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Use of bioassays to assess hazard of food contact material extracts: State of the art.

Isabelle SEVERIN, Emilie SOUTON, Laurence DAHBI, and Marie Christine CHAGNON*
Derttech « Packtox », University of Bourgogne Franche-Comté, INSERM LNC UMR 1231, AgroSupDijon, F-21000 Dijon, France.

*: to whom correspondence should be addressed
Tel: +33 3 80 77 40 19

e-mail: marie-christine.chagnon@u-bourgogne.fr

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Abstract

This review focuses on the use of *in vitro* bioassays for the hazard assessment of food contact materials (FCM) as a relevant strategy, in complement to analytical methods. FCM may transfer constituents to foods, not always detected by analytical chemistry, resulting in low but measurable human exposures. Testing FCM extracts with bioassays represents the biological response of a combination of substances, able to be released from the finished materials. Furthermore, this approach is particularly useful regarding the current risk assessment challenges with unpredicted/unidentified non-intentionally added substances (NIAS) that can be leached from the FCM in the food. Bioassays applied to assess hazard of different FCM types are described for, to date, the toxicological endpoints able to be expressed at low levels; cytotoxicity, genotoxicity and endocrine disruption potential. The bioassay strengths and relative key points needed to correctly use and improve the performance of bioassays for an additional FCM risk assessment is developed. This review compiles studies showing that combining both chemical and toxicological analyses presents a very promising and pragmatic tool for identifying new undesirable NIAS (not predicted) which can represent a great part of the migrating substances and/or “cocktail effect”.
Introduction

The majority of food distributed in developed countries is packed. Packages are barriers to prevent the chemical and microbiological contamination of food, and to prolong their shelf-life maintaining food quality and safety. Packaging contributes also actively to decrease food waste. Food Contact Material (FCM) is a general term to define materials intended to come directly or indirectly into contact with foodstuff. It includes kitchen utensils, packaging, containers, machines and materials used during the production chain, but also the storage and the transport of foodstuff.

The European framework Regulation (EU) No 1935/2004 for materials and articles intended to come into contact with food lists 17 groups of materials and articles which have to be in compliance with the requirements of the EU regulation (authorization of substance, labelling of materials, traceability, declaration of compliance and safeguard/control measures) and may be covered by specific measures. Among the 17 groups of the materials, only 5 are currently covered by specific measures. Depending on FCM physical/chemical parameters and food composition, FCM may interact with food and transfer some constituents (phenomenon called migration) that lead to low but measurable human chemical exposure (FACET project). Then, migration may cause a potential risk for human health that must be measured and controlled.

Regarding food safety, general requirements are set by article 3 of the regulation: “Material and articles in contact with food do not transfer substances in amounts which could endanger human health, or change food composition and/or organoleptic characteristics”.

FCM are manufactured from different base materials and Intentionally Added Substances (IAS) such as monomers, additives, and polymer production aids, essential in the manufacturing or FCM use since they enhance e.g. processibility, shelf life or mechanical properties. However, in addition to these substances of known origin, substances that are Non-Intentionally Added (NIAS) may be released from FCM and it has been pointed out that
they can represent a large part of migrating substances (Grob et al., 2006, 2010). The article 3 of the “Plastic” Regulation (EU) No 10/2011 defines them as impurities, reaction intermediates or decomposition/reaction products. This text points out that the risk assessment of a substance should cover the substance itself but also relevant impurities and foreseeable reactions or degradation products that could be formed under intended use. However, not only plastics but all FCM types are concerned by NIAS presence; (recycled) paper and board, coatings, metal, cork ….

If some NIAS are already known and controlled, some others are difficult to predict with no available toxicological data. Furthermore, for some of them, it is difficult to analytically characterize, identify, quantify and make a risk assessment (Skjevrak et al., 2005) especially for mixture (eg MOAH in mineral oils from recycled paperboard). It is also important to notice that during these last few years, incidents reported by media were mainly due to NIAS.

**Why is risk assessment difficult regarding NIAS?**

The example of mineral oils found in recycled paper and board is characteristic of the problematic of NIAS. In terms of identification and quantification of NIAS, among specific techniques, GC-MS (detection of volatile molecules) or LC-MS (detection of non-volatile molecules) are the most popular analytical tools performed to characterize a FCM extract composition. Unfortunately, a single method is not able to give exhaustive information on molecules present in an extract (each of them can analyze a defined number of compounds present in FCM matrixes). Thus, a battery of analytical methods is complementary and need to be coupled (Nerin et al., 2013). The different reaction products of polymers, their corresponding degradation products (breakdown compounds), the presence of minor impurities, the degradation of IAS during FCM processing or food packaging storage, lead to the so-called ‘forest of peaks’ in chromatography (Grob et al., 2010). These chromatograms are extremely difficult to analyze and it should be emphasized that, if the GC-MS databases are rich, databases concerning LC-MS only begin to be implemented.
Due to the complex nature of FCM and the evolution of formulations (novel polymers, additives and uses), chemical analysis cannot be exhaustive, and structural identification of all FCM substances could not be achievable since they are present sometimes at trace levels (Nerin et al., 2013, Biedermann et al., 2014).

Predictive approaches of additive and polymer degradation have also been attempted to identify NIAS, but are often hindered by the non-disclosure of the initial composition of the material. Reaction schemes are often complex and function of specific process conditions. Moreover, one reaction intermediate can be the product of the degradation of various molecules recombined with others. Therefore, the prediction of reaction scheme in undefined chemical mixtures is very difficult to achieve completely (Castillo et al., 2013) and is time-consuming (Castle et al., 2013).

In term of risk assessment, the scientific literature may help to predict hazard. Quantitative Structure-Activity Relationship (QSAR) software programs, as statistical and expert methods, are able to predict the physicochemical properties and some biological effects of a substance as a function of its molecular structure such as alerts in genotoxicity (Ashby and Tennant, 1988). However, these in silico toxicological prediction tools require the full characterization of the substance chemical structure and then cannot be applied with unidentified NIAS or with a complex mixture such as FCM extract.

The “threshold of toxicological concern” (TTC) is also an interesting predictive risk assessment tool to use. TTC has been developed to assess potential human health concerns for chemicals of unknown toxicity present at low levels in the diet. However, to apply TTC, the defined chemical structure and the accurate exposure is needed (EFSA, 2012). Furthermore, the applicability of the TTC as a tool for mixture evaluation, not fully characterized, is only endorsed if sufficient information or analysis are available to confirm the absence in the mixture of compounds of exclusion classes (EFSA, 2016).

Regarding toxicological data, EFSA requires, whatever the level of migration, genotoxicity assessment of each FCM starting materials (EFSA note for guidance, 2008).
This approach however does not consider other toxicological endpoint such as “Endocrine Disruptors” (ED), which is receiving increasing attention as an emerging public health concern, as it was the case with BPA. Furthermore, possible interactions between chemicals in a FCM extract can occur (Muncke, 2009). Chronic exposure to ED active substances is of toxicological concern and this concern increases if humans are exposed to mixtures of similar acting (additivity, synergism) ED (Kortenkamp, 2009). For example, Silva et al. (2002) showed that a multicomponent mixture of xenoestrogens produce significant effects when each component was combined at concentrations below its individual No Observed Effect Level (NOEL). To date, neither chemical analysis (not predictive for hazard assessment) nor in silico methods (not adapted to mixtures) is able to identify “cocktail effect” of a complex mixture such as FCM extract (ILSI, 2015).

EFSA requires a toxicological assessment of each substance used in the FCM formulation. Thus, the traditional approach based on the identification/quantification of all substances with their full toxicological characterization (using the decision trees currently employed for regulatory purposes) is no longer applicable, neither from a technical point of view (highly resource intensive, because of time and cost constraints), nor useful as many NIAS are likely to be of no safety concern (ILSI, 2015).

**Bioassays: a relevant solution for FCM hazard assessment**

One possible way to face these challenges with NIAS in complex mixtures is to use in vitro biological short-term assays (on yeast, bacteria or cellular models), called “bioassays”, in complement to chemistry, to assess the full biological effects of the finished packaging. By this way, if any process affects the FCM giving rise to by-products, they could be indirectly tested in the mixture (Riquet et al., 2016). Compared to analytical methods, bioassays give comprehensive information on hazard and quality assessment of mixtures because they include possible interactions between the different chemicals present in the mixture, they are able also to identify non-monotonic dose effect.
Historically, bioassays have been performed as alternative methods in order to identify hazard of complex mixtures of low doses of environmental pollutants (Depledge and Fossi, 1994; Walker et al., 1996; Calow, 1998). Bioassays play also a great role in the understanding of the mode of action of a pure substance, participating to the determination of AOP (adverse outcome pathway) concept, an integrated approach, from exposure and molecular initiating event to individual and population response (Vinken et al., 2013). AOP emphasizes linkages of molecular events, toxicity pathways and downstream consequences for toxicity showing the causal linkage for pathway perturbation to conventional in-life responses, providing a basis for using cell-based assays with well-defined in vitro models for safety assessment (Adeleye et al., 2015).

The interest for in vitro bioassays has increased in recent years since the European regulation on chemicals (REACH) recommends, for the risk assessment of chemicals to give priority to the in silico and in vitro methods. Furthermore, the use of bioassays limits the in vivo studies and thus comply with the 3R rule (refine, reduce, replace) (Directive 2010/63/EU). Concerning medical device assessment, in vitro bioassays focusing on cytotoxicological endpoint are used for a long time to ensure device biocompatibility (ISO 10993-5) determining whether a material may contain significant quantities of biologically harmful extractables. Bioassays are also requested to assess the water quality and manage efficiently environment (Directive 2000/60/EC). Genotoxicity testing in vitro is performed in the field of botanical/herbal substances/preparation (EMEA, 2008) and novel foods/ingredients (EFSA, 2016). All these areas have to deal with the chemical assessment of complex mixture, as it is the case for FCM.

However, there is plethora of bioassays, different tests according the toxicological endpoint using several biological models and with different conditions of time of exposure, concentrations, vehicle, medium, presence or not of fetal bovine serum or exogenous metabolizing system …. Furthermore, they are many ways to prepare the FCM sample before testing with bioassays (FCM extraction, evaporation/concentration steps, solubilization in a biocompatible solvent …). All these parameters make difficult to compare
the data of the different studies and could be a subject of controversy. Thus, there is a real need to standardize and recognize reliable bioassays for a properly use.

