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Safety Assessment of Recycled Plastics from Post-Consumer Waste with a Combination of a Miniaturized Ames Test and Chromatographic Analysis

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Abstract: European circular economy goals require the use of recycled polymers in sensitive applications such as food packaging. As plastic recyclates can contain unknown post-consumer substances, the European Food Safety Authority evaluates recycling processes using a worst-case assumption: all contaminants are DNA-reactive mutagens/carcinogens with extremely low safety thresholds. The current data are insufficient to estimate whether this assumption is justified. To provide scientific evidence on the presence of DNA-reactive mutagens in recycled plastics, 119 input and output samples from plastic recycling were tested with a miniaturized Ames test. DNA-reactive mutagens were not detected in recycled polyethylene terephthalate, which is already approved for food contact. However, other types of recycled plastics (polyethylene, polypropylene and polystyrene), which are currently unauthorized for food contact, showed DNA-reactive, mutagenic effects in a total of 51 samples. The DNA-reactive substances that are responsible for the detected mutagenic activity could not be identified by comparison of the bioassay data with analytical results from a chromatographical screening. The data from the Ames test analysis of different independent batches and a comparison of input and output material indicate that the DNA-reactive contaminants are not randomly introduced through the misuse of recycled packaging by consumers, but are systematically formed during the recycling process from precursors in the input. This publication highlights the need to identify the source for this critical contaminant to enable the future use of polyethylene, polypropylene and polystyrene in sensitive applications.

Keywords: exposure; food packaging; food contact materials; miniaturized Ames; polymer contaminants; polyolefins; recycling; regulation; safety

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1. Introduction

Even though plastic food contact materials (FCMs) are made from different types of polymers, mechanical recycling to produce food-grade plastic is currently only possible for post-consumer polyethylene terephthalate (PET) bottles [1]. This is due to the fact that recyclates contain a multitude of different substances. Contaminants can be introduced during the collection and reprocessing of the plastics in addition to substances already present in virgin materials, also including non-intentionally added substances (NIAS) [2,3]. Particular emphasis must be placed on DNA-reactive, mutagenic or carcinogenic substances, for which a very low exposure limit of 0.0025 µg/kg body weight (bw) per day

applies according to the Threshold of Toxicological Concern (TTC) concept [4,5]. Following a worst-case scenario, the European Food Safety Authority (EFSA) currently assumes that all unidentified contaminations have a DNA-reactive, mutagenic function [6] and therefore uses this threshold for all chemicals of unknown identity. Recycled PET (rPET) was approved for food contact applications previously, as migration modeling shows that DNA-reactive, mutagenic substances, even if present in rPET, do not migrate into foodstuff in levels exceeding their threshold [7]. The situation is more complicated for polyolefins like polyethylene (PE) or polypropylene (PP). These highly diffusive materials consist of a different polymeric structure, which mediates an easier transfer of chemicals from packaging into packed content [1]. Recycling processes to produce food-grade recycled polyolefins are, therefore, currently not approved by EFSA [8].

In view of climate targets and the importance of becoming independent of fossil resources, plastic recycling is becoming increasingly important in the European Union [9]. To enable the highest possible recycling rates, the use of recycled polymers in sensitive applications, such as FCMs, is inevitable. A recent proposal of the European Commission foresees a minimum recycled content of 30% for single-use plastic beverage bottles and polyethylene terephthalate (PET) food packaging, as well as 10% for all other plastic FCMs by 2030 [10]. To increase the availability of different types of food-grade recycled plastics in the future and generate the highly needed data on material safety, the European legislation on recycled plastic FCMs was recently revised. With the publication of the recycling regulation (EU) 2022/1616, the concept of novel technologies was introduced which allows yet unauthorized recycled food contact plastics to be temporarily placed on the market. For that, an initial safety report must be submitted and reviewed by a local competent authority before market release. The report provides the contaminant level in the plastic input and recycled output, the decontamination efficiency and data about the transfer of these contaminants to food [11]. Legislation requires that the developer of a recycling technology constantly monitors and regularly reports incidental contaminations during operation. To this end, all produced input and output batches must be analyzed by analytical methods to list all substances with a molecular weight below 1000 Dalton, of which 20 chemicals with the highest occurrence need to be identified. Based on the collected data, EFSA finally decides whether or not the recycling technology proved to be sufficiently safe to be officially authorized and recycled plastics can be continued to be used as FCMs [11]. With this objective in mind, a feasible and clear testing strategy is needed, especially to uncover DNA-reactive, mutagenic contaminations, including currently unknown components, to fulfil the 0.0025 µg/kg bw/day limit [4,12]. As no single analytical method is able to identify all detected substances at the moment [13–15], especially in complex matrices like recycled plastics, an optimization of current testing schemes is urgently needed. As the physico-chemical properties of genotoxins regarding their hydrophobicity and volatility can be quite diverse [16], a combination of analytical methods is necessary to detect a broad spectrum of possible DNA-reactive, mutagenic substances. That said, even such a combination will not result in a complete identification, as analytical reference standards are not yet available for all compounds, especially for as yet unknown NIASs [17]. Further, analytical screening can neither predict the hazard of toxicologically unclassified substances nor consider potential mixture effects. So far, the most promising strategy is, therefore, to combine chromatographic analysis with in vitro bioassays, such as the Ames test, to detect direct DNA reactivity at low levels [15,18–21]. While chromatographic analysis (targeted and non-targeted) aims to identify known and suspected chemicals to assess a potential hazard based on toxicological data, the Ames test determines the DNA-reactive mutagenicity of the overall mixture considering unknown substances as well as potential interactions [15,20]. Even though the Ames test proved to be the most sensitive among different mutagenicity and genotoxicity test systems, the assay was criticized for its insufficient limits of detection (LODs) in the past [15,22,23]. A miniaturized format, the Ames microplate format (MPF^{TM}), a fluctuation assay which uses liquid medium and scores DNA reactivity colorimetrically [24], can reach lower LODs than

the traditional Ames test according to the OECD 471 guideline [25,26]. While the Ames MPF^{TM} reaches LODs of 0.1 (4-NQO) and 5 (BaP) µg/kg food for known DNA-reactive substances such as 4-nitroquinoline 1-oxide (4-NQO) and benzo[a]pyrene (BaP), minimum concentrations of 1 (4-NQO) and 33 (BaP) µg/kg food are required in the plate-based version (LODs calculated based on Rainer et al. [25] assuming a concentration factor of 1000). That said, detection limits in both formats highly depend on the DNA reactivity of the respective substance. A comprehensive comparison of LODs was previously provided by Rainer and colleagues [25]. Even with the miniaturized improved format it is currently not possible to detect all DNA-reactive, mutagenic substances to cover a daily exposure of 0.0025 µg/kg bw, as this translates to a rather low detection limit requirement of 0.15 µg/kg food (60 kg adult, 1 kg daily food consumption). Further improvements of the test systems, including suitable sample preparation techniques [22,25], are required. That said, this miniaturized Ames test has already been used in the screening of migrates/extracts of food contact materials [27] and PET recyclates [19].

The current study aimed to investigate whether or not the Ames test combined with chromatographic analysis can detect DNA-reactive, mutagenic substances in recycled plastics as implied by EFSA, as these potentially migrate into food, especially from highly diffusive polyolefin materials. For that, a wide variety of recycled samples, including different polymer types as well as input (washed flakes from post-consumer waste) and output (recycled granules or finished products produced from these granules) materials, were analyzed using the miniaturized Ames test. Generally, the project focused on the investigation of polyolefins (low-density polyethylene (LDPE), high-density polyethylene (HDPE) and PP), although polystyrene (PS) and PET samples were included for comparison. Initial experiments were set up in a way to enable comprehensive testing using two bacterial strains each in the absence and presence of metabolizing enzymes [27,28]. As it became apparent that a surprisingly high rate of DNA-reactive, mutagenic results was found, the focus was shifted towards high throughput and identifying as many DNA-reactive, mutagenic samples as possible. Most of the DNA-reactive, mutagenic results originated from the tester strain TA98 (for frameshift mutations) after the external supplementation of metabolizing enzymes (i.e., TA98 +S9). For this reason, instead of trying to show the absence of DNA-reactive substances down to the LODs in the miniaturized Ames test, with multiple test runs, only TA98 +S9 was applied for several samples. Thus, the Ames experiments that were performed for this study do not necessarily meet regulatory standards, but were used as a screening tool. Non-targeted and/or targeted GC and high-performance liquid chromatography (HPLC) screening techniques were performed on the same samples in parallel to the in vitro bioassay. These results were already published separately by Rung et al. [29]. To obtain more information about substances causing the DNA-reactive, mutagenic activity, a multifactor analysis was carried out to investigate possible correlations between results from the miniaturized Ames and the (separately published [29]) chromatographical analysis data.

2. Results

2.1. Results from the Miniaturized Ames Test Analysis

2.1.1. Overview of the Screening Results

A total of 119 input and output samples from plastic recycling were analyzed for DNA-reactive, mutagenic effects with a miniaturized version of the Ames test.

A broad overview of the results is shown in Table 1, where samples are classified regarding their DNA-reactive, mutagenic response in each testing condition. In Supplementary Table S1, additional information about the intensity of the DNA-reactive, mutagenic signal (expressed as fold induction) and potential inhibitory effects (expressed as spike recovery), as well as sample specific detection limits are given.

