to note that the EFSA applies worst-case assumptions, particularly in the context of food contact materials. While these evaluations may raise concerns about using recycled plastics for food contact materials, certain cosmetic packaging applications may still be considered acceptable. Many of the underlying findings were generated purely for scientific purposes, such as positive results from miniaturised Ames tests. These do not conform to OECD guidelines and may not reflect regulatory testing standards.

2 Important findings and measures from the SafeCycle project

2.1 Identified risk factors for safe recycling for contact-sensitive packaging

Risk factor: Degradation products from reprocessed printing inks

Printing inks are safe on virgin packaging, but the safety assessment of printing inks does not include potential degradation products after heating to >200 °C. The results from the SafeCycle project have shown that nitrocellulose binder and several organic pigments can form mutagenic degradation products during the heat stress of recycling processes.

Measures: Decontamination processes cannot efficiently remove critical degradation products from printing inks. Measures that avoid printing inks from entering the recycling process are most efficient.

Risk factor: Degradation products from azopigments in colour masterbatches at increased heat stress of recycling

In contrast to printing inks, colour masterbatches are intended to be heated in an extruder. However, the SafeCycle project revealed that some azopigments are sufficiently stable for normal processing of polyethylene, but can form carcinogenic primary aromatic amines (PAAs) at the increased heat stress of 240 °C in recycling processes.

Measures: Sorting of white and uncoloured fractions for especially sensitive applications can avoid this risk. White TiO_2 pigments are no risk factor for safe recycling. Use of more heat stable colour pigments should be recommended as design for safe recycling.

Risk factor: Degradation products from aromatic polyurethane (PU) adhesives

Aromatic polyurethane adhesives can degrade to primary aromatic amines at heat stress. These adhesives are therefore not used for packaging applications with predictable heat stress (autoclaving, microwave packaging). However, in recycling heat stress cannot be prevented.

Measures: Avoiding aromatic PU adhesives in input streams or the removal of the adhesives during the recycling for contact sensitive applications would be the most efficient measure.

No general safety concerns for adhesives, colour masterbatches or printing inks in virgin plastic!

2.2 Technological developments and opportunities

Technological opportunities: Delabelling

Labels often contain a layer of printing to provide surface for information. When these coloured layers remain in the recycling process, these labels can degrade to harmful substances, which are integrated into the recyclate and could potentially migrate into the new article.

Measures: Heavily coloured labels, which might lead to harmful degradation products after recycling should be removed to improve recyclate quality.

Technological opportunities: Deinking

Printing ink residues can form potentially hazardous substances after high thermal processes such as extrusion. Efficient deinking methods prior to extrusion can reduce the formation of unknown NIAS and potentially hazardous degradation products.

Measures: Printing inks that can be easily removed before extrusion should be used in preference and the packaging should be designed as such, that deinking is possible. Challenge tests should be developed for deinking measures and to determine their cleaning efficiencies.

Technological opportunities: Sorting technologies for coloured materials

Since primarily printed or coloured material tends to be an issue after recycling, the input streams could be sorted for non-printed or coloured packaging. Consequently, a sorting of mainly white or transparent material is a possibility.

Measures: Sorting for white or transparent materials.

Technological opportunities: Selection of heat-stable colour masterbatches and adhesives

Currently, the safety assessment of masterbatches and adhesives does not consider the additional heat stress of recycling. Some colour masterbatches and adhesives form mutagenic degradation products (e.g. primary aromatic amines) when heated to 240 °C, but the project results show that safer alternatives exist.

Measures: In a circular economy, safety assessment of masterbatches and adhesives for food packaging should include the increased heat stress of a recycling process. Masterbatches and adhesives that give no indication for critical degradation products after heating to 240 °C should be selected for food packaging to enable safe recycling.

2.3 Recommendations for testing

Testing: Non-target screening

The temperature and holding times have an influence on the formation of critical substances such as PAAs. The choice of process conditions can be critical.

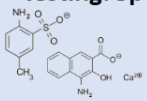
Recommendation: One possibility to define optimal process conditions is a non-target screening approach using high-resolution GC-HRMS or LC-HRMS. This approach offers an opportunity to determine optimized process conditions. This can be used to investigate how chemicals alter during processing, and at which point a critical temperature or holding time is reached.

Testing: Analysis for DNA-reactive mutagens with the Ames test

DNA-reactive substances have 120-fold lower safety thresholds than any other substance class. The Ames test can specifically detect DNA-reactive mutagens including currently unknown substances. This is important as detected critical mutagenic contaminants were exclusively degradation products, that are often unknown and unexpected, that can be easily overlooked in GC screenings alone and can only be detected in HPLC screenings or with targeted methods.

Recommendation: Initial hazard assessment of recycling processes should include the analysis with the Ames test. The method should be optimized for detection of low concentrations in a complex matrix. Concentration of extracts (minimum 100-fold) is important to improve sensitivity. Based on project results, the testing battery can be reduced to the test strains TA98 and TA100. However, a subsequent risk analysis is necessary to fulfill the entire scope of a safety assessment. In this context, it should be emphasized that a standardized sampling procedure is key.

Testing: Specific analysis for primary aromatic amines if printed or coloured input streams cannot be excluded



Primary aromatic amines (PAA) are known DNA-reactive carcinogens with very low safety thresholds that cannot be detected in standard GC-MS non-target screenings. The SafeCycle project showed that recycling of coloured input streams can lead to PAA contaminations.

Testing recommendation: Initial hazard assessment of recycling processes should include testing of different batches for PAAs with specific, target methods for PAAs. Standard PAA screenings cover only a small fraction of possible degradation products during recycling but can be a valuable warning signal. In cases of concentrations close below the safety thresholds, it should be considered that standard PAA screenings can only detects the “tip of the iceberg”.

Testing: Use of advanced chemical methods

Thermal exposure to samples with decorative elements can lead to expected degradation products such as 3,3-dichlorobenzidine and PCB-11.

Recommendation: Suspect target screening with known and suspected substances such as PAA (LC-MS) or PCB (GC-MS) can be used to detect possible impurities. LC and GC should complement each other here.

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3 Background

3.1. Regulatory aspects

In the spirit of a circular economy, products should be reprocessed into new products after they have been used. Short-lived packaging materials like packaging in particular should be recycled into new products. The Packaging and Packaging Waste Regulation (PPWR) published in 2025 (EU, 2025) provides clear quantities for the use of recyclates. However, the use of recyclates must not endanger consumer health. This is particularly important for sensitive applications such as food packaging. This area is therefore subject to a number of regulations that must be complied with. The use of recyclates in food packaging is regulated by Recycling Regulation 2022/1616 (EU, 2022). There are clear specifications for the input of recycling processes and the requirements for the authorisation of processes.

The regulation distinguishes between "suitable technologies" and "novel technologies". Suitable technologies are processes that have already been tested by the European Food Safety Authority (EFSA) and are evaluated as safe. Most recycling processes for PET and recyclates from closed and controlled loops fall into this category. Novel technologies, on the other hand, have not yet been analysed by the EFSA with regard to consumer safety. These are recycling processes for non-PET polymers, i.e. mainly polyolefins (PE, PP) and polystyrene (PS) in the packaging sector. For these polymers, extensive data on the safety of the recycling process (e.g. data on cleaning efficiency) and data on consumer exposure to hazardous substances must be provided. As part of the review, EFSA will analyse this data and, if it is deemed safe enough, recommend the technology for authorisation. In addition to the authorisation process described above, food contact materials (FCMs) containing recyclates must also meet the requirements of Regulation 10/2011 as well as amendments (EU, 2011) and Framework Regulation 1935/2004 (EU, 2004), in the same way as FCMs made from virgin material.