After a short presentation of the bioassay principles, this article aims to review, to date, the in vitro bioassays performed to test FCM extracts regarding cytotoxicity, genotoxicity or endocrine disruption potential endpoints, to highlight their strengths, weaknesses and reliability. They will be discussed concerning their relevance in a strategy for FCM risk assessment especially regarding NIAS and new challenges in food toxicology. This review does not include other endpoints such as neurotoxicity and immunotoxicity as, until now, to our knowledge, only scarce scientific researches have been done to assess this type of toxicity, with in vitro bioassays and FCM extracts.
Cytotoxicity

Cytotoxicity is an essential toxicological endpoint to take into account as it measures disturbances of cell homeostasis that can lead to cell death by necrosis or apoptosis. Cytotoxic effect can be due to disruption of basal cellular mechanisms and cell integrity like cell morphology, cell viability, cell growth, metabolic rates and gene transcription controlling basal functions … (Afssa, 2006).

Numerous cell responses to toxic stress occur before a measurable toxicity, thus monitoring cytotoxicity pathways at sublethal levels and checking specific markers can increase sensitivity (avoiding false negative/positive response due to too high concentrations) and reproducibility (using the same range of concentrations between independent experiments) in the following toxicological studies.

Cytotoxicity is then a prerequisite step before studying specific toxicological endpoints (genotoxicity, agonist or antagonist effect of potential endocrine disruptors) on substances or mixtures leading to define the concentration range for further in vitro testing. This range can be 50% up to 80% according to the applied bioassays, avoiding data misinterpretation.

By establishing the concentration at which 50% (or 30% regarding the endpoint) of the cells are affected (i.e. IC₅₀ or IC₃₀), it is possible to compare quantitatively responses of single compound in different systems or several compounds in individual systems (Eisenbrand et al., 2002). The cytotoxicity assessment has been used as a predictor of acute systemic toxicity in vivo, as in a large study of diverse range of chemicals, a reasonably good correlation was found between basal cytotoxicity and acute toxicity in animals and humans (Clemedson et al., 2000; Hamm et al., 2017).

The endpoints for cytotoxicity need to be sensitive, feasible, consistent and to get a discriminatory power. In the following part, principles of the bioassays performed for assessing the cytotoxic potential of FCM extracts are briefly described.
The most common cytotoxic endpoints focused on the breakdown of the cellular permeability and cell proliferation. Briefly, principles of these assays are based on the incubation of the cells exposed to the FCM mixture, in the presence of an exclusion (trypan blue)/inclusion (neutral red, MTT, WST-1, resazurin) dye that will be incorporated in dead/live cells or in specific organelles for an easy detection of the cell viability. Extracellular release of intracellular “marker” components can also be measured giving information on cell permeability damage. For example, lactate dehydrogenase (LDH), a cytosolic enzyme, is released into the culture medium following a loss of the cell membrane integrity after an exposure to cytotoxic substances.

The methods measuring cell proliferation rely on the principle that any toxic effect, by killing cells or by blocking the cell cycle, reduces the proliferation of an entire population. Biochemical (total protein content or BrdU incorporation) and counting methods (colony forming ability) can also be performed (for review, Adan et al., 2016). The most widespread methods assessed the cell viability. However, before dying, cells go through a state called “morbidity”, prior to death, where homeostasis is disturbed but where cells may still (to some toxic limit), recover initial viability (reversible process). In 1985, Fauris et al. developed a cytotoxicity assay, the “RNA synthesis inhibition test” on HelaS3 cell line for drinking water pipeline assessment in France (AFNOR, 1996 XP 41-250-3). This assay is particularly useful for water samples, since the cell culture medium can be prepared using the test sample as the aqueous base. The principle of this test is to measure the kinetic of RNA synthesis by incorporation of tritiated uridine into cellular RNA after cell treatment; a substance or a mixture is considered cytotoxic if the kinetic of RNA synthesis is reduced by 30% or above in comparison to negative control. The RNA synthesis assay has been adapted to the HepG2 cell line and as a screening method using microtitration plates (Valentin et al., 2001; Valentin-Sèverin et al., 2002). This bioassay was shown reliable, reproducible and very sensitive compared to other sublethal assays (AlamarBlue® assay and ATP measure) when testing a series of water contaminants (Jondeau et al., 2006).
assay has been normalized by the CEN using human cell lines (Hela and HepG2 cells) for paperboard water extracts (EN 15845, 2010; EN 16418, 2014, respectively).

Lastly, the **inhibition of boar spermatozoan motility assay** has to be mentioned despite its differences with others systems (conventional cells) (Andersson *et al.* 1998). This assay measures the boar spermatozoan motility after 1 to 4 days of treatment of extended boar semen (commercial product obtained from an artificial insemination station) at room temperature and in comparison to control sample. Boar spermatozoan motility inhibition is a highly specific indication of impairment of either mitochondrial activity or membrane integrity or both.

**Cytotoxicity bioassays to test FCM extracts (Table 1)**

**Cytotoxicity of papers and boards**

Papers and boards are the main material studied for cytotoxicity. The RNA synthesis inhibition assay was carried out by Fauris *et al.* (1998) to assess the cytotoxic effect of water extracts from 21 samples (six **papers** and fifteen **boards**) used for food packaging. Among them, 11 samples were made from virgin fibers and 10 samples from recycled fibers. The percentages of the RNA synthesis ranged from the highest values (94%), indicating no cytotoxic effect, to the lowest values (17%), indicating severe cytotoxicity with both virgin and recycled fibers. In addition, the authors concluded that the toxicity level was apparently not due to only a single compound but to simultaneous presence of several substances.

Few years later (2001-2005), a battery of bioassays was applied to complex mixture of food contact **papers and boards** in the European Project “Biosafepaper”. Publications issued from this project (Severin *et al.*, 2005, Bradley *et al.*, 2008, 2010, Honkalampi *et al.*, 2010) evaluated several cytotoxicity tests using different endpoints on model substances and on numerous extracts from paperboard: the RNA-synthesis inhibition test using HeLa and HepG2 cell lines, the inhibition of boar spermatozoan motility test, the TPC on the mouse
hepatoma cell line Hepa-1 and the larynx carcinoma human cell line Hep2. NRU and colony forming ability assays were also performed with Hep-2 cells. Twenty-one extracts were prepared using 3 food simulants (water/ethanol/Tenax) and blinded tested. Not any cytotoxic effect was detected with water samples (7) or with Tenax samples (10). In contrast, paperboard samples (4/4) extracted in ethanol were all cytotoxic (TPC on Hepa-1 cells, NRU on Hep-2 cells, RNA synthesis on HepG2 cells and boar spermatozoan motility), included a non-food grade material. Interestingly, comparing with the chemical analysis data, no specific compound could be linked to the positive response, although a correlation appeared between the total amount of extractables and toxicity (Bradley et al., 2008). Interestingly, the non-food material sample induced a high cytotoxic answer in bioassay confirming that it would not comply with food contact.

The available bioassays chosen at the beginning of the project were consistently evaluated against different criteria such as robustness meaning that they can be easily established in different laboratories without change in their sensitivity or specificity. Furthermore, several assays have to be left out from the final test battery due to a low sensitivity regarding model compounds and difficulties in the data interpretation (bioluminescence Vibrio test) (Biosafepaper, 2006).

In the same period, Binderup et al. (2002) carried out the resazurin assay using human fibroblasts on virgin fiber pulp and recovered/recycled papers for food contact. The recovered papers were about three to four times more cytotoxic than the virgin pulp and in accordance with the chemical analyses again, water extracts were less cytotoxic than ethanol extracts. They concluded that the results from the cytotoxicity data showed a clear association between the biological response and the total amount of chemicals detected in the samples, suggesting that a cytotoxicity bioassay could be used as a preliminary screening step for checking the amount of toxic compounds.

Cytotoxicity of coatings
In 2006, Mittag et al. assessed the cytotoxic effect of 95% ethanolic migrates from two can coatings, (epoxy and polyester-based). Cytotoxicity effects were different according to the assay (BrdU-ELISA, WST-1 assay, NRU, RNA synthesis inhibition test) and the cell line used (Hela-S3, Caco-2, HT-29, HepG2). Both coatings showed a cytotoxic effect on Caco-2 cells but only the epoxy coating revealed a significant effect in HepG2 cells. The metabolic activities of some cell lines could modulate cell response against reactive compounds present in the migrate of the polyester based coating in substances. In parallel to bioassays, the amount of the legally regulated substances (Bisphenol A, Bisphenol A diglycidyl ether (BADGE) and derivatives (BADGE-2H₂O, BADGE-HCl-H₂O, BADGE-2HCl) in the migrate of the epoxy coating was analysed and their implication in the cytotoxic effect estimated. However, only about 0.5 % of the migrate effect in the NRU could be imputed to these regulated substances suggesting that NIAS, not predicted or non-identified, could be involved in the observed biologic answer.

**Cytotoxicity of adhesives**

The feasibility of the RNA synthesis inhibition assay using HepG2 cell line was checked in the European project “Migresives” (2007-2010) and applied to adhesives extracts, it was successful regards to realistic exposure for hazard identification, as long as a non-cytotoxic concentration of solvent was respected. Four of the six laminates (paperboard + adhesive layer) were identified cytotoxic in contrast to the adhesive alone, suggesting again that NIAS (issued from process such as lamination) could be formed (Migresives final report, 2010).

**Cytotoxicity of plastics**

Sauvant et al. (1995) published the first study regarding bioassays applied to FCM. They performed a battery of assays (MTT, NRU, Coomassie blue, LDH, Trypan Blue and RNA synthesis) on a murine fibroblast cell line (L-929) to assess the cytotoxicity of PET and PVC
materials at the main steps of bottle manufacturing process (in term of cytotoxicity). They concluded that it was sufficient to control bottles as some abnormalities were detected on raw materials (compound, resin, preform, paraison) which were not anymore observed on finished products.