Table 1. Miniaturized Ames test results of 119 plastic recyclate samples (input and output). A total of 40 rHDPE, 18 rLDPE, 12 rPET, 37 rPP, 10 rPS and two other samples were tested. DNA-reactive, mutagenic results in either of the strain/S9 combinations are highlighted in bold and red. I: input, O: output, +: DNA-reactive, mutagenic, -: non-DNA-reactive, non-mutagenic, -*: non-DNA-reactive, non-mutagenic (an inhibiting sample dilution was tested), nd: no data, eq: equivocal.

Code	Type	I/O	TA98		TA100		Code	Type	I/O	TA98		TA100		Code	Type	I/O	TA98		TA100		Code	Type	I/O	TA98		TA100	
			−S9	+S9	−S9	+S9				−S9	+S9	−S9	+S9				−S9	+S9	−S9	+S9				−S9	+S9	−S9	+S9
H1	HDPE	I	nd	-	nd	nd	H11	HDPE	O	-*	-	-*	-	**H21**	**HDPE**	**O**	**nd**	**+**	**nd**	**nd**	H31	HDPE	O	-	-	-	-
H2	HDPE	I	nd	-	nd	nd	H12	HDPE	O	-*	-	-	-	**H22**	**HDPE**	**O**	**nd**	**+**	**nd**	**nd**	H32	HDPE	O	-	-	-	-
H3	HDPE	I	nd	-	nd	nd	H13	HDPE	O	-	-	-	-	**H23**	**HDPE**	**O**	**-***	**+**	**-**	**-**	H33	HDPE	O	-	-	-	-
H4	HDPE	I	nd	-	nd	nd	H14	HDPE	O	-	-	-	-	**H24**	**HDPE**	**O**	**nd**	**+**	**nd**	**nd**	**H34**	**HDPE**	**O**	**nd**	**+**	**nd**	**nd**
H5	HDPE	I	-	-	-	-	**H15**	**HDPE**	**O**	**-**	**+**	**-**	**-**	**H25**	**HDPE**	**O**	**nd**	**+**	**nd**	**nd**	H35	HDPE	O	-	-	-	-
H6	HDPE	O	nd	-	nd	nd	**H16**	**HDPE**	**O**	**-***	**+**	**-**	**-**	**H26**	**HDPE**	**O**	**-***	**+**	**-**	**-**	H36	HDPE	O	nd	eq	nd	nd
H7	HDPE	O	-	-	-	-	H17	HDPE	O	-*	-	-	-	**H27**	**HDPE**	**O**	**-***	**+**	**-**	**-**	H37	HDPE	O	-	-	-	-
H8	HDPE	O	-	-	-	-	H18	HDPE	O	-*	-	-	-	**H28**	**HDPE**	**O**	**-**	**+**	**-***	**-**	H38	HDPE	O	-	-	-	-
H9	HDPE	O	-	-	-	-	**H19**	**HDPE**	**O**	**-***	**+**	**-***	**-**	**H29**	**HDPE**	**O**	**-***	**+**	**-**	**-**	H39	HDPE	O	-	-	-	-
H10	HDPE	O	-*	-	-	-	**H20**	**HDPE**	**O**	**nd**	**+**	**nd**	**nd**	H30	HDPE	O	-*	-	-	-	**H40**	**HDPE**	**O**	**-**	**+**	**-**	**-**
L1	**LDPE**	**I**	**nd**	**+**	**nd**	**nd**	**L6**	**LDPE**	**O**	**eq**	**+**	**-**	**-**	**L11**	**LDPE**	**O**	**nd**	**+**	**nd**	**nd**	**L16**	**LDPE**	**O**	**-**	**+**	**-**	**-**
L2	**LDPE**	**I**	**nd**	**+**	**nd**	**nd**	**L7**	**LDPE**	**O**	**-**	**+**	**-**	**eq**	**L12**	**LDPE**	**O**	**-**	**+**	**-**	**+**	**L17**	**LDPE**	**O**	**+**	**+**	**-**	**-**
L3	**LDPE**	**O**	**-**	**+**	**-**	**-**	**L8**	**LDPE**	**O**	**-**	**+**	**-**	**+**	**L13**	**LDPE**	**O**	**-***	**+**	**-**	**+**	L18	LDPE	O	-*	-	-	-
L4	**LDPE**	**O**	**-**	**+**	**-**	**-**	**L9**	**LDPE**	**O**	**-**	**+**	**+**	**+**	**L14**	**LDPE**	**O**	**-**	**+**	**-**	**-**							
L5	**LDPE**	**O**	**-**	**+**	**-**	**-**	**L10**	**LDPE**	**O**	**nd**	**+**	**nd**	**nd**	**L15**	**LDPE**	**O**	**-**	**+**	**-**	**-**							
E1	PET	I	nd	-	nd	nd	E4	PET	I	-*	-	-	-	E7	PET	O	-	-	-	-	E10	PET	O	-	-	-	-
E2	PET	I	nd	-	nd	nd	E5	PET	O	-	-	-	-	E8	PET	O	-	-	-	-	E11	PET	O	-*	-	-	-
E3	PET	I	-	-	-	-	E6	PET	O	-	-	-	-	E9	PET	O	-	-	-	-	E12	PET	O	-*	-	-	-
P1	PP	I	nd	-	nd	nd	**P11**	**PP**	**O**	**nd**	**+**	**nd**	**nd**	**P21**	**PP**	**O**	**-**	**+**	**-**	**-**	**P31**	**PP**	**O**	**+**	**+**	**-**	**-**
P2	PP	I	nd	-	nd	nd	P12	PP	O	nd	-	nd	nd	P22	PP	O	-	-	-	-	**P32**	**PP**	**O**	**+**	**+**	**-**	**-**
P3	PP	I	nd	-	nd	nd	P13	PP	O	nd	-	nd	nd	P23	PP	O	nd	-	nd	nd	P33	PP	O	-	-	-	-
P4	PP	I	-	-	-	-	P14	PP	O	-	-	-	-	P24	PP	O	-	-	-	-	P34	PP	O	-	-	-	-
P5	PP	I	-	-	-	-	**P15**	**PP**	**O**	**-**	**+**	**-**	**-**	P25	PP	O	-	-	-	-	P35	PP	O	-	-	-	-
P6	PP	I	-	-	-	-	P16	PP	O	-	-	-	-	P26	PP	O	-	-	-	-	**P36**	**PP**	**O**	**nd**	**+**	**nd**	**nd**
P7	**PP**	**I**	**+**	**eq**	**-**	**-**	P17	PP	O	-	-	-	-	P27	PP	O	-	-	-	-	P37	PP	O	nd	-	nd	nd
P8	**PP**	**O**	**-**	**-**	**-**	**+**	P18	PP	O	-	eq	-	-	P28	PP	O	nd	-	nd	nd							
P9	PP	O	-	-	-	-	P19	PP	O	-	-	-	-	P29	PP	O	-	-	-	-							
P10	**PP**	**O**	**nd**	**+**	**nd**	**nd**	**P20**	**PP**	**O**	**-***	**+**	**-**	**-**	**P30**	**PP**	**O**	**-***	**+**	**-***	**-**							
S1	PS	I	nd	-	nd	nd	S5	PS	I	-	eq	-	-	**S9**	**PS**	**O**	**-**	**+**	**-**	**-**	**O1**	**PE**	**I**	**+**	**+**	**-**	**-**
S2	**PS**	**I**	**-**	**+**	**-**	**-**	**S6**	**PS**	**I**	**+**	**+**	**-***	**-***	**S10**	**PS**	**O**	**-**	**+**	**-**	**-**	**O2**	**PE/PP**	**O**	**-**	**+**	**-**	**eq**
S3	**PS**	**I**	**+**	**+**	**-**	**-**	S7	PS	I	-	-	-	-														
S4	**PS**	**I**	**-**	**+**	**-**	**-**	S8	PS	I	-	-	-	-														

In the initial phase of the study, where 89/119 samples were screened, testing was conducted using two different *Salmonella* strains, TA98 and TA100, which represent different mutagenic modes of action (point and frameshift mutations). To mimic mammalian metabolic processes otherwise not covered by prokaryotic tester organisms such as *Salmonella*, tests were further performed in the presence and absence of externally supplied metabolizing enzymes (rat liver S9). A DNA-reactive, mutagenic activity was identified for 38/89 samples. Interestingly, most of the samples classified DNA-reactive (36/38) triggered an effect in the test condition TA98 +S9. Only two out of 38 DNA-reactive samples were classified as DNA-reactive, as they showed DNA-reactive mutagenicity in other strain/S9 combinations (P7 in T98 −S9, P8 in TA100 +S9; see Table 1). This condition was therefore readily identified as the most sensitive one to detect DNA-reactive responses in the context of recycling. Further tests and evaluations presented in this publication will, therefore, only focus on the TA98 +S9 testing condition. The sample screening for the remaining 30 samples, applying only the testing condition TA98 +S9, uncovered additional 13 DNA-reactive, mutagenic samples. Overall, nearly half of the samples (51/119) scored DNA-reactive, mutagenic activity in the Ames test.

2.1.2. Comparison of Different Polymer Types

To determine whether the TA98 +S9-detected DNA reactivity is equally induced by different polymer types, the sample set included 40 HDPE (H1–H40), 18 LDPE (L1–L18), 12 PET (E1–E12), 37 PP (P1–P37), 10 PS (S1–S10) and two other materials (a PE sample of unknown density (O1) and a PE/PP mix (O2)). Importantly, none of the analyzed PET samples showed DNA-reactive effects, while a DNA-reactive mutagenic classification was found in all other polymer groups, i.e., in 15/40 HDPEs, 17/18 LDPEs, 9/37 PPs, 6/10 PSs and in 2/2 of the as other classified materials (see Figure 1).

