Environment International 207 (2026) 109948

Contents lists available at ScienceDirect

Environment International

journal homepage: www.elsevier.com/locate/envint

Review article

A regulatory perspective on the applicability of NAMs in genotoxicity and carcinogenicity assessment in EU: current practices and future directions

Cecilia Bossa [a,*], Silvia Alivernini [b], Cristina Andreoli [a], Gabriele Aquilina [b], Leonello Attias [b], Emilio Benfenati [c], Maria Dusinska [d], Naouale El Yamani [d], Henriqueta Louro [e,f], Francesca Marcon [a], Giuseppa Raitano [c], Elise Rundén-Pran [d], Maria Teresa Russo [b], Maria João Silva [e,f], Chiara Laura Battistelli [a]

[a] Environment and Health Department, Istituto Superiore di Sanità, Viale Regina Elena 299, 00161 Rome, Italy
[b] National Centre for Chemicals, Cosmetics and Consumer Protection, Istituto Superiore di Sanità, Viale Regina Elena 299, 00161 Rome, Italy
[c] Department of Environmental Health Sciences, Istituto di Ricerche Farmacologiche Mario Negri IRCCS, 20156 Milano, Italy
[d] Health Effects Laboratory, Department of Environmental Chemistry and Health Effects, The Climate and Environmental Research Institute NILU, 2007 Kjeller, Norway
[e] Department of Human Genetics, National Institute of Health Dr. Ricardo Jorge, 1649-016 Lisbon, Portugal
[f] Comprehensive Health Research Centre (CHRC), NOVA Medical School, Universidade Nova de Lisboa, Lisbon, Portugal

ARTICLE INFO

Keywords:
Non animal methods
New approach methodologies
Genotoxicity
Carcinogenicity
Hazard assessment
Chemical regulation
Regulatory science

ABSTRACT

New Approach Methodologies (NAMs) are gaining significant momentum globally to reduce animal testing and enhance the efficiency and human relevance of chemical safety assessment. Even with substantial EU commitment from regulatory agencies and the academic community, the full regulatory adoption of NAMs remains a distant prospect. This challenge is further complicated by the fact that the academic world, oriented toward NAMs development, and regulatory agencies, focused on practical application, frequently operate in separate spheres. Addressing this disconnect, the present paper, developed within the European Partnership for the Assessment of Risks from Chemicals (PARC), provides a clear overview of both the available non-animal tests and current evaluation practices for genotoxic and carcinogenic hazard assessment, while simultaneously highlighting existing regulatory needs, gaps, and challenges toward greater human health protection and the replacement of animal testing through NAMs adoption.

The analysis reveals a complex landscape: while the EU is deeply committed to developing and adopting NAMs, as outlined in its Chemical Strategy for Sustainability and supported by initiatives like PARC, prescriptive regulations such as Classification, Labelling and Packaging (CLP) and Registration, Evaluation, Authorisation and Restriction of Chemicals (REACH) still heavily mandate *in vivo* animal data for hazard classification, particularly for germ cell mutagenicity and carcinogenicity. This reliance creates a "too-short-blanket-problem," where efforts to reduce animal testing may impact human health protection because of the current *in vivo*-based classification criteria. In contrast, sectors such as cosmetics and certain European Food Safety Authority (EFSA)-regulated products demonstrate greater flexibility toward progressive integration of NAMs. While the deep mechanistic understanding of genotoxicity and carcinogenicity has significantly advanced the integration of alternatives to animal tests into regulatory chemical hazard assessment, their broader and full implementation faces considerable challenges due to both scientific complexities (i.e., the development and validation of fit-for-purpose NAMs) and existing legislative provisions.

1. Introduction

The use of chemicals in modern society, in almost all production processes, makes the chemical sector one of the most important and globalized industries. Chemicals are used in every material and all areas: from industry to healthcare, from agriculture to transport, from construction to energy, up to consumer products (detergents, cosmetics, medical and surgical aids, biocides, etc…), helping to improve

* Corresponding author.
E-mail address: cecilia.bossa@iss.it (C. Bossa).

https://doi.org/10.1016/j.envint.2025.109948
Received 24 July 2025; Received in revised form 18 November 2025; Accepted 18 November 2025
Available online 21 November 2025

the quality of life. In parallel, national and international legislations are in place to assess the safety of chemicals and prevent hazardous substances from causing harm to human health and the environment. Currently, the amount and type of information required for chemical risk assessment in different frameworks may vary depending on the wide variety of chemical substances, products and applications. A common aspect in the assessment of many regulatory endpoints refers to the high relevance given to data from animal studies, despite the research on alternatives to animal testing has been active for decades (e.g., starting from Russel and Burch Replacement, Reduction and Refinement (3Rs) principles (Russel and Burch, 1959). Recently, the study and development of new approach methodologies (hereinafter referred to as NAMs), expressly designed to improve human health protection and reduce the use of animals for regulatory purposes, have gained new momentum both at the research and policy levels (Cattaneo et al., 2023; Escher et al., 2022; National Institute for Public Health and the Environment (RIVM), 2022, US EPA, 2018, US EPA, 2021; Westmoreland et al., 2022; European Commission, 2020; US EPA, 2018). The term "NAM" generally refers to a wide range of methods, including *in vitro* tests*, in silico* models (e.g., (Quantitative) structure–activity relationship – QSARs, Physiologically Based Kinetic (PBK) models, *in vitro* to *in vivo* extrapolation (IVIVE) approaches, Artificial Intelligence (AI) based methods), high-throughput screening (HTS) bioassays, omics applications, advanced cell culture systems (e.g., 3D tissue models, organoids, organs-on-a chip) (Żuchowska et al., 2024).

Although there is a perceived meaning of the term NAMs, as methodologies that support toxicity assessment under the 3Rs umbrella, it is worth noting that there is actually no complete agreement on the definition in the scientific community and in different regulatory frameworks, as illustrated in Table A in the Supplementary Material. Differences in interpretation primarily concern whether NAMs should include only novel methods, whether their inclusion requires explicit adherence to the 3Rs principles or strictly non-animal approaches, and whether broader strategies such as read-across, Adverse Outcome Pathways (AOPs), and Integrated Approaches to Testing and Assessment (IATA) fall within the NAMs scope (Escher et al., 2022; ECHA, 2023a; Food and Drug Administration (FDA), 2024; Health Canada, 2023; ICCVAM, 2018; OECD, 2020; US EPA, 2021; US EPA, 2018). In any case, AOPs and IATAs are generally recognized as the foundational means through which information from NAMs can be cohesively integrated, allowing for a comprehensive assessment of the specific endpoint. As can be noted, the different definitions are broad and all-encompassing, such as they might include very different types of methods at very different levels of scientific and technical maturity and overall regulatory acceptability (Table A in the Supplementary Material).

Regarding the integration of NAMs into risk assessment, it is generally expected to bring about a significant change in the way chemical safety is assessed. This change can be conceived as an "evolution," such as an optimization of the current regulatory system by NAMs integration with – and progressive replacement of – traditional toxicological studies, or as a "revolution", adapting legal and regulatory schemes to the use of NAMs (Burgdorf et al., 2019; Knight et al., 2021). In the evolutionary scenario, the focus is on using NAMs alongside traditional tests to provide mechanistic insights, refine hazard characterization, and improve the efficiency of risk assessment. On the other hand, the revolutionary scenario implies a shift in the focus from the observation of broad, macroscopic effects on animals to understanding the precise molecular and cellular mechanisms that drive toxicity in humans, towards a new animal-free paradigm often referred to as Next Generation Risk Assessment (NGRA) (Schmeisser et al., 2023). Actually, the transition to NAMs based risk assessment may involve elements of both evolution and revolution. In fact, there is a need for progressive integration, whilst also preparing for the potential of a more radical change in how risk assessment is carried out.

The European Union (EU), as outlined in its Chemical Strategy for Sustainability, is a key player deeply committed to developing and adopting NAMs (European Commission, 2020), with a lot of resources being implemented. Notably, the European Partnership for the Assessment of Risks from Chemicals (PARC) has been designed to address various challenges associated with innovating chemical risk assessment (Marx-Stoelting et al., 2023). Within PARC, a concrete proposal for a roadmap towards implementation of NGRA (Herzler et al., 2025) as the default risk assessment approach in various EU chemical legislations has been developed (i.e., the NGRA route (European Commission, 2023; PARC, 2023)), which is now supporting the establishment of the European Commission (EC) "Roadmap Towards Phasing Out Animal Testing for Chemical Safety Assessments" (European Commission, 2023).

One of PARC's objectives is to promote innovation in regulatory risk assessment by supporting the adoption of NAMs. This goal unfolds along different tracks, including the development and advancement of NAMs for regulatory hazard assessment and the development of new (quantitative) AOP, IATAs and Defined Approaches (DAs) as building blocks for a future NGRA framework. On the other hand, a bottom-up approach involves assessing and mapping the methodologies currently used for regulatory risk assessment across relevant sectors, through a series of case studies. This line of work will help the identification of potential methodological knowledge gaps and needs, facilitating prioritization of further research and uptake of new approaches. Moreover, the results will support streamlining and harmonizing evaluations of the same chemical substance across different EU laws, i.e., pushing towards a "one substance, one assessment" (OSOA) approach (European Commission, 2020).

The present paper reflects a comprehensive analysis, conducted within WP6.3 workstream of PARC, on current practices for the regulatory evaluation of genotoxic and carcinogenic chemical hazard in several EU frameworks. The overall analysis carried on in the present study provides a comprehensive overview of the current landscape, identifying both the advancements made in integrating non-animal tests and the areas where further development, validation, and regulatory transformation are necessary to achieve a truly protective and animal-free EU regulatory system for genotoxicity and carcinogenicity hazard assessment.

2. Problem formulation and methodological approach

2.1. Problem formulation

Today, we are witnessing a major collaborative effort bringing together different actors, regulatory agencies and the academic community among others, to support the development and implementation of NAMs in regulatory risk assessment. For this effort to be fruitful, it is of the utmost importance that the academic world, who is at the forefront of creating the innovative tools, must be aware of the practical needs and constraints of the regulatory processes (Bearth et al., 2025). This paper aims to address this central theme, focusing on genotoxicity and carcinogenicity hazard assessment, by achieving the following objectives:

- analyzing current regulatory practices in order to highlight the needs, gaps and challenges with regard to greater protection of human health and the replacement of animal testing.
- Understanding if and how current practices could be improved for greater efficiency and reduced reliance on *in vivo* testing through the introduction of NAMs. This in turn implies addressing two main goals: i) getting a clear picture of the available NAMs for the specific context, to determine if any are suitable to fill the gaps and meet existing needs and ii) assessing if and in which instances the use of NAMs is permissible within current legislation.
- Identifying short- and long-term goals for NAMs implementation in this specific context in order to prioritize resources and efforts.

Furthermore, the results of this work could potentially support

streamlining and harmonizing evaluations across different EU legislative frameworks, i.e., pushing towards an OSOA approach. In addition, the information gathered when evaluating these two endpoints, for which the process of integrating alternative methods is already at an advanced stage, can inform a similar transition of other endpoints.

2.2. Methodological approach

This study is based on a mixed method approach: a systematic comparative analysis of genotoxicity and carcinogenicity assessment requirements in key EU chemical legislations, and an expert driven compilation and categorization of available non-animal testing methods and NAMs.

The search methodology adopted for the analysis of the state of the art of regulatory requirements concerning genotoxicity and carcinogenicity assessment within EU regulatory frameworks (Section 4) systematically examined current regulatory evaluation practices by focusing on key EU regulatory bodies and their sector-specific legislations and guidance. The primary sources of information included EU legislative documents and regulations relevant to chemical safety, including horizontal (e.g., Classification, Labelling and Packaging (CLP) and Registration, Evaluation, Authorisation and Restriction of Chemicals (REACH) regulation) and sector-specific (e.g., food, cosmetics) laws as well as guidance documents and scientific opinions issued by relevant EU agencies and official EC scientific advisory bodies (e.g., European Food Safety Authority (EFSA) sectoral guidance documents, Scientific Committee on Consumer Safety (SCCS) Notes of guidance). All relevant legislative texts and official documents were considered eligible, provided they addressed the current state of regulatory practices for hazard assessment of the two endpoints. The search excluded mixtures, unknown or variable composition, complex reaction products or biological materials (UVCBs), nanomaterials, pharmaceuticals and other specific types of substances not mentioned in the text. The assessment of these substances was not discussed in the analysis and comparison. The documents retrieved were searched for sections on genotoxicity and carcinogenicity evaluation, focusing on information requirements, on the potential to avoid *in vivo* testing and the potential for the use of NAMs. We also searched the documents for any impact each piece of legislation has on the others. The authors further discussed the resulting practices, drawing on their expertise across various regulatory contexts. In the specific sub-sections of this paper (i.e., 4.x), narrative descriptions of the practices for each regulatory framework, as well as their schematic tabular and graphical representations, are reported.

In Section 3 of this paper, an expert driven compilation and categorization of relevant non-animal testing methods (including NAMs) for genotoxicity and carcinogenicity assessment, is reported. This analysis was necessary to clarify the meaning of 'NAMs' within a regulatory context that already features a very advanced integration of non-animal tests. The compilation is based on:

- tests required in regulatory risk assessment, as detailed in the different legislative provisions and regulatory guidance, including tests that are no longer in use. The information was searched by accessing legal texts on the EU laws portal (https://eur-lex.europa.eu/homepage.html), the EC public health website (https://health.ec.europa.eu/index_en), the European Chemicals Agency (ECHA, https://echa.europa.eu/home) and EFSA (https://www.efsa.europa.eu/en) websites, the Organisation for Economic Co-operation and Development (OECD) test guidelines (TGs) for the Testing of Chemicals (https://www.oecd.org/en/topics/sub-issues/testing-of-chemicals/test-guidelines.html).
- tests and NAMs discussed in relevant regulatory contexts as reported in e.g., The SCCS Notes of guidance, the OECD work plan, and the reports from the International Workshops on Genotoxicity Testing – IWGT.

Based on their description, the collected tests were categorised to distinguish between well-established non-animal tests for genotoxicity and carcinogenicity assessment, their recent evolutions including technical advancements (e.g., same endpoint in different experimental conditions), and innovative risk assessment methodologies (e.g., different endpoint detected). For each test, information on the level of current regulatory acceptance status, such as availability of OECD TGs or other official validation documents or proposals (e.g., by EU Reference Laboratory for alternatives to animal testing – EURL ECVAM), was documented in Table 1.

3. Non-animal methods and new approach methodologies for genotoxicity and carcinogenicity assessment

Genotoxicity and carcinogenicity are key endpoints for the risk assessment of all types of substances. Exposure to carcinogenic chemicals through the environment, work or consumer products is among cancer risk factors that contribute to the rise in the global burden of cancer, making cancer the second leading cause of morbidity and mortality among non-communicable diseases (Madia et al., 2019). Exposure to genotoxic chemicals may cause DNA lesions that, if not properly repaired, may be fixed into gene mutations and chromosomal aberrations potentially leading to a variety of genetic disorders (Erickson, 2010). Accumulation of DNA damage in somatic cells has been proposed to have a role in degenerative diseases such as cardiovascular and neurodegenerative conditions, premature aging and cancer. If germ cells are affected, spontaneous abortions, infertility or heritable diseases are expected.

Stemming from the somatic mutation theory of cancer (National Research Council US Committee on Chemical Environmental Mutagens, 1983), research on alternatives to animal testing (hereinafter referred to as"alternative tests") for the assessment of chemicals carcinogenicity, based on detecting interactions with and damage to the genetic material, dates back to the 70s (Ames Bruce, 1971; Benigni, 2012; Zeiger, 2004) Over time, this research has led to the development of many short-term *in vitro* tests (Benigni et al., 2010a). Although with mixed success, these alternative tests have been used massively in the genotoxicity and carcinogenicity assessment of all types of substances, as substantiated, *inter alia*, by the availability of data from the dossiers submitted within REACH regulation (Regulation (EC) No 1907/2006, 2006) and EFSA pesticide regulation (Regulation (EU) No 283/2013, 2013) (Fig. 1)). Moreover, the electrophilic theory of chemical carcinogenesis (Miller and Miller, 1977) laid the foundation for understanding the structural determinants of genotoxic carcinogenesis, which later evolved into QSAR models and *in silico* applications (Benigni et al., 2010b).

All these methodologies in their essence can be considered as NAMs *ante litteram* and fit into NAMs definitions (see e.g., the definition of NAMs by ECHA, (ECHA, 2023a)). As a matter of fact, alternative tests, including *in silico*, are non-animal methods that have contributed and still contribute at the global reduction of the use of animal tests in genotoxicity and carcinogenicity risk assessment. These alternative tests assess key mechanistic events in the development of toxicity (genotoxic carcinogenicity in this case). It can also be recognized that these tests "do not produce the same information generated by the traditional animal test method. In fact, they are able to provide biologically relevant information and mechanistic insights that are more useful in the regulatory decision-making process than the animal test method", as per NAMs definition reported in van der Zalm et al. (van der Zalm et al., 2022). As a consequence, alternative tests for genotoxicity, while not entirely 'new' in their development, share common characteristics with NAMs and as such are sometimes included under NAMs umbrella (e.g., (Jagiello et al., 2022; US EPA, 2021)).

As for these premises, to avoid any potential confusion when discussing NAMs, we have compiled, and categorized, a list of available non-animal tests, from established to emerging ones, relevant to genotoxicity and carcinogenicity assessment (Table 1). Well established

Table 1
List of non-animal test methods relevant for genotoxicity and carcinogenicity endpoints.