Recently, Maisanaba et al. (2014) studied in vitro the basal cytotoxicity of extracts from a nanocomposite material with poly (lactic) acid (PLA) and two modified clays on the human intestinal and liver cells (Caco2 and HepG2 cells). Migrations were directly performed using the cellular culture medium as aqueous simulant. MTS, NRU and TPC assays demonstrated the absence of cytotoxic effect whatever the cells and the time of exposure (24h or 48h of treatment).

Riquet et al. (2016) studied the cytotoxicity of polypropylene films prepared with a selection of stabilizers, Irgafos 168, Irganox 1076 and Tinuvin 326, able to generate NIAS (Pospisil, 1993). They showed a significant strong cytotoxic effect using the RNA synthesis inhibition assay of polypropylene film extracts prepared with Irgafos 168 and after two post-treatments: microwave and electron-beam. The authors concluded that it was probably due to the presence of a large quantity of 2,4-di-tert-butyl phenol, a by-product of Irgafos 168, in the extract. When tested alone, the 2,4-di-tert-butyl phenol was indeed very cytotoxic (<10% of viability) at its limit of solubility in the culture medium (100 mg/L).

To summarize, many studies performed cytotoxicity bioassays based on the cellular permeability breakdown. In general, it is preferable to perform sublethal bioassays to detect early change in cells rather than measuring cell mortality. Bioassays measuring cytotoxicity are demonstrated useful for hazard assessment of FCM as a pre-screening and prerequisite step for further in vitro toxicological studies using an adequate concentration range. Combining a battery of bioassays on the same sample appears also relevant to detect an underlying cytotoxic mechanism of action. Through this bibliographic review, it appears that the cytotoxicity did not simply depend on the quantity of the extractable substances but rather more specifically on the composition of the extracts (Bradley et al., 2008). A link between the quantity released by the packaging into the extract and the cytotoxic effect was
observed, although, very often, the effect could not be attributed to a specific identified substance, suggesting either an effect of not identified substances, such as NIAS in the analytical conditions or a “cocktail” effect due to the mixture. While the results indicate a correlation between the total amount of extractables and cytotoxicity, as observed in studies on paper and board toxicity (Fauris et al., 1998; Binderup et al., 2002), they also suggest that potentially harmful compounds could not be always identified by chemical analysis methodologies. Thereby, cytotoxicity could be considered as a biomonitoring tool that could be used, in complement to analytical chemistry for quality control of manufacturing process, as this was the case of the MAFO concept (http://www.papraon.com/fr/vision_valeurs/mafo_concept).

Genotoxicity

Genotoxicity is a broader term that refers to processes, which alter the structure, information content or segregation of DNA (ECHA, 2008). Three levels of mutations (gene mutations, structural and numerical chromosomal alterations) may play a role in cancer, in inherited disorders and congenital defects. Playing a major role in hazard identification, genotoxicity testing can also provide information on mechanism of action, which is pivotal for the characterization of carcinogenic risks and support the use of non-thresholds models for estimation of low doses effects (Eisenbrand et al., 2002). As lot of genotoxic substances need to be metabolized in bioassays to exert toxicity (Guengerich, 2000), an exogenous metabolizing system (S9 Mix) has to be added to avoid false negative data (Ames et al., 1973).

Genotoxicity data are the key information for the majority of evaluations in the area of FCM (Barlow, 2009). A battery of in vitro genotoxicity bioassays is required by EFSA, whatever the level of migration of the substance, to assess the hazard of new food contact starting substance (EFSA, 2008). Historically, three assays were carried out: two tests to detect gene
mutation in bacteria (OECD TG 471) and in mammalian cells (OECD 476) and the third one to study chromosomal aberrations (OECD 473 or 487). In 2011, EFSA published a scientific opinion on genotoxicity testing strategies applicable to food and feed safety assessment and recommended the use of only two in vitro assays, the bacterial reverse mutation test (OECD TG 471) and the in vitro mammalian cell micronucleus test (OECD TG 487). Indeed, this new test combination fulfils the basic requirements to cover the three genetic endpoints with the minimum number of assays, as these two assays are reliable for detection of the majority of genotoxic substances (EFSA, 2011).

In the following part, principles of the bioassays used for assessing the genotoxicity of FCM extracts are briefly described.

The bacterial reverse mutation (Ames test, OECD TG 471) test is always accepted as part of every strategy because of its specificity for detection of genotoxic carcinogens. It is performed on five strains of *Salmonella typhimurium* and/or *Escherichia coli*, and is a well-validated test system; which has been adopted at international level. It has been carried out during the last 40 years as initial screening test in most strategies for mutagenicity testing of new chemicals (Mortelmans and Zeiger, 2000).

A liquid micromethod (*Ames II Test*) can also been applied to only two strains (*S. typhimurium* TA mix and TA 98) with a colorimetric readout. It is an effective screening alternative to the standard Ames test requiring less test material and labor with a low interlaboratory variability (Fluckiger-isler *et al.*, 2004). The Ames II test has been expanded to include other tester strains, called *Ames MPF TM* making now a reliable predictive and rapid tool to assess mutagenicity (Kamber *et al.*, 2009, Fluckiger-isler and Kamber, 2012).

Another genotoxicity bioassay using bacteria could also be chosen, the Rec assay which is a DNA repair test. The rationale of the *B. subtilis* rec-assay is based on the relative difference of survival of a DNA repair-recombination proficient strain and its deficient strain, which is interpreted as genotoxicity. It can detect wide spectra of DNA damage including intercalation, breakage of DNA molecules and chemical changes of DNA bases (Matsui,
and is useful for identification of genotoxic substances in mixed samples such as environmental water samples (Matsui et al., 1992).

However, bacterial repair/mutation assays are unable to detect genotoxic substances, which target chromosomal integrity or segregation, or affect genomic stability by indirect mechanisms (eg disturbance of DNA repair fidelity, cell cycle control or apoptosis). Then, the in vitro micronucleus test (MN) (OECD TG 487) is reliable to identify substances that cause structural and numerical chromosomal damage in cells that have undergone cell division during or after the exposure to the tested substance. The assay detects micronuclei in the cytoplasm of interphase cells employing human or rodent cells lines to detect efficiently both clastogens and aneugens. The in vitro protocol can also be combined with fluorescence in situ hybridization (FISH) to provide additional mechanistic information (eg. on non-disjunction). Numerous efforts were made to automate micronuclei scoring (image analysis, laser scanning cytometry, and flow cytometry). The “In Vitro MicroFlow” assay was evaluated independently by Collins et al. (2008) and demonstrated good performance and portability in an inter-laboratory ring trial using mouse lymphoma L5178Y cells (Bryce et al., 2008). Advances to extend the method to other cell lines and using 96-well microplate format have also been described (Bryce et al., 2009, 2010).

An alternative to the MN assay was the chromosomal aberration test on CHL/IU cells. The principle is to identify agents that cause structural chromosome aberrations in cultured mammalian cells. At predetermined intervals after cell exposure to the substance, cells are treated with a metaphase-arresting substance (colchicine), harvested, stained and metaphase cells are analyzed microscopically to check the presence of chromosome aberrations. However, this bioassay has been rapidly replaced by the MN assay, easier to analyze.

Some other bioassays detected DNA primary lesions, which can be further repaired. Thereby, they are less predictive than a DNA mutagenicity or chromosomal aberration bioassay and are commonly performed as pre-screening tests.
The **sister chromatid exchange assay** (SCE) is a short-term bioassay for the detection of reciprocal exchanges of DNA between two sister chromatids of a duplicating chromosome. SCEs represent the interchange of DNA replication products at apparently homologous loci. The exchange process presumably involves DNA breakage and reunion, although little is known about its molecular basis. Detection of SCEs requires differentially labelling sister chromatids, achieved e.g. by incorporation of bromodeoxyuridine (BrdU) into chromosomal DNA for two cell cycles. This assay described as the OECD guideline 479 was deleted in April 2014, due to no longer use in practice by regulatory authorities and due to more performant assays (with guidelines available) in the existing battery of genotoxicity tests targeting the same endpoints.

The **comet assay** detects DNA single and double strand breaks, alkali-labile lesions, as well as DNA strand breaks arising during the DNA repair. It is considered an indicator test detecting pre-mutagenic lesions and is used for mechanistic studies. Comet assay is a rapid, simple and very sensitive test for measuring DNA damage in mammalian cells or tissues from which single cell suspensions can be prepared (reviewed by Collins et al., 2008). Furthermore, cell division is not required and a low number of cells is sufficient for the analysis. Despite widespread use *in vitro*, there is not yet an available OECD guideline. However, the procedure is consistent with international publications that define the optimal conditions to implement the assay (Tice *et al.*, 2000). Oxidative DNA damages can also be detected using specific enzymes such as the formamido-pyrimidine glycosylase or endonuclease III (Collins, 2009).

A new generation of bioassays based on induction of DNA damage response/stress pathways gene transcription is promising as more sensitive and more specific genotoxicity endpoints. The **BlueScreen HC assay** (Gentronix Ltd) is an adaptation of the GreenScreen HC, performed on TK6 lymphoblastoid cell line genetically modified, which contains the GADD45α promoter (Hastwell *et al.*, 2009). This test uses a luciferase instead of GFP (to generate a luminescent output), the superior signal to noise ratio makes it even more
suitable for adaption to even higher throughput screening in 384-well format. It has been validated with pharmaceutical formulations and flavor and fragrance materials (Etter et al., 2015). Multi-laboratory ring-trials have been coordinated for validation studies (Lynch et al., 2011). This bioassay detects mutagens, clastogens, nucleoside analogues, aneugens and chemicals that interfere with the maintenance and integrity of the genome. EFSA (2011) concluded that these reporter gene assays are promising to gain insight the mechanism of action and to guide a further testing strategy.

Genotoxicity bioassays to test FCM extracts (Table 2)

Genotoxicity testing for FCM extract risk assessment can detect a hazard of a specific substance, but also be predictive when testing a mixture with all chemicals simultaneously present. Furthermore, it contributes to understand the mechanism of action (MOA) of chemical carcinogens.

