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Risk assessment of food contact materials

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Abstract

Food Contact Materials (FCMs), such as plastics, papers, ceramics and inks used in food packaging, containers, kitchen utensils and tableware are subject to scrutiny due to their potential to release toxic compounds into food. In the European Union, materials and articles intended for contact with food must adhere to stringent safety regulations and novel materials not explicitly covered by existing legislation require individual risk assessment. This project focused on the assessment of the genotoxic potential of two substances used in FCMs, specifically neodecanoic acid (NDA) and di(2-ethylhexyl) phthalate (DEHP), for which data gaps have been identified in genotoxicity studies. NDA was selected because it was re-evaluated due to the intention to approve its use in printing inks for FCMs. For DEHP, various studies on genotoxicity are available, which, however, differ in their outcome. DEHP is commonly used as a plasticiser to enhance the flexibility, transparency, durability of plastics and is ubiquitously detected in daily life. The present study followed the EFSA strategy for the assessment of genotoxicity applying in vitro methods in bacterial and mammalian cells as well as in silico approaches. In this context, aneuploidy, a thresholded genotoxic effect, received particular attention since few indications are available on the aneugenic activity of FCMs. The results showed significant findings that require further investigation.

KEYWORDS

aneuploidy, DEHP, food contact material, genotoxicity, neodecanoic acid

CONTENTS

1 | INTRODUCTION

At the EU level, the legislation on Food Contact Materials (FCM) establishes the need of careful safety assessment of FCM to protect consumers since FCM may contain chemicals migrating from the material itself to the foodstuff and raising health concern. The assessment of genotoxicity represents a key step in risk assessment of FCM and it is based on the EFSA Note for Guidance 2008 (EFSA, 2008).

1.1 | Background

Chemical substances used in the production of FCM are several thousand considering both intentionally added as well as known and unknown non-intentionally added substances (NIAS) (Geueke et al., 2014). Migration of these substances or their breakdown or reaction products into foods can occur. To protect the consumer health, an appropriate chemical risk assessment needs to be undertaken. The framework regulation for FCM is the regulation (EC) No 1935/2004 which lays down the general safety principles for all FCM – first, that FCM must not endanger human health. Another EU regulation, which FCM have to comply with, is regulation (EC) No 2023/2006 on 'Good manufacturing practice' (GMP) that applies to all stages in their manufacturing chain. Besides the general legislation, specific European Union (EU) measures exist for some FCM such as plastic materials (also recycled), ceramics, regenerated cellulose films, active and intelligent materials as well as for some substances including bisphenol A, epoxy derivatives and nitrosamines. However, there are many materials and substances not specifically regulated in a harmonised way and in these cases risk assessment has to be conducted on a case-by-case basis. National legislations and lists of substances evaluated by competent authorities represent the main data sources. One of the most important databases are the 'BfR Recommendations on Food Contact Materials' and the German Printing Inks Ordinance (German Printing Inks Ordinance, 2021). The BfR Recommendations are not legal norms. They do, however, represent the current state of the scientific and technical knowledge to produce consumer goods that are safe (i.e compliant to Article 3 (1)(a) of regulation (EC) No 1935/2004). The BfR unit 74 'Safety of Food Contact Materials', where the fellow has been placed in, deals with risk assessment of chemical substances that migrate from FCM into food or food simulant. The unit is part of the Department 7 'Chemicals and Product Safety' of the BfR that also assesses substances in context of the REACH regulation and is involved in the assessment of the health risks relative to chemicals, cosmetics, FCM, toys and other consumer products.

Among FCM, some phthalates, i.e., bis(2-ethylhexyl) phthalate (DEHP), represent substances of very high concern (SVHC) under Article 57 of REACH Regulation (EC) No 1907/2006 due to endocrine disrupting properties and reproductive toxicity (IARC, 2013). In addition, EFSA recently established a protocol for hazard identification and characterisation of phthalates and structurally similar substances and identified the question on the direct or indirect genotoxic activity of phthalates among the first items to be addressed (2022). In particular, DEHP was recently re-evaluated by the EFSA Panel on FCM (2022) and allowed to be used in plastic FCM. In this opinion, EFSA stated that '…In agreement with the ECHA assessment …, the Panel noted that overall evidence from in vitro and in vivo data on mutagenicity or chromosomal damage for DBP, BBP and DEHP do not give rise to a concern for genotoxicity'. On the other hand, in 2013, IARC concluded that "in vitro studies provided evidence that 'di(2-ethylhexyl) phthalate or its primary metabolite, mono(2-ethylhexyl) phthalate', may result in DNA strand breaks or induce cell transformation" and '…studies of in-vivo mutagenicity in two different transgenic mouse models have been conducted, but the results are conflicting, which confounds the interpretation of these findings' (IARC, 2013). In addition, a recent in vitro study in mammalian cells suggests a possible aneugenic effect of DEHP (Giovani et al., 2022). Therefore, no consistent data are available on DEHP genotoxicity.