Type	Title/ content	Output	Cell type/ organism	Category	Regulatory endpoint	Validation status /comment on usability/References
Alternatives (*In vitro*)	Bacterial Reverse Mutation Test (OECD TG No. 471)	Gene mutations in bacterial cells	Bacterial cells	Gene mutation	Mutagenicity (information requirment)	OECD TG adopted 1997; updated 2020 (OECD Test Guideline No. 471, 2020)
Alternatives (*In vitro*)	*In Vitro* Mammalian Cell Gene Mutation Tests using the Hprt and xprt genes (OECD TG No. 476)	Gene mutations in mammalian cells	Mammalian cells (rodents/human)	Gene mutation	Mutagenicity (information requirment)	OECD TG adopted 2016 (OECD Test Guideline No. 476, 2016)
Alternatives (*In vitro*)	*In Vitro* Mammalian Cell Gene Mutation Tests Using the Thymidine Kinase Gene (OECD TG No. 490)	Gene mutations in mammalian cells	Mammalian cells	Gene mutation and Chromosomal damage (structural)	Mutagenicity (information requirment)	OECD TG adopted 2016 Mainly used to investigate gene mutation but the test is also able to provide information on chromosomal damage (i.e. small and large colony) (OECD Test Guideline No. 490, 2016)
Alternatives (*In vitro*)	*In Vitro* Mammalian Chromosome Aberration Test (OECD TG No. 473)	Chromosomal aberrations (structural)	Mammalian cells	Chromosomal damage	Mutagenicity (information requirment)	OECD TG adopted 2016 (Beal et al., 2023; OECD Test Guideline No. 473, 2016)
Alternatives (*In vitro*)	*In Vitro* Mammalian Cell Micronucleus Test (OECD TG No. 487)	Chromosomal aberrations (structural and numerical)	Mammalian cells	Chromosomal damage	Mutagenicity (information requirment)	OECD TG adopted 2016; updated 2023 (OECD Test Guideline No. 487, 2023)
Alternatives (*In vitro*)	*In vitro* Sister Chromatid Exchange Assay in Mammalian Cells (formerly OECD TG No. 479)	Reciprocal exchanges of DNA between two sister chromatids of a duplicating chromosome	Mammalian cells	DNA damage	Mutagenicity (WoE)	OECD TG adopted 1986, Deleted 2014 Indication of DNA damage can be obtained from this test even if the OECD TG 479 has been deleted
Alternatives (*In vitro*)	DNA Damage and Repair, Unscheduled DNA Synthesis in Mammalian Cells *in vitro* (formerly OECD TG No. 482)	Synthesis of DNA outside the S-phase	Mammalian cells	DNA damage	Mutagenicity (WoE)	OECD TG adopted 1986, Deleted 2014 Indication of DNA damage can be obtained from this test, able to detect only bulk DNA adducts
Alternatives (*In vitro*)	*In vitro* Comet assay (Standard and Enzyme-modified)	DNA damage	Mammalian cells	DNA damage	Mutagenicity (WoE)	Useful for Mode of Action clarification. The assay is able to detect primary and secondary DNA damage (both single and double DNA breaks and intermediates in DNA repair processes, with the alkaline protocol); oxidised purines and pyrimidines on DNA with enzyme-modified protocol; double strand breaks with a neutral protocol. Possibility of high throughput 96-well format (CometChip). The test has been recently included in the OECD work plan for OECD TG programme, project n. 4.190 (OECD Work plan, 2025; Scientific Committee on Consumer Safety, 2023).
Alternatives (*In vitro*)	Cell Transformation Assay (Syrian hamster embryo cell transformation assays at pH 6.7 and at pH 7.0)	morphological cell transformation	Syrian hamster embryo (SHE) cells	Carcinogenicity (genotox, non-genotox)	Carcinogenicity (WoE)	OECD Guidance Document No. 214 (OECD Guidance Document No. 214, 2015) OECD detailed review paper 31 (OECD, 2007). DB-ALM Protocol n° 136 (https://tsar.jrc.ec.europa.eu/). The assays provide information about possible genotoxic and non-genotoxic carcinogenicity potential of the substance.
Alternatives (*In vitro*)	*In vitro* BALB/c 3 T3 Cell Transformation Assay	morphological cell transformation	BALB/c 3 T3	Carcinogenicity (genotox, non-genotox)	Carcinogenicity (WoE)	OECD detailed review paper 31 (OECD, 2007). DB-ALM Protocol n° 137 (https://tsar.jrc.ec.europa.eu/)
Alternatives (*In vitro*)	*In Vitro* Bhas 42 Cell Transformation Assay	morphological cell transformation	Bhas 42 Cell	Carcinogenicity (genotox, non-genotox)	Carcinogenicity (WoE)	OECD Guidance Document No. 231 (OECD Guidance Document No. 231, 2017) OECD detailed review paper 31 (OECD, 2007)DB-ALM Protocol n° 156 (https://tsar.jrc.ec.europa.eu/)

(*continued on next page*)

Table 1 (*continued*)

Type	Title/ content	Output	Cell type/ organism	Category	Regulatory endpoint	Validation status /comment on usability/References
NAMs / Evolution of alternatives	*In vitro* mammalian cell gene mutation tests using the Thymidine Kinase Gene	Gene mutations in mammalian cells	Mammalian cells	Gene mutation	Mutagenicity (information requirment)	The sensitivity of the Mammalian Cell Gene Mutation Assay can be improved by the use of XRCC1−/−/XPA−/− TK6 cells (Ibrahim et al., 2020; Scientific Committee on Consumer Safety, 2023)
NAMs / Evolution of alternatives	*In vitro* PIG-A gene mutation assay	mutations at the endogenous phosphatidylinositol glycan anchor biosynthesis, class A gene	Lymphoblastoid human cell lines	Gene mutation	Mutagenicity (WoE)	*In vivo* OECD TG available (OECD Test Guideline No. 470, 2022). Early stage in terms of safety testing and hazard identification. (Rees et al., 2017; Scientific Committee on Consumer Safety, 2023)
NAMs / Evolution of alternatives	TGR cell-based assays	*in vitro* transgenic rodent cell gene mutation assay	Transgenic rodent cells	Gene mutation	Mutagenicity (WoE)	(White et al., 2019; Scientific Committee on Consumer Safety, 2023)
NAMs / Evolution of alternatives	Miniaturized Ames test	Gene mutation	Bacterial cells	Gene mutation/ prescreening	Mutagenicity (WoE)	Mutagenicity in bacterial cells, pre-screening 'miniaturized' liquid version of the Ames test, allow higher throughput and use less test material. Colorimetric detection (Flamand et al., 2001)
NAMs / Evolution of alternatives	Multi-Endpoint Genotoxicity assay (i.e., MEGA-Screen)	The multi-endpoint genotoxicity assay is based on confocal microscopy and image analysis, followed by data processing in the open-source programming language R.	p53-competent A549 cell-line	Structural and Numerical chromosome aberrations and mechanistic information	Mutagenicity (WoE)	The approach is based on the simultaneous use of several biomarkers (i.e. total cell counts, detection of micronuclei, MN kinetochore labeling, γH2AX, cell-cycle analysis) (Dertinger et al., 2019)
NAMs / Evolution of alternatives	3D Reconstructed Human Skin Comet assay	DNA damage	3D Reconstructed Human Skin	DNA damage(dermal exposure)	Mutagenicity (WoE)	OECD TG Ongoing, Project 4.139 (OECD Work plan, 2025; Scientific Committee on Consumer Safety, 2023) TSAR – Tracking System for Alternative methods towards Regulatory acceptance (https://tsar.jrc.ec.europa.eu/)
NAMs / Evolution of alternatives	3D Reconstructed Human Skin MN assay	Chromosomal aberrations (structural and numerical)	3D Reconstructed Human Skin	Chromosomal damage (dermal exposure)	Mutagenicity (WoE)	OECD TG Ongoing, Project 4.139 ECVAM under evaluation TM2020-04 (OECD Work plan, 2025; Scientific Committee on Consumer Safety, 2023) TSAR – Tracking System for Alternative methods towards Regulatory acceptance (https://tsar.jrc.ec.europa.eu/). Considered as a 'tier 2' assay for dermally exposed compounds. To assess the potential of substances primarily associated with dermal exposure to cause DNA damage (SCCS (Scientific Committee on Consumer Safety, 2023).
NAMs / Evolution of alternatives	Hen's egg test HET-MN	Chromosomal aberrations (structural and numerical)	Fertilised chicken egg	Chromosomal damage	Mutagenicity (WoE)	The fertilised chicken egg is a highly complex biological system, which is assumed to be physiologically closer to the *in vivo* situation than the 2D cell systems. The reflection of particular steps of ADME and the intrinsic metabolic capacity of the developing egg are the major advantages in comparison to classical cell culture systems. (JRC, 2020; Reisinger et al., 2019; Scientific Committee on Consumer Safety, 2023)
NAMs	Error-corrected Next-Generation Sequencing (ecNGS) approaches	mutation frequency and characterisation of mutation spectrum	Any cell type	Gene mutations and chromosomal aberrations	Mutagenicity (WoE)	OECD Ongoing, Project 4.175 (OECD Work plan, 2025). Detailed Review Paper on the application ecNGS (Marchetti et al., 2023) for gene mutation evaluation. It can be run in parallel to any other toxicity assay and has the

(*continued on next page*)

Table 1 (*continued*)

Type	Title/ content	Output	Cell type/ organism	Category	Regulatory endpoint	Validation status /comment on usability/References
						potential to augment genotoxicity assessments by complementing the standard toxicity test battery (currently quite expensive and little diffused)
NAMs	ToxTracker	Gene expression	Mammalian stem cells	DNA damage, oxidative stress, protein damage by flow cytometry analysis of fluorescent reporter genes	Mutagenicity, carcinogenicity, NGC (WoE)	OECD TG Ongoing, Project 4.125 (OECD Work plan, 2025). SCCS (Scientific Committee on Consumer Safety, 2023). Insight into both genotoxic and non-genotoxic modes of action (Hendriks et al., 2024)
NAMs	γH2AX/pH3 assay	DNA damage	Mammalian cell	Structural and Numerical chromosome aberrations	Mutagenicity (WoE)	OECD ongoing project n. 4.168 (OECD Work plan, 2025). DRP and a Retrospective Performance Analysis for the *in vitro* γ H2AX/pH3 method. Detection by flow cytometry (Khoury et al., 2013).
NAMs	Transcriptomic (e.g., TGx-DDI and GenoMark)	transcriptomic biomarkers for genotoxicity	Mammalian cells	Gene expression	Carcinogenicity and genotoxicity (WoE)	Level of expression of genes involved in the repair of DNA damage, cell cycle control, cell death, etc (Fortin et al., 2023; Thienpont et al., 2024; Scientific Committee on Consumer Safety, 2023)

alternative tests are reported as "alternatives *in vitro*". Available computational prediction tools are reported as "alternative *in silico*" in Table B in the Supplementary Material. New methods based on variations of existing ones (e.g., different protocols, advanced testing systems, *in vitro* variants of *in vivo* tests) are considered as NAMs and classified as evolution of alternative tests. Tests based on methodologies newly applied in risk assessment (e.g., omics methodologies) or based on new target endpoints, are reported as NAMs. Whilst the OECD TG are not yet available for the following methods, the *in vitro* Comet assay (Standard and Enzyme-modified) and the different types of cell transformation assays (CTAs), are reported among the alternatives *in vitro*. In particular, *in vitro* Comet assay has been recently approved as OECD project for development of OECD TG (OECD Work plan, 2025). CTAs have been on the scene for a long time (OECD Guidance document has been approved in 2015 and 2017 (OECD Guidance Document No. 214, 2015; OECD Guidance Document No. 231, 2017)), and only in 2025 the CTA was approved as OECD project for development of OECD TG. However, their regulatory use is still under discussion. The category of NAMs "evolution of alternatives" comprises the 3D reconstructed human skin Comet and micronucleus assays, both included in the work plan for the OECD TG Programme (OECD Work plan, 2025). With respect to classical cell culture systems, the 3D reconstructed human skin models are engineered tissues mimicking the physiological structure and function of native human skin, including epidermis and sometimes dermis. This allows for more *in vivo*-relevant testing conditions, especially for dermally applied substances. Presently these tests are recommended as "higher tier" or follow-up assays after initial screening with more high-throughput 2D methods (Martus et al., 2020). Among the "evolution of alternatives", emerging mammalian cell gene mutation assays are also included, such as the *in vitro* Pig-a gene mutation assay, *in vitro* assays based on cells from transgenic rodents (TGR), and assays using TK6 cells. The Pig-a assay is the *in vitro* variant of the *in vivo* Mammalian Erythrocyte Pig-a Gene Mutation Assay, whose OECD TG was recently released (OECD TG N. 470). Despite having possible advantages over others mammalian cell gene mutation assay (i.e., implementation on human cells, possible high-throughput capabilities, facility of *in vitro-in vivo* correlation of data obtained with the *in vivo* counterpart), the *in vitro* Pig-a was considered still at an immature stage (Scientific Committee on Consumer Safety, 2023). Mammalian cell gene mutation assays based on transgenic rodent cells are among emerging alternatives to traditional test systems. Presently, many different transgenic rodent derived systems have been employed on many different chemicals (reviewed in (White et al., 2019)). Although these test systems have the potential to augment or replace existing *in vitro* mammalian cell mutagenicity assays, the lack of harmonised protocols has been identified as a critical challenge to their regulatory use, hindering validation efforts (White et al., 2019). To enhance the performance of *in vitro* mutation tests with TK6, over 100 mutant sub-lines from TK6 cells have been studied to augment the sensitivity of the test and allow the investigation of modes of action of genotoxic effects. As an example, the XRCC1−/−/XPA−/− TK6 variant has been categorized among "evolution of alternatives" in Table 1 (Ibrahim et al., 2020). The miniaturized Ames test is placed in the same category, representing an evolution of the Ames test in terms of higher throughput and reduced test material required for the analysis. The "miniaturization" primarily refers to moving from agar plates to liquid cultures in multi-well microplates. This typically involves a fluctuation assay format, where growth (indicating reversion) is detected by a change in a pH indicator dye. Finally, the "evolution of alternatives" category includes the Hen's Egg test for Micronucleus Induction (HET-MN). The HET-MN test is considered a promising tool for genotoxicity assessment, offering a unique "intermediate" position between *in vitro* and full *in vivo* methods. Its ability to incorporate systemic exposure and endogenous metabolism is a significant advantage, particularly for screening purposes, even if its full regulatory acceptance is still evolving (Scientific Committee on Consumer Safety, 2023).

Among NAMs focusing on new endpoints, we reported:

- The γH2AX/pH3 assay: this assay detects an extremely early event in the DNA damage response pathway that is the phosphorylation of histone H2AX. This event occurs very rapidly and extensively around DNA double-strand breaks, which can lead to genomic instability, chromosomal aberrations, and cell death, if not properly repaired. Combination with phosphorylation at histone H3, which indicates mitotic abnormalities or arrest, supports also aneugenicity detection. An OECD project developing a Detailed Review Paper and a Retrospective Performance Analysis for the *in vitro* γH2AX/pH3 method is ongoing (OECD Work plan, 2025).

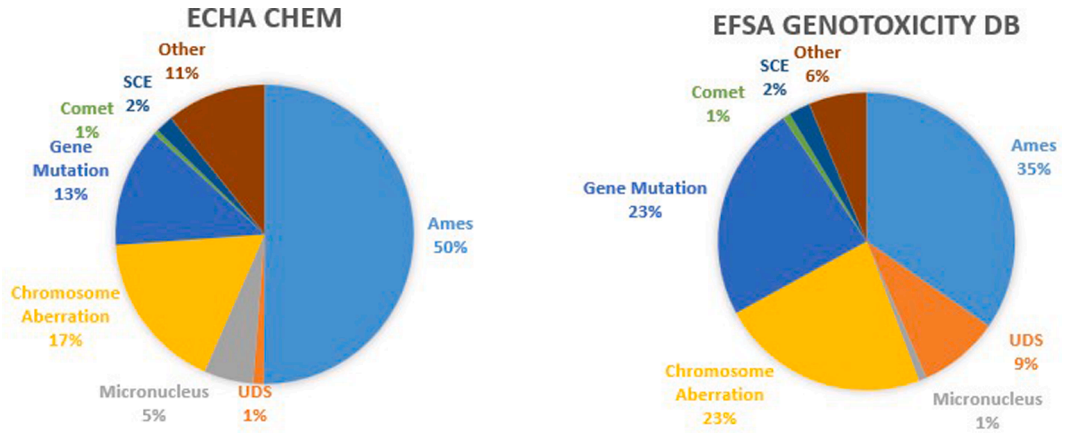

Fig. 1. Availability of *in vitro* test data for substances from REACH (ECHA CHEM database, left side) and b) EFSA pesticide regulation (EFSA genotoxicity database, right side, retrieved from the QSAR Toolbox version 4.6 (OECD, 2025b). Ames: Bacterial Reverse Mutation Test; UDS: Unscheduled DNA Synthesis test with mammalian cells; Micronucleus: *In vitro* Mammalian cell Micronucleus test; Chromosome Aberration: *In vitro* Mammalian chromosome aberration test; Gene Mutation: *In vitro* Mammalian cell gene mutation test; Comet: *In vitro* Mammalian Alkaline Comet Assay; SCE: Mammalian bone marrow Sister Chromatid Exchanges.

- Toxtracker: this assay uses multiple specific green fluorescent protein reporter genes, each linked to a different cellular stress response pathway, allowing for mechanistic insights of the type of damage induced (e.g., direct DNA damage, oxidative stress, cellular stress, aneugenicity/clastogenicity). While some reporters are specific for direct DNA damage, others specifically monitor pathways involved in cellular stress responses that are key modes of action for non-genotoxic carcinogens (NGC). The pattern of activation of multiple green fluorescent signals is measured by flow cytometry. TG for Toxtracker assay is on the OECD Test Guidelines Programme (TGP) (OECD Work plan, 2025).
- Omics based methodologies:
  - o error-corrected Next-Generation Sequencing (ecNGS): this technology is an advanced application of next-generation sequencing that drastically reduces the intrinsic error rate of sequencing. ecNGS can directly quantify mutation frequencies and mutational spectra at endogenous genomic loci, rather than relying on specific reporter genes. This technique holds great promise both for mutation quantification and characterization, and as an early biomarker of carcinogenic potential (Lynch et al., 2023; Marchetti et al., 2023; Schuster et al., 2024; Zhang et al., 2025). A project developing a Detailed Review Paper of ecNGS for gene mutation evaluation is ongoing under the OECD TGP (OECD Work plan, 2025).
  - o transcriptomics biomarkers: GENOMARK and TGx-DDI. These methods are based on transcriptomic profiling to detect gene expression changes resulting from chemical exposure. In particular, transcriptional signatures related to genotoxic effects were developed. The Transcriptomic Genotoxicity – DNA Damage Inducing (TGx-DDI) biomarker, initially developed in TK6 cells, distinguishes DDI from non-DDI chemicals by analyzing the gene expression of 64 biomarker genes. GENOMARK consists of 84 genes that identify genotoxicants in human HepaRG cells (Thienpont et al., 2024).

In general, NAMs listed in Table 1, are not expected singularly to replace animal testing. Among them, a few tackle *in vivo*-like situations, while others focus on higher throughput or augmented human relevance. Some NAMs address direct mutagenicity endpoints, while others measure early cellular responses to DNA damage. Different combinations of NAMs may be exploited together to refine equivocal *in vitro* traditional test results and clarify the mode of action of a genotoxic compound, in order to reduce recourse to *in vivo* testing or select the most suitable *in vivo* follow-up. The use of NAMs, in fact, is often considered within AOP frameworks, widely recognized as a source of information to guide the interpretation, generation and application of data from NAMs. However, especially for regulatory use, the question arises of how to standardize and validate, in addition to the NAM itself, the combination of evidence that generates the result, including the estimation of associated uncertainties. Several initiatives are addressing the problem, formulating concepts and defining frameworks for the integration of NAMs. It is worth mentioning among them, the OECD related efforts towards the development AOP-informed IATAs or DAs (OECD, 2025a). Moreover, within PARC several activities are focusing on development of IATAs and AOPs for mutagenicity and carcinogenicity (e.g., (Demuynck et al., 2025)). Integrated approaches and strategies, published or under development (e.g., OECD, IATAs and AOPs), are collected separately in the Supplementary Material (Table C).