Genotoxicity of papers and boards

As they have no specific EU regulation, paper and boards were the first material tested with bioassays to assess the toxicity as a global response of the mixture leached by the material. Binderup et al. (2002) carried out the Ames assay to compare 4 paper samples, one virgin paper and three recycled paper-boards using the micro-suspension technique. Sample extracts were prepared either in water or in 99% ethanol. None of the water extracts was genotoxic whatever the strains (with or not S9 mix) up to a concentration corresponding to 100 mg paper/plate. With ethanol extracts, 2 samples induced a weak positive effect in TA98 strain (closed to a doubling of revertant/plate at the highest concentration (50 mg paper/plate)) but this tendency was not confirmed when a three fold higher concentration (150 mg/plate) was tested.
Osaki et al. (2004, 2005) performed the Rec assay with *Bacillus subtilis* for testing genotoxicity of **paper-board virgin or recycled samples**. In 2004, 13/28 products possessed a DNA-damaging activity, 75% were issued from the recycled paper-boards and 19% from the virgin samples, suggesting that recycled products exhibited more such biologic response than virgin products. Ten standard substances detected in virgin or recycled paper products were tested alone and BisphenolA (BPA), 1,2-benzisothiazoline-3-one (BIT), 2-(thiocyanomethylthio) benzothiazole, 2,4,5,6-tetrachloro-isophthalonitrile, 2,4,6-trichlorophenol (TCP), and pentachlorophenol caused DNA damage in *B. subtilis*, suggesting that these substances could be at the origin of the biological answer observed with paper-board extracts. In 2005, dehydroabietic acid (DHA) and abietic acid (AA) were detected in the seven recycled products for food-contact use. A good correlation was observed between the total amount of DHA and AA determined in paper extracts and the observed DNA-damaging activity. Moreover, using the REC assay, genotoxic effects in paper products showed a good match with standard compounds, indicating that genotoxic effects of these paper products were mostly attributable to DHA and AA.

Osaki et al. (2004) carried out the comet assay on HL-60 cells to confirm the genotoxicity observed in the Rec assay. Among the eight samples of paper-board positive with the REC assay, six induced also a significant positive response (fivefold or higher) without any cytotoxicity in the comet assay. Standard substances (2,4,6-trichlorophenol, benzophenone, 4,4′-bis(diethylamo) benzophenone and 1,2-benzisochiazoline-3-one) also caused a weak increase in the comet assay when tested alone. Osaki et al. concluded that most recycled paper products contain chemicals with genotoxic activity. However, the genotoxic effect could not be attributed to the quantity of chemicals present in the recycled sample extracts suggesting some interactions between substances present in the extract.

In the European Biosafepaper project (2001-2005), nineteen **food contact papers and boards** and one non-food contact board were extracted with different solvents (hot or cold water, 95% ethanol, Tenax) and tested using the Ames and the comet assays. None of the
water or Tenax extracts was positive. In contrast, the ethanol extract of the non-food grade sample (blind tested) gave a significant positive response in the Ames test (Bradley et al., 2008). Again, individually, none of the substances identified by GC/MS analysis (diisopropynaphthalenes or phthalates) could explain the positive response observed in the genotoxicity test, as these substances are known to be not genotoxic. In accordance with Osaki observations, those data suggest that unknown substances could be present in the extract, as non-detected by GC-MS in the experimental conditions. The positive response observed could also be the result of a combined effect of substances present together in the extract.

Regarding NIAS, Koster et al. (2014) proposed a pragmatic safety assessment approach, in five steps, for unknown substances which could be present at low exposure levels in food contact matrixes. To illustrate the concept, they analyzed three extracts of paperboards. The step 3 consists to “exclude substances with genotoxic potency”. To do so, they performed the BlueScreen HC assay (with or without S9 mix) which was negative whatever the extract. They also demonstrated the ability of the tests to detect genotoxic response in a complex matrix by spiking one sample with known genotoxins (taxol and benzo[a]pyrene, in the presence and absence of a metabolic activation system).

Bengtström et al. (2014) proposed a method for extraction and extracts fractionation and tested two paperboard samples in a AhR reporter gene assay using the rat hepatoma cell line (H4IIE) to detect well-known carcinogens such as polycyclic aromatic hydrocarbons (PAH). A positive response with the raw extracts from recycled fiber was observed on the AhR. After fractionation, 11 fractions (alkaline and acidic) were tested. One fraction induced a similar positive effect in both conditions, suggesting that the substance responsible for the observed answer in the extract was probably neutral.

**Genotoxicity of coatings and adhesives**
Bioassays were also performed to detect genotoxicity with other types of FCM. Mittag et al. (2006) tested 95 % ethanolic migrates of two can coatings (epoxy and polyester based) using the mutagenicity Ames II test: none of extracts exhibited mutagenic effect. However, the test was performed without an exogenous metabolic system (S9 mix), which could lead to false negative data.

In the European Migresives project (2007-2010), the Ames test (with and without S9 mix) and the comet assay (HepG2 cells) were applied on six ethanol extracts of glues and adhesives for paperboard. None of extracts was genotoxic in the experimental conditions.

**Genotoxicity of plastics**

Concerning polymers such as the polyethylene terephthalate (PET), several studies focalized on the genotoxicity of PET bottled water extracts. Biscardi et al., (2003) carried out the comet assay with human leucocytes to study the release of mutagenic compounds into mineral water from PET bottles and suggested that this approach coupled with a characterization by GC/MS could be useful for periodic monitoring of mineral water quality from the spring to the shelf. Ergene et al. (2008) retained the sister chromatide exchange (SCE) assay on human blood lymphocytes to evaluate mutagenicity of various PET bottled drinking waters, these authors did not identify any effect.

Bach et al. (2013, 2014) tested the genotoxicity of PET bottle extracts used for drinking waters after exposure to sunlight or high temperature. The Ames test and the micronucleus assay using the HepG2 cell line, were negative whatever the conditions (sunlight or temperature), suggesting no drastic effect on the PET storage.

Induction of SCE was prior checked with plastic materials such as polystyrene, polypropylene and polyvinylchloride by Hens et al. (1988) and none was genotoxic. In 2014, the genotoxicity of styrene oligomers extracted from polystyrene were analysed by
Nakai et al., with the Ames test and the *in vitro* chromosomal aberration (CA) assay on
CHL/IU cells. Ames and CA bioassays showed negative data with polystyrene extracts, even
with high concentrations of oligomers. More recently, Riquet et al. (2016) studied the
genotoxicity of polypropylene films additivated with three different anti-oxidants (Irgafos 168,
Irgafos 1076, Tinuvin 326) and submitted to physical post treatments such as electron beam
or microwave. In the comet assay on HepG2 cells, none of the 12 extracts was positive.

The mutagenicity of two PLA *nanocomposites* samples was assessed with the Ames test.
Migration test was performed using distilled water (10 days at 40°C). Both extracts were
tested at five different concentrations (20%, 40%, 60%, 80%, 100%). The revertant number
was not increased compared to non-exposed bacteria, suggesting that extracts were not
mutagenic whatever the strain (Maisanaba et al., 2014).

To summarize, most of the time, this is the Ames test, which was carried out to check the
FCM extract genotoxicity. Positive results were observed with ethanol extracts of
paperboard, only one with a water extract of PET.
Negative data could also be attributed to a lack of sensitivity of the bioassays (in terms of
lowest concentration detected). Indeed, detection threshold of the bioassay is rarely or never
mentioned. It is currently well-known that the regulatory bioassays applied to FCM
assessment and developed in the 70's are not enough sensitive (ILSI, 2015).
However, some bioassays are promising in term of sensitivity such as the BlueScreen HC.
Transcriptional activation assays have been widely characterized with a good
sensitivity/specificity confirmed in independent studies (Hastwell *et al.*, 2009, Jagger *et al*.,
2009, Birrel *et al*., 2010). These assays, to date, are promising mostly as a prescreening step
to gain insights into the mechanisms of action of substances and guide the testing strategy
(EFSA, 2011).
Concerning the positive data, the main conclusion is that it appears impossible to establish a link between the genotoxic effect observed in the extract and the presence of identified genotoxic substance in this extract (Bengtström et al., 2016). As seen before, the low level of chemicals present in the extracts could not explain the genotoxic effect (Osaki et al., 2004). This suggests either that unknown substances are present (NIAS), and not detected by chemical analysis, and/or that a combined effect of two or more of substances is behind the positive response. The results emphasize, however, the importance of testing the whole extract for toxicity as well as considering the individual substances (Bradley et al., 2010). Indeed, since substances are tested for regulatory purposes on an individual basis at generally high dose levels, there is only limited data available on mixture effects especially in a low dose range. The analysis of combined effects of substances in a broad dose range represents a key challenge to current experimental and regulatory toxicology.

In the current practice, there is a need of more information; such as testing the toxicity of the overall mixture representing a worst case scenario of what the finished FCM is able to release.

**Endocrine disruption**

Endocrine Disruptors (ED) defined as “an exogenous substance (or mixture) that alters function(s) of the endocrine system and consequently causes adverse health effects in an intact organism, or its progeny, or (sub)populations” (Weybridge, 1996). ED may lead to detrimental health effects including interferences with male or female reproductive systems, hormone related cancers, reproductive tract abnormalities, neurodevelopmental disorders and obesity (UNEP/WHO, 2013). *In vivo* studies have demonstrated adverse effects after exposure to ED, such as changes in mammary gland development with known estrogenic compounds (Mandrup et al., 2012) and male sex organ abnormalities after treatment with known anti-androgenic compounds (McIntyre et al., 2001).
In 2011, the European Centre for Ecotoxicology and Toxicology of Chemicals (ECETOC) proposed a new approach in accordance to the definition of Weybridge by integrating knowledge of adverse effects and modes of action to better screen endocrine disrupting substances (Bars et al., 2011). According to the EFSA Scientific Committee (EFSA, 2013), an endocrine disruptor can be defined by three criteria: endocrine activity, adversity of effects and a plausible link between endocrine activity and adverse effect.

Endocrine disruption is of high concern for researchers as there is increasing scientific, media and public attention regarding packaging migration as a source of food chemical contaminations. Chemicals leaching from packaging (bisphenol A, phthalates...) into food contribute to human ED exposure (Muncke, 2011; Yang et al., 2011) ED represent also a high concern regarding Developmental Origins of Health and Disease (DOHAD) (Barker, 2003). In addition, exposure to many chemicals, even if they are present at/or below their individual effect level (NOAEL), can lead to adverse effects (Kortenkamp et al., 2007).