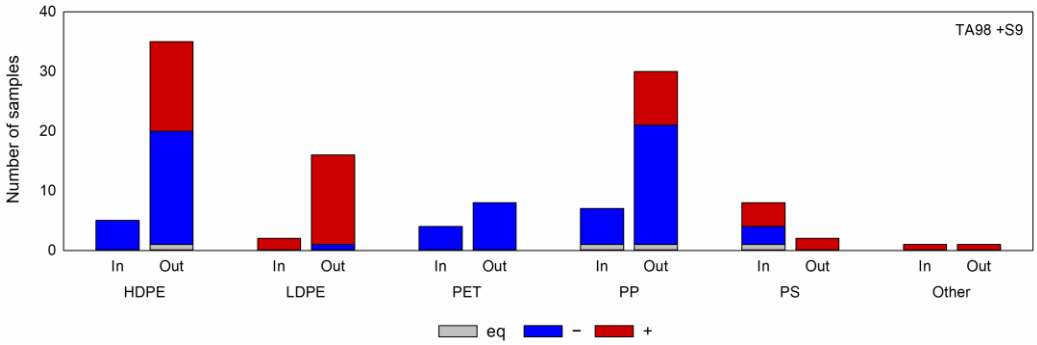

Figure 1. Miniaturized Ames test results of recycled plastics (input and output) in test condition TA98 +S9 sorted by polymer type. The number of DNA-reactive, mutagenic (+, red), non-DNA-reactive, non-mutagenic (−, blue) and equivocal (eq, grey) recyclates sorted by processing stage (in: input, out: output) is presented.

2.1.3. Comparison of Different Processing Stages

Further, the impact of different processing stages was investigated by analyzing TA98 +S9-detectable DNA reactivity in input (i.e., flakes, 27 samples) and output materials (i.e., re-extruded granules or finished products, 92 samples). Overall, the portion of DNA-reactive, mutagenic samples was higher in the output (42/92 samples DNA-reactive, mutagenic) than in the input (7/27 samples DNA-reactive, mutagenic; see Figure 2). Except for PET, DNA-reactive, mutagenic activity was found in output samples of all polymer types, i.e., 15/35 HDPEs, 15/16 LDPEs, 9/30 PPs, 2/2 PSs and 1/1 PE/PP mix (classified as other). Among the input flakes, only materials of the polymer types PS and LDPE (DNA-reactive, mutagenic samples: 2/2 (LDPE), PS: 4/8(PS)) and 1/1 PE sample of unknown density showed DNA reactivity, while no DNA reactivity was found for HDPEs and PPs (DNA-reactive, mutagenic samples: 0/5 (HDPE), 0/7 (PP)). That said, the number of tested input flakes was much lower than that of the output samples (27 input vs. 92 output samples). A

pair of input (L1) and output (L10) materials was analyzed by sampling the input flakes and their corresponding output granules after processing, resulting in a clear increase in DNA-reactive, mutagenic activity after the extrusion process (see Figure 2).

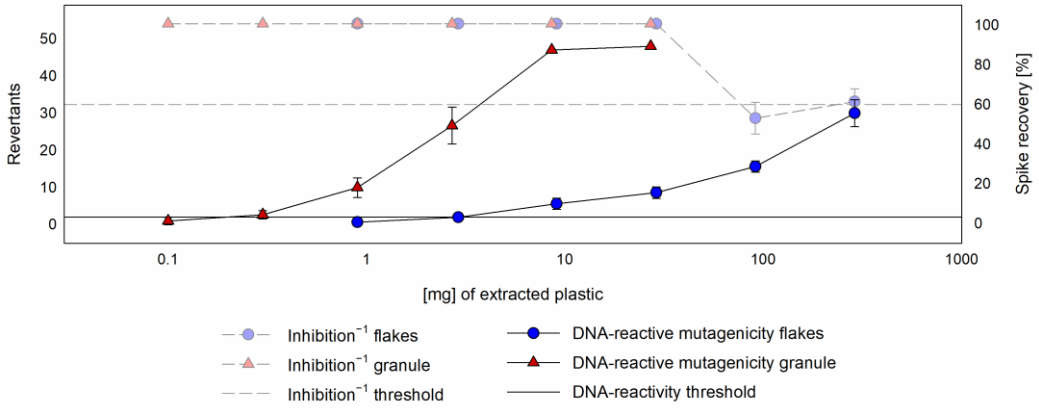

Figure 2. Comparison of DNA-reactive effects of corresponding input (L1, flakes) and output (L10, granule) LDPE materials in the miniaturized Ames test. Tests were carried out in the condition TA98 +S9. The term "[mg] of extracted plastic" refers to the amount of extracted plastic per 10 µL portion of concentrated DMSO extract (i.e., the volume required for one miniaturized Ames test). Black, solid curves represent the DNA reactivity response (expressed as revertants): blue circles—flakes, red triangles—granule. Grey, dashed curves represent the inhibition^{-1} (expressed as spike recovery): light blue circles—flakes, light red triangles—granule.

2.1.4. Comparison of Different Polymer Batches

To analyze whether DNA-reactive responses were batch-specific or appeared reproducibly over different batches, the sample set partially contained different batches of samples, all produced on the same production line, from the same input source, by a common manufacturer (e.g., H20 and H21). Indeed, a systematic contamination independent of the production date was confirmed: the rLDPE samples L6, L7 and L8 (different batches of sample group 1) as well as the rHDPE samples H20, H21 and H22 (different batches of sample group 2) and H24 and H25 (different batches of sample group 3) each showed strong DNA reactivity with similar signal intensities within a sample group (see Figure 3). As these batch-wise comparisons focused on DNA-reactive recyclates, however, the overall high portion of DNA reactivity presented in Table 1 overestimates the actual level of Ames-positive samples. If different batches were grouped together and only a common result per sample group was considered, the number of DNA-reactive samples was reduced to 38 samples.

2.2. Correlation of Miniaturized Ames Test Results with Analytical Data

To identify the substances responsible for the DNA-reactive, mutagenic activity in the test condition TA98 +S9, the same samples tested by the miniaturized Ames test (in this study) were analyzed using non-targeted GC and targeted HPLC by Rung et al. [29]. The data of the chromatographic evaluation from Rung and colleagues were already published previously [29]. Although numerous toxicologically suspicious substances were identified and toxicologically classified, the data could not fully explain the reason for the detected DNA reactivity. Further, the correlation between a DNA-reactive, mutagenic classification and detected substances could be multifactorial. To analyze such complex relationships and to reduce the dimensionality of the original dataset, a principal component analysis (PCA) was performed, considering TA98 +S9 Ames data and the most relevant detected substances (V1–V42, Table 2) (for data selection criteria see Section 4.3.2).