3.2. Challenges in the use of recycled plastics for food contact materials

The use of post-consumer recycled (PCR) plastics in FCMs is still a major challenge due to the unknown levels of non-intentionally added substances (NIAS) (Geueke, 2018). The limits of detection (LOD) of current analytical techniques are insufficient to identify all NIAS, particularly in PCR plastic packaging. Nevertheless, the PPWR sets very strict requirements for the use of recycled materials in contact sensitive packaging such as FCM. Specifically, a minimum recycled content of 30% for PET and 10% for plastic materials other than PET shall be incorporated until 2030 (EU, 2025). According to EU Regulation 1935/2004, FCMs should be manufactured in such a way that they do not endanger human health However, the risk posed by unidentified NIAS remains high, particularly for highly diffusive materials such as polyolefins (PO). Polyethylene terephthalate (PET) remains the only recycled plastic that can be used as a direct FCM because it has demonstrated compliance with the set requirements.

Therefore, there is an urgent need to identify unclassified NIAS in order to exclude critical substances that could endanger human health, and to increase the use of recycled plastics in FCM or cosmetic applications. EFSA sets very strict requirements for FCM materials and generally recommends the use of the Threshold of Toxicological Concern (TTC) concept (EFSA, 2019). EFSA also employs a worst-case approach, whereby unknown substances are deemed potentially genotoxic in the absence of analytical methods to prove otherwise (EFSA,

2016). Typically, GC-MS is used to screen for critical substances in PCR materials. However, due to the large number of peaks ("forest of peaks"), it is not feasible to identify and toxicologically evaluate all NIAS using a non-targeted GC-MS method. HPLC-HRMS is also recommended to screen for non-volatile substances, as GC-MS alone has not been sufficient to detect all relevant NIAS in previous investigations (Mayrhofer et al., 2023, Prielinger et al., 2024).

An alternative test method to chemical analysis is the Ames test, an *in vitro* bioassay which specifically screens for direct DNA-reactive effects. The Ames test can be used to test pure substances or complex mixtures such as FCM extracts (Rainer et al., 2019) and is also recommended by the International Life Sciences Institute (ILSI) (Koster et al., 2015). The Ames test has the additional advantage of being able to detect cumulative effects (a sum of different toxicants) and that the whole mixture can be evaluated in one run. However, the limits of biological detection (LOBD) of the miniaturised Ames test are not sufficient to detect all substances at the TTC threshold for genotoxins of 0.15 µg per person per day (Schilter et al., 2019, Rainer et al., 2021). Therefore, it is essential to gain a better understanding of the exact composition of packaging materials and to identify potential hazards along the value chain. In this context, Design for Recycling is an important step towards a functional circular economy that makes PCR materials safer to use (Ulla Gürlich, 2024).

3.3. Differences in the use of plastic recyclates for food and cosmetic packaging

For cosmetic packaging, a published guideline from the Cosmetics, Packaging and Toxicology (CosPaTox) consortium provides a clear framework regarding the use of post-consumer plastic recyclates in cosmetic packaging (CosPaTox, 2024). One of the main differences is that there are several use-cases for cosmetic packaging, as there are three main categories, which are leave-on, rinse-off cosmetics and detergents, with decreasing requirements for safety and recyclate quality. Furthermore, DIN SPEC 91521 provides recommendations for using recycled plastics, categorized by three levels of suitability (leave-on, rinse-off and home care products), as well as recommendations for analytical test methods (Deutsches Institut für Normung (DIN), 2025). In addition, the application amounts are often much lower compared to food (on average 17.4 g/day of cosmetics compared to 1 kg of food per day (SCCS et al., 2024). The routes of exposure (oral, dermal, respiratory) and the contact time (especially for rinse-off cosmetics) must also be considered.

For dermal exposure (e.g. leave-on cosmetics), only about 1% of the substance is absorbed through the skin compared to oral digestion. On the other hand, skin sensitizers and skin allergens are also important substances that need to be investigated and critically assessed. Worst-case exposure scenarios can be used to calculate the use of recyclates for defined applications, depending on the type of exposure, the amount applied and the contact time. If it can be demonstrated that the maximum acceptable consumer exposure (MACE) is below 0.0025 µg/kg bw/day (the TTC threshold for genotoxic substances) even in the presence of unknown NIAS, a specific amount of recyclate (usually expressed as a percentage) can be used at a tolerable level for a defined use case. This simplifies the implementation of plastic recyclates for cosmetic packaging compared to food packaging. Further specifications and substances prohibited in cosmetics can be found in Annex II of EU Regulation 1223/2009 (EU, 2009).

4 Methodological research work in the SafeCycle project

4.1 Chemical analysis

HPLC-MS and HPLC-HRMS were used in the project for suspected or unknown semi-volatile substances. HPLC-HRMS provides the retention time, the molecular peak, the corresponding fragments and the exact mass. This allows for a more in-depth investigation of substances that are suspected or need to be investigated in more detail. This analytical method provides a fingerprint that offers more detailed information about the sample. Statistical alignment and prioritisation of the results enables the influence of factors such as temperature on polymers to be investigated. HPLC-HRMS measurement is consequently a method for identifying substances. If these substances need to be confirmed and quantified, HPLC-MS/MS is employed. Here, selected masses are defined and measured sensitively using an adapted method. If further degradation products are suspected that are no longer LC-capable, GC or GC-MS analysis can be employed, meaning these techniques complement each other.

4.2 Bioassays: Methodology for the miniaturised Ames test

4.2.1 Miniaturised Ames test

Due to the typically low analyte concentrations and complex sample matrices, sensitive and efficient testing methods are required to assess DNA-reactive mutagens in FCMs. The Ames test is a regulatory accepted method to assess genotoxicity. The OECD guideline 471 (OECD, 2020) describes a standard testing procedure based on agar plates designed for pure substances and pharmaceuticals. But due to high detection limits and high sample consumption, this method is rather unsuited for complex mixtures like plastic extracts. To counteract this, the miniaturised Ames test can be combined with an extract pre-concentration. The miniaturised Ames test is a liquid-based version of the traditional agar-based Ames test. It enhances sensitivity and lowers detection limits by integrating a pre-concentration step, allowing the identification of mutagenic substances at trace levels. It also accommodates small sample volumes and is well-suited for complex mixtures, offering a practical advantage for FCM testing. The microplate-based system enables higher throughput and improved efficiency relative to the conventional agar plate method.

Sample preparation for the miniaturised Ames test was performed according to EU Regulation No 10/2011 (EU, 2011) and Rainer et al. (Rainer et al., 2019). As food simulant, 95% ethanol was used and to assume a worst-case scenario the samples were migrated at 60 °C for 10 days. For foils, a surface to volume ratio of 3 dm^2/300 mL ethanol was used. Samples with a high density, such as granules, were migrated at a ratio of 30 g/100 mL. Samples with a low density or a high surface area, such as milled powders or mixed flakes, were migrated with a ratio of 15 g/300 mL. After migration, the samples were concentrated to approximately 1 mL to achieve a concentration factor of 100 or 300, respectively. The concentration step is necessary to increase sensitivity and to reach lower limits of biological detection (LOBD) with the Ames test. Lower lowest effect concentration (LEC) values in the Ames test are important as many genotoxins cannot be detected at the TTC value of 0.0025 µg/kg body weight per day (Rainer et al., 2021). In general, EU Regulation 10/2011 states that a full risk assessment is required for unknown substances that migrate into food at concentrations of 0.01 mg/kg or higher (EU, 2011). As a minimum requirement, LEC values comparable to this threshold are needed (Rainer et al., 2018). The final step in sample preparation was a solvent exchange

from 95% ethanol to DMSO. DMSO is a more suitable solvent, as ethanol can be harmful to bacteria, potentially distorting the validity of the results of the assay due to the masking of inhibitory or toxic effects.

The sample extracts were tested in accordance with the Xenometrix Ames TA98/TA100 manual (Xenometrix, 2018). Samples were tested with *Salmonella* Typhimurium strains TA98 and TA100. The sample extracts were also tested with and without metabolic activation. S9, a rat liver extract, was used to mimic the human metabolism. Some chemicals are biologically inactive unless metabolising enzymes in the human body convert them to mutagens (e.g. benzo[a]pyrene). Additionally, the samples were spiked with a known mutagenic substance. The spike is used to determine whether the extract has any inhibitory or cytotoxic effects on the bacteria. Inhibition would distort results for mutagenicity due to false negative results.