Therefore, endocrine disruption is an important toxicological endpoint to evaluate as FCM can be a potential source of ED (Muncke, 2009). ED have numerous action mechanisms; direct (genomic or non-genomic pathways) and indirect such as an inhibition of enzymes involved in the steroid synthesis. A battery of different bioassays is available and a lot have been carried out to screen hormonal activities in FCM extracts, regarding direct action direct mechanisms.

The **E-Screen assay**, was among the first bioassay developed for estrogenic activity screening. It measures cellular estrogen-dependent proliferation of a human breast cell line (MCF7 cells). This quantitative assay compares the cell number achieved by similar seeding of MCF-7 cells in the absence of estrogens (negative control), in the presence of 17 beta-estradiol (positive control) or in presence of a new substance using a range of concentrations (Soto et al., 1995). This assay is very sensitive but not specific; as cell proliferation can be mediated through other pathways than by a transcriptional activation of estrogen responsive genes. Then, the use of antagonist is advised to validate a positive answer. E-Screen has
not been not recommended by the Interagency Coordinating Committee on the Validation of Alternative Methods ICCVAM (2003).

Numerous in vitro tests for screening hormonal activities are based on transcriptional activation assays using different models (mammary or human cell lines/yeast) transfected with a specific nuclear receptor, involved in endocrine disruption (ER, AR, GR ...) coupled to a reporter gene allowing to detect agonist or antagonist activities. Reporter gene assays are recommended by OECD as level 2 screening test. They can provide data about selected endocrine mechanism(s)/pathway(s) described in the “Conceptual Framework of the OECD for the testing and assessment of endocrine disrupting chemicals” (OECD, 2012).

Indeed, bioassays based on transcriptional activation with numerous systems/models have been developed.

The YES (Yeast Estrogen Screen) and the YAS (Yeast Androgen Screen) assays are based on this principle. In the YES assay, the yeast strain contains the stably integrated human estrogen receptor (hERα) gene and an expression plasmid containing the reporter gene lacZ under the control of estrogen responsive elements (ERE). For the YAS assay, the yeast strain hosts an integrated gene coding for human androgen receptor (hAR) in its genome and expression plasmids carrying the reporter gene lac-Z (encoding the enzyme β-galactosidase). Then, hormonal activities of tested substances are detected with the sensitive chlorophenol red-β-D-galactopyranoside substrate leading to a color change in the medium from yellow to purple. It measures agonistic interactions on the human receptor of estrogens alpha (hERα) or on the human receptor of androgens (hAR) respectively, but also antagonist activities corresponding to Yeast Antiestrogen (YAES) and Antiandrogen Screen assays (YAAS). One important advantage of this assay is that the yeast has not endogenous receptor, leading to the absence of nuclear receptor cross-talk.

An OECD guideline (OECD 455) has been established for stably transfected transactivation (STTA) in vitro assays to detect estrogen receptor agonists and antagonists based on two fully validated test methods, the STTA assay using ERα-HeLa-9903 cell line and the
BG1Luc ER TA assay using the BG1Luc-4E2 cell line (now named VM7Luc4E2 cell line). Following ligand binding, the receptor-ligand complex translocates to the nucleus where it binds to specific DNA response elements and transactivates a firefly luciferase reporter gene, resulting in an increased cellular expression of luciferase enzyme activity (converting luciferin), which is quantitatively measured.

Some reporter gene assays with human cell lines are specific androgens receptors. Different protocols exist using several transfected cell lines (MDA-kb2 (Wilson et al., 2002), PALM (Real et al., 2015), T47D (Willemsen et al., 2004).

The Chemical Activated LUciferase gene eXpression bioassays (CALUX®) commercialized by BioDetection Systems are also screening tools based on the same principle using mammalian cells. This system permits to measure the activation of a wide range of biological activities on nuclear receptors such as activation of ERα/β (estrogen receptor alpha or beta), AR (androgen receptor), GR (glucocorticoid receptor), TR (thyroid receptor) and AhR (arylhydrocarbons receptor).

Bioassays for testing endocrine disruption potential of FCM extracts (Table 3)

Hormonal activities of plastics

- PET bottled drinking waters

Among plastic, the PET was the most FCM studied for its endocrine disruption potential. Estrogenicity of PET bottled water was first reported by Pinto and Reali (2009). They carried out the YES bioassay to investigate the estrogenicity of 30 extracts of water samples from nine brands of PET bottles available in Italy. This preliminary study showed that more than 90% of the water samples did not exhibit any appreciable estrogenic activity. The highest estrogenic activity detected in water extracts was equivalent to the activity induced by 23.1 ng/L of the natural hormone 17β-estradiol (E2).
Concomitantly, Wagner and Oehlmann (2009) analyzed 20 brands of commercially available mineral waters packaged in glass or PET using the YES assay and detected estrogenic effect in 60% of samples with a maximum activity Equivalent to 75.2 ng/L of E2. Later, two articles (Wagner and Oehlmann, 2011, Wagner et al., 2013) completed this work. In 2011, they used an improved extraction procedure with the E-screen bioassay and detected an estrogenic response in 11/18 samples analysed corresponding to an estrogenic activity of 1.9-12.2 pg Equivalent E2/L of bottled water. In 2013, they studied the anti-estrogenic and anti-androgenic activities of 18 extracts of bottled waters with the YAES and YAAS assays and demonstrated that the majority of glass or PET bottled water extracts inhibited significantly both human estrogen and androgen receptor. In the YAES, 13 products were anti-estrogenic (inhibition of 19.2 up to 61.1%) and 16 samples in the YAAS were anti-androgenic (inhibition of 19.0 up to 92.3%). In contrast, Maggioni et al. (2013) analysed the estrogenicity of 5 PET bottled mineral waters bought in local Italian markets using HELN-ERα cells without detecting any significant hormonal activities in the extracts.

In 2013, Plotan et al. studied also the endocrine disruption potential of fourteen brands of bottled mineral waters in triplicate (42 samples) with a battery of reporter gene assays coupled with a single solid phase extraction step. Estrogenic (ER), androgenic (AR), progestagenic (PR) and glucocorticoid (GR) activity was found in 78% of the samples, with an average concentration of 10 ng Equivalent E2/L, 26 ng Equivalent testosterone/L, 123 ng Equivalent progesterone/L and 13.5 ng hydrocortisone Equivalent/L. However, as activity levels were low, they concluded that it was not a matter of concern for the consumers’ health.

In 2013 and 2014, Bach et al. studied the toxicity of water packaged in PET or glass bottles after treatment such as exposure to sunlight (2, 6, 10 days) or to high temperatures (40, 50, 60°C). Anti-androgenic activity was assessed with a reporter gene assay using the human MDA-MB453-kb2 cell line and the estrogenic activity was evaluated with a transcriptional activation assay using the HepG2 cell line. Data didn’t show any hormonal activities of the
water extracts, suggesting that sunlight and temperature exposure did not generate new NIAS with ED potential.

Real et al. (2015) investigated the hormonal activity (estrogen and androgen agonist and antagonist effects) of extracts prepared from 29 waters packaged in PET or glass bottles commercially available in southern Spain. E-Screen and PALM cell luciferase assays were applied. All samples evidenced at least one of the four hormonal activities measured. Three-quarter of the bottled waters were estrogenic ranging from 11.2 to 77.9 pg Equivalent E2 /L. They found anti-estrogenic activity in 37.9% of the samples, 38.4% issued of plastics bottles 33.3% of glass bottles. They also detected anti-androgenic activity in 41.4% of the extracts (5 to 40.1% of inhibition of the hAR). Weak androgenic activity was also detected in around 30% of the samples. This study was interesting as hormone-like activities observed in waters from both plastic and glass bottles suggest that plastic packaging was not the only source of contamination and that water and bottling process may play a role.

In 2016, Chevolleau et al. studied a large panel of hormonal activities (ER, AR, PR, GR, TR) of 7 PET bottled waters and 4 glass bottled waters after different ageing conditions using stable reporter gene cell lines derived from Hela cells. No anti or agonist activities were detected using 5 reporter cell lines indicating the absence of receptors ligands in the extracts.

- Other plastics

Concerning the endocrine disruptor potential of polystyrene (PS), E-screen assay was performed by Hirano et al., (2001) to test a novel styrene trimer extracted from polystyrene food containers, they concluded that the trimer was not estrogenic. Fail et al. (1998) demonstrated also the absence of estrogenicity when testing mixtures of styrene oligomers extracted from PS, using a reporter gene assay and a human breast cancer cell line (MCF7). Yang et al. (2011) checked a broad range (455) of commercially available plastics resins (HDPE, PP, PS, PLA, PC, PET, …) and product types (flexible packaging, food wrap, rigid
packaging, baby bottle component, deli containers and plastic bags) from different retailers/producers using an accurate robotized version of the E-Screen assay to detect estrogenic activity of chemicals leached into saline or ethanol extracts. Most of the plastics products released chemicals with a potential endocrine activity. Among the 28 PS resins tested, 14 released chemicals with an estogenic activity. They also showed that even when a “simple” polymer (without additives) such as polyethylene or polyvinyl chloride did not exhibit hormonal activity, commercial resins and products from these polymers often leached chemicals with hormonal effects. Those data point out, again, that it is very important to test the finished packaging instead of the raw materials. These data were consistent with a second study published by Bittner et al. (2014a) who showed using more than 1000 in vitro assays, that many BPA-free PC-replacement products made from various types of resins, including PETG, PS, PES, and Tritan™ resins, could leach chemicals with hormonal activity (13/25). Agonist responses observed using ER assay with the BG1Luc cell lines or E-Screen were confirmed as the answers were stopped with a specific ER antagonist, such as ICI 182-780.