Currently, among tests listed in Table 1, only TG methods (i.e., methods described in an OECD TG) are generally accepted by regulatory agencies for the assessment of genotoxicity and carcinogenicity. Driven by the need to detect most of the types of DNA damage possibly induced by chemicals, the tests are implemented in strategies that show common elements among regulatory agencies. In general, an *in vitro* basic test battery combining mutagenicity tests which can detect both gene mutation and chromosomal damage represents the first step in the assessment (OECD, 2016). At this level, the most requested tests currently are:

- *in vitro* bacterial reverse mutation test (OECD Test Guideline No. 471), the Ames test;
- *in vitro* gene mutation test in mammalian cells (OECD Test Guideline No. 476, 2016; OECD Test Guideline No. 490, 2016) (in case the Ames test is not applicable to the substance e.g. for substances with significant toxicity to bacteria, not absorption by bacteria or for nanoforms);
- *in vitro* mammalian cell micronucleus test (MN, (OECD Test Guideline No. 487, 2023)) or Chromosomal Aberration tests (CA, (OECD Test Guideline No. 473)). The preferred request is for MN *in vitro* due to its ability to reveal, using the appropriate experimental conditions, both CA and aneuploidy.

As for the need to conduct *in vivo* tests, it varies considerably depending on the regulatory scope under which the substance under scrutiny falls, ranging from pesticides, where *in vivo* testing is always required, to cosmetics, where all *in vivo* tests have been banned in EU. In general, if there is some evidence of genotoxicity *in vitro*, *in vivo* assays should address the mutagenic endpoint found to be positive in the *in vitro* testing battery.

The assays recommended for the *in vivo* testing are the following:

- *in vivo* Micronucleus Test (OECD Test Guideline No. 474, 2016), able to reveal both aneugenicity and clastogenicity, or *in vivo* Chromosomal Aberration Test (OECD Test Guideline No. 475, 2016), only for clastogenicity.
- TGR mutation assay, (OECD Test Guideline No. 488, 2022)) able to reveal gene mutation, or *in vivo* Comet assay (OECD Test Guideline No. 489, 2016), which reveals both clastogenicity and gene mutation.

This series of results, in addition to being required for the evaluation of genotoxicity, also serve as a pre-screening for carcinogenesis. The actual evaluation for carcinogenesis, which is triggered by these initial results or other findings (e.g., preneoplastic lesions observed in a subchronic toxicity study), is primarily based on the two-year rodent bioassays (OECD Test Guideline No. 451, 2018; OECD Test Guideline No. 453, 2018; OECD Test Guidelines No. 452, 2018). The criteria indicating the effective need to carry them out differ substantially between sectors. These studies, in addition to requiring a very large number of laboratory animals, are extremely time- and resource-consuming and their predictive capacity has been strongly questioned (ECHA, 2023b; Madia et al., 2019).

Based on these premises, it can be recognized that the regulatory assessment of mutagenicity and carcinogenicity represents a context where the evolutionary scenario hypothesized above, i.e., non-animal tests are implemented alongside traditional animal experimentation, is already in place. In the following sections we will analyze the details of the processes in key EU regulatory contexts, to understand if and how they can be improved in terms of greater efficiency and less use of *in vivo* testing through the introduction of NAMs. Insights derived from analyzing a context that has already been in the evolution process for years will inform the evolution of other endpoints.

4. Genotoxicity and carcinogenicity regulatory assessment in EU

The EU regulatory framework consists of several pieces of cross-cutting/horizontal (e.g., (Regulation (EC) No 1272/2008, 2008; Regulation (EC) No 1907/2006, 2006) and sector-specific legislations (e.g., for worker safety, products control, specific chemicals, environmental protection). The following analysis critically examines the current regulatory evaluation practices for genotoxicity and carcinogenicity hazard assessment within key EU regulatory bodies and sector-specific legislations, focusing on the current possibilities and potential needs for implementing NAMs.

4.1. Chemicals under ECHA's remit

ECHA is primarily responsible for the implementation of the EU's cornerstones in chemicals legislation, the CLP and REACH Regulation. Its remit covers a vast range of industrial chemicals and chemical substances across their entire lifecycle, ensuring broad industrial safety and environmental protection.

4.1.1. The CLP regulation

The CLP Regulation (Regulation (EC) No 1272/2008, 2008) establishes legally binding hazard identification and classification rules. All chemical substances which are placed on the EU market must be self-classified by manufacturers, importers or downstream users according to CLP (Article 4.1). This obligation applies regardless of the tonnage and involves identifying the hazards of the substance or mixture and comparing the hazard information with the criteria laid down in CLP. If a substance already has a harmonised classification (i.e., an entry in Annex VI to CLP), this is legally binding and must be applied by companies.

The CLP Regulation serves as a horizontal reference point for most of the EU chemicals and chemicals-related legislations and, as such, ensures a high degree of consistency of chemical hazard identification and classification (Fig. 2). Notably, CLP incorporates the classification criteria and labelling rules agreed at UN level, the Globally Harmonised System of Classification and Labelling of Chemicals (GHS). The mechanism is such that any changes at the GHS level should also be transposed at the CLP level and *vice versa*.

It is worth noting that, whilst CLP applies to most industrial sectors, areas not covered by this regulation exist, mainly relating to radioactive substances and mixtures, cosmetics, medicines and certain medical devices, food and the transport of dangerous goods.

Information on intrinsic properties of substances, coming from REACH and other sectorial legislations could activate the classification process in CLP. Therefore, type and amount of information required in REACH processes of Registration and Evaluation and in sectorial legislations (e.g., pesticides regulation), highly interrelate with information requested to fulfil the criteria for classification established by CLP. Moreover, classification could trigger risk management measures as Authorization and Restriction within REACH and impact also on sectorial legislations. Indeed, CLP is the basis for many legislative provisions on the risk management of chemicals. For example, the supply of substances classified as CMR (Carcinogen, Mutagen and Reprotoxic substances) category 1A/1B to the general public is restricted (entry 28, 29 and 30 of the Annex XVII to REACH). Moreover, CMR substances (category 1A/1B) are present in Annex II to Regulation (EC) 1223/2009 (Regulation (EC) No 1223/2009, 2009), where substances prohibited in cosmetic products are listed. In addition, the Toy Safety Directive (Directive 2009/48/EC, 2009) ensures that substances classified as a known, presumed or suspected carcinogen, mutagen or reproductive toxicant are not allowed in accessible parts of toys. CLP regulation can affect likewise the rules on Health and Safety at Work (Fig. 2).

This intimate interrelation among CLP and other pieces of legislation is such that information on hazard, collected for instance during REACH registration and evaluation processes, should allow for the (self)-classification of the substance, also because it is not expected that any missing information could be generated within the classification procedure.

Presently, the different hazard classes for genotoxicity and carcinogenicity, are mainly based on effects on animals (Table 2).

As shown in Table 2, to classify a substance as mutagen, the mutagenicity potential to germ cells has to be investigated through appropriated *in vivo* assays. At present the *in vitro* tests are conclusive (no classification is requested) only in case of negative results for gene mutation, chromosomal aberration and/or aneugenicity. In case of positive results even in only one of those assays the appropriated *in vivo* follow-up is mandatory to reach a conclusion. Currently, the revision of the criteria of germ cell mutagenicity classification is ongoing under the GHS umbrella, with support by the Organisation for Economic Cooperation and Development (OECD) (Project 4.157: Support to UN GHS for modification of germ cell mutagenicity criteria, (OECD Work plan, 2025). This process can trigger a revision of the current CLP criteria.

Similarly, classification criteria for carcinogenicity are based on *in vivo* data (Table 2). In Annex I of the CLP it is reported that the classification as carcinogen is based on human evidence, animal studies or data from a structural analogue. The critical point of the carcinogenicity evaluation is the evaluation of the strength of evidence of the data. In line with the terminology defined by the International Agency for Research on Cancer (IARC), evidence of carcinogenicity can be *sufficient*

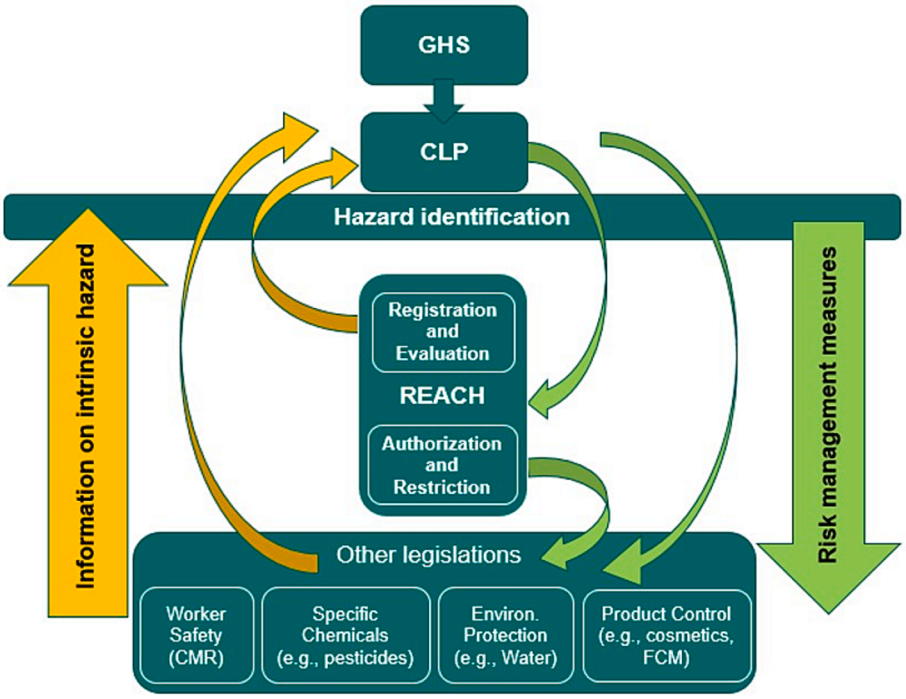

Fig. 2. Interrelationships between CLP and other legislation in the EU. FCM: Food Contact Materials

or *limited*. In general, if there are positive studies in different species (i.e., mouse and rat), this is considered by default as *sufficient*[1] evidence that can trigger category 1B. In some cases, it is possible to classify in category 1B even with a single study, if the tumor incidence is very high and observed in both sexes. Only if there are serious doubts on the reliability of these studies or about the relevance of the observed tumors for humans, the data are downgraded to *limited* evidence and category 2 is applied. In any case, to classify a substance as carcinogen at least one *in vivo* carcinogenicity study performed according to the appropriate OECD guidelines is needed.

According to CLP criteria, there are currently no NAMs that could replace an *in vivo* mutagenicity or carcinogenicity test for classification purposes. An exception can be made for the application of read-across to a closely related substance or metabolite, that is, when available, the data on similar substances can also be used to classify the substance in the same category or in a lower category based on the robustness of the read-across prediction. In these cases, NAMs (e.g., *in vitro* CTAs or *in silico* predictions) could be used to provide information about the potential carcinogenicity/mutagenicity of the test substance when compared to a similar substance already classified.

While this option still implies the availability of *in vivo* studies on the analogue(s) substance(s), there is room for greater development and testing of NAMs, which can be used to strengthen the justification of read-across and increase its regulatory acceptability.

[1] Sufficient evidence of carcinogenicity: a causal relationship has been established between the agent and an increased incidence of malignant neoplasms or of an appropriate combination of benign and malignant neoplasms in (a) two or more species of animals or (b) two or more independent studies in one species carried out at different times or in different laboratories or under different protocols. An increased incidence of tumours in both sexes of a single species in a well-conducted study, ideally conducted under Good Laboratory Practices, can also provide sufficient evidence. A single study in one species and sex might be considered to provide sufficient evidence of carcinogenicity when malignant neoplasms occur to an unusual degree with regard to incidence, site, type of tumour or age at onset, or when there are strong findings of tumours at multiple sites.

4.1.2. The REACH regulation

To comply with REACH regulation (Regulation (EC) No 1907/2006, 2006), the primary cross-cutting legislation to manage the risks of industrial chemicals manufactured or imported into the EU, companies must provide to ECHA a registration dossier containing information on the properties and hazards of substances. The standard information requirements, which are requested as a minimum to meet the registration obligations of REACH, depend on the quantity of the substance that is manufactured or imported into the EU. The data required by REACH regulation for different tonnages, together with appropriate adaptations, are reported in the annexes VII-XI to REACH. In general, the information requirements increase with increasing tonnage. Moreover, the REACH strategy for mutagenicity assessment implies that *in vivo* testing is foreseen only in the presence of *in vitro* positive results (Fig. 3).

This strategy has enabled great progress towards reducing animal testing in the assessment of genotoxicity endpoints. On the other hand, some areas of potential toxicity remain not covered. Notably, for low tonnage substances, amounting to nearly 50 percent of total registered substances (i.e., from 1 to 10t/y, Fig. 4), only Ames test is required as standard information. If the result is negative, no other information is requested. Consequently, a potential limit of this approach is the lack of data about the ability of the substance to exert other genotoxicity endpoints, such as structural or numerical chromosome aberrations, as Micronucleus test or Chromosome Aberration test *in vitro* are requested only from 10t/y. Indeed, as reported in the REACH regulation (annexes VIII-X), tests for these endpoints are mandatory for substances produced or imported in quantities starting from 10t/y and above.

Concerning carcinogenicity evaluation, as reported in the Annex X to REACH regulation, a carcinogenicity study is requested only for substances produced or imported in EU at very high tonnage (>1000 t/y), in case the substance has widespread dispersive use or with frequent/long term human exposure, is classified as germ cell mutagen category 2 or there is evidence from the repeated dose study(ies) (the latter are required by annex IX) that the substance is able to induce hyperplasia and/or pre-neoplastic lesions. If the substance is classified as germ cell mutagen category 1A or 1B, the assumption is that a genotoxic mechanism for carcinogenicity is likely. In these cases, a carcinogenicity test

Table 2
Classification criteria for genotoxicity and carcinogenicity.

Mutagen Category 1 (Table 3.5.1 of CLP) **Substances known to induce heritable mutations or to be regarded as if they induce heritable mutations in the germ cell of humans. Substances known to induce heritable mutations in the germ cells of humans**		**Carcinogen Category 1 (Table 3.6.1 of CLP)** **Substances known or presumed human carcinogens** **A substance is classified in Category 1 for carcinogenicity on the basis of epidemiological and/or animal data**	
1 A **Signal Word: DANGER** **H340: May cause genetic defects**	The classification in this category is based on positive evidence from human epidemiological studies. Substances to be regarded as if they induce heritable mutations in the germ cells of humans.	**1 A** Signal Word: DANGER H350: May cause cancer	Category 1A, known to have carcinogenic potential for humans, classification is largely based on human evidence.
1 B **Signal Word: DANGER** **H340: May cause genetic defects**	-Positive result(s) from *in vivo* heritable germ cell mutagenicity tests in mammals; or -positive result(s) from *in vivo* somatic cell mutagenicity tests in mammals, in combination with some evidence that the substance has potential to cause mutations to germ cells. It is possible to derive this supporting evidence from mutagenicity/genotoxicity tests in germ cells *in vivo*, or by demonstrating the ability of the substance or its metabolite(s) to interact with the genetic material of germ cells; or -positive results from tests showing mutagenic effects in the germ cells of humans, without demonstration of transmission to progeny; for example, an increase in the frequency of aneuploidy in sperm cells of exposed people.	**1B** Signal Word: DANGER H350: May cause cancer	Category 1B, presumed to have carcinogenic potential for humans, classification is largely based on animal evidence. Such evidence may be derived from: - human studies that establish a causal relationship between human exposure to a substance and the development of cancer (known human carcinogen); or - animal experiments for which there is sufficient [1] evidence to demonstrate animal carcinogenicity (presumed human carcinogen)
Mutagen Category 2		**Carcinogen Category 2**	
Signal Word: Warning **H341: Suspected of causing genetic defects**	positive evidence obtained from experiments in mammals and/or in some cases from *in vitro* experiments, obtained from: -somatic cell mutagenicity tests *in vivo*, in mammals; or -other *in vivo* somatic cell genotoxicity tests which are supported by positive results from *in vitro* mutagenicity assays. Note: Substances which are positive in *in vitro* mammalian mutagenicity assays, and which also show **chemical structure activity relationship** to known germ cell mutagens, shall be considered for classification as Category 2 mutagens.	Signal Word: Warning H351: Suspected of causing cancer	Suspected human carcinogens. The placing of a substance in Category 2 is done on the basis of evidence obtained from human and/or animal studies, but which is not sufficiently convincing to place the substance in Category 1A or 1B, based on strength of evidence together with additional considerations (see section 3.6.2.2). Such evidence may be derived either from limited evidence of carcinogenicity in human studies or from limited evidence of carcinogenicity in animal studies. In the 3.6.2.2.6. are indicated some important factors which may be taken into consideration when assessing the overall level of concern. One of them is: structural similarity to a substance(s) for which there is good evidence of carcinogenicity.

will normally not be required (Annex X to REACH regulation). The strategy as such highlights a potential protection gap, especially for NGC (Madia et al., 2019). Moreover, the fact that carcinogenicity tests are not standard information requirements under REACH, limits availability of carcinogenicity information that could inform CLP classifications. While the information can be retrieved through the substance evaluation process, this occurs rarely and it is very time consuming (Karamertzanis et al., 2019; Woutersen et al., 2019).

As a matter of fact, after the first ten years of REACH application, a lack in availability of data required to potentially classify substance in CLP class Mutagenicity/Carcinogenicity (Muta/Carc) 1A/1B has been reported (Karamertzanis et al., 2019; Woutersen et al., 2019).This issue may have partly been overcome, in the case of mutagenicity, by a recent amendment of REACH (Regulation (EU) 2022/477, 2022), which extended and clarified the information requirements of this endpoint. For instance, situations in which the Ames test can be replaced by an *in vitro* gene mutation test in mammalian cells (e.g. for substances with significant toxicity to bacteria, not taken up by bacteria, or for nano-forms), have been detailed. Importantly, to make *in vivo* studies compulsory from a legal point of view, some of the mutagenicity information requirements have been slightly reworded, and some of the studies have been moved from column 2 of the Annexes (VII-X), which outlines specific rules for adapting the standard information requirements, to column 1 that establishes the standard information required. These apparently minor modifications are expected to greatly impact on the future availability of *in vivo* data for substances with *in vitro* mutagenicity positive results. As a consequence, although mutagenicity alternatives to animal testing are well developed, they are not sufficient to completely rule out the recourse to animal testing and indeed we are even moving towards an increase of *in vivo* tests requested under REACH for this endpoint.