4.2.2 Plate Ames test according to OECD 471

Samples that tested positive in the miniaturised Ames test and showed a strong initial colour were additionally tested in the plate Ames test according to OECD Guideline No 471 (OECD, 2020). The miniaturised Ames test is evaluated based on a colour change in the indicator medium from purple to yellow, triggered by a pH indicator. Wells that turn yellow are counted as positive wells. To rule out any interferences from the inherent colour of the samples, plate Ames tests were performed to confirm positive results. Samples that demonstrated strong positive results in the miniaturised Ames test were also tested positive in the plate Ames test. This rules out the possibility of misinterpreting positive results due to barely visible colour changes (e.g. pigment samples). In addition, the probability of false positive results due to statistical outliers or too low positive thresholds were also reduced.

5 Findings from the previous project PolyCycle

In the scope of the FFG PolyCycle project, a study from Mayrhofer et al. (Mayrhofer et al., 2023) indicated that 51 out of 119 PCR plastic samples were tested positive in a miniaturised Ames test. Input (flakes) and output (granules) materials were tested from collected post-consumer plastic waste in the EU. Recycled PET was the only material which did not demonstrate any positive Ames test results. In particular, very high-diffusive PCR plastics such as low-density polyethylene (LDPE) showed a very high rate of positivity (around 90%) and other polyolefins such as high-density polyethylene (HDPE) and polypropylene (PP) demonstrated a positivity rate of around 30%. The tested samples showed higher n-fold inductions in the Ames test after extrusion (in 42 out of 92 samples) compared to the input materials.

Certain residues in the input material may degrade to hazardous substances that increase mutagenic activity in the Ames test. In other words, specific additives in the plastic materials or residues of printing inks which are not heat stable may form degradation products after high thermal stress. A clear identification or the exact source of contamination is still unknown (unidentified NIAS), but these high numbers of Ames test positive samples have only been detected in recycled plastics, not in virgin materials. This means that NIAS that are introduced along the value chain (from consumer to finished recyclate) or specific components in the packaging materials are not suitable for current mechanical recycling processes. Extrusion processes typically use temperatures of 160 °C to 260 °C or even higher to completely melt the input material and to achieve the desired material quality.

6 Results from the SafeCycle project

6.1 Identified risk factors for safe recycling for contact sensitive packaging

6.1.1 Degradation products from reprocessed printing inks

In the previous PolyCycle project, many recycled polyolefin and polystyrene samples demonstrated mutagenic effects that could not initially be explained. To find the sources of this mutagenic activity, several risk factors were evaluated by a systematic screening of well-defined input materials. One of the first potential risk factors being evaluated was the effect of printing. For this purpose, defined printed plastic samples, that have never been in contact with food and consumers, were recycled and the results from printed and unprinted plastic were compared (Figure 1). Mutagenic activity could only be observed in the printed PP film after the recycling process. The printed PP film did not give any indications for mutagenic contaminants before the recycling process. The non-printed PP film did not show any mutagenic effects after recycling at the same conditions.

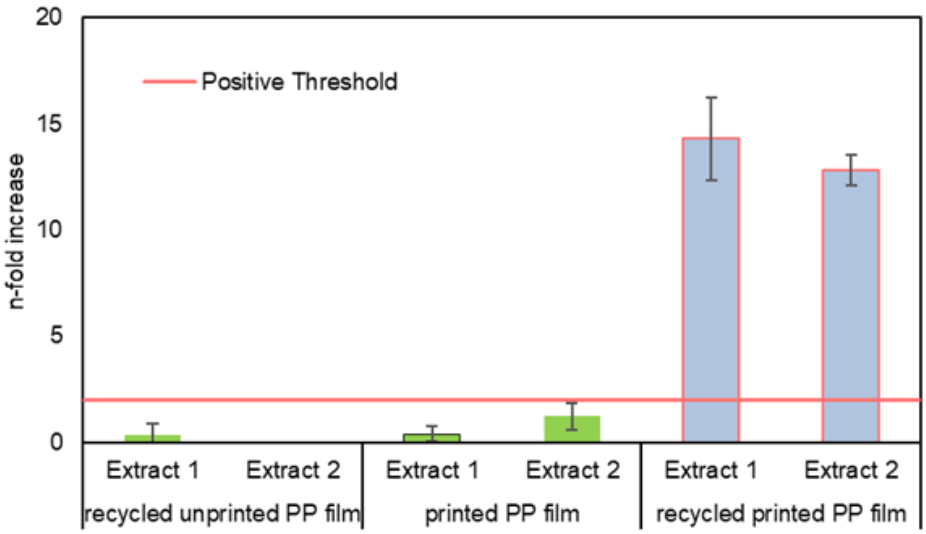

Figure 1: Comparison of miniaturised Ames test results (TA98+S9) for printed and unprinted PP film after industrial scale recycling.

Further experiments confirmed the above results. In a screening, films that were printed with different inks (water-based, UV-treated) and colour shades and pigments were tested in the Ames test after controlled thermal treatments. A worst-case heat treatment (240 °C for 30 minutes) was applied under a nitrogen atmosphere to prevent oxidation. The results indicated that many printed films and pigments showed higher n-fold inductions in the Ames test compared to the untreated samples. The unprinted samples showed no increase in mutagenic activity after heating. This led to the assumption that specific components in printing inks and pigments might form hazardous degradation products at high temperatures.

The results from this experiment clearly show that recycled printed plastic can be a risk factor for mutagenic effects in the Ames test. To get more information on the sources of the detected mutagenic activity, systematic evaluations of different printed samples were performed after the heat stress of a recycling process.

6.1.1.1 Degradation products from nitrocellulose-based printing inks

To evaluate the influence of the binders, printed PE films with three different types of binders for printing inks were compared. To avoid interference from colour pigments, white printed films containing only the pigment TiO_2 were used. TiO_2 was tested negative in the Ames test and was deemed heat stable. Subsequently, a mutagenic effect caused by the coloured pigment was ruled out.

In Figure 2, results of the different binder systems of printing inks after recycling at industrial scale are presented. The nitrocellulose binder based printed film demonstrated a strong mutagenic activity after the heat stress of the recycling process, while the two other binders PVB and polyurethane showed no indications for mutagenic degradation products in the recycling process.

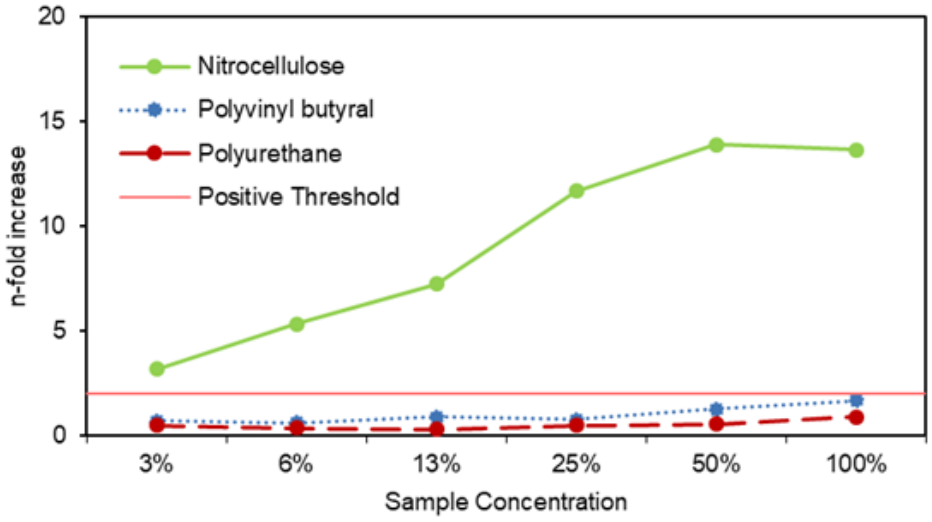

Figure 2: Comparison of Ames MPF results of different binders in printing inks using the test strain TA98 with metabolic activation (+S9).