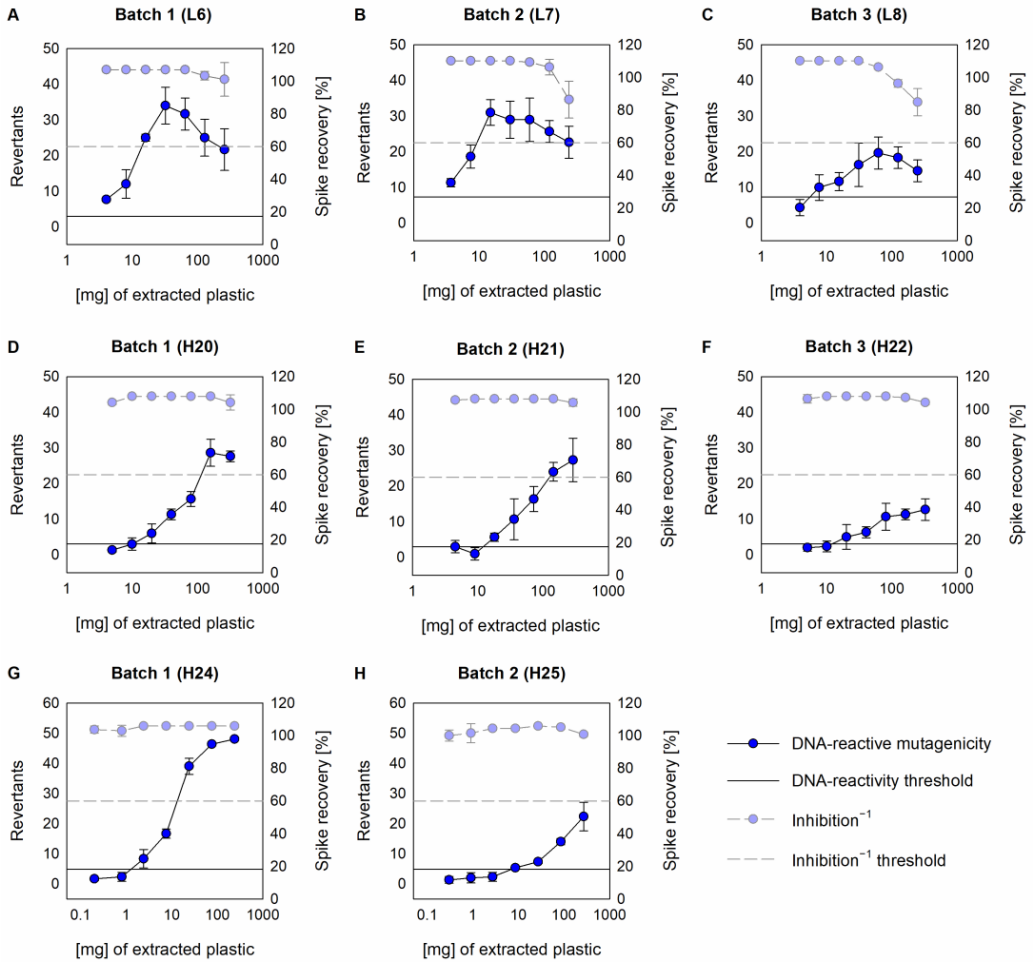

Figure 3. Comparison of the signal intensity of different batches of DNA-reactive, mutagenic samples. The following materials represent different batches of one sample: 1. L6, L7 and L8 ((**A**–**C**); sample group 1), 2. H20, H21 and H22 ((**D**–**F**); sample group 2) and 3. H24 and H25 ((**G**,**H**); sample group 3). Tests were carried out in the condition TA98 +S9. The term "[mg] of extracted plastic" refers to the amount of extracted plastic per 10 µL portion of concentrated DMSO extract (i.e., the volume required for one miniaturized Ames test). Black, solid curves with blue circles represent the DNA reactivity response (expressed as revertants). Grey, dashed curves with light blue circles represent the inhibition^{-1} (expressed as spike recovery).

The pool of tested rPET materials (E1–E12) did not contain any samples classified as DNA-reactive, and thus no correlation analysis with the substances V1–V42 was required.

Further, the chromatographic data for rPS (S1–S4 and S6–S10) were not normally distributed. As PCA is only applicable for normally distributed data, no statement can be made about the correlations between miniaturized Ames test and chromatographic screening results in this work.

Also, for rPP (P1–P6, P8–P9, P11–P17, P21–P22 and P24–P27) no correlation with the miniaturized Ames test results was discernible using PCA, since the DNA-reactive, mutagenic and the non-DNA-reactive, non-mutagenic samples were not separable from each other within the cluster plot.

While the rHDPE group (H1–H33, H35 and H37) contained both non-DNA-reactive, non-mutagenic and DNA-reactive, mutagenic samples in the testing condition TA98 +S9, all of the chromatographically analyzed rLDPE (L3–L8 and L12–L17) and rPE/PP (O2) materials were characterized as DNA-reactive, mutagenic (in TA98 +S9). Correlation analysis is, however, only possible if both endpoints (DNA reactivity and non-DNA reactivity) are represented in the miniaturized Ames dataset to distinguish the DNA-reactive effect in

the presence of a substance from the lack of a DNA-reactive effect in the absence of the substance. To also analyze the rLDPE and rPE/PP materials using principal component analysis, all PE-based materials (rLDPEs, rHDPEs and the rPE/PP mix) were combined into one rPE group due to their similar diffusion properties.

Table 2. Selected substances, their molecular mass (in brackets; in g/mol) and CAS number for principal component analysis. V: variable, M: molecular mass, NA: not available.

V	Substance (M in g/mol)–CAS	V	Substance (M in g/mol)–CAS
V1	Irgafos 168 (646)–31570-04-4	V22	2-Phenyl-1,2,3,4-tetrahydronaphthalene (208)–29422-13-7
V2	Oxidized Irgafos 168 (662)–95906-11-9	V23	N,N-dimethyltricyclo [5.3.1.04,9]undec-5-en-2-amine (191)–NA
V3	Irganox 245 (587)–36443-68-2	V24	1,3,5-Triphenylcyclohexane (312)–28336-57-4
V4	Irganox 1010 (1178)–6683-19-8	V25	2-Methylanthraquinone (222)–84-54-8
V5	Irganox 1076 (531)–2082-79-3	V26	(2E)-2-(3-Oxo-1-benzothiophen-2-ylidene)-1-benzothiophen-3-one (296)–522-75-8
V6	Irganox 1330 (775)–1709-70-2	V27	4-Isopropyl-N-(2-(2-methyl-1 H-indol-3-yl)-ethyl)-benzenesulfoamide (356)–NA
V7	Limonene (136)–138-86-3	V28	2,2′-Thiobis(6-tert-butyl-p-cresol) (358)–90-66-4
V8	Acetaldehyde (44)–75-07-0	V29	Bumetrizole (316)–3896-11-5
V9	1,3-Dioxolane (74)–646-06-0	V30	(Z)-Docos-13-enamide (337)–112-84-5
V10	Ethylene glycol (62)–107-21-1	V31	Octocrylene (361)–6197-30-4
V11	Oxidized Irgafos 168 (662)–95906-11-9	V32	1-Phenyl-1,2,3,4-tetrahydronaphthalene (208)–3018-20-0
V12	Irgafos 168 (646)–31570-04-4	V33	Triphenyl phosphate (326)–115-86-6
V13	(1-Methyl-2,2-diphenylcyclopropyl)sulfanylbenzene (317)–56728-02-0	V34	Tributyl citrate (360)–77-94-1
V14	(2,3-Diphenylcyclopropyl)methyl phenyl sulfoxide (332)–131758-71-9	V35	Tinuvin 770 (480)–52829-07-9
V15	1,3-Diphenylpropane (196)–1081-75-0	V36	2-Butyl-5-hexyloctahydro-1H-indene (264)–55044-33-2
V16	(3-Cyclopent-2-en-1-yl-2-methyl-1-phenylprop-1-enyl)benzene (274)–NA	V37	S-[(E)-1,3-Diphenylbut-2-enyl] N,N-dimethylcarbamothioate (311)–NA
V17	Di-p-xylylene (208)–1633-22-3	V38	4-tert-Butylcyclohexyl acetate (198)–10411-92-4
V18	4-Cyano-1,2,3,4-tetrahydro-1-naphthaleneacetonitrile (210)–57964-40-6	V39	Oleamide (282)–301-02-0
V19	4-(1-Cyanoethyl)-1,2,3,4-tetrahydronaphthalene-1-carbonitrile (210)–57964-39-3	V40	Docosanamide (340)–3061-75-4
V20	3,6,13,16-Tetraoxatricyclo [16.2.2.2(8,11)]tetracosa-1(20),8,10,18,21,23-hexaene-2,7,12,17-tetrone (384)–24388-68-9	V41	1-Iodo-2-methylundecane (296)–73105-67-6
V21	1,2-Diphenylpropane (196)–1081-75-0	V42	Bornyl acetate (196)–76-49-3

The cluster plot (see Figure 4) showed a partial division of the DNA-reactive, mutagenic and the non-DNA-reactive, non-mutagenic rPE samples. The DNA-reactive, mutagenic cluster (red) extended from the zero point into the first quadrant, while the non-DNA-reactive, non-mutagenic cluster (blue) extended orthogonally into the second quadrant. The visual separation of these two areas showed that the examined data provide a basis for differentiating these groups. That said, a full separation of both groups was not possible

based on the analyzed data as indicated by the overlapping zone in the middle of the clusters. Further, the cluster plot showed that there were outliers in both the non-DNA-reactive and the DNA-reactive groups. The first two PCs caused 23.32% of the variation of the Ames test results.

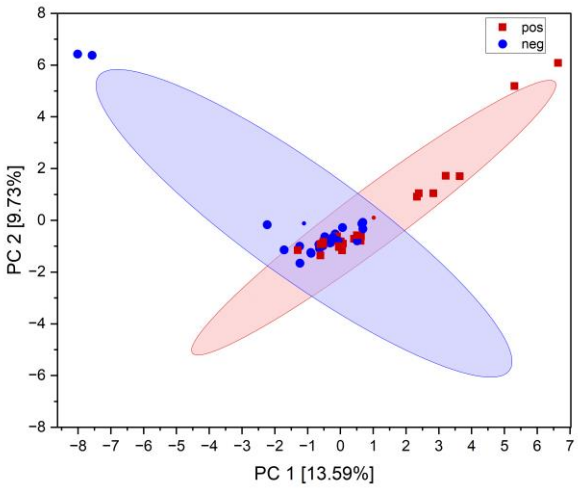

Figure 4. Cluster plot of the principal component analysis of DNA-reactive, mutagenic effects (red squares/red area: samples with DNA-reactive, mutagenic Ames test result (pos), blue circles/blue area: samples with non-DNA-reactive, non-mutagenic Ames test result (neg)) depending on the variables V1–V42 of all rPE samples (significance 0.05; principal component 1 (PC 1): 13.59%; principal component 2 (PC 2): 9.73%).

By displaying the principal components in the biplot (see Figure 5) it was possible to see which variables (i.e., substances represented by vectors) contributed strongly to explaining the variance in the data. Vectors with positive PC1 and PC2 values (quadrant 1, top right, Figure 5) colocalized with the cluster of samples classified as DNA-reactive in the cluster plot (quadrant 1, top right, Figure 4). This shows that the DNA-reactive, mutagenic classification was associated with the vectors of Irgafos 168 (CAS 31570-04-4, V1/V12), oxidized Irgafos 168 (CAS 95906-11-9, V2/V11), Irganox 1010 (CAS 6683-19-8, V4), Irganox 1330 (CAS 1709-70-2, V6), N,N-dimethyltricyclo[5.3.1.04,9]undec-5-en-2-amine (V23), 4-tert-Butylcyclohexyl acetate (CAS 10411-92-4, V38), Oleamide (CAS 301-02-0, V39) and Bornyl acetate (CAS 76-49-3, V42) for rPE samples. Importantly, PCA does not prove causality. Therefore, a discussion about the plausibility of causal relationships is given in Section 3.2.