An additional task of Unit 74 at BfR is the evaluation of substances for inclusion in the positive list of the German Ordinance for Printing Inks. This is carried out according to the specifications of the EFSA "Note for Guidance for the Preparation of an Application for the Safety Assessment of a Substance to be used in Plastic Food Contact Materials". Despite the fact that neodecanoic acid (NDA) is listed in regulation (EU) No 10/2011, the Unit 74 highlighted a gap-of-knowledge in the assessment of genotoxicity of NDA that is relevant for printing inks used for FCM.

In this context, aneuploidy, a thresholded genotoxic effect, has gained particular attention based on the recently published EFSA Guidance on aneugenicity assessment (EFSA Scientific Committee, 2021). This EU-FORA programme contributed to the evaluation of two selected FCM, i.e., DEHP and NDA, for their potential to induce aneugenic effects since few indications are available on the aneugenic activity of FCM or data gap have been identified in studies not performed in line with the EFSA SC Guidance on aneugenicity assessment (EFSA Scientific Committee, 2021). Data generated in this project will close data gaps and improve the scientific risk assessment of Food Contact Materials of the hosting institution (BfR).

2 | DATA AND METHODOLOGIES

2.1 | Methodologies

The work programme was conducted within the framework of the European Food Risk Assessment (EU-FORA) Fellowship Programme at two institutions: the fellow's home institution, Italian Institute of Health (ISS), Dept. Environment and Health (Rome, Italy) and the hosting institution, German Federal Institute for Risk Assessment (BfR), Department of Chemical

and Product Safety – Safety of Food Contact Material (Berlin, Germany). Dr. Thomas Tietz, head of Unit 74 (BfR), provided supervision. It was planned to start the training programme at the ISS in Italy with experimental work focused on in vitro studies referring the EFSA strategy for the assessment of genotoxicity.

2.1.1 | Ames test

The Ames test is a method used to assess the mutagenic potential of a substance, i.e. its ability to cause gene mutations in DNA. This test uses bacteria (usually Salmonella Typhimurium and *Escherichia coli*) that have a mutation that makes them unable to synthesise an essential amino acid (such as histidine). Bacteria are exposed to the test substance and then placed on a culture medium lacking that amino acid: if the substance induces mutations, bacteria will regain the ability to synthesise the amino acid and form visible colonies (Figure 1).

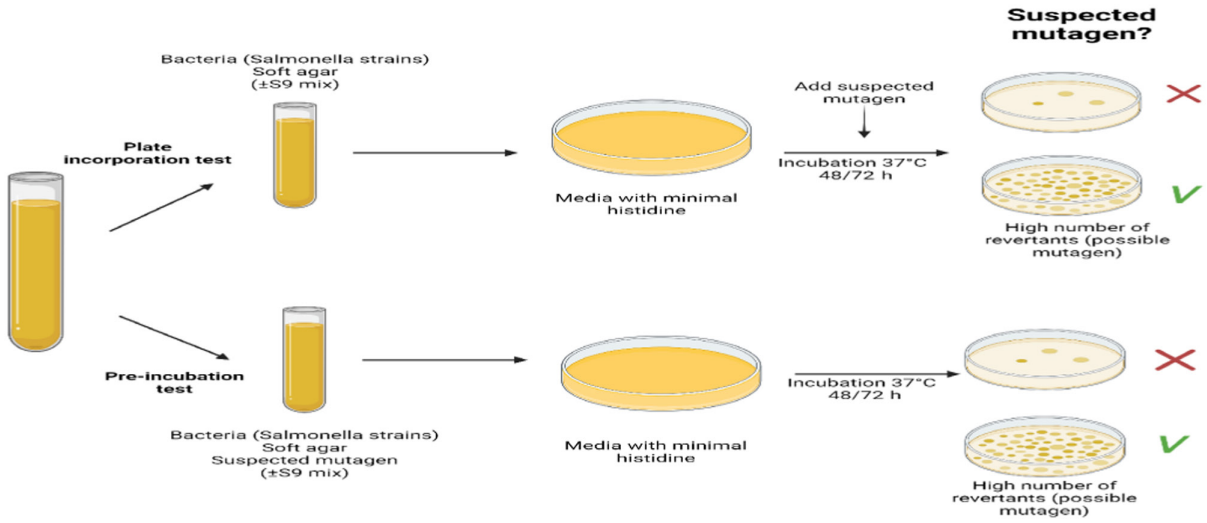

FIGURE 1 Scheme of the Ames test – Created with BioRender.com.