The mutagenic effects of nitrocellulose-based printing inks after recycling could be observed for several independent samples. There were no indications for mutagenic activity in the nitrocellulose binder in virgin printed samples. The effects could only be observed after the heat stress induced by recycling.

In a laboratory scale experiment laboratory grade nitrocellulose and TiO_2 applied on a PE film confirmed that nitrocellulose subjected to heat stress showed mutagenic activity in the Ames test. The effect was already observed at temperatures of 160 °C, as shown in Figure 3, therefore milder processing conditions cannot fully prevent the formation of mutagenic degradation products. TiO_2 itself is no risk factor for the formation of mutagenic degradation products but uncoated laboratory-grade TiO_2 seemed to catalyse the degradation and resulted in increased mutagenic activity in the Ames test after heating to 160 °C.

Figure 3 shows the Ames test results of nitrocellulose in this experiment. Before heating, neither the unprinted nor the nitrocellulose printed samples showed genotoxic activity. After heating to 160 °C in a hydraulic press, the unprinted samples remained negative while the nitrocellulose-based plastic samples tested positive. According to published literature (European Printing Ink Association (EuPIA), 2016, Lisiecki et al., 2024), nitrosamines were identified as a potential degradation product of nitrocellulose in the presence of secondary

amines but only after high-temperature treatment. In this experiment however, mutagenic activity could be observed also in the absence of an amine source.

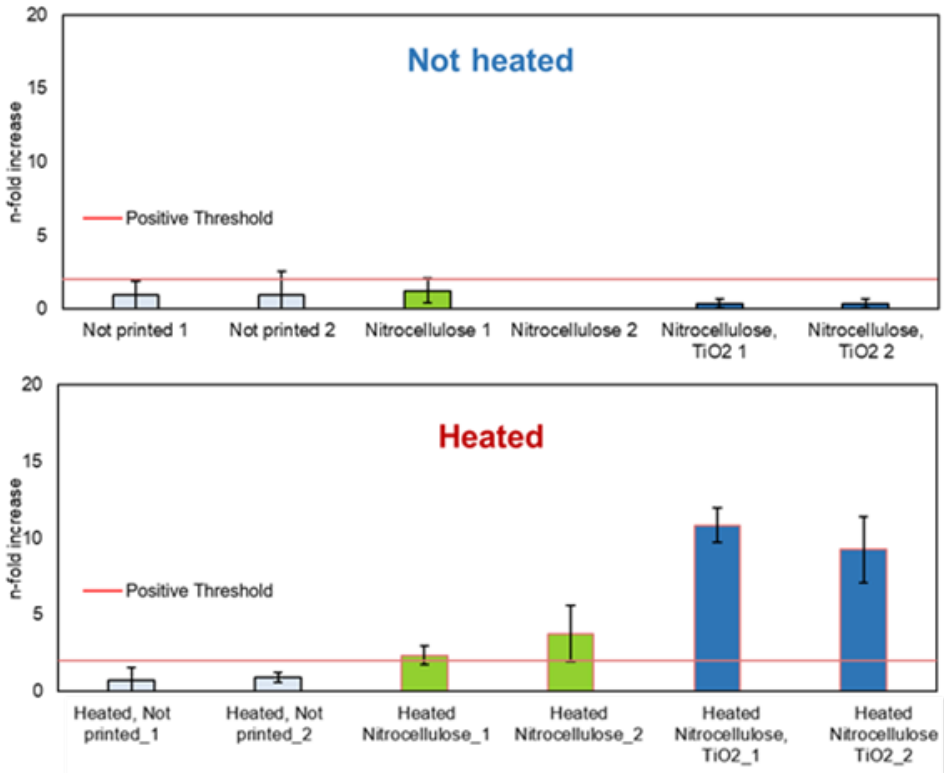

Figure 3: Comparison of miniaturised Ames test (TA98+S9) outcomes of samples with nitrocellulose binder with TiO_2 and non-printed sample before and after heating to 160°C. The y-axis indicates the two tested sample migrates while the x-axis depicts the n-fold increase of the bioassay indicating the genotoxic effect. The positive threshold, above which a sample is considered Ames positive, is indicated by a red line.

6.1.1.2 Degradation products from organic pigments in printing inks

Systematic testing of recycled plastic produced from defined printed input material showed that degradation products from the nitrocellulose binder are not the only risk factors for recycled printed plastic. Figure 4 shows the Ames test results for recycled plastic produced from different printed PP films, with a PU-based binder. While no mutagenic activity was observed in the TiO_2 printed PP film, a higher mutagenic activity was observed for different coloured printed input materials with the same binder and additional organic colour pigments. The results clearly indicate that not only the nitrocellulose binder, but also organic pigments can be a source for mutagenic degradation products after the heat stress of the recycling process. Furthermore, PE films printed in various colours and heated up to 240 °C for 30 minutes were tested positive in the Ames test, while the white printed reference yielded a negative result (Figure 5). All tested PE films printed in various colours and heated to 240 °C for 30 minutes were tested positive in the Ames test while the white printed reference did not indicate any mutagenic activity.

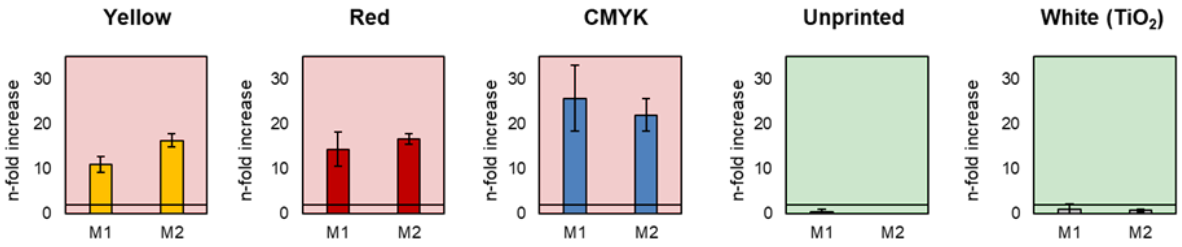

Figure 4: Comparison of the mutagenicity of recyclates produced from PP films, printed in different colours with a PU-binder based printing ink in the Ames MPF™ TA98+S9. The n-fold increase giving the strength of genotoxic activity of two migrates M1 and M2 are shown for each pigment on the y axis. The positive threshold above which the sample is evaluated as positive is indicated with a grey line.

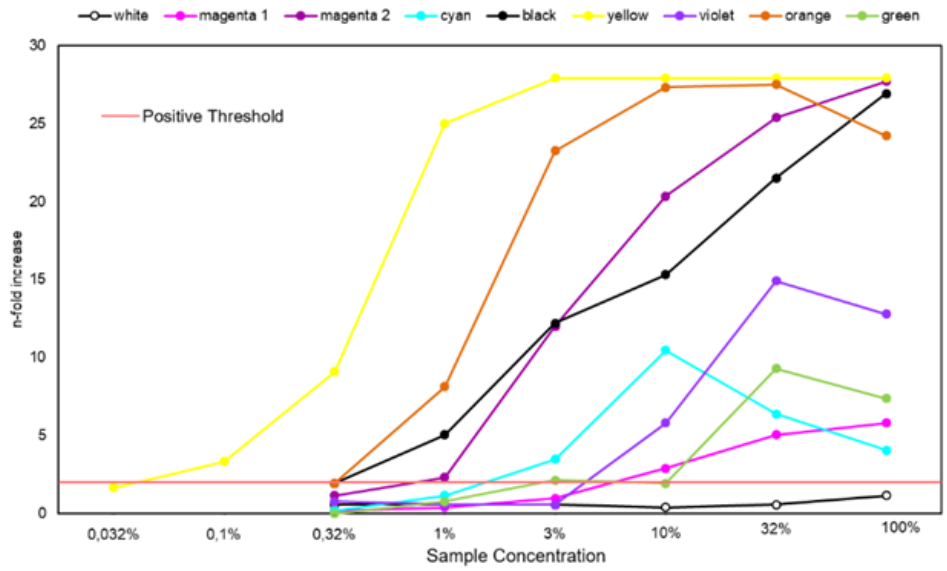

Figure 5: Miniaturised Ames test results (TA98+S9) of different coloured printed PE films in dilution series from 100% sample concentration to 0.032%. The y axis shows the n-fold increase indicating the genotoxic effect of the sample. Above the positive threshold at the value of 2 indicated by a red line, the sample dilution is Ames positive.