Moreover, while testing of genotoxic carcinogens has a clear strategy and a similar approach across legislations, there is a regulatory gap for the identification of NGC which acts through a large variety of specific

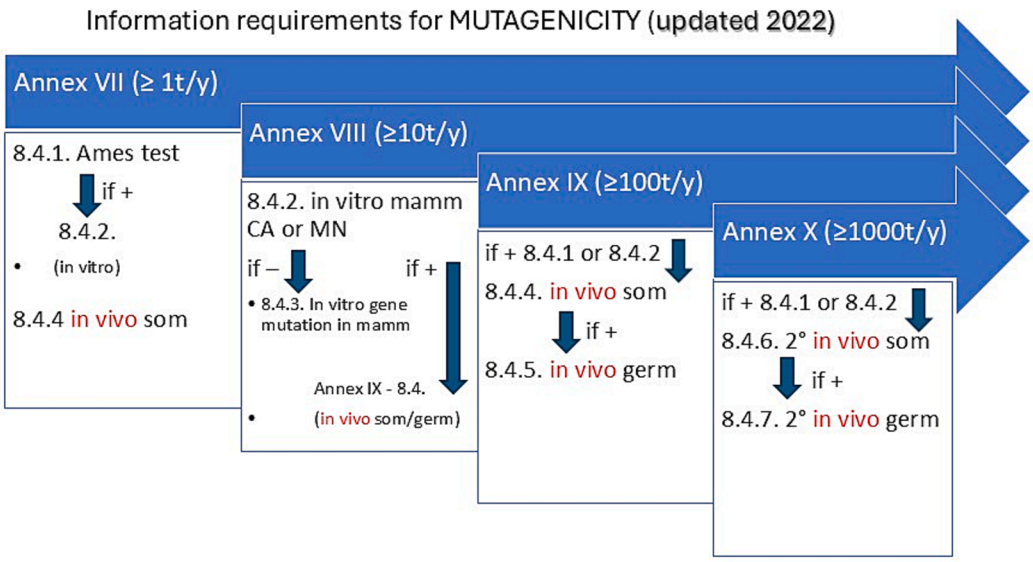

Fig. 3. Information requirements for mutagenicity according to REACH updated (Regulation (EU) 2022/477, 2022). Mamm: mammalian cells test; Som: somatic cells test; Germ: germ cells test. Text box accompanying figure 3: For substances above 1 tonne per year (t/y) (annex VII-X) an *in vitro* gene mutation study in bacteria (i.e., Ames test) should be performed. The recent REACH amendment (Regulation (EU) 2022/477, 2022) clarifies the situations in which the assay can be replaced by an *in vitro* gene mutation test in mammalian cells (e.g. for substances with significant toxicity to bacteria, not taken up by bacteria, or for nanoforms). From 10t/y and above (annexes VIII-X), additionally to the Ames test, an *in vitro* mammalian cell micronucleus test (OECD Test Guideline No. 487, 2023) or Chromosomal Aberration tests (OECD Test Guideline No. 473, 2016) must be conducted. As reported in the MSC 75 min (ECHA, 2021) the preferred request is for MN *in vitro* due its ability to reveal, using the appropriate experimental condition, both CA and aneuploidy. Furthermore, if these tests give negative results, an *in vitro* gene mutation test in mammalian cells (OECD Test Guideline No. 476, 2016; OECD Test Guideline No. 490, 2016) is also required. Depending on the positive/negative results of the above-mentioned *in vitro* tests, other studies should be performed or proposed by the registrant or may be requested by the Agency. For substances above 1t/y (annexes VII-X), if the Ames test gives positive results, an *in vitro* mammalian cell micronucleus test or Chromosomal Aberration test have to be conducted and adequate *in vivo* follow-up must be proposed or may be required by the Agency, to address the gene mutation and/or the chromosomal aberration concern (introduced in column 1 as information requirement from 100t/y and up, i.e. annexes IX and X). *In vivo* tests on germ cells mutagenicity, to address the chromosomal aberration concern and/or the gene mutation concern, should be submitted if there is a positive result in an available *in vivo* mammalian somatic cell genotoxicity study, which gives rise to concern, for substances above 10t/y (a second *in vivo* somatic or germ cells test could be envisaged in Annex X). Specifically, the Transgenic Rodent assay (OECD Test Guideline No. 488, 2022) can be used to reveal gene mutations in germ cells, whilst a Dominant Lethal (OECD Test Guideline No. 478, 2016) or a mammalian spermatogonial chromosomal aberration test (OECD Test Guideline No.483, 2016) can be used to reveal chromosomal aberrations. At present no test to reveal aneugenicity on germ cells is available (adaptation of OECD TG 489 *in vivo* comet assay for gonads is under development, OECD Project 4.156 (OECD Work plan, 2025). In case of clear toxicokinetic evidence that neither the substance nor its metabolites reach the germ cells, the *in vivo* study on germ cells is not required. On the other hand, if the substance is already classified as carcinogen category 1A or 1B and appropriate risk management measures are in place, the *in vitro* and *in vivo* assays for genotoxicity are not required.

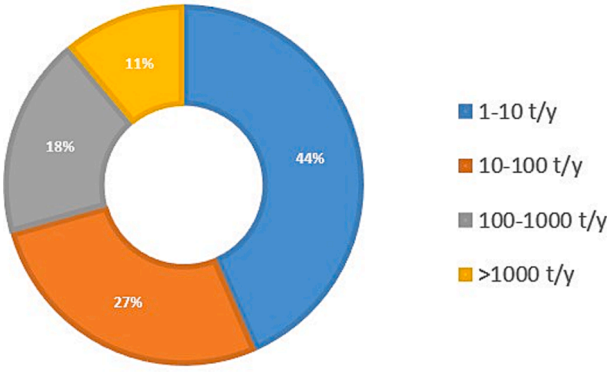

Fig. 4. Number of REACH registered substances (01.06.2008–30.09.2024) by tonnage bands (around 21,000 substances in total) (ECHA, 2025).

mechanisms. The common hallmarks of cancer include cell proliferation, evading growth suppressors, resisting cell death, enabling replicative immortality, inducing angiogenesis, and activating invasion and metastasis (Hanahan, 2022). There is no agreed approach or test battery based on *in vitro* models as first screening for NGC. The OECD addressed this gap by establishing an expert group to develop an IATA of non-genotoxic carcinogens (Jacobs et al., 2020). Using the AOP concept, various cancer models were developed, and overarching mechanisms and modes of action were identified (Luijten et al., 2020). Among non-animal methods and NAMs for non-genotoxic carcinogens, CTAs and proliferation assays have been identified as pivotal downstream events in the process of tumorigenesis and suggested to enter in the OECD work plan towards establishment of OECD TG.

4.1.3. NAMs in ECHA

As under REACH testing on vertebrate animals is considered as a last resort to meet the information requirements for registration, the possibility of adapting the information requested is foreseen, rules for adaptations being part of the legal text (REACH Annex XI).

Indeed, the possibility of using NAMs is already explicitly foreseen in REACH as *in vitro* tests, QSARs, as well as strategies like grouping and read-across, or weight of evidence (WoE) approach are among adaptations allowed to fulfill the registration requirements. It should be noted, however, that in all cases "the information provided shall be adequate for the purpose of classification, labelling and/or risk assessment" (REACH Annex XI), which in practice translates into requiring information equivalent to that requested in the annexes VII-X.

From the report on alternatives, published by ECHA every three years, it can be deduced that registrants in general and especially for genotoxicity make extensive use of the possibilities provided by annex XI, with read-across being the most frequent option (ECHA, 2023c). From the report it is not possible to infer which is the rate of acceptance

of read-across and other adaptations proposals. However, it is reported that adaptations provided by registrants often fail to comply with the legal requirements and are inadequate to ensure the safe use of chemicals.

A detailed analysis of how many, which and why adaptations are accepted or rejected would help clarify and address their failures. In parallel, it would also help to outline room to manoeuvre within which to move, in order to increase acceptability of NAMs.

In the context of *in silico* models regulatory acceptability, a significant step forward has recently been made with the development of the QSAR Assessment Framework (QAF) (Gissi et al., 2024; OECD, 2024). The QAF, developed within an OECD project, provides a comprehensive and transparent workflow for models and predictions assessment, allowing a systematic and consistent evaluation. The application of QAF is expected to establish confidence around QSARs, ultimately improving their acceptability in the regulatory context.

While there are presently no NAMs that can replace current information requirements on a one-to-one basis, as a short-term goal, emerging science can be leveraged to support adaptations within the framework of Annex XI such as grouping, read-across, or in the context of WoE.

In most recent years, IARC has considered the key characteristics of carcinogens, including their *in vitro* genotoxicity, as a stream of evidence for the carcinogenicity of agents under evaluation (Supplementary Figure A). This illustrates the rising relevance of integrating the knowledge generated through non-animal methods. The IARC considers and integrates findings from three evidence streams: cancer in humans, cancer in animals and mechanistic evidence. For the two first streams the existent evidence is classified in sufficient, limited, inadequate or suggesting lack of carcinogenicity whereas regarding the mechanistic evidence the categories considered comprise: strong, limited or inadequate mechanistic evidence. Concerning the strong mechanistic evidence, results in several different experimental systems are consistent, and the overall mechanistic database is coherent, further support can be provided by studies that demonstrate experimentally that the suppression of key mechanistic processes leads to the suppression of tumour development. Quantitative structure–activity considerations, *in vitro* tests in non-human mammalian cells, and experiments in non-mammalian species may provide corroborating evidence but typically do not in themselves provide strong evidence. The considerations can go beyond quantitative structure–activity relationships to incorporate similarities in biological activity relevant to common key characteristics across dissimilar chemicals (e.g. based on molecular docking or –omics data). However, consistent findings across a number of different test systems in different species may provide strong evidence. More detailed information is provided by IARC (IARC, 2019). The overall evaluation by IARC for an agent classification is as follows: Group 1 (carcinogenic to humans), group 2A (probably carcinogenic to humans), group 2B (possibly carcinogenic to humans, and group 3 (not classifiable as to its carcinogenicity) (Supplementary Figure B).

Another process, currently adopted in ECHA, which involves the use of NAMs is embedded in the ECHA Integrated Regulatory Strategy (IRS, Fig. 5, (ECHA, 2024)). IRS is an informal decision-making process which aims at accelerating the identification of chemicals that need regulatory action. To efficiently select substances that raise potential concern, IRS has shifted its focus to groups of structurally related substances rather than individual substances (indicated as Grouping in the Fig. 5). In this context, NAMs (currently mainly QSARs) are exploited to screen more efficiently the pool of chemicals (from registrations and classification and labelling intentions). While structural similarity is the starting point, additional information, potentially coming from NAMs (e.g., using omics profiling), may be considered when forming the groups to delineate for example bioactivity similarity (Patlewicz et al., 2025; Rovida et al., 2021; Zhu, 2016).

4.2. Chemicals under EFSA remit

EFSA oversees the scientific safety assessment of products associated with the food chain, including substances used in feed and food (such as additives, enzymes, flavourings, nutrient sources), food contact materials and pesticides as well as genetically modified organisms, chemical substances generated by food manufacturing processes and processing aids.

Under current legislation applicable in EU to foodstuff, no reference is made to any amount of substance that is exempted from testing, thus it is assumed that every substance should follow a safety assessment by EFSA, where genotoxicity and carcinogenicity are included. For evaluating the different types of substances, EFSA follows sectoral guidances in line with regulatory requirements by EU laws on foodstuffs (food enzymes (Regulation (EC) No (1332)/2008, 2008); food additives (Regulation (EC) No 1333/2008, 2008); food flavourings (Regulation (EC) No 1334/2008, 2008); novel foods (Regulation (EU) 2015/2283, 2015); food contact materials (Regulation (EC) 1935/2004, 2004)). In some contexts, such as for Plant Protection Products (PPP) active substances, testing requirements (including those for genotoxicity and carcinogenicity) are agreed by the European Commission and the Member States and incorporated into EU legislation (Regulation (EU) No 283/2013, 2013). EU regulations and guidance documents relevant to EFSA regulated products are reported in Supplementary Table D.

With the intention of harmonizing as much as possible the genotoxicity testing for risk assessment across EFSA's Scientific Panels, EFSA Scientific Committee (SC) provided recommendations on testing strategy for genotoxicity assessment (EFSA Scientific Committee, 2021a, 2017a; EFSA Scientific Committee, 2011). This genotoxicity guidance framework is currently under revision to incorporate the most up to date knowledge and define the role of new and updated non-animal approaches, including NAMs (EFSA PC-1237, 2024). In addition, EFSA has announced a public consultation on its revised guidance concerning the margin of exposure approach for chemicals that exhibit both genotoxic and carcinogenic properties (EFSA PC-1047, 2024).

Currently, the minimum data requirements for genotoxicity evaluation, which is always required for food and feed safety assessment, are represented by the basic battery of *in vitro* tests (Fig. 6).

In the event of positive results from the basic battery, the SC recommends 'a documented WoE approach for the evaluation and interpretation of genotoxicity data', taking into account not only the quality and availability of the data on genotoxicity itself, but also all other relevant data that may be available (EFSA Scientific Committee, 2017a; EFSA Scientific Committee, 2011). This process implies consideration of all evidence, such as physico-chemical characteristics, structure–activity relationships (including structural alerts for genotoxicity and read-across from structurally related substances), Absorption, Distribution, Metabolism, and Excretion (ADME) studies, and the outcomes of any repeated-dose toxicity and carcinogenicity studies, including evaluation of pre-existing or non-standard data. If, after such a review, a decision is taken that *in vivo* testing is necessary, tests should be selected on a case-by-case basis using expert judgement, with flexibility in the choice of test, guided by the full data set available for the substance. *In vivo* tests should relate to the genotoxic endpoint(s) identified as positive *in vitro* and to appropriate target organs or tissues.

Deviations from this common approach are requested for the assessment of PPP active substances, for which at least an *in vivo* test is always needed independently from the results obtained *in vitro* (Regulation (EU) No 283/2013, 2013). The use of structure–activity relationships methodology to raise a warning for *in vitro* negative substances is mentioned as a trigger for further *in vitro* testing. Furthermore, specific guidance exists for assessing the genotoxicity of nanomaterials in food and feed (EFSA Scientific Committee, 2021b). Details on regulatory practices on the genotoxicity testing of nanomaterials are fully discussed in a recent paper (Andreoli et al., 2025).

Concerning the assessment of genotoxicity in germ cells, specific

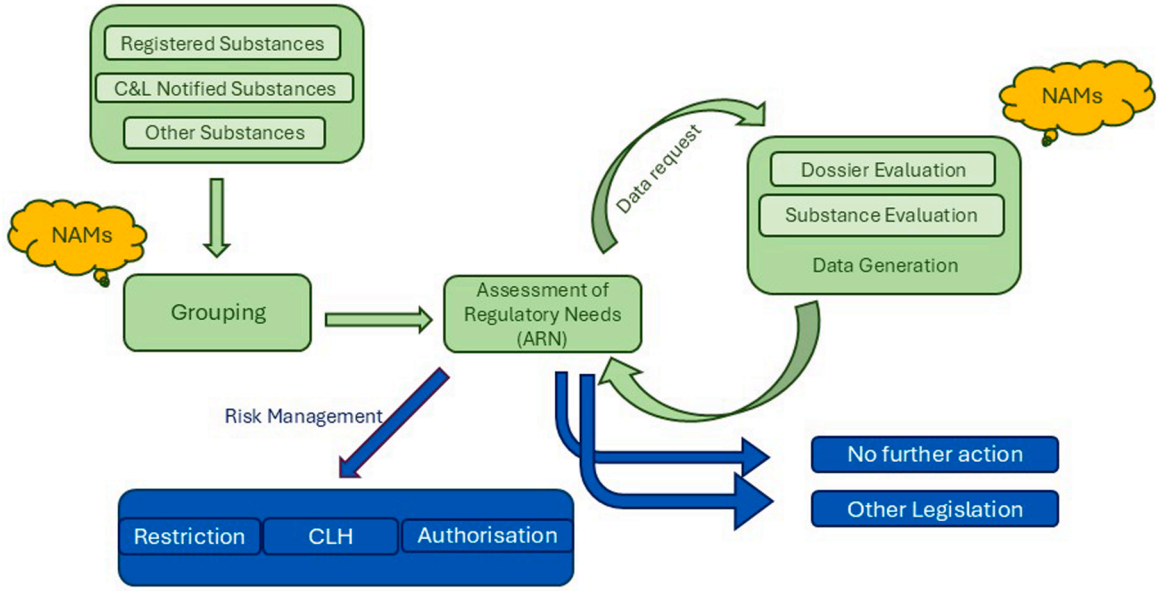

Fig. 5. ECHA's Integrated Regulatory Strategy (modified from https://echa.europa.eu/irs-infographic).

tests are not required by EFSA and the results obtained *in vivo* in somatic tissues are extrapolated to the germ cells (EFSA Scientific Committee, 2011). A conservative approach is applied assuming that a compound showing systemic availability can also reach the germ cells. Therefore, a compound showing genotoxic activity *in vivo* is considered a germ cell mutagen being potentially hazardous to future generations, while a substance that is negative in somatic tissues *in vivo* is assumed to be negative in germ cells. In the case of PPP active substances, the necessity for conducting *in vivo* germ cells studies is considered on a case-by-case basis (Regulation (EU) No 283/2013, 2013).

A tiered approach is applied in general also regarding carcinogenicity assessment. Initially, together with the *in vitro* genotoxicity battery, a modified 90-day toxicity test (OECD TG 408 (OECD Test Guideline No. 408, 2018) with extended parameters from the OECD TG 407 (OECD Test Guidelines No. 407, 2008), is often requested (EFSA CEP Panel (EFSA Panel on Dietetic Products, Nutrition and Allergies (NDA) et al., 2021; EFSA Panel on Food Additives and Nutrient Sources added to Food (ANS), 2021; EFSA CEP Panel, 2021; Regulation (EC) No 1333/2008, 2008). Critical results of the subchronic study and/or the (geno)toxicity tests could trigger the need for carcinogenicity studies.

This approach does not apply to PPP active substances, for which a long-term carcinogenicity study in rat is always required (together with a second carcinogenicity study in mice, "unless it can be scientifically justified that this is not necessary" (Regulation (EU) No 283/2013, 2013). In case of flavourings, the need for the subchronic study (as well as other *in vivo* studies, except genotoxicity) is conditional to the genotoxicity outcome, as it should only be performed if the genotoxicity concern has been ruled out (EFSA FAF Panel (EFSA Panel on Food Additives and Flavourings), 2022).