2.1.2 | In vitro micronucleus test

The in vitro micronucleus test is a method used to assess the genotoxic potential of a substance, meaning its ability to cause chromosomal DNA damage. This damage can lead to the formation of micronuclei, which are small additional nuclei formed when chromosome fragments or whole chromosomes are not incorporated into the main nucleus during cell division. Cytochalasin B, a substance blocking cytoplasmic division but not nuclear division, can be added to the cell cultures to induce the formation of cells with two nuclei (binucleated cells) (Fenech, 2020). This protocol focuses the analysis of micronuclei in cells divided only once after treatment thus increasing the sensitivity of the assay. The binucleated cells are examined under a microscope to count the number of micronuclei present per thousand binucleated cell (Figure 2).

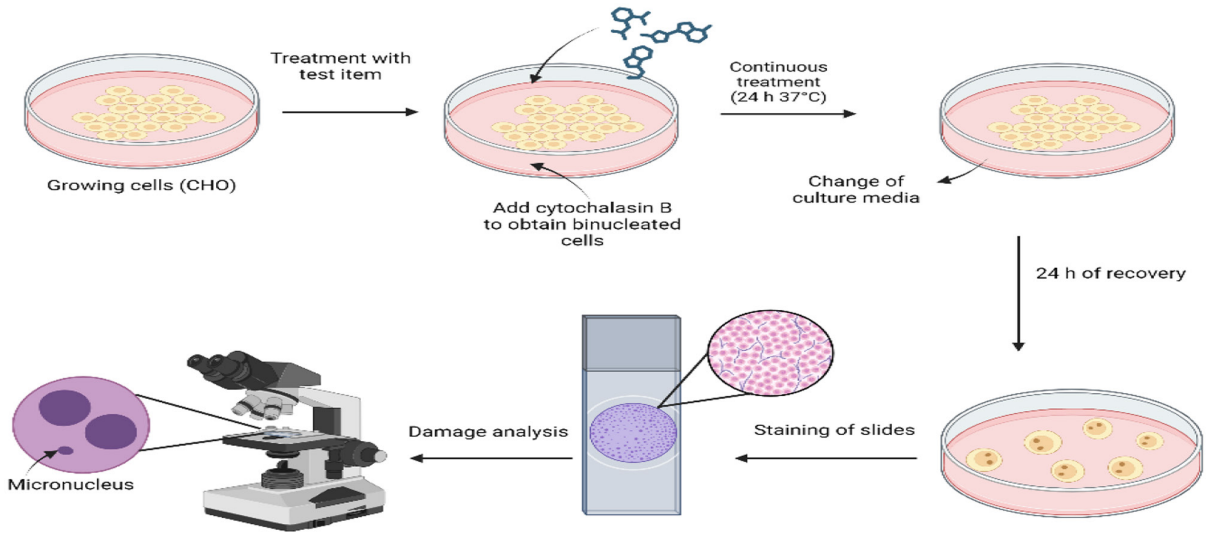

FIGURE 2 Scheme of the in vitro micronucleus test – Created with BioRender.com.

2.1.3 | Characterisation of the content of micronuclei

CREST immunostaining is a technique used to detect the centromere, the region where microtubules of the mitotic spindle attach during cell division. Antibodies against the centromeric proteins (CREST antibodies) are conjugated with a fluorescent dye and allow to visualise the centromeres under a fluorescence microscope. This technique helps characterise the content of micronuclei by detecting the presence or absence of centromeric proteins, which indicates whether the micronucleus contains whole chromosomes or chromosome fragments, respectively. The characterisation of the content of micronuclei allows to clarify the mode of action of the test item and classify a substance as clastogenic (causing DNA strand breaks) or aneugenic (affecting chromosome number) (Figure 3).