Thermal analyses using differential scanning calorimetry (DSC) were carried out to identify critical temperatures that led to increased n-fold inductions. In DSC, a sample of known mass is heated and changes in heat capacity are observed by monitoring the heat flow compared to a reference cell. Changes in heat capacity were observed in UV-treated inks in a temperature range of 160–180 °C and in water-based inks in a range of 120–150 °C. Pigment samples also exhibited changes in heat capacity between 140 -150 °C. Pigment samples also demonstrated heat capacity changes between 140 – 150 °C.

The temperatures at which thermal events occurred were used to determine the conditions for additional heat treatment of printed films and pigments that tested positive after heat treatment at 240 °C. Samples were tested after being treated at 120 °C, 160 °C, 200 °C and 240 °C for 30 minutes in a nitrogen atmosphere. The samples were specifically treated below and above the temperatures at which the heat capacity changes in form of a clear peak were detected by DSC. It was observed that treating samples above the critical temperature ranges resulted in higher n-fold inductions in the Ames test compared to samples below the critical temperatures. At temperatures above 160 °C, the printed films and pigments showed an increased rate of Ames test positive results, indicating that certain ink components such as pigments or binders, are not heat-stable and may degrade during mechanical recycling processes.

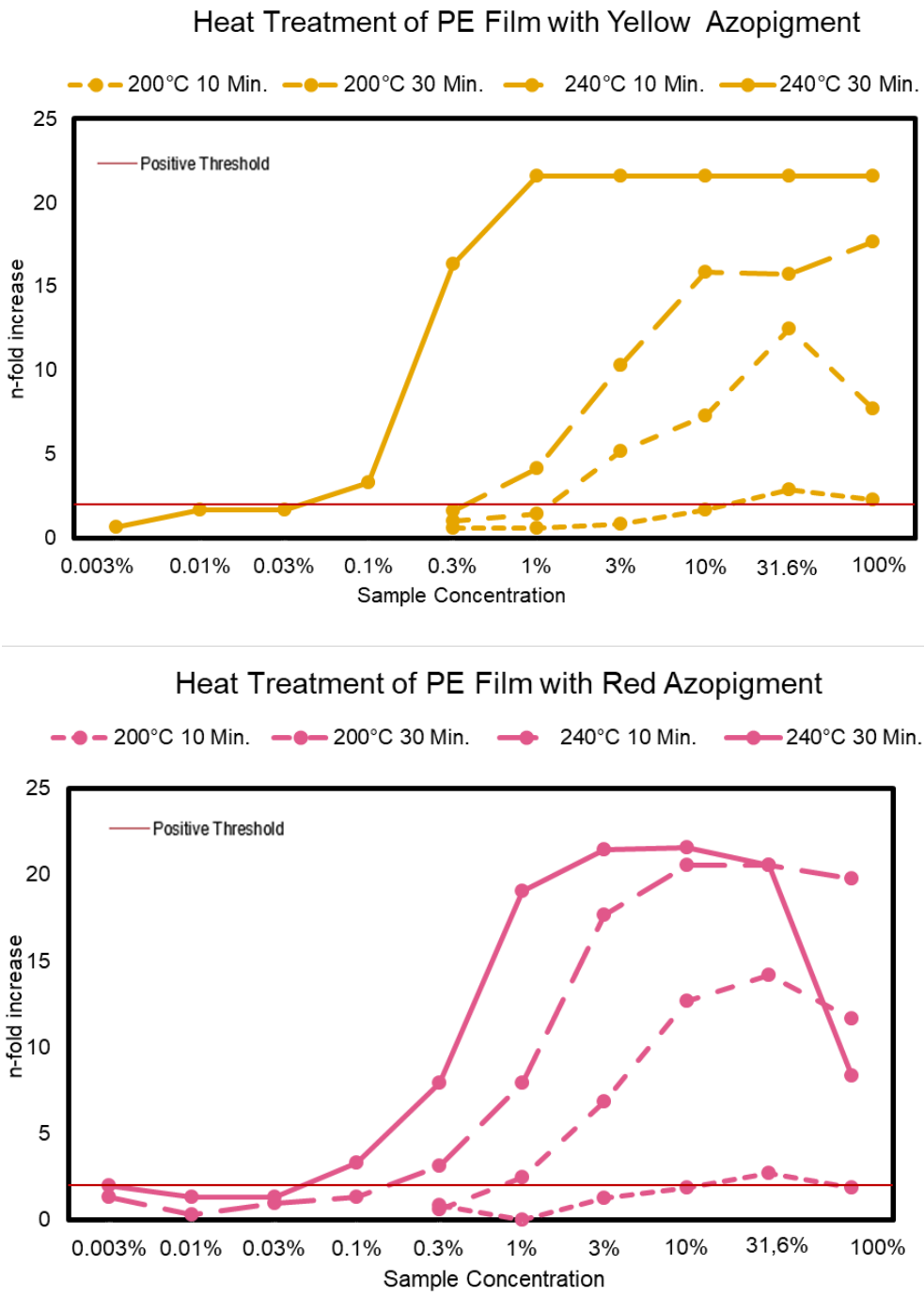

Figure 6: Ames MPF results of PE films with red and yellow azopigment heated with different conditions tested in the bacterial strain TA98 with metabolic activation. While the x axis shows the sample dilution from undiluted to 0.003%, the y axis shows the n-fold increase indicating the genotoxic effect. The positive thresholds are shown with red lines at a value of 2 and the different textures of the lines indicate the four tested heating conditions of the samples.

Variations in temperature and duration of the heat treatment were found to affect the results. Figure 6 shows a dilution series of two different PE films in the bacterial strain TA98+S9, demonstrating the dependence on heating time and especially temperature. Hereby, the temperature reduction from 240 °C to 200 °C greatly reduced the genotoxic effects. It should be noted that printing inks are usually not intended for high temperature applications and processes. Therefore, the safety assessment of printing inks does not include potential degradation products after heating to >200 °C.

Due to the strong increase in mutagenicity observed in some printed and coloured plastic samples when subjected to strong heat stress, despite no activity being observed prior to heating, it is assumed that degradation products originating from the printing inks may be the cause. One component of printing inks is pigments, whereby especially, but probably not only, azopigments could form primary aromatic amines under heat treatment and in presence of amines (e.g. from food). Some of those PAAs are or can be genotoxic according to literature, therefore, further investigations within the SafeCycle project were done.

One pigment commonly used in food contact materials is pigment yellow 13. Figure 7 shows a theoretical reaction the pigment may undergo, especially under high heat stress.

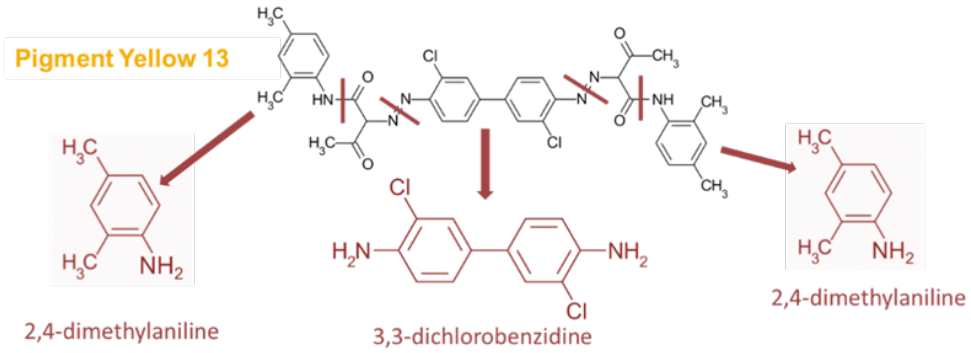

Figure 7: Possible reactions of pigment yellow 13 potentially enhanced or enabled by heat. With the indicated bond breaks the primary aromatic amines 2,4-dimethylaniline and 3,3-dichlorobenzidine could emerge as degradation products.