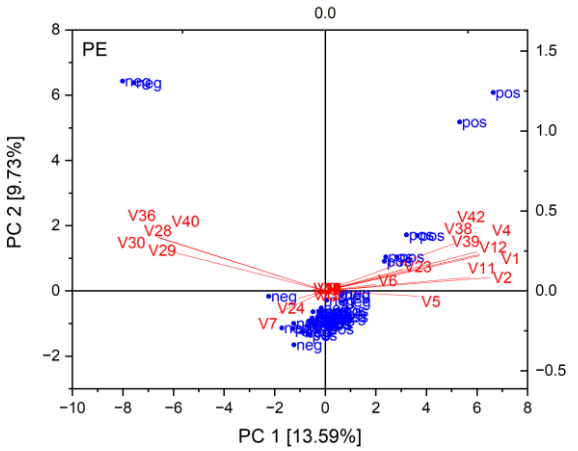

Figure 5. Biplot of the principal component analysis of DNA-reactive, mutagenic effects (blue: samples with DNA-reactive, mutagenic (pos) and non-DNA-reactive, non-mutagenic (neg) Ames test result, red: variables V1–V42 shown as vectors) of all rPE samples (significance 0.05; principal component 1 (PC 1): 13.59%; principal component 2 (PC 2): 9.73%).

3. Discussion

3.1. Interpretation of the Miniaturized Ames Test Results

Overall, 119 samples from plastic recycling were tested in the miniaturized Ames test (see Table 1). Of these, 51 samples tested positive for DNA-reactive mutagenicity. The vast majority of the DNA-reactive, mutagenic results were found in the condition TA98 in presence of rat liver S9. This repeating pattern indicates that the effects might be caused by a specific substance or substance group that requires metabolic activation [30]. A random contamination with many different post-consumer substances seems unlikely, as this would result in a more equal distribution of DNA-reactive, mutagenic effects over all testing conditions, which would not reflect the observed focus on TA98 +S9. To follow up this theory, comparison studies for different batches belonging to one sample group were performed. As similar DNA reactivity independent of the production date and a specific input was detected (see Figure 3); these experiments supported a systematic contamination with DNA-reactive, mutagenic substances from a common source. Importantly, a broad introduction of false positive artefacts, e.g., through contamination with the amino acid histidine originating from protein residues of packed food, could be ruled out, as genotoxicity was confirmed by additional in vitro bioassays targeting a different mode of action. For that, a HepGentox assay based on the p53 pathway [31] and an umuC test based on the SOS pathway [32] were used to test selected recyclate extracts. A Petri-dish-based Ames test (based on OECD guideline 471 [26]) with selected samples confirmed that the DNA-reactive behavior was independent of the miniaturized format applied here. The miniaturized Ames tests were performed in two different laboratories (OFI, FH Campus Vienna) for some selected positive and negative samples in this study and gave very similar results.

Very high-fold inductions were detected for multiple samples (see Figure 3, Supplementary Table S1), which were, e.g., significantly higher than signals from an equally tested DNA-reactive aromatic hydrocarbon fraction of a mineral oil [33]. Such high activity is usually only achieved for very active standard substances such as DNA-reactive positive controls. An extract of the recycled sample H24 even showed TA98 +S9-detected DNA reactivity up to a 1:100 dilution. If, for instance, a primary aromatic amine (PAA) such as 2,4-diaminotoluene was the source of the detected activity in H24, a concentration as high as 2193 mg/kg polymer would be necessary to explain the detected DNA-reactive mutagenicity (calculated based on the substance's lowest effective concentration (LEC) from Rainer et al. [25] by dividing the LEC of the pure substance in DMSO (658 mg/L) by the M/V ratio in the DMSO extract (30 kg/L) and multiplying the result by the dilution factor of 100). For the highly active PAA 2-aminoanthracene, which is used as a common positive control in the miniaturized Ames test due to its strong DNA reactivity [34,35], 1.1 mg/kg polymer would still be required (calculated based on Rainer et al. [25]; LEC: 0.3 mg/L, M/V: 30 kg/L). As chromatographic analysis could not identify the source of the measured signals, these substances only serve as an example. The actual amount of the contaminating mutagen per mass unit polymer depends on the DNA-reactive potential of the respective chemical. That said, the target substance is likely a potent DNA-reactive chemical in the Ames test, as the overall amount in the recycled polymer must meet a plausible concentration range.

The portion of TA98 +S9-detected DNA-reactive, mutagenic samples varied based on the type of plastic that was tested (e.g., 15/40 DNA-reactive, mutagenic samples for rHDPE, 0/12 DNA-reactive, mutagenic samples for rPET) (see Figure 1). However, the overall sample size was too small to derive significant conclusions on the differences between polymer types, except for rPET, which never scored DNA reactivity. Samples were taken from input (washed flakes) and output streams (granules and final products). The positivity rate for the output materials appeared to be higher (42/92 DNA-reactive, mutagenic samples for output vs. 7/27 DNA-reactive, mutagenic samples for input). Further, DNA-reactive, mutagenic samples were represented among all polymer types (except rPET) in the output, but were only present in rPS, rLDPE and a PE sample of unknown density for the input. It has to be mentioned, however, that the number of

analyzed input samples was generally low and that input and output materials were not necessarily taken from the same recycling plants or process batches. Therefore, an overall comparison of DNA-reactive mutagenicity in the in- and output must be viewed critically. In fact, a direct comparison between corresponding input (L1) and output (L10) materials was only possible for one sample pair (see Figure 2), which confirmed higher signals intensities in the output though.

While 66 out of 119 samples did not show DNA reactivity in TA98 +S9, it must be stated that the miniaturized Ames test is not capable of detecting most DNA-reactive substances down to the required target LOD of 0.15 µg/L, derived from the TTC value of 0.0025 µg/person/kg bw/day, as was demonstrated by previous publications [22,23,27]. In addition, not all sample extracts were tested with all strain/S9 combinations to increase the screening throughput. Therefore, the overall rate of samples that contain DNA-reactive mutagens might be even higher than reported in this study. However, it must be considered that for this particular work, the samples were not selected to represent the highest possible quality of recycled materials, but rather a broad spectrum of currently available materials was intended to be covered, including worst-case samples. Additionally, when DNA-reactive, mutagenic results were detected from a particular recycling plant, an increased effort in testing the corresponding batches was made. This means that the sample selection is strongly biased towards DNA reactivity.

3.2. Identification of the Sources for DNA-Reactive, Mutagenic Activity

In vitro bioassay results can give no information about the chemical nature of the substances that cause the mutagenic effects. The results of the miniaturized Ames test were therefore compared with results from a chromatographical screening, which was performed on the same samples, but was separately published by Rung et al. [29]. In their study, Rung and colleagues analyzed the samples by generally available, high-throughput non-targeted HS-GC-FID, liquid-injection GC-FID and GC-MS methods. In addition, a routine targeted reversed phase HPLC method was used to quantify a small number of defined plastic additives and their oxidation products to consider also some non-volatile substances [29]. A comparison of chromatographical results and bioassay data did not give an obvious explanation for the detected DNA-reactive, mutagenic effects by single substances. In most of the samples that showed mutagenic activity, no substance with a known mutagenic effect or a structural alert for genotoxicity could be detected in Rung's chromatographical screening. The detected substances mostly originated from known plastic additives (e.g., antioxidants), the polymer itself (e.g., monomers such as polyethylene glycol) or contaminants from previous fill goods (e.g., the aroma compound limonene). The origin of the substances, as well as all chemical analysis data, were already discussed previously [29].

To evaluate whether any correlations between the analytical data and a DNA-reactive response in the miniaturized Ames test can be found by reducing the dimensionality of the dataset, the bioassay data from this study and the separately published chemical analysis data (from the same samples) were analyzed with a principal component analysis.

As the group of rPETs contained only non-DNA-reactive, non-mutagenic samples, PCA was not required for this polymer class.

For the rPS and rPP classes, no further conclusions could be obtained through PCA unfortunately. As the chromatographic data for rPS materials were not normally distributed, PCA was not applicable for this group. In a normally distributed data collection, values are concentrated around the average (mean), and the probability of finding values close to the mean is higher than the probability of finding extreme values. A high number of extreme values can arise from a too-small database, when mean values are randomly underrepresented, or from uncategorized subgroups that differ too much from one another. Indeed, the number of samples was limited for polystyrene (nine samples) and most came from one source. Therefore, the formulation of the analyzed samples might not have been representative of recycled polystyrenes in general.

For the tested rPP materials, the DNA-reactive, mutagenic and the non-DNA-reactive, non-mutagenic groups were not separable in the cluster plot of the PCA, making any conclusions about a correlation with chromatographically identified substances impossible. A possible explanation for the colocalization could be that other substances were responsible for the DNA-reactive, mutagenic effect than the substances of high and medium volatility detectable by the mostly GC-focused screening analysis conducted by Rung et al. [29].