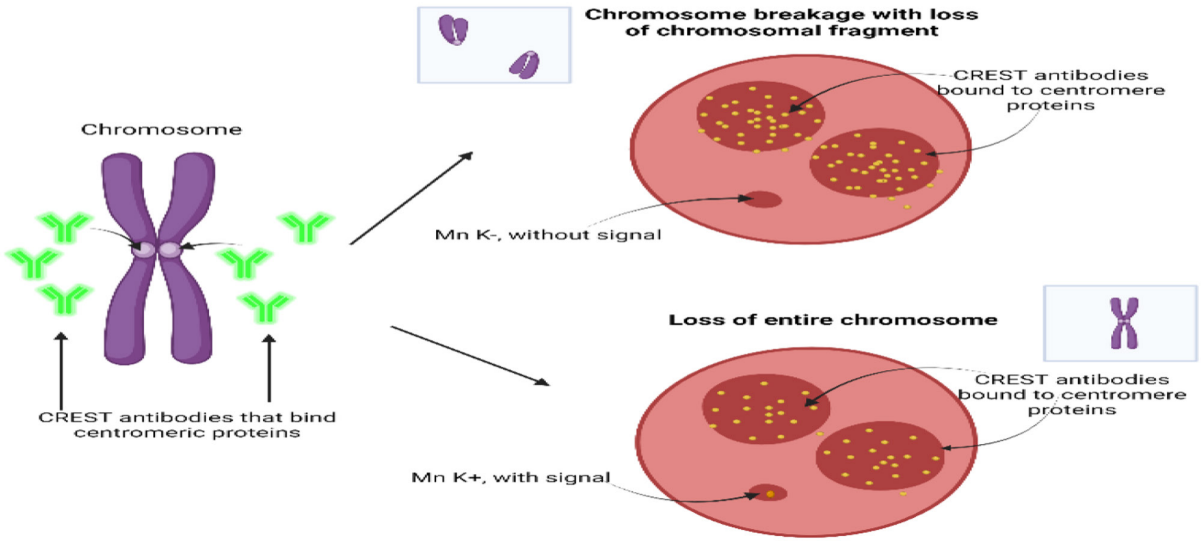

FIGURE 3 Scheme of the CREST immunostainig – Created with BioRender.com.

2.1.4 | In vitro chromosomal aberration test

The in vitro chromosomal aberration test is a well-established method used to assess the genotoxic potential of a substance, which means its ability to cause DNA damage. In this test, mammalian cells are exposed to the substance under assessment and are analysed under a microscope to detect chromosomal aberrations, such as breaks, exchanges or losses of chromosomal segments.

2.1.5 | Dose–response analysis (EFSA software PROAST)

EFSA has made available an online application implementing statistical methods for Benchmark dose modelling using the R-package PROAST (version 66.20) or BMABMDR (version 0.1.5), respectively. It allows to estimate the dose that corresponds with the benchmark response of interest. The estimated benchmark dose (BMD) is reported along with its lower and upper limit of confidence or credible interval, respectively. When fitting a set of models, a weighted average of the model-specific BMD estimates can be obtained. The confidence/credible interval for the BMD is then estimated using parametric bootstrap sampling or iterative Markov Chain Monte Carlo (MCMC) sampling.

3 | ASSESSMENT

3.1 | In vitro genotoxicity tests with NDA

3.1.1 | Bacterial cell mutagenicity assay (the Ames test)

To evaluate the potential of NDA to induce gene mutations in bacterial cells, an Ames test was conducted using five bacterial strains of *S.* Typhimurium (TA98, TA100, TA97a, TA1535 and TA1537). Two independent experiments were performed applying the plate incorporation and the preincubation assay both with and without metabolic activation. No cytotoxicity was observed in a preliminary concentration-finding assay; therefore, five concentrations of NDA were tested up to 5000 µg/plate. No significant changes in the number of revertant colonies were induced by NDA treatment at any concentration

and in any experimental condition. These results show that NDA does not induce gene mutations in bacterial cells under the experimental conditions applied in this study.

3.1.2 | In vitro mammalian chromosomal aberration and micronucleus tests

To assess the potential of NDA to induce structural chromosome damage in mammalian cells, an in vitro chromosomal aberration assay was conducted in Chinese Hamster Ovary (CHO) cells applying a continuous treatment (24 h). At least four concentrations of NDA were selected for the chromosome aberration test, based on the results of a preliminary cytotoxicity test (range from 100 to 500 µg/mL). No increase in the frequency of structural chromosomal aberrations was observed at any tested concentrations of NDA compared to the negative control cultures.

To evaluate the potential of NDA to induce structural and numerical chromosomal damage in mammalian cells, an in vitro micronucleus test was conducted in CHO cells. Three independent experiments were performed applying a continuous treatment (24 h) in the presence and absence of metabolic activation. At least four concentrations of NDA were selected for the analysis of micronuclei in binucleated cells, based on the results of a preliminary cytotoxicity test (range from 100 to 600 µg/mL). Cytotoxicity was detected at the highest concentration tested. A reproducible, statistically significant and concentration-related increase in the frequency of micronuclei was observed at 200 µg/mL and above ($p < 0.05$). These positive results indicate that NDA induces chromosomal damage in mammalian cells under the experimental conditions applied in the study.