Among others, those two PAAs as potential degradation products from pigment yellow 13 were assessed in more detail to investigate whether or not they may cause mutagenicity in the heated pigment.

Heated samples were analysed using LC-MS/MS, resulting in the detection and quantification of 35 different PAAs.. Both of the theoretical degradation products of pigment yellow 13, being 2,4-dimethylaniline and 3,3-dichlorobenzidine, were detected in heated samples. Genotoxicity assessment of those PAAs as pure substances revealed mutagenicity of 3,3-dichlorobenzidine which is in accordance with literature while 2,4-dimethylaniline tested negative.

6.1.2 Degradation products from organic pigments in colour masterbatches at increased heat stress of recycling

Printing inks are not designed for high temperature applications, therefore heat stability of colour pigments is typically not an important requirement for printing inks. Coloured masterbatches however, are generally intended for use at higher temperatures, particularly those encountered in an extruder. The heat stress experienced during the recycling process can also be considerably stronger for coloured masterbatches than for the initial extrusion step used to produce virgin coloured plastic.

Mutagenic activity was detected in coloured recycled HDPE samples, from two different industrial scale recycling processes that had coloured HDPE bottle caps as an input material. Based on previous findings for heat stressed azopigments in recycled printed plastic, the colour pigments were identified as a potential risk factor at the increased heat stress of recycling. Some of the azopigments used in printing inks, that demonstrated mutagenic effects after heating to 240 °C, are also reported to be used in coloured plastic and would therefore be a plausible explanation for mutagenic effects. Therefore, in a follow-up experiment, different

coloured HDPE screw caps (market samples or samples provided by project partners) were tested before and after heating to 240 °C. Most screw caps did not show any indications for mutagenic effects after the heating process, but one of the two tested yellow screw caps showed a notable mutagenic activity in the Ames test after heating to 240 °C, and mutagenic effects could also be observed for mixed collected screw caps. The results indicate that some pigments used in HDPE are not yet designed to withstand the increased heat stress of the recycling process. However, for most of the tested coloured screw caps, there were no indications of critical degradation products following increased heat stress (Figure 8).

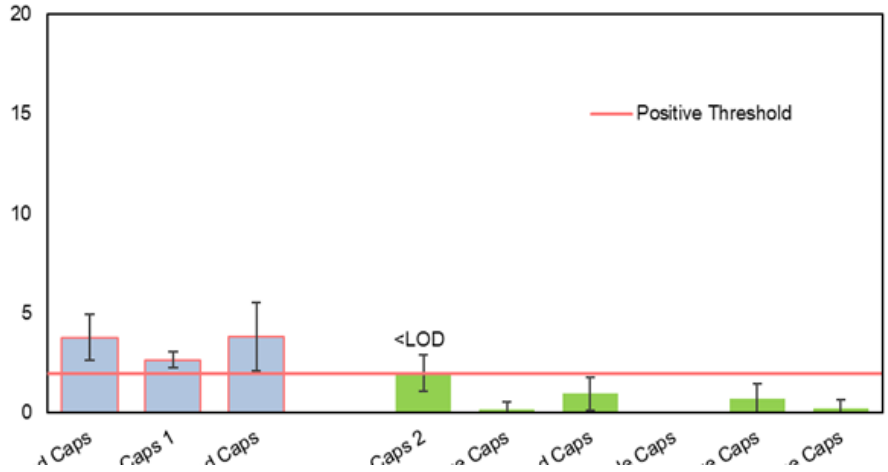

Figure 8: Genotoxic effects of heated samples tested in the miniaturised Ames test condition TA98+S9. Values are depicted as bars indicating the n-fold increase with positive, genotoxic outcomes being above the threshold of 2 in blue with red frame. Ames test negative samples are shown in green on the right side of the graph.

To enable circular recycling of HDPE bottle caps, it would be beneficial if the safety assessment of colour pigments accounted for the increased heat stress of the recycling process, and if measures were taken to ensure that only colour pigments that remain safe after thermal treatment were used. Results from the SafeCycle project show that some azo pigments can form primary aromatic amines at temperatures as low as 240 °C during the recycling process.

The degradation of certain azopigments to primary aromatic amines was identified as one of the main mechanisms that lead to positive Ames test results after PE samples were thermally treated. Some PAAs are not detectable with standard GC-MS screening analyses, but show strong mutagenic effects when tested as pure substances. Positive miniaturised Ames test results were also confirmed by a regulatory approved variant of the Ames test (OECD 471), reducing the probability of false-positive results.

Although primary aromatic amines (PAA) were not initially the main focus of the project they were detected and quantified in numerous heated samples. A total of 35 different PAAs were detected in the heat-treated samples. The compound 3,3'-dichlorobenzidine (CAS No. 91-94-1) was found particularly frequently in the tested materials. 3,3’-dichlorobenzidine is a degradation product of azopigments, which is formed after exposure to high temperatures. Further testing showed a correlation between the presence of 3,3'-dichlorobenzidine and positive Ames test results. This substance was detected in samples prepared specifically for the project, but also in PCR samples of unknown origin. In addition, further PAA were identified that are not included in the REACH regulation. A variety of PAAs were analysed in the Ames test yielding in results ranging from non-mutagenic to strongly-mutagenic (Figure 9). Those

results indicate that PAAs are a potential hazard for genotoxicity, but not all of them tested positive in the miniaturised Ames test.

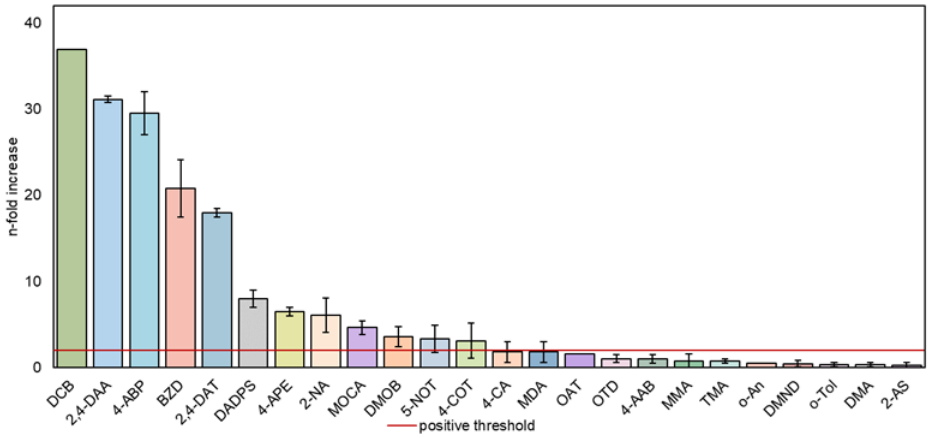

Figure 9: Genotoxicity of different primary aromatic amines tested in the Ames test TA98+S9. List of tested PAAs: 3,3′-dichlorobenzidine (DCB), 2,4-diaminoanisole (2,4-DAA), 4-aminobiphenyl (4-ABP), benzidine (BZD), 2,4-diaminotoluene (2,4-DAT), 4,4′-diaminodiphenylsulfide (DADPS), 4,4'-oxydianiline (4-APE), 2-naphthylamine (2-NA), 4,4'-methylene-bis-(2-methylaniline) (MOCA), o-dianisidine (DMOB), 2-methyl-5-nitroaniline (5-NOT), 4,4'-methylene-bis-(2-methylaniline) (4-COT), 4-chloroaniline (4-CA), 4,4′-diaminodiphenylmethane (MDA), o-aminoazotoluene (OAT), 3,3'-dimethylbenzidine (OTD), 4-aminoazobenzene (4-AAB), 2-methoxy-5-methylaniline (MMA), 2,4,5-trimethylaniline solution (TMA), 2-anisidine (o-An), 2,2‘-dichloro-4,4’-methylenedianiline (DMND), o-toluidine (o-Tol), 2,4-dimethylaniline (DMA), 2-amino-5-methylbenzenesulfonic acid (2-AS)

6.1.3 Degradation products from aromatic PU adhesives

Experiments indicated that both printed but also unprinted multilayer pouches led to positive Ames test results, while pouches without adhesives yielded in negative results. In a direct comparison of aliphatic and aromatic PU adhesives, it was observed that only the aromatic adhesives showed mutagenic effects. In this case, a primary aromatic amine is considered to be the suspected degradation product. Figure 10 demonstrates that heated aromatic PU adhesives showed an increased rate of positive results in the Ames test, which is why they were identified as risk factors for safe recycling.