In the PCA cluster plot of rPE, two areas were formed for the DNA-reactive, mutagenic and the non-DNA-reactive, non-mutagenic classified samples, which overlap in the middle and separate diagonally against each other (see Figure 4). In the non-DNA-reactive, non-mutagenic group several samples deviate far from the group in the middle diagonally towards PC2. These could be outliers, which have a very high purity or deviate very strongly from the majority in their material properties. In the DNA-reactive, mutagenic group, some samples overlap with the non-DNA-reactive, non-mutagenic group. It seems plausible that in this subgroup the strength of the Ames test is reduced compared to other DNA-reactive samples. A separation of DNA-reactive, mutagenic from non-DNA-reactive, non-mutagenic classified samples diagonally towards PC1 is also apparent. This could be caused due to particularly contaminated samples, which may also show particularly high Ames test results. That said, the miniaturized Ames test setup in the current study was not designed to make quantitative comparisons, especially as extraction conditions varied partially (e.g., different solvents) and two labs were involved in the experimental analysis. The bioassay data, therefore, cannot confirm (or falsify) the predictions from PCA. In general, the weak separation of the DNA reactivity groups in the cluster plot and the low cumulative variance (23.32%) indicates that other variables (i.e., substances) have an influence on the DNA-reactive mutagenicity, which have not been considered here.

The PCA biplot showed that the DNA-reactive, mutagenic classification of PE-based recycling input and output materials correlated with the substances Irganox 1010 (CAS 6683-19-8, Code V4), Irganox 1330 (CAS 1709-70-2, V6), Irgafos 168 (CAS 31570-04-4; V1/V12), oxidized Irgafos 168 (CAS 95906-11-9, V2/V11), 4-tert-butylcyclohexyl acetate (CAS 10411-92-4, V38), N,N-dimethyltricyclo[5.3.1.04,9]undec-5-en-2-amide (V23), oleamide (CAS 301-02-0, V39) and bornyl acetate (CAS 76-49-3, V42) (see Figure 5).

Irgafos 168 (V1/V12), Irganox 1010 (V4) and Irganox 1330 (V6) are approved additives, which were clearly shown to be non-DNA-reactive in the Ames test in previous studies [36–39]. Although this rules out a causal relationship, a correlation could arise because additives are likely re-dosed during multiple processing cycles to compensate for high mechanical and thermal stress. The thermal and mechanical stress would serve as a common factor and could lead to further substances causally related to the Ames test result.

Also, the correlation with oxidized Irgafos 168 (V2/V11) could indicate a general bias towards increased oxidative stress in DNA-reactive, mutagenic recycled samples and that other oxidized substances with a possible DNA-reactive, mutagenic function could be present. One possible reason might be the high proportion of output materials within the analyzed sample pool which suffer from higher thermal and mechanical stress.

In case of N,N-dimethyltricyclo[5.3.1.04,9]undec-5-en-2-amide (V23), no CAS number could be determined. Therefore, the substance might be either too new or a wrong allocation of the database. The literature search provided insufficient results to interpret possible causal relationships with Ames test results.

Oleamide (V39) is used in the compounding and further processing of rubber and plastics by extrusion or injection molding. Its correlation with a DNA-reactive, mutagenic classification may indicate that a high degree of processing promotes the DNA-reactive, mutagenic effect. Oleamide itself has no proven DNA-reactive, mutagenic effect [40,41].

4-tert-Butylcyclohexyl acetate (V38) is widely used in perfumery. It is a versatile fragrance in toilet soaps, detergents, colognes, shampoos, creams, incense and other low-cost fragrances [42–44]. Bornyl acetate (V42) is the acetic acid ester of the terpene borneol, and is also used as an odor component, e.g., in bath preparations, sprays and soaps, certain foods and as an ingredient in pharmaceuticals [45–47]. Both of these substances are

typical post-consumer contaminations without a DNA-reactive, mutagenic effect. However, both may occur simultaneously with other mutagenic substances due to their common post-consumer-source.

Unfortunately, none of the detected substances could explain the DNA reactivity response obtained with bioassay analysis, and an adaption of the analytical screening approach will be necessary in future work. That said, the analytical test program by Rung et al. [29] was not specifically designed for the detection of DNA-reactive mutagens. Recycled plastics could contain known DNA-reactive contaminants such as primary aromatic amines, nitrosamines, polyaromatic hydrocarbons or aflatoxins. These substances would probably not have been detectable using the applied chromatographical methods and would require a more specialized targeted screening approach. Such an extension of the analytical battery would not guarantee a successful identification either, as the activity might be caused by an as yet unknown DNA-reactive mutagen. To identify substances of unknown structure, non-target screenings could be of help, especially when combined with in silico tools for DNA-reactive mutagenicity. However, a GC-MS non-target screening, as performed by Rung et al. [29], can only detect unknown mutagens if they are in the volatile or intermediate volatility range. An additional HPLC-MS non-target screening is necessary to cover low-volatile or involatile contaminants as well.

Based on the results of the miniaturized Ames test, whether the DNA-reactive, mutagenic effects are caused by a single substance or by a mixture of many different mutagens cannot be judged. Potential mixture effects could further complicate the identification with chemical analysis. Indeed, an additive mixture effect in the Ames test can be assumed if several mutagens acting via comparable mode of actions are present [48].

As DNA-reactive substances are widely banned in the general public (e.g., food grade plastics, etc.), it seems unlikely that the responsible substance is intentionally used, but could rather be a degradation product formed during the recycling process. Comparative testing of a pair of an input (L1) and an output sample (L10) with the miniaturized Ames test showed that the melting and regranulation process did not reduce the levels of contamination, but seemed to induce higher DNA reactivity instead (see Figure 2). The formation of DNA-reactive substances is therefore likely to be triggered during the recycling process, probably by heat-induced, side- or degradation reactions during the extrusion. This would make an identification even more difficult, as such a degradation product has a probably unknown chemical structure that is neither available as an analytical standard, nor included in current analytical databases.

Therefore, new strategies will be necessary for identification. To reduce complexity, it would be favorable to work with well-defined artificially produced simulated recycling samples, rather than with real-world post-consumer materials. This could help to evaluate, e.g., the possible effects of reprocessed printing inks or adhesives. If an activity is detected in a less complex, well-defined sample, chemical analysis is simplified. In addition, fractionation protocols and an effect-directed analysis, where fractions, rather than the whole extract, are analyzed in the miniaturized Ames test, could facilitate identification. If only one of several fractions shows DNA-reactive, mutagenic activity, a chemically less complex input could be used for chemical analysis.

3.3. Potential Impact on Plastic Recycling for Food Contact

As already mentioned in a previous chapter, the recycled plastic materials that were tested for this study were in no way representative of the overall market and are biased towards a selection of strongly DNA-reactive, mutagenic samples. None of the recycled polyolefin and PS materials are currently in use as food contact materials. However, as already discussed, there is clear evidence that these materials are systematically contaminated with DNA-reactive, mutagenic substances in a concentration that can be readily detected with the miniaturized Ames test. The only exception seems to be rPET, where no DNA-reactive, mutagenic results could be found. If the source of the activity cannot be identified, assuming total migration, many of the tested materials are clearly not suitable

for high-value applications, such as food contact materials, according to current European legislation [12,15]. In addition, even their use in less critical applications should be questioned. One example is the sector of cosmetic packaging, where polyolefins are often used for flexible tubes or stable bottles. Leave-on products, especially, support substance uptake via the dermal route due to prolonged product exposure. Since not only the duration of products' exposure on the skin, but also the application on a large surface of skin (whole-body products such as shower gel) and their use at low body weights (products for children and babies such as baby shampoo) can lead to higher exposures, even rinse-off products may require a higher quality of packaging materials [49]. While polyolefins are highly diffusive materials resulting in high migration, polystyrene products are less diffusive, and the migration of contaminants from PS is much slower at typical storage temperatures [1]. As the current study aimed to extract the highest bioassay-compatible concentrations, the applicability of miniaturized Ames test results from rPS extracts on realistic use cases must be critically reviewed. It has already been shown that, due to the low diffusivity of polystyrene, recycled polystyrene is safe in food contact in certain layered structures working as a functional barrier, even if residues of DNA-reactive, mutagenic contaminants are in the inner layer [50].

In addition to consumers, occupational health and safety measures of workers in recycling plants should be considered since the current results cannot confirm or exclude an interaction of volatile compounds in the recyclate with employee health. Even though the final recycled materials might only be used in non-critical applications by consumers (e.g., flower pots or garbage bags), where the threshold of DNA-reactive substances is met, exhaust fumes from the production machinery should be specifically analyzed and tested for its safety for the health of employees and the environment.

When interpreting the results generated in the current work, it should be acknowledged that the Ames test is an in vitro bioassay which detects DNA reactivity based on bacterial cultures and that chromatographic analysis provides a framework to assign the measured effects to toxicologically critical substances causing such effects. Although the results suggest mutagenic potential and the Ames test has high predictivity of rodent carcinogenicity [51] (e.g., about 77% of Ames positive chemicals are indeed carcinogenic in rodents according to an analysis of the national toxicology program database [52]), not every mutation in a living organism results in lasting DNA damage or cancer development in humans [53–55]. For transferability to humans, further parameters have to be considered, such as the route of intake (e.g., oral, dermal, and inhalation), the amount of exposure over defined periods of time and protective or detoxification mechanisms. Moreover, the parameters used for sample preparation might represent a worst-case scenario for some materials, as migration with 95% ethanol for 10 days at 60 °C could be exaggerated for certain use cases. Currently, there are insufficient data on the impact of recycled packaging on humans. Thus, the results can neither validate nor invalidate the concern for human health. However, to the best of the authors' knowledge, it is currently the appropriate and sole approach to identify potentially DNA-reactive compounds and to exclude critical materials from the food and cosmetics sector.