3.1.3 | Characterisation of micronucleus content by CREST immunostaining

Based on the positive outcome of the in vitro micronucleus test, specific staining was performed to characterise the content of micronuclei and clarify the mode of action of the test item (clastogenic or aneugenic). The content of micronuclei was characterised in binucleated cells by immunostaining with anti-kinetochore (CREST) antibodies. The results showed comparable percentages of centromere positive and negative micronuclei in NDA-treated and in vehicle control cultures. However, NDA treatment was associated with a significantly higher frequency of positive micronuclei containing multiple spots, suggesting the loss of groups of chromosomes. These data indicate that NDA might have an aneugenic mode of action. Additional experiments are needed to verify whether these results are confirmed under different experimental conditions (e.g. with and without the use of cytochalasin B) and in different cell lines.

3.2 | Investigating the point of departure for DEHP

The aneugenic mode of action for DEHP was previously demonstrated (Amadio et al., 2024). In the frame of the present project, additional in vitro experiments were performed to investigate whether a Point of Departure for the induction of micronuclei after DEHP treatment could be identified. The micronucleus test was carried out in CHO cells applying a continuous treatment in the absence of metabolic activation and testing eight concentrations of DEHP ranging from 0.25 to 5 µM. A statistically significant increase of the frequency of micronuclei was observed at 2 µM and above. At BfR, these data were used for Benchmark Dose Modelling. A dose–response relation was confirmed. However, the increase in micronuclei was too small to derive a Benchmark dose and respective confidence/credible interval corresponding to a relevant effect size in line with the respective EFSA Guidance (2022). The results suggested that further experiments have to be performed to get additional data, both on the shape of the dose–response curve and the experimental variability.

3.3 | Fellow training in risk assessment

The EU-FORA dedicated training modules, attended by the fellow during the EU-FORA programme, offered her the possibility to gain general information about food safety risk assessment. The EU-FORA training programme was supplemented by the 'learning by doing' activities realised by the fellow during the collaboration between the two institutes BfR and ISS.

In particular, the fellow conducted scientific activities at the 'Safety of Food Contact Materials' unit (Unit 74) of the BfR. The main task of this unit is the evaluation of substances for inclusion in the BfR recommendations on food contact materials and the positive list of the German Printing Inks Ordinance. This evaluation is carried out according to the specifications of the EFSA's 'Note for Guidance for the Preparation of an Application for the Safety Assessment of a Substance to be used in Plastic Food Contact Materials'. The fellow benefited from the knowledge and experience of the experts in the EFSA working group on food contact materials who work in Unit 74 of the BfR. Additionally, the fellow enhanced knowledge of tools for health risk assessment related to substances migrating from food contact materials. The fellow had the opportunity to assist in the evaluation of dossiers for the inclusion of new substances in the 'BfR Recommendations on Food Contact Materials' database and the positive list of the German Printing Inks Ordinance. This also included active participation in the Panel Toxicology of the BfR Commission on Consumer Products.

Furthermore, thanks to this EU-FORA project, the fellow had the opportunity to participate in a symposium held from 30 April to 2 May, 2024, in Geneva, Switzerland. At this symposium, organised by the IAFP's European Symposium of Food Safety, the fellow gave a presentation on their EU-FORA project in collaboration with two other fellows from the same cohort, on Materials Science and Applied Chemistry in Food Safety.

4 | CONCLUSIONS

4.1 | Overall conclusions on the genotoxic activity of NDA

NDA did not induce gene mutations in bacterial cells and in chromosomal aberration assays, while increased the frequency of micronuclei as observed by the in vitro micronucleus test. The characterisation of the content of micronuclei indicated that most of the NDA-induced micronuclei seem to have resulted from chromosome loss and that aneugenicity may be the prevalent mode of action of NDA. This conclusion is supported by the negative results observed for the induction of structural chromosomal damage by the in vitro chromosome aberration assay. However, further studies are needed to confirm these results in different experimental conditions and with different cell types.

4.2 | Conclusions on aneugenicity of DEHP

Preliminary dose–response analysis of increased micronucleus formation by DEHP via Benchmark Dose Modelling indicates that further experiments are needed to get additional data, both on the shape of the dose–response curve and the experimental variability.

5 | DISCLAIMER

The results of these studies are intended to be published in other scientific journals. To avoid copyright claims, they were described only very briefly in this report.

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