Other exception to the general tiered approach concerns plastic Food

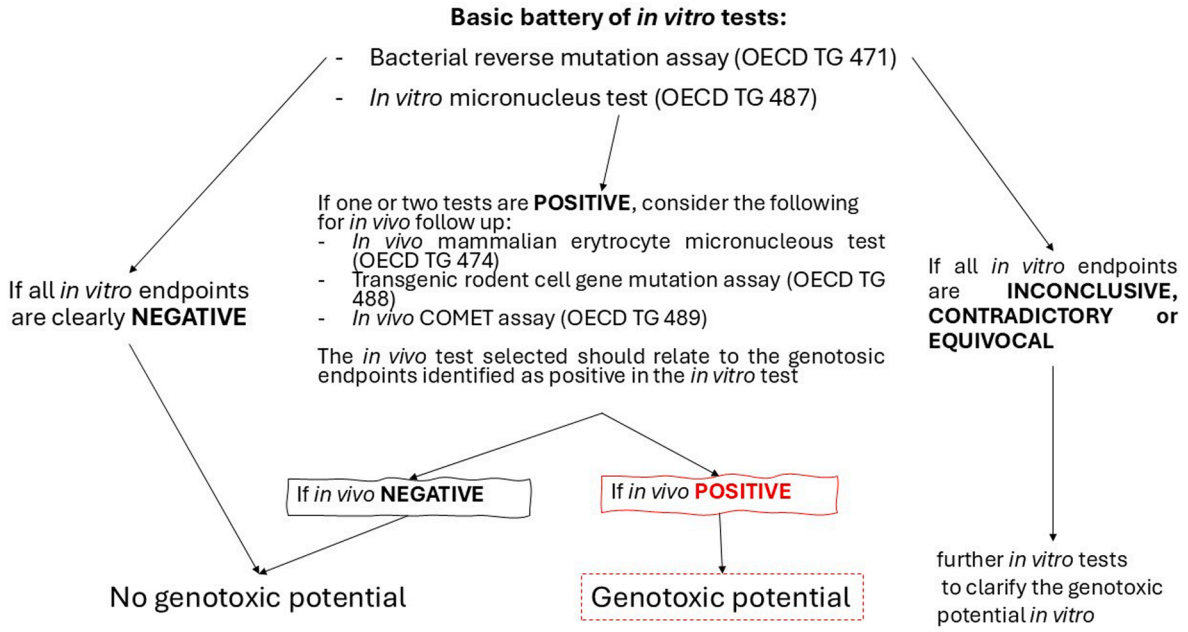

Fig. 6. Scheme of genotoxicity testing strategy recommended by EFSA Scientific Committee (adapted from (EFSA Scientific Committee, 2011)).

Contact Materials, where the need of toxicology tests depends on the amount of migration, going from a minimal dataset (the basic *in vitro* genotoxicity battery) for low migration (<0.05 mg/kg/food), to a full dataset, including carcinogenicity testing, in case of high migration (from 5 mg/kg/food to 60 mg/kg/food) (EFSA CEF Panel (EFSA AFC Panel EFSA Panel on Food Additives, Flavourings, Processing Aidsand Materials in contact with Food, 2008).

From what has been reported, it is worth pointing out that the information collected by EFSA on regulated products is generally not intended to feed classification purposes, except in the case of PPP active substances. This is a non-negligible element with respect to the possibility of using assessment methods that are not strictly those foreseen by the hazard classes of the CLP regulation.

4.2.1. NAMs in EFSA

To date, compliance with the guidelines is the standard requirement for tests used in EFSA risk assessment. However, in practice EFSA is gradually adopting a more flexible/pragmatic approach. Thus, data from not formally validated tests will be still considered in a weight of evidence approach (EFSA Scientific Committee, 2017b; Escher et al., 2022). A roadmap for action was recently proposed by EFSA to identify priorities towards the incorporation of NAMs into regulatory hazard and risk characterisation of chemicals in food and feed (Escher et al., 2022).

The short/medium term goal is to include NAMs in the standard battery of tiered testing strategies (Fig. 6) and assessment approaches for different endpoints in order to improve the knowledge on the mechanisms of action, reduce uncertainties associated with *in vitro* results and better design *in vivo* studies, to be considered in the end, as a last resource to complete the risk assessment (Corvi and Madia, 2017). As a longer-term goal, EFSA intends to move towards mechanistic risk assessment, using non-animal-based approaches for hazard and exposure assessment (Escher et al., 2022; Tarazona et al., 2022).

Presently, new methods not yet formally validated can be used in the WoE approach to reduce uncertainties and support conclusions on genotoxicity (EFSA Scientific Committee, 2017b). To this aim, indicator endpoints can be used to gain supporting information on modes of action (MoA), e.g, through detection of oxidative DNA damage (Collins et al., 2023) or characterization of the content of micronuclei (EFSA Scientific Committee, 2021a). Moreover, several other methods are considered such as those detecting the interference with the machinery of chromosome segregation (e.g., through the analysis of the organization of the mitotic spindle), changes in the expression of genes involved in the repair of DNA (toxicogenomic methods) that may provide supporting information on the main genotoxicity effects (i.e., gene mutation, structural and numerical chromosomal damage). The evaluation of the results from non-guidelines methods is based on expert judgment on a case-by-case approach, considering the reliability and relevance of the studies (EFSA et al., 2023).

In addition, for the assessment of genotoxicity and carcinogenicity, EFSA allows the application of *in silico* methods (e.g., QSAR models) in the initial steps of hazard identification QSARs are typically used in combination with other non-testing approaches (such as Grouping and read-across) and testing methods (such as *in vitro* methods) in the context of WoE assessments.

In specific circumstances, QSARs may play a major role. In smoke flavourings assessment, if *in silico* predictions for an identified component and for its predicted or reported metabolites, do not identify structural alerts for genotoxicity endpoints and these endpoints are negative in a combination of independent and multiple QSAR models, no experimental genotoxicity testing is required (EFSA FAF Panel (EFSA Panel on Food Additives and Flavourings), 2021).

Concerning the toxicological potential (including mutagenicity/genotoxicity and carcinogenicity) of contaminants potentially present in new botanical products introduced into the European food market, recently a database has been released by EFSA, i.e. the Compendium of botanicals (Nicova et al., 2025). Notably, the Compendium includes predictions from different QSAR tools supporting the identification of priority substances for further assessment.

Regarding the pesticide risk assessment, metabolites and degradation products are often poorly characterized from a toxicological perspective, and *in silico* approaches have been proposed to identify those relevant to dietary risk assessment in the EFSA guidance (Guidance on the establishment of the residue definition for dietary risk assessment, (EFSA, 2016)). The EFSA guidance outlines a three-step procedure starting with genotoxicity assessment using QSAR models, grouping, and read-across approaches to determine whether further testing or general toxicity assessment is required (Benigni et al., 2019; EFSA, 2016).

4.3. Chemicals regulated under the EU cosmetics framework and NAMs in SCCS note of guidance

Cosmetic products are regulated under Regulation (EC) No 1223/2009 which imposes animal testing ban for cosmetics ingredients, or combinations, and on finished cosmetic products (Regulation (EC) No 1223/2009, 2009). Integral to the regulation are the Annexes, which detail specific lists of substances either prohibited or restricted in cosmetic products. For instance, in Annex 2 to the regulation, prohibited substances are listed, including substances classified as CMR under CLP.

The SCCS is responsible for carrying out safety evaluations of annexed substances, and of substances with safety concerns, possibly concluding on their safe cosmetic use. SCCS also develops and keeps up to date the 'Notes of Guidance for the Testing of Cosmetic Ingredients and Their Safety Evaluation'(NoG), containing a set of guidances to be considered by Applicants when developing safety dossiers for cosmetic ingredients.

In compliance with the restrictions on animal testing, all available scientific data must be considered and integrated following the recommendations reported in the NoG. Indeed, animal studies on mutagenicity or genotoxicity are considered acceptable when data are already available from tests conducted prior to the animal testing ban, or when they are generated to comply with other legislative frameworks unrelated to cosmetics. Notably, in the last update of the NoG (Scientific Committee on Consumer Safety, 2023) special attention is paid to NAMs.

For mutagenicity assessment, SCCS recommended strategy is depicted in Fig. 7. In the initial step, data from predictive methods (e.g. read-across, chemical categories, QSARs or other *in silico* predictions), and other available toxicological data must be considered. The core *in vitro* testing strategy reflects the strategy also implemented in other regulations. In fact, the base-level testing of cosmetic substances is represented by a Bacterial Reverse Mutation Test (OECD TG No.471) or, if not suitable, a Mammalian cell gene mutation assay (OECD TG No. 476, OECD TG No. 490) and an *in vitro* Micronucleus Test (OECD TG No. 487).

In case of clearly negative results in both tests, the substance is considered not genotoxic/mutagenic, whilst if at least one test is clearly positive, the substance will be considered as positive, and no further testing is considered necessary in both cases. If equivocal or in case of indication of false negative (e.g., lack of cellular uptake) or false positive (e.g., bacteriotoxicity) results in any of the main tests are highlighted, a Toolbox for the follow up *in vitro* is applied, for further evaluation.

The Toolbox considers additional *in vitro* tests, such as the 3D reconstructed human skin Comet and micronucleus assays, the enzyme-linked comet assay, or the Hen's Egg test for Micronucleus Induction (HET-MN), in a WoE approach. Other tools supporting this approach are the reporter gene assays (i.e., GreenScreen HC, BlueScreen HC, Toxtracker), the transcriptomics analysis in TK6 cells, HepG2 cells or HepaRG™ cells, the γH2AX assay, or assays that simultaneously analyse different biomarkers (i.e., the MultiFlow and the Multi-Endpoint Genotoxicity Assay – MEGA-Screen system) (Scientific Committee on Consumer Safety, 2023). The OECD is developing guidelines for 3D skin comet assays and micronucleus assay as well as for the Toxtracker

(OECD Work plan, 2025).

Advanced exposure models allow to test compounds using specific routes of exposure. Advanced models, such as 3D reconstructed skin models simulate human skin's response to dermally applied cosmetic products mimicking the complexity of human skin, including interactions between different skin layers and cells. Studies, such as the one conducted by Pfuhler et al. (Pfuhler et al., 2021), revealed a high correlation with *in vivo* outcomes, demonstrating sensitivity, specificity, and overall accuracy in assessing genotoxicity. An analysis of a testing strategy using both the reconstructive skin micronucleus as a follow-up test for substances positive in standard *in vitro* chromosomal aberration assays and a comet assay for substances with positive results in standard gene mutation assays, resulted in a sensitivity of 89 %. The skin model demonstrated sufficient metabolic activity to activate pro-mutagens. Additionally, more complex tissue-like structures allow to detect not only primary but also secondary genotoxicity which is mediated by other cells (i.e., macrophages), confirming the assay's reliability for dermal exposure genotoxicity testing. The 3D reconstructed skin models, together with results from Toxtracker, recently helped to reach the conclusion for the evaluation of a substance used in oxidative hair coloring (Scientific Committee on Consumer Safety SCCS, 2025).

Given the many possibilities offered by the toolbox, expert judgement may be required to reach a conclusion. Much emphasis is placed also on other approaches, such as AOPs, IATAs and DAs, NGRA, and the possibility of integrating NAMs for hazard identification therein.

In the absence of validated *in vitro* approaches for carcinogenicity assessment, in addition to the *in vitro* mutagenicity tests as a pre-screening, SCCS NoG examines NAMs useful to indicate potential genotoxic as well as NGC, to be applied in an overall WoE approach. In particular, last SCCS NoG (Scientific Committee on Consumer Safety, 2023) includes the possibility to combine (e.g., under a IATA approach) data from different tests, such as *in vitro* and *in silico* assays (Jacobs et al., 2020; Smith et al., 2020), some of which are listed below:

- Well developed *in vitro* mutagenicity/genotoxicity tests especially on advanced *in vitro* models (skin, liver, lung models, etc.in combination with micronucleus and comet assay);
- Cell Transformation Assays (OECD Guidance Document No. 214, 2015; OECD Guidance Document No. 231, 2017);
- Toxicogenomics assays (Schmitz-Spanke, 2019) such as the TGx-DDI biomarker, developed to identify chemicals that can cause DNA damage in human cells in culture and useful to distinguish genotoxic from NGC (Li et al., 2019; Thienpont et al., 2024).
- Assays with endpoints capturing early key event mechanisms (Jacobs et al., 2020; Oku et al., 2022), such as dysregulation of gap junction intercellular communication (GJIC) or the scrape loading-dye transfer (SL-DT) technique;
- *In silico* methods for genotoxicity and carcinogenicity, including structure–activity relationships-based analyses.

Special attention is devoted to the recently developed IATA for NGC (Jacobs et al., 2020; Louekari and Jacobs, 2024) as a means to integrate in a structured manner different type of information to ultimately draw a conclusion on the carcinogenic potential.

5. Discussion

Reflecting their crucial role in risk assessment of chemicals, genotoxicity and carcinogenicity endpoints have historically occupied a central position in the longstanding challenge towards the replacement of animal testing in regulatory toxicology. As a result, many alternative tests (both *in vitro* and *in silico*) were developed and implemented in legislation and guidelines, contributing to reduce *in vivo* tests for the evaluation of genotoxicity and carcinogenicity (Table 3). Nevertheless, high relevance is still given to data from animal studies, especially for the confirmation of positive *in vitro* mutagenicity results (Table 3). As some *in vitro* positive results are not translated to *in vivo* effects, and thus do not raise a concern for human health, this confirmation is necessary. Available NAMs could take over to resolve equivocal *in vitro* outcomes, by contributing to improve the accuracy of traditional *in vitro* tests. Thus, although non-animal approaches are not new to genotoxicity and carcinogenicity assessment, advancements in mechanistic understanding and new techniques, implemented as NAMs (Table 1), offer new opportunities: i) to further globally reduce the use of *in vivo* testing, which, as documented through the paper, is still crucial to most regulations; ii) to help filling existing protection gaps that have become apparent over the years.

In this process, the regulatory readiness of the NAM under scrutiny will obviously be given a prominent place (see details on validation status in Table 1). This includes, on the one hand, the NAM validation/qualification (the discussion of which is beyond the scope of this paper), that is to generate objective evidence demonstrating its relevance and reliability in meeting specific requirements. On the other hand, as the validation/qualification systems are time and resource intensive, and the scientific research on NAMs is flourishing, it is necessary to determine whether the NAM under scrutiny is fit for purpose. This implies analysing both the regulatory context to establish the actual feasibility of NAM's implementation and the specific regulatory need that the NAM could address. To this purpose, we analysed the current assessment practices of genotoxicity and carcinogenicity in key EU legislations, highlighting, within each legislation, gaps and needs towards greater protection of human health as well as short and long-term goals for the introduction of NAMs and the replacement of animal testing (summarized in Table 4).

As reported in section 4, while the scientific foundation for carcinogenesis and mutagenesis assessment in regulatory contexts is shared, strategies are not fully harmonized. As a matter of fact, the legislative machinery for risk assessment and management requires also differentiation across sectors to ultimately guarantee the highest possible level

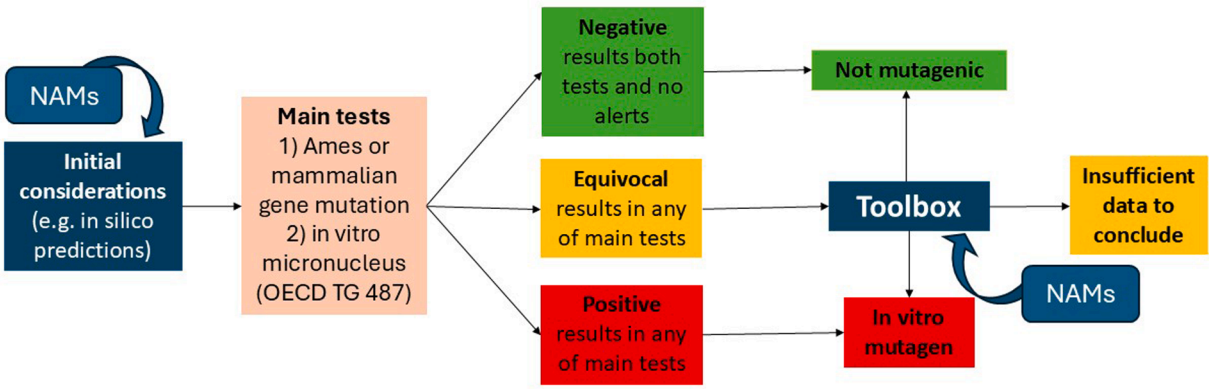

Fig. 7. Testing strategy for genotoxicity/mutagenicity of cosmetic ingredients (adapted from (SCCS, 2023)).

of protection while balancing industrial competitiveness in the global market. Across the various legislations, important differences have been highlighted both in terms of *in vivo* testing required and the possibility of integrating NAMs results within current regulatory landscape (Table 3). While these differences in principle represent many potential opportunities for harmonization towards the OSOA approach, in practice the information requirements prescribed by the legislative provisions pose limits. In fact, in the analysis were considered both prescriptive regulations, which mandate specific tests (e.g. CLP, REACH, PPP regulation) and less prescriptive, principle-based regulations which offer more flexibility in how objectives are met (e.g., many EFSA regulated products and cosmetics regulation). In the latter case, sector specific guidance often exists with recommended testing strategies. These differences in legislation have a practical impact on the feasibility of using NAMs in the short term, due to a foreseeable difference in procedures and timing on the possible implementation of changes in guidance rather than in legal tests. This in turn may affect one of the primary objectives of the OSOA package, i.e., to streamline and harmonize assessments of the same chemical substance across different EU laws.

As many chemicals in the EU must undergo a hazard classification process, which is independent of the quantity of the substance and its intended use, the analysis of classification criteria deserves a prominent

Table 3
Testing requirements for mutagenicity and carcinogenicity (Carc) assessment in different EU legislations. The final column shows the acceptance of NAMs.

		in vitro mutagenicity			*in vivo* mutagenicity		Carc	NAMs
	Test	Gene mut. in bacteria	Chrom Aberrat	Gene mut. in mamm. cells	Somatic cells	Germ cells	2 year rodents	
ECHA-REACH	>1 t/y	y (SIR)	n (only if triggered by pos)	n (only if Ames NA)	n (only if triggered by pos)	n (only if triggered by pos)	n	Annex XI
	> 10 t/y	y (SIR)	y (SIR)	n (only if triggered by neg)	n (only if triggered by pos)	n (only if triggered by pos)	n	Annex XI
	> 100 t/y	y (SIR)	y (SIR)	n (only if Ames NA)	SIR (only if triggered by pos)	SIR (only if triggered by pos)	n	Annex XI
	> 1000 t/y	y (SIR)	y (SIR)	n (only if Ames NA)	SIR (only if triggered by pos)	SIR (only if triggered by pos)	n (only if triggered)[a]	Annex XI
ECHA - CLP					y (Muta 2)	y (Muta 1B)	y	Read-Across
EFSA	Regulated products	y (basic *in vitro* battery)	y (basic *in vitro* battery)	if Ames NA	Follow up of *in vitro* pos	n (conservative approach)	Only if triggered[b]	WoE
	Pesticides AS	y	y	if Ames NA	y	Case-by-case decision	y	n
SCCS	Cosmetics	y	y	if Ames NA	n	n	n	y

Notes: Carc Carcinogenicity; y yes; n no; SIR Standard Information Requirement; Muta: Germ cell mutagenicity CLP class; Pos: positive results; Neg: negative results. AS: Active substances; NA: Not Applicable; WoE Weight of Evidence.
in vivo testing requirement: yes (red); no (green); when triggered (yellow).
[a]Widespread dispersive use or frequent / long-term human exposure and is Muta 2 or evidence from the repeated dose study(ies). [b]Critical results of the subchronic study and/or the (geno)toxicity tests could trigger the need for carcinogenicity studies.

place. As stated in Section 4.1.1, current criteria to conclude on the substance classification as carcinogens and/or mutagens are based on *in vivo* studies. In this context, NAMs can only be used as supporting information of a read-across approach to a 'similar' chemical. This similarity, for instance, could be based on NAMs evidence. Despite being a limitation in the direct use of NAMs for classification purposes, it also offers an opportunity for the use of NAMs in the short term within the CLP regulation (short-term goal, Table 4). On the other hand, this approach still implies availability of *in vivo* results on the analogue(s) chemical(s).