3.4. Outlook and Future Perspective

More research is needed to fully understand the nature of the DNA-reactive contaminations and their source. In order to highlight research areas of interest, some hypotheses to explain the origin of the DNA-reactive, mutagenic signals will be discussed here.

Although the use of CMR substances for household use is strictly limited if technically avoidable [56–58], consumer misuse of food packaging, which may result in the accidental contamination of packaging waste with CMR substances, and the transfer of CMR substances from the packaging of technical products should be investigated. That said, the DNA-reactive responses of recycled polymers were shown to be highly reproducible over several batches by the authors of this work. If a contamination (with low quality

packaging or post-consumer substances) is responsible for the detected activity, it must occur systematically.

Further, food and, if technically unavoidable, cosmetic products may also contain CMR substances directly [58,59]. An example is formaldehyde in self-tanning products. However, these products were shown to be safe for the consumer regardless [60], and formaldehyde, which is Ames-negative using the tester strain TA98 and only weakly positive with TA100, cannot explain the activity reported here.

One potential source of DNA-reactive substances might derive from the reactions and degradation of printing inks. It has been shown previously that nitrocellulose, which is a very common binder of printing inks, can react with amines to form nitrosamines at high temperature [61–63]. Therefore, nitrocellulose-based printing inks cannot be used for high-temperature applications. However, in recycling these, high temperatures cannot be avoided. Further, the thermal degradation of some azo pigments in printing inks triggers the release of PAAs [64]. Even though both nitrosamines and PAAs are known Ames-positive substance groups, neither is exclusively positive in testing condition TA98 +S9 [65–68]. Therefore, the strong DNA reactivity that has been detected in this study is likely caused by other degradation or reaction products during the recycling process which might be currently unknown.

Further investigations into the collection, washing and recycling processes of polymer packaging could shed light on the conditions that promote the formation of the as yet unknown DNA-reactive, mutagenic contaminants. This could include examining the effects of temperature, light exposure and the presence of other chemicals on the degradation of, e.g., polymer additives, printing inks and adhesives. In contrast to the current study, which aimed to provide a broad overview, the authors suggest a more systematic selection of samples to evaluate whether different recycled sample classes (e.g., printed vs. non-printed samples, food-grade vs. non-food grade materials, etc.) differ in their DNA-reactive, mutagenic output. This will help to focus analytical efforts on the most relevant sample category. As none of the substance groups discussed above would have been detectable with the mostly GC-focused analytical approach carried out in this work, the analytical screening strategy should be extended using additional techniques. As a possible connection to non-volatile substances seems likely, the earlier-mentioned non-targeted LC-HRMS screening is a promising possibility.

4. Materials and Methods

4.1. Sample Material

Within the scope of this study, recycled plastic samples (input and output) were directly received from industry partners. The materials represented different polymer types. Recycled polyolefins were mainly analyzed, including 18 recycled LDPEs (rLDPEs; L1–L18), 40 recycled HDPEs (rHDPEs; H1–H40), 37 recycled PPs (rPPs; P1–P37), one recycled polyethylene (rPE) sample with unknown density (O1) and one sample containing a mixture of PE and PP (O2). A further 10 recycled PS (rPS) materials (S1–S10) and 12 rPETs (E1–E12) were tested. Input (flakes; in total 27 samples) and output (granules and final products; in total 92 samples) materials of every polymer type were tested. A detailed overview of the samples is provided in Table 1. Importantly, the tested sample materials were not representative of the overall market, as additional samples from the same supplier (e.g., additional batches) were selected for further testing if DNA reactivity was shown initially.

4.2. Evaluation of DNA-Reactive, Mutagenic Effects with the Miniaturized Ames Test

To detect DNA-reactive, mutagenic substances, recycled polymers were extracted; the extracts were pre-concentrated and analyzed by a miniaturized Ames test.

4.2.1. Sample Extraction and Pre-Concentration

For extraction experiments, three solvents were used initially: an isopropanol/cyclohexane (9:1) mixture (IPA/CHx) (isopropanol: Merck, Darmstadt, Germany; cyclohexane: ≥99.5%, VWR International, Fontenay-sous-Bois, France) (based on [69]), dichloromethane (DCM, ≥99.5%, Merck, Darmstadt, Germany) (based on [7,19]) and 95% ethanol (diluted from 99.9%, LiChrosolv®, Merck, Darmstadt, Germany) (based on [12,27]). The recycled polymer samples were stored fully immersed in solvent within glass bottles closed tightly with polytetrafluoroethylene-coated screw caps (VWR International, Leuven, Belgium). Depending on the solvent, different time and temperature conditions were applied during extraction: 3 days at 70 °C for IPA/CHx, 3 days at 37 °C for DCM and 1 to 10 days at 60 °C for 95% ethanol (safety drying chamber FDL 115 (Binder, Tuttlingen, Germany)). Extraction with 95% ethanol at 60 °C for 1 to 10 days showed the highest responses in the miniaturized Ames test, as less inhibition was scored than for any of the other procedures and was adopted as the standard protocol. Nevertheless, initial results produced with the other solvents were also shown in this study to provide additional data. Polymer extracts were concentrated to a residual volume of about 1 mL by evaporation using a Syncore® Analyst parallel evaporator (Büchi, Flawil, Switzerland). The concentrated extracts were then mixed with dimethyl sulfoxide (DMSO, ≥99%, Merck, Darmstadt, Germany) and the residual solvent was evaporated to reach a final concentration of 15–30 g polymer per mL DMSO. Substance recovery rates of this method using suitable model substances were published previously [27,70]. At least two independent extracts from each sample were prepared and each tested in triplicates with the miniaturized Ames test. In some cases, e.g., if a sample scored an equivocal result, additional extracts were prepared and tested for clarification. To control for contaminations, a procedural blank was prepared for each round of extraction and treated identically to the prepared extracts. In total, 81 procedural blanks were prepared for the current study. Prior to analysis with the miniaturized Ames test, the samples were stored at −20 °C to 4 °C. Further details on the sample preparation (including information about the extraction solvent, temperature and time, the mass-to-volume (M/V) ratio applied during extract and the final DMSO volume after pre-concentration) are given in Supplementary Table S1. It should be noted that extraction instead of migration, as would occur in contact with filling goods such as food, cosmetics or detergents, was intended in this work. An extraction approach was chosen to recover to highest possible amount of DNA-reactive substances from the materials to (at least partially) compensate for the insufficient detection limits of the miniaturized Ames test.

4.2.2. The Miniaturized Ames Test

To test pre-concentrated polymer extracts with the miniaturized Ames test, two histidine auxotrophic *Salmonella* Typhimurium strains, TA98 (frameshift mutations) and TA100 (point mutations) (Xenometrix, Allschwil, Switzerland), were used. Bacterial overnight cultures were grown in nutrient broth No. 2 (Oxoid Ltd., Basingstoke, UK) supplemented with 50 µg/mL ampicillin sodium salt (Sigma-Aldrich, Steinheim, Germany) to a cell density of about $1.5-2 \cdot 10^9$ CFU/mL. The testing procedure was based on instructions by Xenometrix [35] with minor adaptations [27]. In short, 10 µL of the sample extract was applied in triplicates to 24-well plates (VWR International, Radnor, PA, USA), and mixed in each well with 240 µL of exposure medium supplemented with 10% (TA98) or 5% (TA100) of bacterial overnight culture. After an incubation period of 90 minutes at 37 °C and 250 rpm (0.87 rcf; shaker 3032 (GFL, Burgwedel, Germany, 2.5 cm shaking amplitude)), 2.6 mL reversion indicator medium was added and the content of each well was distributed to 48 wells of a 384-well plate (VWR International, Radnor, PA, USA). Exposure and reversion indicator medium were prepared according to ISO 11350:2012 or obtained from Xenometrix (Xenometrix, Allschwil, Switzerland) [71]. The plates were incubated for 48–72 h at 37 °C (incubator 3501 (Rubarth Apparate GmbH, Laatzen, Germany)), before they were scored for revertant wells, i.e., wells where bacterial growth was triggered by a mutation that

restores histidine prototrophy. Bromocresol purple (bromocresol purple sodium salt, 90 %, Sigma-Aldrich, St. Louis, MO, USA), a pH indicator in the indicator medium, monitors revertant well formation by inducing a color shift from purple to yellow upon growth-induced production of acidic metabolites. A baseline was calculated by summing up the mean number of the negative control (DMSO) revertants and the standard deviation of the negatwive control (DMSO) revertants. Extracts with a fold induction of $\geq$2 compared to baseline were regarded as DNA-reactive, mutagenic (i.e., Ames positive), while extracts showing a fold induction of <2 were classified non-DNA-reactive, non-mutagenic (i.e., Ames negative). An equivocal classification was chosen if different extracts from the same sample showed contradicting results, e.g., one extract scored DNA-reactive, mutagenic, while the second one was non-DNA-reactive, non-mutagenic. To mimic metabolic processes, the samples were tested without (−S9) and with (+S9) metabolizing enzymes present in an S9 mixture. For that, the exposure mixture was supplemented with 4.5% of phenobarbital/β-naphthoflavone-induced S9 (Xenometrix, Allschwil, Switzerland) and an S9-co-factor mixture in the +S9 condition. Inhibiting effects of the samples (e.g., bacteriotoxic/bacteriostatic effects or inhibition of metabolic S9 enzymes) were evaluated by spiking the exposure mix with Ames-positive substances, i.e., 2-nitrofluorene (2-NF, 98%, Sigma Aldrich, Steinheim, Germany) for TA98 -S9, 4-nitroquinoline 1-oxide (4-NQO, $\geq$98%, Sigma Aldrich, Steinheim, Germany) for TA100 −S9 and 2-aminoanthracene (2-AA, 96%, Sigma Aldrich, Steinheim, Germany) for TA98 and TA100 +S9. The final concentrations of the positive controls in the exposure media used for spike experiments were as follows: 2-NF: 2 µg/mL (TA98 −S9), 2-AA: 0.5 µg/mL (TA98 +S9), 4-NQO: 0.1 µg/mL (TA100 −S9), and 2-AA: 1.25 µg/mL (TA100 +S9). Inhibition was determined based on the spike recovery (SR) of a sample, which was calculated by dividing the mean number of revertants of the spiked sample extract by the mean number of revertants of an equally spiked DMSO control multiplied by 100%. A sample was considered inhibiting if its spike recovery was <60%, a threshold previously established by Rainer and colleagues [27]. The detection limits of the miniaturized Ames test were calculated for each positive control (4-NQO, 2-NF, 2-AA) based on Rainer et al. (see Supplementary Table S1) [27].