Migration from Tritan™ BPA replacement resins and polycarbonates bottles was studied by Guart et al. (2013) using the Y(A)ES and Y(A)AS bioassays. None of the samples was positive after 10 days at 40°C in water. In contrast, Bittner et al. (2014b) studied the chemical leached into 10%, 50% or 100% ethanol or water/saline extracts of 14 thermoplastic resins with the E-Screen and the ER assay on BG1Luc human cell line. Among the tested conditions, 3 Tritan™ resins exhibited significant ER agonist activity (mean answer of 25% of maximal E2 response) after saline extraction when using the BG1Luc assay.

In 2014, the same team (Kirchnawy et al., 2014, Mertl et al., 2014) published studies on different plastic food packaging extracts. In the first study, they analysed the estrogenic effect of extracts from PET, PP, PE, PS and composite films using the YES assay and then, after a preselection, they characterized samples for their estrogen and androgen activity using a battery of bioassays (YES, YAS, ERα and AR CALUX on human osteoblast cells) in combination with chemical trace analysis by GC-MS and HPLCMS.
The first study showed an estrogenic activity ranging from 0.7 to 59 ng Equivalent E2/L for 7/42 samples. All PET samples (11) were negative regarding estrogen activity, which is in accordance with Bach et al. studies (2013, 2014).

In the second study, 4 (two composite films, one polystyrene and one polyethylene) of the 18 samples showed an estrogen activity in a similar range in both YES and ERα CALUX assays. Two other samples (polystyrene) were positive but only in the ERα CALUX due to the lower limits of detection of this bioassay. Androgenic activity was not detected in any of the tested samples. When testing for antagonists, significant differences between yeast and human cell-based bioassays were observed, with a higher number of samples positive in the Y(A)ES and Y(A)AS assays than in the ER/AR CALUX assays, despite a better sensitivity of the human based bioassay to receptor antagonists as demonstrated with reference standards tested by the authors. Using YES and YAS, many samples showed a strong antagonistic activity (3 anti-ER and 8 anti-AR) which was not observed when using the human cell-based CALUX assays (no anti-ER and 1 anti-AR). The high occurrence of antagonistic effects in YES and YAS shows the necessity to check specificity of the response, which would decrease the toxicological measured activity. It is also very important with antagonist activity to verify first if the answer is not due to a cytotoxic effect.

Furthermore, to analyze FCM for hormone activities, human cells based reporter gene assays have been mentioned as more suitable model than yeast cell-based test systems (OECD, 2012).

- ED potential of plastics when applying post-treatments

The leaching of monomers and additives from a plastic item into its content is often accelerated if the product is exposed to common-use stresses such as ultraviolet (UV) radiation in sunlight, microwave radiation, and/or moist heat via boiling or dishwashing, these conditions generating compounds with hormonal activities (Nerin et al., 2002).
Yang et al. (2011) using 455 commercially plastics materials showed that, for example, one unstressed sample of an HDPE resin with no detectable estrogenic activity in saline or ethanol extracts was able to release chemicals with an estrogenic activity equivalent to 47% of the E2 activity when extracted (in ethanol) after an UV light stress on the resin. Similarly, two samples of low-density PE resins and PETG resins with no detectable estrogenic activity subsequently exhibited estrogenicity when stressed, especially by UV. Two articles related to thermoplastic resins extracts after stress such as autoclaving, microwaving and UV radiation (A or C) were published. Fifty (Bittner et al., 2014a) and fourteen (Bittner et al., 2014b) reusable PC-replacement products made from seven types of resins (acrylic, COC, COP, PES, PETG, PS, Tritan™) were also tested. The answers were dependent on the type of resins, the conditions of extractions or the applied post-treatment. Using the E-Screen assay, PS resins saline extracts showed a significant estrogenic activity when autoclaved or exposed to UVC. The same conclusion was made with four BG1Luc assays performed on ethanol extract of Tritan™ plaque when exposed to UVC or UVA (Bittner et al., 2014b).

In contrast, the glycol-modified polyethylene terephthalate (PETG), cyclic olefin polymer (COP) or copolymer (COC) thermoplastic resins did not release chemicals with hormonal activity in the same experimental conditions.

More recently, Riquet et al. (2016) investigated the effects of physical post-treatments (electron-beam or microwave) on PP films prepared with a selection of additives. The authors choose the STTA assay according to the OECD guideline 455 with hERα-HeLa-9903 cells to check estrogenic activity in the extracts. Among the 12 extracts, one extract of FCM prepared with Irganox 1076 and post processed with a microwave treatment, showed a significant and concentration dependent estrogenic activity (representing 20% of the E2 activity). This positive effect was suggested to be due to the 2,6-Di-tert-butylphenol, a by-product of Irganox 1076 (Biedermann et al., 2014), identified by analytical method in the extract.

Hormonal activities of papers and boards
**Food contact paperboards** extracts were also tested for their hormonal activities. First, Binderup *et al.* (2002) studied, with two recombinant reporter gene assays (YES with *Saccharomyces cerevisiae* and AhR reporter gene assay with H4IIE rat hepatoma cells), the activity of paper samples produced from virgin or recycled fibres. Both ethanol and water extracts of the paper samples were cytotoxic for the yeast leading to non-usable data. One ethanol extract showed a positive dose-response in the AhR assay.

Lopez-Espinosa *et al.* (2007) carried out the E-Screen assay to detect estrogenicity of forty paper and food container cardboard extracts. Estrogenic activity was detected in 90% of extracts with a mean activity of 3.2 ng Equivalents E2/g of paper extract.

Mertl *et al.* (2014) studied 3 food paper boards extracts, two exhibited anti-androgenic activity in the yeast screen (Y(A)AS) but the answer could not be reproduced using the anti-AR-CALUX. The source of the observed antagonistic effects, specific to the yeast model, remains unclear but could be due to a cytotoxic effect of extracts on the yeast, leading to false positive data.

To conclude concerning the potential endocrine activities of FCM extracts, numerous studies have been performed. Positive answers when expressed in Equivalent of E2 were very low suggesting a weak effect of FCM. Nevertheless, these observed activities demonstrate the sensitivity of bioassays. Furthermore, it is important to check if antagonist answer (which is a decrease of activity) is not due to an underlying cytotoxic effect.

Another bioassay, the H295R steroidogenesis assay (OECD guideline 456), focuses on ED indirect mechanism of action, appears also an interesting and additional endpoint to check with finished FCM (Bengtström, 2014), when a direct mechanism is not identified.

The other point is that finished FCM appear more interesting to test, as it is often positive compared to the raw material, suggesting a role and impact of the process.

In contrast to genotoxicity, for ED detection, bioassays were usually performed on cell lines without taking into account the metabolism. Recently, Mollergues *et al.* (2016) incorporated an exogenous activation system into the CALUX® assay to check if endocrine activity could
be influenced by phase 1 enzymatic activities in the model, which is relevant and important to avoid any false negative/positive answers.

**Discussion and key points for a properly use of bioassays for hazard assessment of FCM extracts**

As illustrated by this review and regarding the studied toxicological endpoints, numerous bioassays have been applied to FCM extracts. However, the different experimental conditions when performing bioassays make very difficult comparison between the data. This is particularly the case with the FCM extract preparation and the vehicle used for testing (Tables 4 and 5). It points out the need to harmonize and to use standardized bioassays. Indeed, the case of plastic PET bottled mineral waters is of interest, as some authors detected ED activities, whereas some did not (Sax, 2010; Bach *et al*., 2012, Chevolleau *et al*., 2016).

For a properly use of bioassays, the first step which is of great importance is to harmonize and validate the extraction step. This review clearly shows that conditions of sample extraction and preparation before testing followed different protocols (Table 4 and 5). Concerning extraction solvent, simulants required by the current plastic regulation (EU) No 10/2011 in migration studies are: 10% ethanol for aqueous food, 3% acetic acid for acidic foods, 20% ethanol for alcoholic foods, 50% ethanol or vegetable oil for fatty foods and Tenax for dry foods. Food simulants can be replaced by substitute food simulants if it is based on scientific evidence that the substitute overestimates migration compared to the regulated simulants.

To note, the majority of studies were performed before the application of regulation (EU) No 10/2011, that is why water was often used instead of 10% ethanol. Nevertheless, to date, to
demonstrate compliance with the overall migration limit for all types of foods, 10% ethanol can be replaced by distilled water or water of equivalent quality.

With simulators used for the extraction such as water or ethanol, the extraction conditions are various in terms of exposure time and temperature. The extract protocol step before testing in bioassays differs extremely; from “Just an evaporation until dryness” to “Solid Phase Extraction” (SPE) with different solvents. The SPE technique appears to be the main methodology searching for ED properties while for the genotoxicity endpoints, conditions were more various (evaporation, lyophilization, SPE, ASE ...).

Lastly, to dissolve the substance in the cell culture media, the principal vehicles are water for wet food contact and DMSO or ethanol for fatty contact. However, ethanol, even at low concentrations has been shown to induce metabolic enzymes (CYP2E1) and to be clastogenic in vitro (Kayani and Parry, 2010). It is of first importance to take into account vehicle effect and to systematically compare the control cells exposed to it versus cells incubated only with culture medium.

Extraction procedures are currently optimized for analytical detection of specific chemicals. But, in food, FCM do not release just one substance but several. The leaching substances are not only IAS but also NIAS, which are a challenge for testing. Analytical methods have to be adapted and efficient to identify new/unknown NIAS. To notice, extraction procedures should not dilute the chemical concentration before testing: the extract must be representative of a realistic FCM migration into the food and consequently the human exposure. Furthermore, another important point is to not loss chemicals during the extract preparation and before testing (volatile or high vapor pressure compounds). Samples prepared by freeze-drying could generate false negative data due to the possible loss of active volatile compounds (Chevolleau et al., 2016). Wagner et Oehlman (2011) demonstrated that inappropriate sample preparation techniques (SPE method with complete evaporation) may also lead to false-negative results in bioassays due to a loss of volatile
compounds during sample treatment. They conclude that the SPE method employing a traditional silica-based sorbent (C18) and a sample preparation that minimizes evaporation steps appear to be the most effective for the extraction of estrogen-like compounds from bottled waters. Moreover, particular attention is needed to avoid sample contamination due to laboratory equipment. All experiments should be performed using exclusively glass, Teflon, and stainless steel material and excluding any plastic material (such as vials, caps, tubes, and pipette cones) (Chevolleau et al., 2016).