In the context of REACH (Section 4.1.2), tiered strategies for collecting evidence on mutagenicity/genotoxicity and carcinogenicity endpoints while reducing the necessity to perform *in vivo* assays, are in place from its start (as animal testing should be a last resort in REACH, according to article 25 (Regulation (EC) No 1907/2006, 2006). However, although on the one hand they have allowed significant progress toward animal testing reduction, on the other hand diverse challenges have been highlighted: a) *in vitro* positives need *in vivo* confirmation, b) carcinogenesis detection has low efficiency for NGC, c) data are often not sufficient for classification purposes, d) *in vitro* negatives results may be not enough protective for human health (Table 4). The latter point has been raised as a critical issue in REACH for substances below 10t/y, for which the genotoxicity concern (which would also be the only trigger for the carcinogenicity assessment of this tonnage band) can be ruled out by a negative result in the Ames test, thus neglecting other genotoxicity endpoints (i.e., clastogenicity and aneuploidy) (Berggren and Worth, 2023). Another critical aspect is related to germ cell mutagenicity classification. The need to demonstrate mutagenic effects on germ cells is under debate, as well as the definition of class 1A mutagens, which is impossible to populate in practice. To date, for the classification of chemicals as mutagens and/or carcinogens, information currently necessary for the fulfilment of the CLP criteria, especially in categories 1A/B, has hardly been submitted under REACH, highlighting a possible protection gap (Karamertzanis et al., 2019; Woutersen et al., 2019). Failure to classify under CLP in turns has a negative impact on the level of human health protection, as CM (carcinogens and mutagens) harmonised classification (together with R, reprotoxicants) is an essential step for several risk management options which guarantee a high level of protection (Woutersen et al., 2019). The lack of sufficient available information, at least for mutagenicity classification, has been potentially fixed by one of the latest REACH updates in 2022 (Regulation (EU) 2022/477, 2022). This update does not change the substance of the testing strategy but in fact modifies legal aspects of the standard information requirements. From now on, an increase in availability of *in vivo* results can be expected and in relation to that, also an increased possibility to classify chemicals in the most hazardous categories

Table 4
Challenges, short-term and long-term goals towards improvement of human health protection and implementation of NAMs.

Regulatory area	Gaps & needs (Challenges)	Short-term goals	Long-term goals
CLP	Lack of data to satisfy classification criteria, including *in vivo* data, information on germ cells, and non-genotoxic cercinogens (NGC). Current classification criteria for mutagens and carcinogens are primarily based on *in vivo* animal studies, limiting direct NAM replacement. The reduction of animal testing can currently lead to a decrease in human health protection because it limits the ability to classify substances in the most hazardous categories (i.e., CMR), a "too-short-blanket-problem".	NAMs to support read-across classification. NAMs can be used to strengthen the justification of read-across (e.g., supporting biological similarity) for classification purposes and its regulatory acceptability, although this still implies *in vivo* data on analogue substances.	Update of CLP criteria, especially for germ cell mutagenicity and criteria for Category 1A classification. This requires a change in the CLP criteria that trigger Mutagen and Carcinogen classifications.
REACH	For low-tonnage substances (<10t/y), only the Ames test is required, potentially missing other genotoxicity endpoints (i.e., structural or numerical chromosome aberrations). A protection gap exists for NGC, as carcinogenicity studies are typically only required for very high tonnage (>1000t/y) under specific conditions. Frequent recourse to *in vivo* testing, as *in vitro* positive results require *in vivo* confirmation.Data is often insufficient for CLP purposes, hindering classification in hazardous categories (e.g., Muta/Carc 1A/1B). Challenges in germ cell evaluation.	NAMs in adaptations (Annex XI). REACH explicitly allows NAMs (*in vitro* tests, QSARs, grouping, read-across, WoE) as adaptations. Improve transparency on alternatives acceptability and understand reasons for past failures in adaptations. NAMs exploitation to strengthen and refine ECHA's Integrated Regulatory Strategy (IRS), especially for detecting potential NGC. NAMs reducing *in vivo* follow up in equivocal cases.	NAMs as new info requirements to: i) better characterize low tonnage subsantces; ii) lower *in vitro* False Positives rate and reduce *in vivo* follow up. NAMs for NGC and germ cell mutagenicity. REACH information requirements to be modified according to changes in CLP criteria.
EFSA	Recourse to *in vivo* experimentation is still frequent, especially for PPP active substances, where an *in vivo* test is *always* required regardless of *in vitro* results. No agreed approach or test battery based on *in vitro* models for the first screening of NGC.	NAMs in WoE approaches. EFSA is gradually adopting a more flexible approach, considering data from not formally validated NAMs within WoE. Possibility to submit "other relevant data," even if not directly replacing *in vivo* results. Integrating NAMs in the standard battery of tiered testing strategies to improve mechanistic understanding and reduce uncertainties.	Change of information requirements in Plant Protection Product (PPP) regulation.
Cosmetics Regulation	Refinement/standardization of *in vitro* follow-up of positive results. Slow validation of NAMs Absence of validated *in vitro* approaches for carcinogenicity assessment.	Integration of different NAMs for genotoxicity and carcinogenicity assessment.	Operationalization of AOP and IATA frameworks, towards NGRA
Overall/ Common Challenges	Harmonization of NAMs adoption through legislations (e.g., OSOA) is limited by a lack of complete harmonization across different EU legislations. Slow validation of NAMs; an urgent need to speed up the validation of relevant NAMs to ensure safety decisions are based on reliable evidence. Standardization in evidence integration: a major challenge is how to standardize and validate the combination of different NAMs evidence that generate the final result, including estimating associated uncertainties. Need for NAMs that can reliably address non-genotoxic carcinogenicity		

(Mutagenicity categories 1B/2, and consequently potentially Carcinogenicity categories). To simplify, this has so far turned out to be a "too-short-blanket-problem", as the reduction of animal testing impacts the human health protection, in consequence of *in vivo* criteria for classification. The current setup is such that the more animal welfare increases, by use of existent or new non-animal methods, the less it is possible to classify substances in Carcinogenicity and/or Mutagenicity categories, and thus trigger those risk management measures that allow a high level of protection of human health. Based on these premises it is clear that a change of REACH (and other data generating legislation) information requirements towards the use of NAMs, must be accompanied (if not preceded) by a change of the CLP criteria that trigger C and M classifications.

At present, the possibility to implement NAMs (i.e., SAR based approaches QSAR, WoE, read-across, *in vitro* tests) in the context of REACH, apart from alternative assays already in place, relies on their exploitation within adaptations of the information requirements, providing equivalent information is given (Annex XI, cf. Section 5.1.3). Also, NAMs not directly associated with the apical endpoint to which the information requirement refers, could be envisaged as supporting evidence to strengthen WoE, read-across or grouping approaches (short-term goal, Table 4). To push in the direction of using NAMs under Annex XI of REACH, it would be useful to know the acceptance rate of the adaptations submitted so far, detailing strengths and weakness of submissions (i.e., beyond information reported under Article 117(3) of the REACH (ECHA, 2020). Such knowledge would give the opportunity to learn from previous experience on how to integrate data from NAMs, supporting their increasingly acceptable use at regulatory level (short-term goal, Table 4).

In other frameworks, where regulatory decisions do not need to comply with CLP, NAMs implementation may have a faster route. This is the case of most EFSA regulated products, where the transition to the use of NAMs is gradually taking place (cf. Section 4.2.1). Data from not formally validated NAMs in fact may be already considered in a WoE approach on which to base regulatory decisions/recommendations (short-term goal, Table 4). Many of the laws regulating substances under EFSA's remit allow for a more flexible assessment approach, as they do not prescribe specific test methodologies. This inherent flexibility unlocks significant opportunities for the development and implementation of NAMs, and ultimately for their regulatory promotion, as demonstrated for instance by the QSARs applications for the risk assessment on botanicals and PPP residues definition (cf. Section 4.2.1). On the other hand, there is a risk of increasing differences between evaluations carried out in different contexts, moving away from the OSOA objectives (see Common Challenges in Table 4).

The difference in the feasibility of NAMs regulatory implementation is even more pronounced in the case of cosmetics, where NAMs increasingly represent an indispensable resource to complete the risk assessment (cf. Section 4.3). Animal testing ban in the cosmetics sector constitutes a challenge but also a unique opportunity to lead progress in the regulatory application of NAMs (short-term goal, Table 4). Cosmetic Regulation is a leading example of how the early use of NAMs has been integrated into safety evaluation practices. NAMs, such as *in vitro* tests including advanced 3D models and *in silico* modeling, play a critical role in assessing the safety of cosmetic products without relying on animal testing. Validated NAMs are vital for cosmetic regulation. Expediting the validation of a wide array of relevant NAMs is crucial in this sector to guarantee that safety decisions are grounded in reliable evidence, thereby providing comprehensive human health protection (see Challenges in Table 4).

6. Concluding remarks

In conclusion, although at the forefront for the use of alternative to animal tests, genotoxicity and carcinogenicity endpoints can greatly benefit from the adoption of NAMs, towards more efficient assessment and greater protection of human health, further contributing to the decrease of the number of laboratory animals. Some NAMs are already used in some legislations, while promising methods exist for which confidence still needs to be strengthened and the regulatory groundwork needs to be prepared. On the other hand, The NAMs currently available are not yet able to fill all the gaps (e.g., NGC and germ cells mutagenicity assessment). Still a challenge is represented by the need for standardized and reproducible frameworks for the integration of evidence.

The possibilities highlighted in this paper for using NAMs already within existing legislation will help increase awareness and confidence in these methods. Meanwhile, achieving a truly protective and animal-free EU regulatory system for genotoxicity and carcinogenicity assessment requires, in addition to validated and fit-for-purpose NAMs, a fundamental transformation of existing regulatory procedures. This pivotal shift necessitates the strategic integration of both established and emerging NAMs to enhance human health protection while simultaneously fulfilling the commitment to animal welfare and addressing current protection gaps.

CRediT authorship contribution statement

Cecilia Bossa: Writing – review & editing, Writing – original draft, Supervision, Investigation, Conceptualization. **Silvia Alivernini:** Writing – review & editing, Writing – original draft, Investigation, Conceptualization. **Cristina Andreoli:** Writing – review & editing, Writing – original draft, Investigation. **Gabriele Aquilina:** Writing – review & editing, Investigation. **Leonello Attias:** Writing – review & editing, Investigation. **Emilio Benfenati:** Writing – review & editing, Investigation. **Maria Dusinska:** Writing – review & editing, Writing – original draft, Investigation, Conceptualization. **Naouale El Yamani:** Writing – review & editing. **Henriqueta Louro:** Writing – review & editing, Writing – original draft, Investigation, Conceptualization. **Francesca Marcon:** Writing – review & editing, Writing – original draft, Investigation, Conceptualization. **Giuseppa Raitano:** Writing – review & editing, Writing – original draft, Investigation. **Elise Rundén-Pran:** Writing – review & editing. **Maria Teresa Russo:** Writing – review & editing, Writing – original draft, Investigation, Conceptualization. **Maria João Silva:** Writing – review & editing, Writing – original draft, Investigation, Conceptualization. **Chiara Laura Battistelli:** Conceptualization, Writing – review & editing, Writing – original draft, Supervision, Investigation.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

Authors developed the work in the course of their employment at their respective organizations and under the scope of the Partnership for the Assessment of Risks from Chemicals (PARC) that has received funding from the European Union's Horizon Europe Research and Innovation Programme under Grant Agreement No. 101057014. The authors would like to thank the PARC WP6 leaders and partners for fruitful discussions. HL and MJS acknowledge the support from the Comprehensive Health Research Centre (CHRC), NOVA Medical School, Universidade NOVA de Lisboa, Lisbon, Portugal.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.envint.2025.109948.

Data availability

Data sharing is not applicable to this article as no new data were created or analyzed in this study.

References

Ames Bruce, N., 1971. The Detection of Chemical Mutagens with Enteric Bacteria. Chemical Mutagens. In: Hollaender, A. (Ed.), Chemical Mutagens. Springer, Boston, MA, pp. 267–282. https://doi.org/10.1007/978-1-4615-8966-2_9.

Andreoli, C., Dusinska, M., Bossa, C., Battistelli, C.L., Silva, M.J., Louro, H., 2025. Regulatory practices on the genotoxicity testing of nanomaterials and outlook for the future. Regul. Toxicol. Pharm. 162, 105881. https://doi.org/10.1016/j.yrtph.2025.105881.

Beal, M.A., Audebert, M., Barton-Maclaren, T., Battaion, H., Bemis, J.C., Cao, X., Chen, C., Dertinger, S.D., Froetschl, R., Guo, X., Johnson, G., Hendriks, G., Khoury, L., Long, A.S., Pfuhler, S., Settivari, R.S., Wickramasuriya, S., White, P., 2023. Quantitative in vitro to in vivo extrapolation of genotoxicity data provides protective estimates of in vivo dose. Environ. Mol. Mutagen. 64, 105–122. https://doi.org/10.1002/em.22521.

Bearth, A., Roth, N., Jansen, T., Holden, L., Čavoški, A., Di Consiglio, E., Hauzenberger, I., Lee, R., Mombelli, E., Tcheremenskaia, O., Wendt-Rasch, L., Wilks, M.F., 2025. New approach methodologies in human health risk assessment across European regulatory frameworks: status quo, barriers and drivers for regulatory acceptance and use. Environ. Int. 196, 109279. https://doi.org/10.1016/j.envint.2025.109279.

Benigni, R., 2012. Alternatives to the carcinogenicity bioassay for toxicity prediction: are we there yet? Expert Opin. Drug Metab. Toxicol. https://doi.org/10.1517/17425255.2012.666238.

Benigni, R., Bossa, C., Tcheremenskaia, O., Giuliani, A., 2010a. Alternatives to the carcinogenicity bioassay: in silico methods, and the in vitro and in vivo mutagenicity assays. Expert Opin. Drug Metab. Toxicol. 6, 809–819. https://doi.org/10.1517/17425255.2010.486400.

Benigni, R., Bossa, C., Worth, A., 2010b. Structural analysis and predictive value of the rodent in vivo micronucleus assay results. Mutagenesis 25, 335–341. https://doi.org/10.1093/mutage/geq010.

Benigni, R., Laura Battistelli, C., Bossa, C., Giuliani, A., Fioravanzo, E., Bassan, A., Fuart Gatnik, M., Rathman, J., Yang, C., Tcheremenskaia, O., 2019. Evaluation of the applicability of existing (Q)SAR models for predicting the genotoxicity of pesticides and similarity analysis related with genotoxicity of pesticides for facilitating of grouping and read across. EFSA Supporting Publ. 16. https://doi.org/10.2903/sp.efsa.2019.EN-1598.

Berggren, E., Worth, A.P., 2023. Towards a future regulatory framework for chemicals in the European Union – Chemicals 2.0. Regulatory Toxicology and Pharmacology. https://doi.org/10.1016/j.yrtph.2023.105431.

Burgdorf, T., Piersma, A.H., Landsiedel, R., Clewell, R., Kleinstreuer, N., Oelgeschläger, M., Desprez, B., Kienhuis, A., Bos, P., de Vries, R., de Wit, L., Seidle, T., Scheel, J., Schönfelder, G., van Benthem, J., Vinggaard, A.M., Eskes, C., Ezendam, J., 2019. Workshop on the validation and regulatory acceptance of innovative 3R approaches in regulatory toxicology – Evolution versus revolution. Toxicol. In Vitro 59, 1–11. https://doi.org/10.1016/j.tiv.2019.03.039.

Cattaneo, I., Astuto, M.C., Binaglia, M., Devos, Y., Dorne, J.L.C.M., Fernandez Agudo, A., Fernandez Dumont, A., Garcia-Vello, P., Kass, G.E.N., Lanzoni, A., Liem, A.K.D., Panzarea, M., Paraskevopulos, K., Parra Morte, J.M., Tarazona, J.V., Terron, A., 2023. Implementing new approach methodologies (NAMs) in food safety assessments: strategic objectives and actions taken by the European food safety authority. Trends Food Sci. Technol. https://doi.org/10.1016/j.tifs.2023.02.006.

Collins, A., Møller, P., Gajski, G., Vodenková, S., Abdulwahed, A., Anderson, D., Bankoglu, E.E., Bonassi, S., Boutet-Robinet, E., Brunborg, G., Chao, C., Cooke, M.S., Costa, C., Costa, S., Dhawan, A., de Lapuente, J., Bo', C.D., Dubus, J., Dusinska, M., Duthie, S.J., Yamani, N.E., Engelward, B., Gaivão, I., Giovannelli, L., Godschalk, R., Guilherme, S., Gutzkow, K.B., Habas, K., Hernández, A., Herrero, O., Isidori, M., Jha, A.N., Knasmüller, S., Kooter, I.M., Koppen, G., Kruszewski, M., Ladeira, C., Laffon, B., Larramendy, M., Hégarat, L Le, Lewies, A., Lewinska, A., Liwszyc, G.E., de Cerain, A.L., Manjanatha, M., Marcos, R., Milić, M., de Andrade, V.M., Moretti, M., Muruzabal, D., Novak, M., Oliveira, R., Olsen, A.K., Owiti, N., Pacheco, M., Pandey, A.K., Pfuhler, S., Pourrut, B., Reisinger, K., Rojas, E., Rundén-Pran, E., Sanz-Serrano, J., Shaposhnikov, S., Sipinen, V., Smeets, K., Stopper, H., Teixeira, J.P., Valdiglesias, V., Valverde, M., van Acker, F., van Schooten, F.J., Vasquez, M., Wentzel, J.F., Wnuk, M., Wouters, A., Žegura, B., Zikmund, T., Langie, S.A.S., Azqueta, A., 2023. Measuring DNA modifications with the comet assay: a compendium of protocols. Nat. Protoc. https://doi.org/10.1038/s41596-022-00754-y.

Corvi, R., Madia, F., 2017. In vitro genotoxicity testing–can the performance be enhanced? Food Chem. Toxicol. 106, 600–608. https://doi.org/10.1016/j.fct.2016.08.024.