4.3. Correlation Analysis of Miniaturized Ames Test and Chromatographic Analysis Data

4.3.1. Chromatographic Analysis

To identify the substance(s) triggering a DNA-reactive, mutagenic effect in the miniaturized Ames test, a subset of the same recycled materials analyzed in the current study, i.e., 12 rLDPEs (L3–L8 and L12–L17), 35 rHDPEs (H1–H33 and H35, H37), 24 rPPs (P1–P6, P8–P9, P11–P17, P21–P22 and P24–P27), one PE/PP mix (O2), 10 rPS (S1–S10) and 12 rPETs (E1–E12), was analyzed in parallel through chromatographic analysis. The data from this chromatographic analysis (including a profound description of the applied methodology) were previously already published by Rung et al. [29].

In short, Rung and colleagues analyzed the samples using a set of GC-based non-target screening approaches including (1) liquid-injection GC-flame ionization detector (FID), (2) liquid-injection GC–mass spectrometry (MS), (3) headspace (HS)-GC-FID and (4) HS-GC-MS. The HS-GC measurements allowed the identification and quantification of volatiles (substances V7–V10 in Table 2) such as limonene. Liquid injection GC measurements of plastic extracts were carried out to identify and quantify medium volatile substances, including oxidized and non-oxidized additives (substances V11–V42 in Table 2). Further, Rung et al. applied a targeted reversed phase HPLC-MS method to analyze polyolefins extracts for known additives (substances V1–V6 in Table 2).

In the current publication, the previously published chromatographic analysis data (from Rung et al. [29]) and the in vitro bioassay data generated here by the miniaturized Ames test were combined for a principal component analysis as described in Section 4.3.2.

4.3.2. Principal Component Analysis

To investigate which substances are responsible for DNA reactivity in recycled polymer extracts, miniaturized Ames test results (from this study) were correlated with chromatographically detected chemicals (study from Rung et al. [29] on the same samples) through a multivariate data analysis tool using principal component analysis.

The following approach was chosen for the statistical evaluation: 1. Appropriate PCA input data were selected from the chromatographic data (Rung et al. [29]) and the miniaturized Ames test data (from this study) (see selection criteria below). 2. Normal distribution of the chromatographic data was checked to test whether principal component analysis was applicable. 3. PCA was applied to identify substances correlating with a DNA-reactive, mutagenic Ames classification. 4. The possibility of a causal relationship between a correlating substance and a DNA-reactive response was discussed based on literature data.

1. Selection of input data for PCA: a. Chromatographic data set: In total, 205 different substances were detected in the recycled materials by Rung et al., of which 175 were identified [29]. To reduce this substance pool, the following criteria were applied: (I) the abundance of the occurring substances (occurrence in number of samples: $\geq$2), (II) their quantifiability (concentration above the detection limit) and identifiability (semi-quantitatively assigned via internal standards and via NIST database (NIST/EPA/NIH Mass Spectral library, version 2.0 d:2005-04, MS Search 2.0, Gaithersburg, MD, USA)) and (III) their toxicological evaluation (Cramer classes II and III, determined by Toxtree (Estimation of Toxic Hazard-A Decision Tree Approach, version 3.1.0-1851-1525442531402, Sofia, Bulgaria)). This resulted in a set of 42 substances. These substances, including their concentrations, were defined as input variables (V1–V42) for PCA and are listed in Table 2. Among these substances, some (e.g., Irgafos 168, see Table 2) were considered twice in PCA, as they were detected in different concentrations by GC- and HPLC-based screenings. While the authors acknowledge that each sample can contain each substance only at one concentration, such differences can be explained by different sample/extract preparation methods, the susceptibility of the material to different sample treatment steps, different chromatographic columns, different detection/quantification limits of the analysis methods or if a substance is inhomogeneously distributed throughout a polymer sample. To take these effects into account, all substance concentrations were considered. b. Miniaturized Ames test data set: Only miniaturized Ames test data obtained in the testing condition TA98 +S9 were used for PCA (column "TA98 +S9" in Table 1). Further, only samples clearly classified as DNA-reactive, mutagenic (labelled with "+") or non-DNA-reactive, non-mutagenic (labelled with "−") were included. Equivocal samples (labelled with "eq") were excluded from the evaluation.

2. Test for normal distribution: The chromatographic data were tested for normal distribution with the Shapiro–Wilk Test [72], which offers a comparatively high test-strength when testing samples with $n < 50$. The analysis was performed using OriginPro (version 2022b, OriginLab Corporation, MA, USA) [73].

3. Principal component analysis: PCA is a method for dimensionality reduction and representing data in a new coordinate system in which the principal components are uncorrelated. The principal components are linear combinations of the original variables (i.e., the chromatographically detected substances, V1–V42) that explain the variance in the data. The maximum number of extracted principal components is equal to the number of variables. The first principal component (PC1) captures the largest variance in the data, the second principal component (PC2) captures the next largest variance, etc. In PCA, the individual variables are vectors in a multidimensional space. To visualize the data in 2D, two principal components (PC1 and PC2) were selected, and a biplot (see Figure 5) and a cluster plot (see Figure 4) were created. The biplot shows DNA reactivity results from the miniaturized Ames test and the chromatographically detected substances (i.e., variables) in the same coordinate system as function of PC1 and PC2. Each sample is represented by one data point with the description of its result from the miniaturized

Ames test. Detected substances (V1–V42) are represented by vectors protruding from the zero point. The direction and length of the vectors in relation to the position of the datapoints is used to determine the relationship between miniaturized Ames test results and the detected substances. A visual colocalization between the vector and a group of datapoints indicates a correlation. Cluster plots were created to visually indicate the areas of sample groupings (group 1: DNA-reactive, mutagenic samples, group 2: non-DNA-reactive, non-mutagenic samples) as a function of PC1 and PC2. In both graphs, the axis labels and the plot description give the percentages which show the proportion of the variance explained by each principal component normalized to the total variance [74]. The principal component analysis was carried out using the OriginPro software (version 2022b, OriginLab Corporation, MA, USA) [73].

4. Literature confirmation: Substances correlating with DNA reactivity were checked by a literature search to investigate a possible causal relationship.

5. Conclusions

In the current work, recycling input and output materials, including PET, PS and various polyolefins, were investigated using a miniaturized Ames test. In recycled PET, which is already authorized for food contact at the European level, no DNA-reactive, mutagenic contaminants were detected in any of the tested samples. However, materials containing DNA-reactive, mutagenic substances were identified in all other recycled polymer classes. Considering the detection limits of mutagenicity and genotoxicity in in vitro bioassays, which are currently insufficient to meet relevant safety thresholds, additional DNA-reactive, mutagenic effects cannot currently be ruled out. The reported findings, therefore, seem concerning and could hinder the progress in plastic recycling, especially in sensitive applications like food contact materials. Attempts to correlate the DNA-reactive, mutagenic activity with substances detected by a mainly GC-focused analytical screening could not identify relevant chemicals which explain the detected effects. The data indicate that the DNA-reactive, mutagenic target substance is not randomly introduced by consumers. Instead of decontaminating the input stream, the recycling process itself seems to trigger the formation of these critical compounds. While a strictly consumer-related contamination can hardly be controlled, a systematic contamination might be easier to prevent if the source of the contamination can be identified. Future work will therefore focus on the identification of these compounds and their sources. Investigations must include additional analytical methods like LC-HRMS analysis and specific analysis for critical substance classes such as PAAs and nitrosamines, since the mainly GC-focused screening applied here was insufficient, possibly because the target substances are of low volatility or nonvolatile. Further, a systematic testing of well-defined laboratory-scale recycled plastics (e.g., printed/unprinted, packed with defined food/empty) with the miniaturized Ames test could help to identify sources for the DNA-reactive contaminants and also reduce complexity in chemical analysis. Ultimately, a better understanding of the sources of contamination in recycled polymer products could enable the development of effective strategies for preventing and minimizing their occurrence in order to guarantee the safe use of recycled plastics.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/recycling8060087/s1, Table S1: Detailed miniaturized Ames test results of 119 input and output materials of plastic recycling.

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