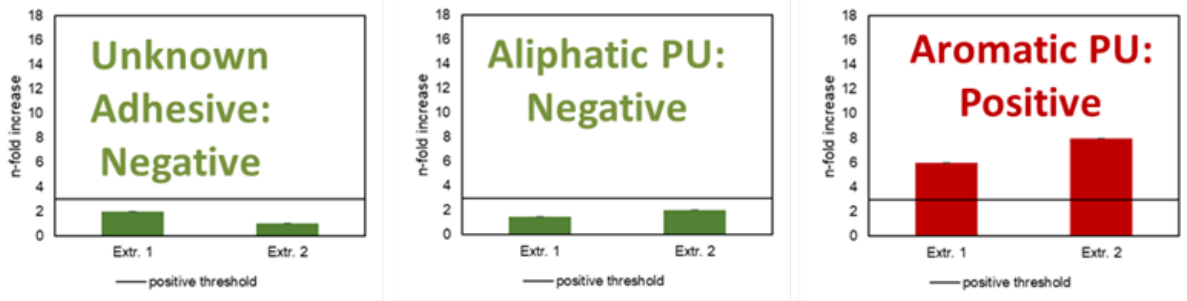

Figure 10: Miniaturised Ames test results (TA98+S9) of different PU adhesives after recycling simulation at 240 °C. The x-axis of the figure shows the n-fold increase of each sample extract indicating the strength of genotoxic effect. Ames test negative results are in green and are below the positive threshold (grey line). Ames test positive results are indicated in red and are above the positive threshold.

6.2 Technological developments and opportunities

Another aspect of the project involved developing mitigation strategies to avoid the critical risk factors identified when recycling back to food contact materials. Three possible solutions were suggested: delabelling, deinking and sorting. These strategies are intended specifically for use in the recycling of food and cosmetic contact materials. These are not general

recommendations for all recycling applications. For less critical applications, these measures might not be cost-effective or environmentally friendly.

6.2.1 Delabelling

Efficient delabelling methods have been used since the early stages of PET recycling. Established recycling guidelines ensure easy separation of bottle and label via a swim/sink principle, making use of the density differences between the materials. Data collected within the PolyCycle project never showed any indication for mutagenic contaminants in recycled PET bottles. PP and PS cups, which contained either non-removable or removeable sleeves and labels were mechanically recycled and prepared for migration and miniaturised Ames tests. The results indicated that sleeves or labels which were removed before shredding led to much cleaner granules after extrusion and showed lower mutagenic activity in the Ames test (Figure 11). It is notable, that all the de-labelled PP and PS cups yielded negative results in the Ames test. In contrast, the cups with non- or hardly removable sleeves and labels showed higher n-fold inductions in the Ames test, alongside an increased number of Ames test positive results.

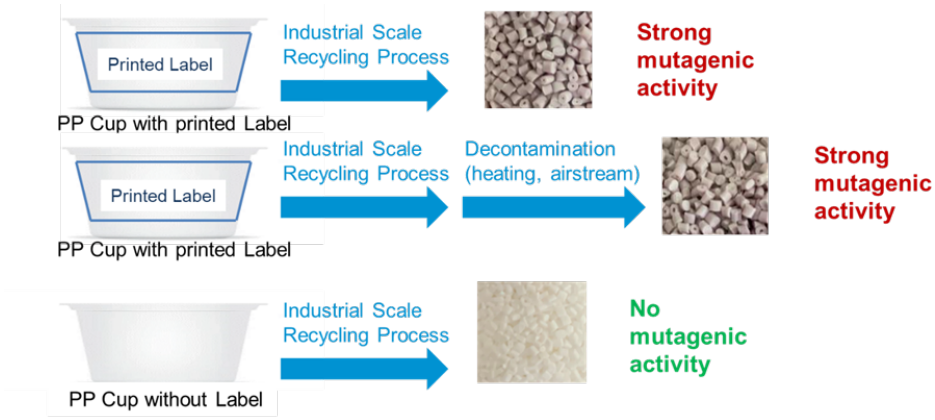

Figure 11: Delabelling as a possible solution to avoid genotoxic effects in recycled plastics. Defined, post-industrial PP cups were recycled and tested with the miniaturised Ames test (TA98+S9). Cups with printed labels showed strong mutagenic activity after recycling, those without label were Ames test negative. An additional decontamination step did not change the positive results of the printed cups.

6.2.2 Deinking

Another promising mitigation strategy identified within the project is deinking. In a laboratory scale experiment, printed films, which previously tested positive in the Ames test, were deinked by the use of subsequent washing steps. Different washing solutions were used to remove the inks from the films before recycling, to prevent the inks from entering the recycling process. While no mutagenic activity was detected in the printed virgin samples, after recycling simulations at 240 °C for 30 minutes, genotoxic effects were observed in the printed film samples. Figure 12 shows that the deinking process reduces this genotoxic effect by 99% compared to a sample that was not deinked.

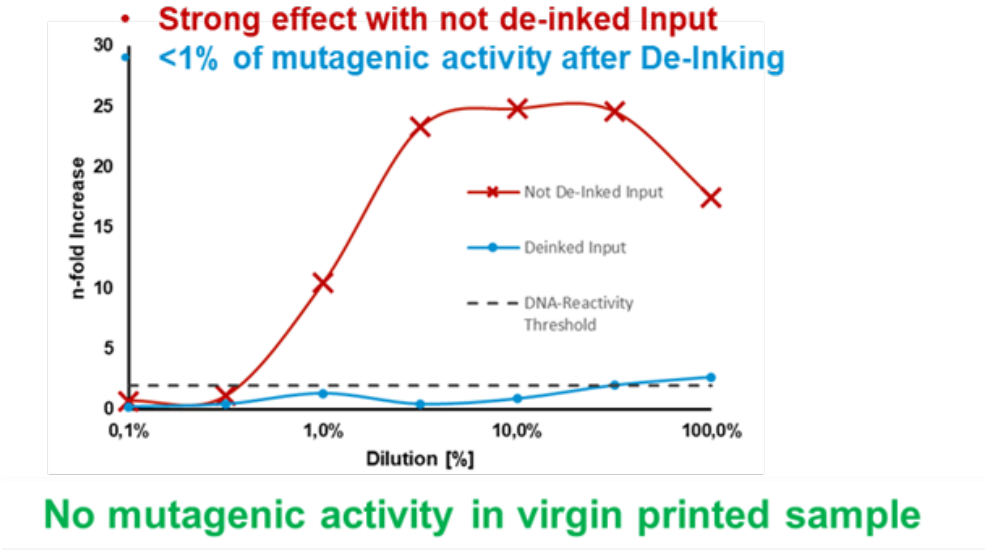

Figure 12: Comparison of the results of the miniaturised Ames test (TA98+S9) of a deinked and a non-deinked sample after a simulated recycling process (30 min, 240 °C).

6.2.3 Sorting technologies for coloured materials

Within the SafeCycle Project, it was found that uncoloured and light-coloured recyclates produced fewer genotoxic effects in the Ames test. To evaluate the validity of this trend, a sorting experiment with yoghurt and milk product cups as input stream was executed. Unsorted flakes, which were partly printed and partly unprinted, resulted in mutagenic effects in the Ames test after the recycling process. In contrast, sorted white flakes demonstrated no mutagenic activity after recycling. Sorted printed flakes yielded a strongly positive result in the Ames test after recycling. Apart from that, many other samples shown in previous chapters support the trend that mutagenic substances often emerge in the recycling of coloured materials, whereas this is not as commonly observed in the recycling of uncoloured or white plastics. Therefore, sorting the white and uncoloured fractions appears to be a possible solution for safe recycling.