Demuynck, E., Vanhaecke, T., Thienpont, A., Cappoen, D., Van Goethem, F., Winkelman, L., Beltman, J., Murugadoss, S., Olsen, A.-K., Marcon, F., Bossa, C., Shaikh, S.-M., Nikolopoulou, D., Hatzi, V., Pennings, J.L.A., Luijten, M., Adam-Guillermin, C., Paparella, M., Audebert, M., Mertens, B., 2025. Advancing genotoxicity assessment by building a global AOP network. Environ. Mol. Mutagen. 66(6-7), 341-366. https://doi.org/10.1002/em.70022.

Dertinger, S.D., Totsuka, Y., Bielas, J.H., Doherty, A.T., Kleinjans, J., Honma, M., Marchetti, F., Schuler, M.J., Thybaud, V., White, P., Yauk, C.L., 2019. High information content assays for genetic toxicology testing: A report of the International Workshops on Genotoxicity Testing (IWGT), in: Mutation Research - Genetic Toxicology and Environmental Mutagenesis. Elsevier B.V. https://doi.org/10.1016/j.mrgentox.2019.02.003.

Directive 2009/48/EC, 2009. DIRECTIVE 2009/48/EC OF THE EUROPEAN PARLIAMENT AND OF THE COUNCIL of 18 June 2009 on the safety of toys (Text with EEA relevance).

ECHA, 2020. The use of alternatives to testing on animals for the REACH Regulation. https://doi.org/10.2823/092305.

ECHA, 2021. Minutes of the 75 th Meeting of the Member State Committee (MSC-75) Summary Record of the Proceedings and Conclusions and action points. https://echa.europa.eu/documents/10162/2166307/minutes_msc-75_en.pdf/f22eeded-6f0a-6ca7-747b-a9503542f17c?t=1634729500249.

ECHA, 2023a. Report on the European Chemicals Agency's "New Approach Methodologies Workshop: Towards an Animal Free Regulatory System for Industrial Chemicals" 31 May – 1 June 2023, Helsinki, Finland. https://doi.org/10.2823/7494.

ECHA, 2023b. Key Areas of Regulatory Challenge. https://doi.org/10.2823/568850.

ECHA, 2023c. The use of alternatives to testing on animals for the REACH Regulation Fifth report under Article 117(3) of the REACH Regulation. https://doi.org/10.2823/805454.

ECHA, 2024. Integrated Regulatory Strategy – Past successes and future outlook. https://doi.org/10.2823/6708113.

EFSA, 2016. Guidance on the establishment of the residue definition for dietary risk assessment EFSA Panel on Plant Protection Products and their Residues (PPR). EFSA J. 14. https://doi.org/10.2903/J.EFSA.2016.4549.

EFSA AFC Panel (EFSA Panel on Food Additives, Flavourings, Processing Aidsand Materials in contact with Food), 2008. Note for guidance for the preparation of an application for the safety assessment of a substance to be used in plastic food contact materials. EFSA J. 6. https://doi.org/10.2903/j.efsa.2008.21r.

EFSA, Andreoli, C., Aquilina, G., Bignami, M., Bolognesi, C., Crebelli, R., Dusinska, M., Gürtler, R., Louro, H., Marcon, F., Nielsen, E., Schlatter, J., Vleminckx, C., Astuto, M. C., Nathanail, A.V., Benford, D., 2023. Harmonised approach for reporting reliability and relevance of genotoxicity studies. EFSA Supporting Publ. 20. https://doi.org/10.2903/sp.efsa.2023.EN-8270.

EFSA FAF Panel (EFSA Panel on Food Additives and Flavourings), 2021. Scientific Guidance for the preparation of applications on smoke flavouring primary products. EFSA J. 19. https://doi.org/10.2903/j.efsa.2021.6435.

EFSA FAF Panel (EFSA Panel on Food Additives and Flavourings), 2022. Scientific Guidance on the data required for the risk assessment of flavourings to be used in or on foods. EFSA J. 20. https://doi.org/10.2903/j.efsa.2022.7673.

EFSA Panel on Dietetic Products, Nutrition and Allergies (NDA), Turck, D., Bresson, J.L., Burlingame, B., Dean, T., Fairweather-Tait, S., Heinonen, M., Hirsch-Ernst, K.I., Mangelsdorf, I., McArdle, H.J., Naska, A., Neuhäuser-Berthold, M., Nowicka, G., Pentieva, K., Sanz, Y., Siani, A., Sjödin, A., Stern, M., Tomé, D., Vinceti, M., Willatts, P., Engel, K.H., Marchelli, R., Pöting, A., Poulsen, M., Salminen, S., Schlatter, J., Arcella, D., Gelbmann, W., de Sesmaisons-Lecarré, A., Verhagen, H., van Loveren, H., 2021. Guidance on the preparation and submission of an application for authorisation of a novel food in the context of Regulation (EU) 2015/22831 (Revision 1)2. EFSA J. 19. https://doi.org/10.2903/j.efsa.2021.6555.

EFSA Panel on Food Additives and Nutrient Sources added to Food (ANS), 2021. Guidance on safety evaluation of sources of nutrients and bioavailability of nutrient from the sources (Revision 1)1. EFSA J. 19. https://doi.org/10.2903/j.efsa.2021.6552.

EFSA CEP Panel (EFSA Panel on Food Contact Materials, Enzymes andProcessing Aids), Lambre, C., Barat Baviera, J.M., Bolognesi, C., Cocconcelli, P.S., Crebelli, R., Gott, D. M., Grob, K., Lampi, E., Mengelers, M., Mortensen, A., Riviere, G., Steffensen, I.-L., Tlustos, C., Van Loveren, H., Vernis, L., Zorn, H., Glandorf, B., Herman, L., Aguilera, J., Andryszkiewicz, M., Gomes, A., Kovalkovicova, N., Liu, Y., Rainieri, S., Chesson, A., 2021. Scientific Guidance for the submission of dossiers on Food Enzymes. EFSA J. 19(10), 6851, 35 pp. https://doi.org/10.2903/j.efsa.2021.68.

EFSA PC-1047, 2024. Scoping paper on the revision of the opinion on the Margin of Exposure for chemicals which are both genotoxic and carcinogenic. https://connect.efsa.europa.eu/RM/sfc/servlet.shepherd/document/download/069Tk0000047hfRIAQ.

EFSA PC-1237, 2024. Scoping Paper on the Revision of the Guidance on Genotoxicity Testing Strategy. https://connect.efsa.europa.eu/RM/sfc/servlet.shepherd/document/download/069Tk000007jIWJIA2.

EFSA Scientific Committee, 2011. Scientific opinion on genotoxicity testing strategies applicable to food and feed safety assessment. EFSA J. 9. https://doi.org/10.2903/j.efsa.2011.2379.

EFSA Scientific Committee, 2017a. Clarification of some aspects related to genotoxicity assessment. EFSA J. 15. https://doi.org/10.2903/j.efsa.2017.5113.

EFSA Scientific Committee, 2017b. Guidance on the use of the weight of evidence approach in scientific assessments. EFSA J. 15. https://doi.org/10.2903/j.efsa.2017.4971.

EFSA Scientific Committee, 2021a. Guidance on aneugenicity assessment. EFSA J. https://doi.org/10.2903/j.efsa.2021.6770.

EFSA Scientific Committee, 2021b. Guidance on risk assessment of nanomaterials to be applied in the food and feed chain: human and animal health. EFSA J. 19. https://doi.org/10.2903/j.efsa.2021.6768.

Erickson, R.P., 2010. Somatic gene mutation and human disease other than cancer: an update. Mutat. Res./Rev. Mutat. Res. 705, 96–106. https://doi.org/10.1016/j.mrrev.2010.04.002.

Escher, S.E., Partosch, F., Konzok, S., Jennings, P., Luijten, M., Kienhuis, A., de Leeuw, V., Reuss, R., Lindemann, K., Bennekou, S.H., 2022. Development of a

roadmap for action on new approach methodologies in risk assessment. EFSA Supporting Publ. 19. https://doi.org/10.2903/sp.efsa.2022.en-7341.
European Commission, 2020. Chemicals Strategy for Sustainability Towards a Toxic-Free Environment. Brussels.
European Commission, 2023. COMMUNICATION FROM THE COMMISSION on the European Citizens' Initiative (ECI) 'Save cruelty-free cosmetics – Commit to a Europe without animal testing'.
Flamand, N., Meunier, J.-R., Meunier, P.-A., Agapakis-Caussé, C., 2001. Mini mutagenicity test: a miniaturized version of the Ames test used in a prescreening assay for point mutagenesis assessment. Toxicol. In Vitro 15, 105–114. https://doi.org/10.1016/S0887-2333(01)00003-0.
Food and Drug Administration (FDA), 2024. Potential Approaches to Drive Future Integration of New Alternative Methods for Regulatory Decision-Making.
Fortin, A.-M.-V., Long, A.S., Williams, A., Meier, M.J., Cox, J., Pinsonnault, C., Yauk, C. L., White, P.A., 2023. Application of a new approach methodology (NAM)-based strategy for genotoxicity assessment of data-poor compounds. Front. Toxicol. 5. https://doi.org/10.3389/ftox.2023.1098432.
Gissi, A., Tcheremenskaia, O., Bossa, C., Battistelli, C.L., Browne, P., 2024. The OECD (Q) SAR assessment framework: a tool for increasing regulatory uptake of computational approaches. Comput. Toxicol. 31. https://doi.org/10.1016/j.comtox.2024.100326.
Hanahan, D., 2022. Hallmarks of cancer: new dimensions. Cancer Discov. 12, 31–46. https://doi.org/10.1158/2159-8290.CD-21-1059.
Health Canada, 2023. Use of new approach methods (NAMs) in risk assessment. https://www.canada.ca/content/dam/hc-sc/documents/services/chemical-substances/fact-sheets/use-new-approach-methods-risk-assessment/use-of-new-approach-methods-in-risk-assessment.PDF.
Hendriks, G., Adriaens, E., Allemang, A., Clements, J., Cole, G., Derr, R., Engel, M., Hamel, A., Kidd, D., Kellum, S., Kiyota, T., Myhre, A., Naëssens, V., Pfuhler, S., Roy, M., Settivari, R., Schuler, M., Zeller, A., van Benthem, J., Vanparys, P., Kirkland, D., 2024. Interlaboratory validation of the ToxTracker assay: an in vitro reporter assay for mechanistic genotoxicity assessment. Environ. Mol. Mutagen. 65, 4–24. https://doi.org/10.1002/em.22592.
Herzler, M., Luijten, M., Marx-Stoelting, P., Rivière, G., 2025. The European Partnership PARC's role in actively promoting the uptake of new approach methodologies and next-generation risk assessment into regulatory risk assessment practice. Curr. Opin Toxicol. 42, 100517. https://doi.org/10.1016/j.cotox.2025.100517.
IARC, 2019. IARC Monographs on the Identification of Carcinogenic Hazards to Humans PREAMBLE. IARC Monographs Preamble. https://monographs.iarc.who.int/iarc-monographs-preamble-preamble-to-the-iarc-monographs/.
Ibrahim, M.A., Yasui, M., Saha, L.K., Sasanuma, H., Honma, M., Takeda, S., 2020. Enhancing the sensitivity of the thymidine kinase assay by using DNA repair-deficient human TK6 cells. Environ. Mol. Mutagen. 61, 602–610. https://doi.org/10.1002/em.22371.
ICCVAM, 2018. A Strategic Roadmap for Establishing New Approaches to Evaluate the Safety of Chemicals and Medical Products in the United States. Durham, NC. https://doi.org/10.22427/NTP-ICCVAM-ROADMAP2018.
Jacobs, M.N., Colacci, A., Corvi, R., Vaccari, M., Aguila, M.C., Corvaro, M., Delrue, N., Desaulniers, D., Ertych, N., Jacobs, A., Luijten, M., Madia, F., Nishikawa, A., Ogawa, K., Ohmori, K., Paparella, M., Sharma, A.K., Vasseur, P., 2020. Chemical carcinogen safety testing: OECD expert group international consensus on the development of an integrated approach for the testing and assessment of chemical non-genotoxic carcinogens. Arch. Toxicol. 94, 2899–2923. https://doi.org/10.1007/s00204-020-02784-5.
Jagiello, K., Sosnowska, A., Stępnik, M., Gromelski, M., Płonka, K., 2022. Nano-specific alternative methods in human hazard/safety assessment under different EU regulations, considering the animal testing bans already in place for cosmetics and their ingredients. https://euon.echa.europa.eu/view-article/-/journal_content/title/new-study-identifies-challenges-of-animal-free-test-methods-application-for-nanomaterials.
JRC, 2020. EURL ECVAM Status Report on the Development, Validation and Regulatory Acceptance of Alternative Methods and Approaches (2019) EUR 30100 EN. https://doi.org/10.2760/25602.
Karamertzanis, P.G., Atlason, P., Nathanail, A.V., Provoost, J., Karhu, E., Rasenberg, M., 2019. The impact on classifications for carcinogenicity, mutagenicity, reproductive and specific target organ toxicity after repeated exposure in the first ten years of the REACH regulation. Regul. Toxicol. Pharm. 106, 303–315. https://doi.org/10.1016/j.yrtph.2019.05.003.
Knight, D.J., Deluyker, H., Chaudhry, Q., Vidal, J.-M., de Boer, A., 2021. A call for action on the development and implementation of new methodologies for safety assessment of chemical-based products in the EU – a short communication. Regul. Toxicol. Pharm. 119, 104837. https://doi.org/10.1016/j.yrtph.2020.104837.
Li, H.H., Yauk, C.L., Chen, R., Hyduke, D.R., Williams, A., Frötschl, R., Ellinger-Ziegelbauer, H., Pettit, S., Aubrecht, J., Fornace, A.J., 2019. TGx-DDI, a transcriptomic biomarker for genotoxicity hazard assessment of pharmaceuticals and environmental chemicals. Front. Big Data. https://doi.org/10.3389/fdata.2019.00036.
Louekari, K., Jacobs, M.N., 2024. A modular strategy for the testing and assessment of non-genotoxic carcinogens. Arch. Toxicol. 98, 2463–2485. https://doi.org/10.1007/s00204-024-03753-y.
Luijten, M., Corvi, R., Mehta, J., Corvaro, M., Delrue, N., Felter, S., Haas, B., Hewitt, N.J., Hilton, G., Holmes, T., Jacobs, M.N., Jacobs, A., Lamplmair, F., Lewis, D., Madia, F., Manou, I., Melching-Kollmuss, S., Schorsch, F., Schütte, K., Sewell, F., Strupp, C., van der Laan, J.W., Wolf, D.C., Wolterink, G., Woutersen, R., Zvonar, Z., Heusinkveld, H., Braakhuis, H., 2020. A comprehensive view on mechanistic approaches for cancer risk assessment of non-genotoxic agrochemicals. Regul. Toxicol. Pharm. https://doi.org/10.1016/j.yrtph.2020.104789.
Lynch, A.M., Zanoni, T.B., Salk, J.J., Martincorena, I., Young, R.R., Kucab, J., Valentine, C.C., Yauk, C., Escobar, P.A., Witt, K.L., Frötschl, R., Reed, S.H., Ashford, A., 2023. Next generation sequencing workshop at the royal society of medicine (London, May 2022): how genomics is on the path to modernizing genetic toxicology. Mutagenesis 38, 192–200. https://doi.org/10.1093/mutage/gead012.
Madia, F., Worth, A., Whelan, M., Corvi, R., 2019. Carcinogenicity assessment: addressing the challenges of cancer and chemicals in the environment. Environ. Int. https://doi.org/10.1016/j.envint.2019.04.067.
Marchetti, F., Cardoso, R., Chen, C.L., Douglas, G.R., Elloway, J., Escobar, P.A., Harper, T., Heflich, R.H., Kidd, D., Lynch, A.M., Myers, M.B., Parsons, B.L., Salk, J.J., Settivari, R.S., Smith-Roe, S.L., Witt, K.L., Yauk, C., Young, R.R., Zhang, S., Minocherhomji, S., 2023. Error-corrected next-generation sequencing to advance nonclinical genotoxicity and carcinogenicity testing. Nat. Rev. Drug Discov. https://doi.org/10.1038/d41573-023-00014-y.
Martus, H.-J., Froetschl, R., Gollapudi, B., Honma, M., Marchetti, F., Pfuhler, S., Schoeny, R., Uno, Y., Yauk, C., Kirkland, D.J., 2020. Summary of major conclusions from the 7th International Workshop on Genotoxicity Testing (IWGT), Tokyo, Japan. Mutat. Res./Genet. Toxicol. Environ. Mutagen. 852, 503134. https://doi.org/10.1016/j.mrgentox.2020.503134.
Marx-Stoelting, P., Rivière, G., Luijten, M., Aiello-Holden, K., Bandow, N., Baken, K., Cañas, A., Castano, A., Denys, S., Fillol, C., Herzler, M., Iavicoli, I., Karakitsios, S., Klanova, J., Kolossa-Gehring, M., Koutsodimou, A., Vicente, J.L., Lynch, I., Namorado, S., Norager, S., Pittman, A., Rotter, S., Sarigiannis, D., Silva, M.J., Theunis, J., Tralau, T., Uhl, M., van Klaveren, J., Wendt-Rasch, L., Westerholm, E., Rousselle, C., Sanders, P., 2023. A walk in the PARC: developing and implementing 21st century chemical risk assessment in Europe. Arch. Toxicol. https://doi.org/10.1007/s00204-022-03435-7.
Miller, J.A., Miller, E.C., 1977. Ultimate chemical carcinogens as reactive mutagenic electrophiles, ColdSpringHarbor Lab. ed. Cold Spring Harbor, NY.
National Institute for Public Health and the Environment (RIVM), 2022. Landscape New Approach Methodologies (NAMs) safety assessment chemical substances. https://www.rivm.nl/sites/default/files/2024-06/Landscape%20New%20Approach%20Methodologies%20%28NAMs%29%20for%20the%20safety%20assessment%20of%20chemical%20substances.pdf.
National Research Council (US) Committee on Chemical Environmental Mutagens, 1983. Quantitative Relationship between Mutagenic and Carcinogenic Potencies: A Feasibility Study. Washington (DC): the National Academies Press (US). https://doi.org/10.17226/747.
Nicova, K., Dibusz, K., Vejvodova, P., Novotny, T., Nic, M., Angelini, J., Castoralova, M., Gamaoun, R., Macurkova, A., Neubauerova, T., Plodiková, P., Seryak, L., Sulakova, M., 2025. Transformation and further development of the Compendium of Botanicals reported to contain naturally occurring substances of possible concern for human health when used in food and food supplements. EFSA Supporting Publ. 22. https://doi.org/10.2903/sp.efsa.2025.EN-9388.
OECD, 2007. DETAILED REVIEW PAPER ON CELL TRANSFORMATION ASSAYS FOR DETECTION OF CHEMICAL CARCINOGENS, OECD Environment, Health and Safety Publications Series on Testing and Assessment No. 31, ENV/JM/MONO(2007)18.
OECD, 2016. Overview of the set of OECD Genetic Toxicology Test Guidelines and updates performed in 2014-2015 Series on Testing & Assessment No. 238. JOINT MEETING OF THE CHEMICALS COMMITTEE AND THE WORKING PARTY ON CHEMICALS, PESTICIDES AND BIOTECHNOLOGY. Paris. https://doi.org/10.1787/9789264274761-en.
OECD, 2020. ENVIRONMENT DIRECTORATE JOINT MEETING OF THE CHEMICALS COMMITTEE AND THE WORKING PARTY ON CHEMICALS, PESTICIDES AND BIOTECHNOLOGY Overview of Concepts and Available Guidance related to Integrated Approaches to Testing and Assessment (IATA) Series on Testing and Assessment No. 329.
OECD, 2024. (Q)SAR Assessment Framework: Guidance for the regulatory assessment of (Quantitative) Structure Activity Relationship models and predictions, Second Edition. Series on Testing and Assessment No. 405.
OECD, 2025a. Integrated Approaches to Testing and Assessment (IATA) [WWW Document]. https://www.oecd.org/en/topics/sub-issues/assessment-of-chemicals/integrated-approaches-to-testing-and-assessment.html. (accessed 7.23.25).
OECD, 2025b. QSAR Toolbox [WWW Document]. URL https://www.oecd.org/en/data/tools/oecd-qsar-toolbox.html (accessed 7.23.25).
OECD Guidance Document No. 214, 2015. GUIDANCE DOCUMENT ON THE IN VITRO SYRIAN HAMSTER EMBRYO (SHE) CELL TRANSFORMATION ASSAY. Series on Testing & Assessement No. 214, ENV/JM/MONO(2015)18.
OECD Guidance Document No. 231, 2017. GUIDANCE DOCUMENT ON THE IN VITRO BHAS 42 CELL TRANSFORMATION ASSAY Series on Testing & Assessment No. 231, ENV/JM/MONO(2016)1.
OECD Test Guideline No. 408, 2018. Test Guideline No 408: Repeated dose 90-day oral toxicity study in rodents. OECD Guidelines for the Testing of Chemicals.
OECD Test Guideline No. 451, 2018. Test Guideline No. 451: Carcinogenicity Studies. OECD Guideline for testing of chemicals. https://doi.org/10.1787/9789264071186-en.
OECD Test Guideline No. 453, 2018. Test Guideline No. 453: Combined Chronic Toxicity/Carcinogenicity Studies. OECD Guidelines for the Testing of Chemicals. https://doi.org/10.1787/9789264071223-en.
OECD Test Guideline No. 470, 2022. Test Guideline No. 470 Mammalian Erythrocyte Pig-a Gene Mutation Assay. OECD Guidelines for the Testing of Chemicals.
OECD Test Guideline No. 471, 2020. Test Guideline No. 471: Bacterial Reverse Mutation Test. OECD Guideline for testing of chemicals. Paris. https://doi.org/10.1787/9789264071247-en.