Moreover, the choice of the solvent or vehicle and its compatibility with the biological model is a prerequisite step. Most organic solvents are not suitable for bioassays as they are highly cytotoxic for cells, giving false negative/positive data, thus the candidate solvents are often limited to ethanol and dimethyl sulfoxide (DMSO). Nevertheless, the final concentration of solvent in culture medium should not exceed 1% (the highest non-cytotoxic concentration for most of the cell lines), then, a pre-concentration of the extract before testing is also required. Ethanol and DMSO have a similar log P value, making them capable of extracting similar classes of substances. Moreover, due to its high boiling point, DMSO is able to keep volatile substances during evaporation (Körner et al., 1999). However, in contrast to ethanol, DMSO cannot be easily evaporated if the solution needs to be further concentrated.

The choice of the adequate biological model is also challenging as it determines the quality and reliability of the results. Bioassays must be performed on pertinent in vitro models and the cellular environment has a crucial impact on the experimental results. Most of the models are mammary cells rodents and/or human cell lines and bacterial/yeast. The retained model has to reflect biological effects of concern. Immortalized cell lines often originate from well-known tissue-type. For example, intestinal cell lines like Caco-2 offers interesting opportunities for measuring chemical absorption and mimicking intestinal sensitivity, as intestine is the first barrier of the organism after oral exposure (Van Breemen and Li, 2005).
Regarding genotoxic endpoints, bacteria are prokaryotic cells presenting major genotypic and phenotypic differences with eukaryotic ones making a human extrapolation particularly difficult. They are also not suitable for the search of clastogenic effects (EFSA, 2011). Then, a lot of bioassays are carried out on mammalian cells, mostly on human cell lines. Indeed, during an ECVAM Workshop on genotoxicity testing (2007), scientists agreed that the cell lines commonly used in genotoxicity assays should have certain characteristics; being preferably of human origin, p53 and DNA-repair proficient. P53 tumor suppressor gene, called “the guardian of the genome”, encodes for a pivotal protein involved in the response to DNA damage, mitotic spindle disruption and activation of oncogenes (Shackelford et al., 1999), then P53 is essential for a faithful response of the genotoxicity assay.

Furthermore, information on the metabolic activities of the test systems is also needed, cells with defined Phase 1 and Phase 2 metabolism are considered more relevant in mammalian genotoxicity assays to reduce false positive results (Kirkland et al., 2007). Indeed, the metabolic capability of cellular models for genotoxicity testing remains a prerequisite step for sound interpretation of the results. When the biological model does not possess metabolic activities (for example bacteria), the use of an exogenous metabolic activation system (S9mix) is required to improve the reliability and comparability of bioassays, avoiding false negative data. Although many of the cell lines used in genotoxicity assays fulfil some of these criteria (HepG2, MCL-5, TK6 cell lines), none fulfils all the criteria. The HepaRG cell line is to date considered as a promising model as it retains numerous xenobiotic metabolic activities (Aninat et al., 2006). The same comments can be applied to potential ED. Recently, Mollergues et al. (2016) incorporated an exogenous activation system to check if endocrine activity could be influenced by enzymatic activities, as most of the cell lines are metabolically incompetent and do not include a metabolizing step. To illustrate the importance of the biological models, in 2014, Mertl et al. compared the results of the bioassays performed on yeasts and human cells. They showed that antagonistic effects occurring in the yeast cell based test systems (YES, YAS) could not be observed in other bioassays such as CALUX® using human cell lines. For a FCM screening for hormone activity, the primary purpose is to
cover potential impacts on humans whereby testing for antagonists in YES and YAS can lead to false positive results, (model too sensitive for human exposure). Yeasts suffer from limitations with substances that have fungicidal activity which inhibit cell proliferation, solubility, permeability or transport issues across the cell membrane (ICCVAM, 2003). It has also been reported that the YES assay is not sensitive for anti-estrogenic chemicals (Fang et al., 2000). These limitations could explain why the yeast was not retained by OCDE in the level 2 of OECD Conceptual Framework for Testing and Assessment of Endocrine Disrupters (OECD, 2012). Consequently, human cell based reporter gene assays are more suitable to screen FCM extract for hormone activities than the yeast cell based test systems.

The sensitivity of in vitro bioassays should be sufficient to detect biological effects at a relevant concentration and should not result in false positive/negative data. Endocrine disruptor bioassays are recognized as very sensitive, they give specific answers at low threshold getting down to activities in the range of pg Equivalent E2 /L. This is not always the case for genotoxicity assays, especially regulatory assays. Indeed, the current generation of in vitro genotoxicity bioassays cannot reach down to the (sub) ppb level for genotoxic substances to exclude their presence (ILSI, 2015). Extracts should also be tested up to their limit of solubility (as long as it is not cytotoxic) but not higher as it could generate data which could not be reproduced due to a heterogeneous dissolution.

Therefore, bioassays need to be performed by using a proper sample preparation and the sensitivity and specificity checked by spiking reference substances (well-known toxic substances or not) in order to avoid false negative or positive data for hazard assessment. Furthermore, a limit of biological detection (threshold) of the bioassays should be defined using reference substances in order to compare their sensitivity, according to the toxicological endpoint studied.

Another crucial parameter is the use of the proper concentrations in in vitro experiments to avoid false positive responses; extracts have to be tested at not cytotoxic concentrations. This point is essential, for ED when an antagonist effect is observed in bioassays to avoid
misinterpretation. Concerning genotoxicity, a certain range of cytotoxicity is accepted (50-80% depending to the assay) to validate the outcome of the assay.

Quenching of the reading systems is also an important parameter to check using positive references. Artefacts due to the detection procedure of the bioassay often based on spectroscopic measurements such as fluorescence (self-fluorescence of the mixture, turbidity effect...) or luminescence could occur in the presence of mixture.

Concerning specificity, the use of well-known agonists/antagonists (active hormonal substances) is a current and widespread practice for searching ED activities, e.g. the agonist answer would disappeared when spiking with an antagonist substance. For genotoxicity assay, specificity can be checked using non-active reference substances.

Bioassay strengths for hazard assessment of FCM extracts

The identification of unknown compounds in a complex mixture described as forest of peaks in chromatograms such as for food contact papers is both time consuming and painstaking and it does not give information on its potential hazard. Therefore, some interdisciplinary studies have tried to screen FCMs using both chemical analysis and in vitro tests (Binderup et al., 2002; Lopez-Espinosa et al., 2007; Bradley et al., 2008; Bach et al., 2013; Koster et al., 2014; Bengtström et al., 2016). This process is applied as a fast screening, excluding irrelevant samples and enabling further investigations only on relevant samples (with toxicological concerns). In case of positive response, fractionation of the extract can be performed to identify which substance(s) is (are) responsible for a further risk assessment with the relevant choice of toxicological endpoints (ILSI, 2015).

The expected purpose of in vitro methods is to be at the level of inclusion of first tier of a hierarchical approach (decision tree) in the context of regulatory guidelines and when full
replacement of the *in vivo* methods (3R rules) is possible. Short-term bioassays, if relevant, are less expensive and time consuming than *in vivo* experiments. 

*In vitro* bioassays can be more sensitive, as they isolate the target organ or tissues of interest from the rest of the organism; when performed properly, they provide unequivocal results free from confounding effects of other processes with a controlled concentration and in controlled conditions. Furthermore, it is possible to extrapolate *in vivo* getting the circulating chemical levels in the body (for example in plasma, urine). 

It is important to point out that the observed toxicities in the different studies could not be always explained by the chemical analysis data as chemical analysis is never exhaustive (Honkalampi-Hämäläinen *et al.*, 2010; Ozaki *et al.*, 2004, Nerin *et al.*, 2013). Indeed, if some identified substances are recognized to have a role in the toxicity observed with some samples, very often their identification and quantification did not explain the toxicological response of the tested extracts (Mittag *et al.*, 2006, Bengtström *et al.*, 2016) suggesting that testing the whole leachate therefore offers an opportunity to reduce uncertainty (Muncke, 2011). Several papers were published demonstrating the relevance of *in vitro* bioassays to detect the toxicity of a FCM extract blinded tested, for example, they allowed distinguishing non-food grade from food grade product (Bradley *et al.*, 2008, 2010).

Furthermore, unlike analytical methods or even QSAR methodologies, the *in vitro* bioassays allowed the detection of an overall hazard, generated by one or several unknown substances, released or extracted from the packaging. The interest of bioassays is then to provide a global biological response taking also into account “cocktail” effect as it could occur with ED (Kortenkamp, 2007, SCHER, 2011). 

Bioassays can detect potential harmful activities that cannot be predicted by chemical analysis alone especially concerning NIAS which are not detected/identified by chemical analysis taking also into account a potential “cocktail effect” of the FCM mixture. Then, these approaches, in combination together with *in silico* screening may complement each other (Schilter, 2013; ILSI, 2015).
Bioassays should be a cost effective, rapid and robust approach to identify the substances of concern and to set the right priorities for further investigations in terms of food safety to remove bioactivity due to undesirable substances and to support decision-making. Efforts are currently increasingly focused on non-targeted screening using both analytical methods and in vitro bioassays (Schilter et al., 2014; Koster et al., 2011, 2015). Then, bioassays, when applied early in the development of new packaging, could play an important role in the efficient design of novel and safe packaging materials.

In vitro assays permit also the implementation of toxicity pathways based risk assessment and can be scaled for the evaluation of mixtures of chemicals, in a relative short amount of time, across a wide range of concentrations, diverse cellular responses such as non-monotonic answer. Non-Monotonic Dose-Response (NMDR) describes a dose-response relationship where the slope of the dose-response curve changes direction within the range of tested doses. NMDR therefore challenges traditional toxicology, particularly the common saying of “the dose makes the poison”. Such NMDRs are common for hormones, and have been observed for several ED (Vandenberg et al., 2012).

Recently, to corroborate this idea, Warner and Ludwig (2016) published in Nature, a comment entitled “Rethink how chemical hazards are tested” and they proposed approaches that would help inventors to produce safer chemicals and products. The first one is to standardize chemical safety tests and the second to test the finished product, which is currently the need for FCM testing. To note, the report 2015/2259/(INI) of the European Parliament, published in October 2016, mentioned that “Biotesting should be encouraged as an optional premonitory measure to ensure the safety of chemically complex FCMs and also research on the development of both analytical and toxicological testing to ensure robust and cost-effective safety assessments of FCMs for the benefit of consumers, the environment and manufacturers”.