6.2.4 Selection of heat-stable colour masterbatches and adhesives

Colour pigments in coloured plastic, that are incorporated in the plastic (e.g. by use of colour masterbatches) cannot be removed by common mechanical recycling steps. To remove these colours, a solvent-based or chemical recycling process would be necessary. In contrast to printing inks, colour pigments in colour masterbatches are already selected to be sufficiently heat stable for normal processing steps during packaging production. However, the heat stress during recycling can be substantially stronger than in the first production step of a virgin plastic packaging, especially for PE. Results from the project SafeCycle show that the degradation of colour pigments is strongly temperature dependent and that a rise of temperature from 200 °C to 240 °C can have a huge impact on the formation of primary aromatic amines from some azopigments. Experiments with coloured HDPE screw caps indicate that some colours used in these screw caps can form mutagenic degradation products at increased temperatures, while most coloured screw caps appeared to be sufficiently heat-stable and gave no indications of mutagenic degradation products.

To enable circular recycling of coloured plastic in contact-sensitive packaging, a promising approach would be to specifically select colour pigments that are not forming any critical DNA-reactive degradation products at the increased heat stress of recycling. Therefore, the safety

assessment for critical mutagenic impurities like primary aromatic amines or other degradation products should not only include the initial heat step of the production process but also the increased heat stress of recycling. A similar approach could make sense for adhesives which cannot be removed during the recycling process. Only a small set of defined adhesive samples could be tested after the heat stress of recycling. The results indicate that some adhesives degrade to critical mutagenic degradation products. This could not be observed for all adhesives however, and first results indicated that there may be alternatives that could be safe after recycling. Facilitating circular recycling for contact sensitive packaging with adhesives requires measures to ensure that adhesives are either removeable (e.g. adhesives for labels) or still safe after the heat stress of a recycling process. The safety of colour pigments and adhesives that cannot be removed in recycling processes should therefore be assessed after a simulated recycling process, especially with regards to DNA-reactive, mutagenic degradation products with the lowest safety threshold in the EFSA evaluation of recycling processes.

6.3 Recommendations for testing

6.3.1 Use of advanced chemical methods

The project has shown that the use of printed plastics, followed by a heating step, may result in the presence of primary aromatic amines (PAAs). PAAs are organic substances that can have a negative impact on human health and the environment. Consequently, PAAs generated through heating can also adversely affect the quality of the plastic recyclate. Therefore, it is of great importance to screen for PAAs in the plastic recyclate to confirm their absence.

Unfortunately, the REACH list does not encompass all possible PAAs, which may lead to potential underreporting. For instance, the possible degradation products of PR 57:1 are not accounted for in the REACH list, resulting in the omission of PAA 2-Amino-5-methylbenzene-sulfonic acid as a degradation product. Therefore, it is essential to universally expand the REACH list to include potential PAAs to eliminate possible multicomponent effects in the Ames test. PAAs are substances that are readily ionizable and can be effectively detected using HPLC-MS/MS. A high-resolution mass spectrometer can be advantageous for enabling more in-depth analyses. A variety of methods for the analysis of PAAs are available in the literature.

Furthermore, heating plastics may lead to the formation of additional degradation products that are not LC-compatible. For example, the degradation product 3,3'-dichlorobenzidine can be generated from PY 13 or PO 34 under heat, which in turn can result in the formation of polychlorinated biphenyl PCB-11. The latter can only be detected using GC-MS. Therefore, PCB screening via GC-MS is also recommended to assess the quality of the recyclate. In conclusion, when heating printed plastics, one must consider the worst-case scenario of polycyclic aromatic hydrocarbons (PAHs) being formed. These screenings can contribute to enhancing the safety of recyclates and should be carefully evaluated based on the input materials.

6.3.2 Non-target screening

Non-target screening requires two essential elements: A specific research question and high-resolution mass spectrometers to detect accurate masses. However, due to the vast number of signals, it is realistically impossible to identify all substances. Thus, a concrete research question is necessary to guide the non-target screening process. For instance, it can be utilized to determine the optimal process parameters in recycling, such as identifying the conditions

that result in the fewest degradation products or examining different batches where deviations occur in specific cases. Using accurate masses and statistical models, critical substances can be identified and utilized as markers. Another approach involves measuring inconspicuous samples and establishing these as fingerprints. These fingerprints serve as references and benchmarks for unknown and potentially critical samples. To achieve this effectively, a sufficient number of samples is necessary.

6.3.3 Specific analysis for PAAs if printed or coloured input streams cannot be excluded

Most primary aromatic amines cannot be detected in standard GC-MS screenings. Therefore, based on the project results, a specific target analysis for PAAs in addition to non-target screening is recommended for recycled contact-sensitive plastic where printed and coloured input materials cannot be excluded in the input streams. It has to be considered that many common azopigments in printing ink and colour masterbatches can form PAAs that are not covered by the standard primary aromatic amine screenings for PAAs referred to in the plastics regulation EU 10/2011 (EU, 2011) and listed in Annex XVII to Regulation (EC) No 1907/2006 (EU, 2006). Therefore, the detected PAAs might only represent a fraction of the total amount of PAAs present in the recyclate but can never-the-less serve as an important warning signal for critical degradation products from colour pigments.

6.3.4 Analysis of DNA-reactive effects using a miniaturised Ames test with two strains

The OECD guideline No. 471 (OECD, 2020) recommends using at least five different bacterial strains for the standard pre-incubation Ames test. Studies by Escobar et al. (Escobar et al., 2013) and Williams et al. (Williams et al., 2019) showed using only the *Salmonella* Typhimurium strains TA98 and TA100 with and without metabolic activation was sufficient to detect most mutagenic substances. To verify these results, miniaturised Ames tests were carried out using five test strains with already existing results of the test strains TA98 and TA100.

Plastic extracts from virgin and recycled materials from the FFG COIN project Migratox and the FFG CORNET project PolyCycle were tested. The results with the three additional strains *Salmonella* Typhimurium TA1535 and TA1537 as well as *E. coli* WP2 uvrA[pKM101] were compared to the already existing results. In total, 94 samples were tested using the bacterial strains TA98 and TA100, with mainly negative results. 83 of those samples tested negative with the strains TA98 and TA100 and indicated no positive results in any of the three additional strains. Within the 11 samples that tested positive, 9 also showed positive results with at least one of the three additional strains. The results showed that the three additional strains could not detect any additional positive samples and that TA98 and TA100 detected a higher number of positive samples.

Therefore, for the screening of mutagenic effects in plastic extract samples, the use of only two strains, namely *Salmonella* Typhimurium TA98 and TA100 with and without metabolic activation (±S9), is recommended.

7 Conclusions and outlook

The results of the SafeCycle project have shown that components from printing inks such as nitrocellulose binders and organic pigments like azopigments can lead to undesirable degradation products, resulting in positive Ames tests after plastics are recycled. Some of the substances responsible have been identified, which means that the cause of the negative effects in recycling can be reduced. The results also show that printing inks or pigments in the input of recycling processes should be reduced in order to minimize the negative impact of printing ink components and pigments. Processes such as delabelling and deinking play a major role here.

Reviewing and optimising the efficiency of such processes will be the focus of another forthcoming project: ColourCycle. Within this project recycling simulations and measuring the effectiveness of deinking and delabelling will be investigated. Moreover, a challenge test for deinking with model contaminants will be developed to evaluate and compare the efficiency of deinking processes. Further screening of critical ink components will be part of the ColourCycle project and analytical testing strategies will be developed.

In addition to measures that improve recycling processes, such as delabelling, deinking, and better sorting, it is important to also consider Design for Recycling from the early stages. By already avoiding certain problematic substances during product design, many hazards in recycling can be prevented. This approach plays a key role in making materials more recyclable and supporting a sustainable circular economy.

Finally, as an additional option, the use of non-degradable materials for inks and lacquers, which remain stable under mechanical recycling conditions, should be further investigated to avoid the formation of potentially harmful degradation products during high-thermal processes.

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