OECD Test Guideline No. 473, 2016. Test Guideline No. 473: In Vitro Mammalian Chromosomal Aberration Test. OECD Guidelines for the Testing of Chemicals. https://doi.org/10.1787/9789264264649-en.
OECD Test Guideline No. 474, 2016. Test Guideline No. 474: Mammalian Erythrocyte Micronucleus Test, OECD Guidelines for the Testing of Chemicals. Paris. https://doi.org/10.1787/9789264264762-en.
OECD Test Guideline No. 475, 2016. Test Guideline No. 475: Mammalian Bone Marrow Chromosomal Aberration Test. OECD Guidelines for the Testing of Chemicals. Paris. https://doi.org/10.1787/9789264264786-en.
OECD Test Guideline No. 476, 2016. Test Guideline No. 476 In Vitro Mammalian Cell Gene Mutation Tests using the Hprt and xprt genes. OECD Guidelines for the Testing of Chemicals.
OECD Test Guideline No. 478, 2016. Test Guideline No. 478: Rodent Dominant Lethal Test. OECD Guidelines for the Testing of Chemicals. https://doi.org/10.1787/9789264264823-en.
OECD Test Guideline No. 487, 2023. Test Guideline No. 487: In Vitro Mammalian Cell Micronucleus Test. OECD Guidelines for the Testing of Chemicals. https://doi.org/10.1787/9789264264861-en.
OECD Test Guideline No. 488, 2022. Test Guideline No. 488: Transgenic Rodent Somatic and Germ Cell Gene Mutation Assays. OECD Guidelines for the Testing of Chemicals, Section 4. https://doi.org/10.1787/9789264203907-en.
OECD Test Guideline No. 489, 2016. Test Guideline No. 489: In Vivo Mammalian Alkaline Comet Assay. OECD Guidelines for the Testing of Chemicals. https://doi.org/10.1787/72d77764-en.
OECD Test Guideline No. 490, 2016. Test Guideline No 490: In Vitro Mammalian Cell Gene Mutation Tests Using the Thymidine Kinase Gene. OECD Guidelines for the Testing of Chemicals.
OECD Test Guideline No.483, 2016. Test Guideline No. 483: Mammalian Spermatogonial Chromosomal Aberration Test. OECD Guidelines for the Testing of Chemicals. https://doi.org/10.1787/9789264264847-en.
OECD Test Guidelines No. 407, 2008. Test Guidelines No 407: Repeated Dose 28-Day Oral Toxicity Study in Rodents. OECD Guidelines for the Testing of Chemicals.
OECD Test Guidelines No. 452, 2018. Test Guidelines No. 452: Chronic Toxicity Studies. OECD Guidelines for the Testing of Chemicals. https://doi.org/10.1787/9789264071209-en.
OECD Work plan, 2025. Work plan for the OECD Test Guidelines Programme (TGP). https://www.google.com/url?sa=t&source=web&cd=&ved=2ahUKEwjjsuih7ouRAxV1gP0HHczFC6EQFnoECBUQAQ&url=https%3A%2F%2Fwww.oecd.org%2Fcontent%2Fdam%2Foecd%2Fen%2Ftopics%2Fpolicy-sub-issues%2Ftesting-of-chemicals%2Fwork-plan-test-guidelines.pdf&usg=AOvVaw2nBhOpVXldyFQ3Jj6wOhlx&opi=89978449.
Oku, Y., Madia, F., Lau, P., Paparella, M., McGovern, T., Luijten, M., Jacobs, M.N., 2022. Analyses of transcriptomics cell signalling for pre-screening applications in the integrated approach for testing and assessment of non-genotoxic carcinogens. Int. J. Mol. Sci. https://doi.org/10.3390/ijms232112718.
PARC, 2023. Partnership for the Assessment of Risks from Chemicals Deliverable D2.3 PARCroute roadmap1 WP 2 – T2.2. https://www.eu-parc.eu/sites/default/files/2023-10/PARC_D2.3.pdf.
Patlewicz, G., Judson, R., Paul Friedman, K., Wetmore, B.A., DeVito, M.J., Harrill, J.A., Carstens, K.E., Houck, K.A., Wambaugh, J.F., Padilla, S., Britton, K.N., Shafer, T.J., Degitz, S., Nyffeler, J., Kreutz, A., Richard, A.M., Williams, A.J., Coutros, K., Hornung, M.W., Cowden, J., Everett, L.J., Willis, C.M., Smeltz, M.G., Clifton, M.S., Feshuk, M., Wall, J.T., Sayre, R.R., Brown, J., Thomas, R.S., 2025. Insights derived from testing a library of per- and polyfluoroalkyl substances (PFAS) in a battery of new approach methods (NAMs). J. Toxicol. Environ. Health, Part B 1–69. https://doi.org/10.1080/10937404.2025.2521615.
Pfuhler, S., Downs, T.R., Hewitt, N.J., Hoffmann, S., Mun, G.C., Ouedraogo, G., Roy, S., Curren, R.D., Aardema, M.J., 2021. Validation of the 3D reconstructed human skin micronucleus (RSMN) assay: an animal-free alternative for following-up positive results from standard *in vitro* genotoxicity assays. Mutagenesis 36, 1–17. https://doi.org/10.1093/mutage/geaa035.
Rees, B.J., Tate, M., Lynch, A.M., Thornton, C.A., Jenkins, G.J., Walmsley, R.M., Johnson, G.E., 2017. Development of an in vitro PIG-A gene mutation assay in human cells. Mutagenesis 32, 283–297. https://doi.org/10.1093/mutage/gew059.
Regulation (EC) 1935/2004, 2004. Food Contact Materials - Regulation (EC) 1935/2004 European Implementation Assessment. https://doi.org/10.2861/162638.
Regulation (EC) No 1223/2009, 2009. Regulation (EC) No 1223/2009 of the European Parliament and of the Council of 30 November 2009 on cosmetic products (recast) (Text with EEA relevance).
Regulation (EC) No 1272/2008, 2008. Regulation (EC) No 1272/2008 OF THE EUROPEAN PARLIAMENT AND OF THE COUNCIL of 16 December 2008 on classification, labelling and packaging of substances and mixtures, amending and repealing Directives 67/548/EEC and 1999/45/EC, and amending Regulation (EC) No 1907/2006.
Regulation (EC) No 1332/2008, 2008. Regulation (EC) No 1332/2008 of the European Parliament and of the Council of 16 December 2008 on food enzymes and amending Council Directive 83/417/EEC, Council Regulation (EC) No 1493/1999, Directive 2000/13/EC, Council Directive 2001/112/EC and Regulation (EC) No 258/97 (Text with EEA relevance).
Regulation (EC) No 1333/2008, 2008. Regulation (EC) No 1333/2008 of the European Parliament and of the Council of 16 December 2008 on food additives (Text with EEA relevance).
Regulation (EC) No 1334/2008, 2008. Regulation (EC) No 1334/2008 of the European Parliament and of the Council of 16 December 2008 on flavourings and certain food ingredients with flavouring properties for use in and on foods and amending Council Regulation (EEC) No 1601/91, Regulations (EC) No 2232/96 and (EC) No 110/2008 and Directive 2000/13/EC (Text with EEA relevance).
Regulation (EC) No 1907/2006, 2006. Regulation (EC) No 1907/2006 of the European Parliament and of the Council of 18 December 2006 concerning the Registration, Evaluation, Authorisation and Restriction of Chemicals (REACH), establishing a European Chemicals Agency, amending Directive 1999/45/EC and repealing Council Regulation (EEC) No 793/93 and Commission Regulation (EC) No 1488/94 as well as Council Directive 76/769/EEC and Commission Directives 91/155/EEC, 93/67/EEC, 93/105/EC and 2000/21/EC. Regulation.
Regulation (EU) 2015/2283, 2015. Regulation (EU) 2015/2283 of the European Parliament and of the Council and repealing Regulation (EC) No 258/97 of the European Parliament and of the Council and Commission Regulation (EC) No 1852/2001 (Text with EEA relevance).
Regulation (EU) 2022/477, 2022. Commission Regulation (EU) 2022/477 of 24 March 2022 Amending Annexes VI to X to Regulation (EC) No 1907/2006 of the European Parliament and of the Council concerning the Registration, Evaluation, Authorisation and Restriction of Chemicals (REACH).
Regulation (EU) No 283/2013, 2013. Commission Regulation (EU) No 283/2013 of 1 March 2013 setting out the data requirements for active substances, in accordance with Regulation (EC) of the European Parliament and of the Council concerning the placing of plant protection products on the market Text with EEA relevance.
Reisinger, K., Dony, E., Wolf, T., Maul, K., 2019. Hen's Egg Test for Micronucleus Induction (HET-MN). pp. 195–208. https://doi.org/10.1007/978-1-4939-9646-9_10.
Rovida, C., Escher, S.E., Herzler, M., Bennekou, S.H., Kamp, H., Kroese, D.E., Maslankiewicz, L., Moné, M.J., Patlewicz, G., Sipes, N., van Aerts, L., White, A., Yamada, T., van de Water, B., 2021. NAM-Supported Read-Across: From Case Studies to Regulatory Guidance in Safety Assessment, in: Altex. ALTEX Edition, pp. 140–150. https://doi.org/10.14573/altex.2010062.
Russel, W.M.S., Burch, R.L., 1959. The Principles of Humane Experimental Technique. Methuen, London.
Schmeisser, S., Miccoli, A., von Bergen, M., Berggren, E., Braeuning, A., Busch, W., Desaintes, C., Gourmelon, A., Grafström, R., Harrill, J., Hartung, T., Herzler, M., Kass, G., Kleinstreuer, N., Leist, M., Luijten, M., Marx-Stoelting, P., Poetz, O., van Ravenzwaay, B., Roggeband, R., Rogiers, V., Roth, A., Sanders, P., Thomas, R.S., Marie Vinggaard, A., Vinken, M., van de Water, B., Luch, A., Tralau, T., 2023. New approach methodologies in human regulatory toxicology – not if, but how and when! Environ. Int. https://doi.org/10.1016/j.envint.2023.108082.
Schmitz-Spanke, S., 2019. Toxicogenomics – what added value do these approaches provide for carcinogen risk assessment? Environ Res. https://doi.org/10.1016/j.envres.2019.03.025.
Schuster, D.M., LeBlanc, D.P.M., Zhou, G., Meier, M.J., Dodge, A.E., White, P.A., Long, A.S., Williams, A., Hobbs, C., Diesing, A., Smith-Roe, S.L., Salk, J.J., Marchetti, F., Yauk, C.L., 2024. Dose-Related mutagenic and clastogenic effects of benzo[b]fluoranthene in mouse somatic tissues detected by duplex sequencing and the micronucleus assay. Environ. Sci. Technol. 58, 21450–21463. https://doi.org/10.1021/acs.est.4c07236.
Scientific Committee on Consumer Safety, SCCS 2023. Notes of Guidance for the Testing of Cosmetic Ingredients and their Safety Evaluation 12th revision, SCCS/1647/22.
Scientific Committee on Consumer Safety SCCS, 2025. OPINION on Hydroxypropyl p-phenylenediamine and its dihydrochloride salt (A165). https://doi.org/10.2875/9829351.
Smith, M.T., Guyton, K.Z., Kleinstreuer, N., Borrel, A., Cardenas, A., Chiu, W.A., Felsher, D.W., Gibbons, C.F., Goodson, W.H., Houck, K.A., Kane, A.B., La Merrill, M.A., Lebrec, H., Lowe, L., McHale, C.M., Minocherhomji, S., Rieswijk, L., Sandy, M.S., Sone, H., Wang, A., Zhang, L., Zeise, L., Fielden, M., 2020. The key characteristics of carcinogens: relationship to the hallmarks of cancer, relevant biomarkers, and assays to measure them. Cancer Epidemiol. Biomark. Prevent. https://doi.org/10.1158/1055-9965.EPI-19-1346.
Tarazona, J., Kass, G., Dorne, J., Liem, D., Paraskevopoulos, K., Kleiner, J., Heppner, C., Hugas, M., 2022. Theme (concept) paper - new approach methodologies. EFSA Support. Publ. 19. https://doi.org/10.2903/sp.efsa.2022.e200502.
Thienpont, A., Cho, E., Williams, A., Meier, M.J., Yauk, C.L., Rogiers, V., Vanhaecke, T., Mertens, B., 2024. Unlocking the power of transcriptomic biomarkers in qualitative and quantitative genotoxicity assessment of chemicals. Chem. Res. Toxicol. 37, 465–475. https://doi.org/10.1021/acs.chemrestox.3c00318.
US EPA, 2018. Strategic Plan to Promote the Development and Implementation of Alternative Test Methods Within the TSCA Program. Washington, DC. https://www.epa.gov/assessing-and-managing-chemicals-under-tsca/alternative-test-methods-and-strategies-reduce.
US EPA, 2021. List of Alternative Test Methods and Strategies (or New Approach Methodologies [NAMs]). Washington, DC. https://www.epa.gov/sites/default/files/2021-02/documents/nams_list_second_update_2-4-21_final.pdf.
van der Zalm, A.J., Barroso, J., Browne, P., Casey, W., Gordon, J., Henry, T.R., Kleinstreuer, N.C., Lowit, A.B., Perron, M., Clippinger, A.J., 2022. A framework for establishing scientific confidence in new approach methodologies. Arch. Toxicol. https://doi.org/10.1007/s00204-022-03365-4.
Westmoreland, C., Bender, H.J., Doe, J.E., Jacobs, M.N., Kass, G.E.N., Madia, F., Mahony, C., Manou, I., Maxwell, G., Prieto, P., Roggeband, R., Sobanski, T., Schütte, K., Worth, A.P., Zvonar, Z., Cronin, M.T.D., 2022. Use of new approach methodologies (NAMs). In: Regulatory Decisions for Chemical Safety: Report from an EPAA Deep Dive Workshop, in: Regulatory Toxicology and Pharmacology. Academic Press Inc. https://doi.org/10.1016/j.yrtph.2022.105261
White, P.A., Luijten, M., Mishima, M., Cox, J.A., Hanna, J.N., Maertens, R.M., Zwart, E.P., 2019. In vitro mammalian cell mutation assays based on transgenic reporters: a report of the international workshop on genotoxicity testing (IWGT). Mutat. Res.

Genet. Toxicol. Environ. Mutagen. https://doi.org/10.1016/j.mrgentox.2019.04.002.
Woutersen, M., Beekman, M., Pronk, M.E.J., Muller, A., de Knecht, J.A., Hakkert, B.C., 2019. Does REACH provide sufficient information to regulate mutagenic and carcinogenic substances? Hum. Ecol. Risk Assess. 25, 1996–2016. https://doi.org/10.1080/10807039.2018.1480351.
Zeiger, E., 2004. History and rationale of genetic toxicity testing. Environ. Mol. Mutagen 44, 363–371. https://doi.org/10.1002/em.20062.
Zhang, S., Parsons, B.L., Fitzgerald, D., Ashford, A., Auman, J.T., Chen, T., Dodge, A., Elhajouji, A., Pfaller, L., Harris, S., Higgins, J., Hobbs, C.A., Marchetti, F., Meier, M. J., Myers, M.B., Salk, J., Sahroui, R., Schuster, D., Settivari, R., Smith-Roe, S.L., Yauk, C.L., Yan, J., Williams, A., Chen, C.L., 2025. Transferability, reproducibility and sensitivity of mutation quantification by duplex sequencing. Environ. Mol. Mutagen. https://doi.org/10.1002/em.70020.
Zhu, H., 2016. Supporting read-across using biological data. ALTEX 167–182. https://doi.org/10.14573/altex.1601252.
Żuchowska, A., Baranowska, P., Flont, M., Brzózka, Z., Jastrzębska, E., 2024. Review: 3D cell models for organ-on-a-chip applications. Anal. Chim. Acta. https://doi.org/10.1016/j.aca.2024.342413.