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FCM are a relevant source of widespread exposure to chemical mixture and bioassays provide reliable strategy to check hazard assessment of NIAS of potential health relevance. To date, the best way to test finished FCM appears to use screening reporter gene assays as very sensitive tools.

Regarding cytotoxicity, a good correlation appeared between the total amount of extractables and toxicity and sublethal assays are preferable.

Human cell lines according to the target tissue, the studied endpoint and metabolic activity will be preferred for a better human extrapolation.

Concerning the sample preparation, cautions must be taken: use a biocompatible solvent and avoid the sample dilution before testing. The SPE protocol that minimizes evaporation step appears the most effective methodology for a proper extraction. Extracts should also be tested on an adapted range of concentrations and up to their limit of solubility.

Conclusion

Bioassays are relevant powerful tools to study hazard assessment of FCM extracts. Some of them are already standardized and validated. Concerning hormonal activities, the sensitivity is clearly demonstrated. However, bioassays should be performed with caution as several parameters could interfere on the result (models, sample preparation, dilution, interference with bioassay signals …). Bioassays generate also important information on the mode of action. Bioassay data will feed the pathways-based approaches described by AOP; an alternative method for risk assessment, which is under development and integrated to IATA (Integrated Approaches to Testing and Assessment) studies. Furthermore, the use of bioassays limits the in vivo studies and thus complies with the 3R rule. Due to the complex nature of FCM and the difficulties for their risk assessment (analytical chemistry is never exhaustive), in vitro bioassays targeting specific toxicological endpoints are promising tools for hazard identification of FCM extracts. Moreover, using bioassays in complement to the
chemical analysis, to study the finished FCM (involving NIAS) is pertinent and makes sense as it takes into account the properties of the FCM extract composition.

The approach, more and more used, which consists of assessing the toxicity of extracted substances alongside their chemical characterization, is likely to enable a real technological leap into the assessment of the health concern of the finished FCM. It may also help to comply with the regulations concerning FCM, regarding NIAS and increase the consumer confidence.
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<td>PET (YES)</td>
<td>Real et al., 2015</td>
<td>Positive response for 8/29 samples</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PET (YES)</td>
<td>Real et al., 2015</td>
<td>Positive anti-androgenic response for 12/29 samples</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PET (YES)</td>
<td>Chevolleau et al., 2016</td>
<td>Positive response for 6/12 samples</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PET (YES)</td>
<td>Maligno et al., 2013</td>
<td>Positive estrogenic response for 5/5 samples</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PET (YES)</td>
<td>Plotan et al., 2013</td>
<td>Positive response for 16/42 samples</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PET (YES)</td>
<td>Plotan et al., 2013</td>
<td>Positive anti-androgenic response for 12/42 samples</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PS (stryren oligomers) (estrogenicity on MCF-7 cells)</td>
<td>Riquet et al., 2016</td>
<td>Positive response for 1/12 samples</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PS (stryren oligomers) (estrogenicity on MCF-7 cells and Hela cells)</td>
<td>Ohno et al., 2001, 2003</td>
<td>Negative response</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PET, PP, PE, PS, composite films, and paper board (ERx and AR CALUX)</td>
<td>Mertl et al., 2014</td>
<td>Positive response for 6/18 samples</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>BPA replacement resins (PS, Tritan TM, acrylic, COC, COP, PES, PETG) (estrogenic activity on BG1Luc 4E2 cells)</td>
<td>Bittner et al., 2014 a,b</td>
<td>Positive response for 2/2 samples</td>
<td></td>
<td></td>
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<tr>
<td>Paper board</td>
<td>Paper board (AhR on H4IIE cells)</td>
<td>Binderup et al., 2002</td>
<td>Positive response for 1/4 samples</td>
<td></td>
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</tbody>
</table>
Table 2: Bioassays used with FCM extracts to detect genotoxicity

<table>
<thead>
<tr>
<th>Bioassay</th>
<th>FCM</th>
<th>Reference</th>
<th>Results</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Ames test</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Paper board</td>
<td>Binderup et al., 2002</td>
<td>Negative response</td>
<td></td>
</tr>
<tr>
<td>Paper board</td>
<td>Bradley et al., 2008</td>
<td>Positive response for 1/20 ethanol extracts</td>
<td></td>
</tr>
<tr>
<td>Can Coatings</td>
<td>Mittag et al., 2006</td>
<td>Negative response</td>
<td></td>
</tr>
<tr>
<td>Adhesives for FCM</td>
<td>« Migresives » final report, 2010</td>
<td>Negative response</td>
<td></td>
</tr>
<tr>
<td>PET</td>
<td>Bach et al., 2013; 2014</td>
<td>Negative response</td>
<td></td>
</tr>
<tr>
<td>PLA with nanocomposites</td>
<td>Maisanaba et al., 2014</td>
<td>Negative response</td>
<td></td>
</tr>
<tr>
<td>PS</td>
<td>Nakai et al., 2014</td>
<td>Negative response</td>
<td></td>
</tr>
<tr>
<td>PP</td>
<td>Riquet et al., 2016</td>
<td>Negative response</td>
<td></td>
</tr>
<tr>
<td><strong>Rec Assay</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Paper board</td>
<td>Osaki et al., 2004</td>
<td>Positive response for 13/28 ethanol extracts</td>
<td></td>
</tr>
<tr>
<td>Paper board</td>
<td>Osaki et al., 2005</td>
<td>Positive response for 9/12 ethanol extracts</td>
<td></td>
</tr>
<tr>
<td><strong>Comet assay</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Paper board</td>
<td>Osaki et al., 2004</td>
<td>Positive response for 6/8 ethanol extracts</td>
<td></td>
</tr>
<tr>
<td>Paper board</td>
<td>Bradley et al., 2008</td>
<td>Negative response</td>
<td></td>
</tr>
<tr>
<td>PET</td>
<td>Biscardi et al., 2003</td>
<td>Positive response for 10/24 water extracts</td>
<td></td>
</tr>
<tr>
<td>PP</td>
<td>Riquet et al., 2016</td>
<td>Negative response</td>
<td></td>
</tr>
<tr>
<td><strong>Micronucleus assay</strong></td>
<td>PET</td>
<td>Negative response</td>
<td></td>
</tr>
<tr>
<td><strong>SCE assay</strong></td>
<td>PS, PP, PVC</td>
<td>Negative responses</td>
<td></td>
</tr>
<tr>
<td>Chromosomal aberration test</td>
<td>PET</td>
<td>Negative response</td>
<td></td>
</tr>
<tr>
<td>BlueScreen HC assay</td>
<td>Paper board</td>
<td>Negative response</td>
<td></td>
</tr>
<tr>
<td>AhR reporter gene assay</td>
<td>Recycled paper board</td>
<td>Positive response for 2/2 ethanol extracts</td>
<td></td>
</tr>
</tbody>
</table>
Table 1: Bioassays used with FCM extracts to detect cytotoxicity.

<table>
<thead>
<tr>
<th>Endpoint</th>
<th>Assay</th>
<th>Food contact materials</th>
<th>References used with FCM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Breakdown of cellular permeability</td>
<td>Trypan Blue</td>
<td>Polyvinylchloride/PET</td>
<td>Sauvant et al., 1995</td>
</tr>
<tr>
<td></td>
<td>Neutral red uptake (NRU)</td>
<td>Polyvinylchloride/PET</td>
<td>Sauvant et al., 1995</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Paper-board</td>
<td>Bradley et al., 2008</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Paper-board</td>
<td>Honkalampi et al., 2010</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Can coating</td>
<td>Mittag et al., 2006</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Nanomaterials based on PLA</td>
<td>Maisanaba et al., 2014</td>
</tr>
<tr>
<td></td>
<td>Alamar Blue®, resazurin</td>
<td>Paper-board</td>
<td>Binderup et al., 2002</td>
</tr>
<tr>
<td></td>
<td>MTT and derivatives (MTS, WST-1,...)</td>
<td>Polyvinylchloride/PET</td>
<td>Sauvant et al., 1995</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Can coating</td>
<td>Mittag et al., 2006</td>
</tr>
<tr>
<td></td>
<td>Lactate deshydrogenase (LDH)</td>
<td>Polyvinylchloride/PET</td>
<td>Sauvant et al., 1995</td>
</tr>
<tr>
<td>Cell proliferation</td>
<td>Total protein content (TPC)</td>
<td>Paper-board</td>
<td>Bradley et al., 2008</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Paper-board</td>
<td>Honkalampi et al., 2010</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Nanomaterials based on PLA</td>
<td>Maisanaba et al., 2014</td>
</tr>
<tr>
<td></td>
<td>Bromodeoxyuridine (BrdU)</td>
<td>Can coating</td>
<td>Mittag et al., 2006</td>
</tr>
<tr>
<td>RNA synthesis</td>
<td>Kinetic of inhibition of RNA synthesis (RNA)</td>
<td>Polyvinylchloride/PET</td>
<td>Sauvant et al., 1995</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Paper-board</td>
<td>Fauris et al., 1998</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Can coating</td>
<td>Mittag et al., 2006</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Paper-board</td>
<td>Bradley et al., 2008</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Paper-board</td>
<td>Honkalampi et al., 2010</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Adhesives</td>
<td>« Migresives » final report, 2010</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Polypropylene</td>
<td>Riquet et al., 2016</td>
</tr>
<tr>
<td>Cell motility</td>
<td>Boar spermatozoan motility assay</td>
<td>Paper-board</td>
<td>Bradley et al., 2008</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Paper-board</td>
<td>Honkalampi et al., 2010</td>
</tr>
</tbody>
</table>
• Food contact materials can release into food intentionally added substances or not.
• FCM hazard assessment is currently performed only with single started substances.
• Released chemicals are difficult to predict and identify/quantify.
• Bioassays used cytotoxicity, genotoxicity and hormonal activity are described.
• Bioassays are relevant tools to assess hazard of the finished packaging.