Trends in Food Science & Technology 133 (2023) 277–290

Contents lists available at ScienceDirect

Trends in Food Science & Technology

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

Implementing New Approach Methodologies (NAMs) in food safety assessments: Strategic objectives and actions taken by the European Food Safety Authority

Irene Cattaneo *, Maria Chiara Astuto, Marco Binaglia, Yann Devos, Jean Lou C.M. Dorne, Ana Fernandez Agudo [1], Antonio Fernandez Dumont, Pilar Garcia-Vello, George E.N. Kass, Anna Lanzoni, A.K. Djien Liem, Martina Panzarea, Konstantinos Paraskevopulos, Juan Manuel Parra Morte, Jose V. Tarazona [2], Andrea Terron

European Food Safety Authority (EFSA), Via Carlo Magno 1/A, 43126, Parma, Italy

ARTICLE INFO

Handling Editor: Dr. J O'Brien

Keywords:
Animal-free testing
Preparedness
Risk assessment
Food
Feed
EFSA

ABSTRACT

Background: New Approach Methodologies (NAMs) comprise *in silico* and *in vitro* methods applied as alternative to animal testing. Even though NAMs are already fully implemented as research tools, their use in regulatory risk assessments (RA) is limited currently. To promote the regulatory uptake/acceptance of NAMs, a paradigm shift in risk assessment approaches, and a proper dialogue between risk assessors and risk managers is needed.

Scope and approach: Several reviews addressed the use of NAMs for chemical RA in generic terms, but without providing specific considerations on their use for food/feed safety assessments. Therefore, in this review, we give insights on the potential use of NAMs for regulatory purposes in the EU. We summarise relevant projects and activities on NAMs coordinated by the European Food Safety Authority (EFSA), which is the agency of the European Union that contributes to the safety of the European food and feed chain. The review informs on future developments on the use of NAMs in human health chemical RA, and touches on their use for the assessment of protein toxicity and allergenicity, as well as environmental risks.

Main findings and conclusions: Reducing animal testing and filling some RA gaps via NAMs is almost a reality. Moreover, there is a growing body of evidence confirming that the inclusion of mechanistic information improves risk assessments. EFSA's projects address the main challenge of using intermediate effects observed in non-animal models for safety assessments, especially those linked to adverse effects that are insufficiently covered or uncovered by animal apical endpoints.

1. Introduction

New Approach Methodologies (NAMs) represent 21st century scientific developments in toxicology, food safety, and related sciences, as they enable us to increase our understanding of the interaction between chemicals and biological systems exposed to such chemicals. NAMs include different non-animal-based testing approaches, covering *in silico* and *in vitro* methods, such as quantitative structure-activity relationship (QSAR) and read-across, computational analytical methods, such as physiologically-based kinetic (PBK) models and quantitative *in vitro in vivo* extrapolations (QIVIVE), "high throughput" omic technologies, advanced 3D models based on human cells, and microphysiological

* Corresponding author.
E-mail addresses: irene.cattaneo@ext.efsa.europa.eu (I. Cattaneo), mariachiara.astuto@efsa.europa.eu (M.C. Astuto), marco.binaglia@efsa.europa.eu (M. Binaglia), yann.devos@efsa.europa.eu (Y. Devos), jean-lou.dorne@efsa.europa.eu (J.L.C.M. Dorne), ana.fernandezagudo@echa.europa.eu (A. Fernandez Agudo), antonio.fernandezdumont@efsa.europa.eu (A. Fernandez Dumont), Pilar.GARCIAVELLO@ext.efsa.europa.eu (P. Garcia-Vello), georges.kass@efsa.europa.eu (G.E.N. Kass), anna.lanzoni@efsa.europa.eu (A. Lanzoni), djien.liem@efsa.europa.eu (A.K.D. Liem), martina.panzarea@efsa.europa.eu (M. Panzarea), konstantinos.paraskevopoulos@efsa.europa.eu (K. Paraskevopulos), juanmanuel.parramorte@efsa.europa.eu (J.M. Parra Morte), jtarazona@isciii.es (J.V. Tarazona), andrea.terron@efsa.europa.eu (A. Terron).
[1] Current address: European Chemical Agency, Telakkakatu 6, 00150 Helsinki, Finland.
[2] Current address: National Centre for Environmental Health, Instituto de Salud Carlos III, 28220 Madrid, Spain.

https://doi.org/10.1016/j.tifs.2023.02.006
Received 16 November 2022; Received in revised form 5 February 2023; Accepted 6 February 2023
Available online 8 February 2023

systems (Pistollato et al., 2021). The use of NAMs in the regulatory context (i.e., risk assessment of chemicals and food and feed products) has received growing attention, mostly because NAMs allow to achieve the 3R principles – replace, reduce and refine – in animal testing by minimising the use of animals, improve the mechanistic understanding of toxic effects of chemicals on biological systems, and ease the extrapolation of results gathered in animal studies to humans (Zuang et al., 2022). In 2021, the US Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM) published a status report providing recommendations on qualitative and quantitative metrics to monitor progress in the development and promotion of the use of NAMs (ICCVAM, 2021). This report has been complemented by specific individual work plans from some of the seventeen US Federal Regulatory and Research Agencies that make up this Committee (e.g., US EPA, 2021; US FDA, 2021). It reports on achievements made, and provides objectives for developing and applying NAMs, as well as long-term and short-term strategies to achieve such goals. In the European Union (EU), the Joint Research Centre (JRC) has recently published its 2022 report on research, development and validation activities, as well as initiatives that promote the uptake and use of non-animal methods and approaches in science and regulation (Zuang et al., 2022). In addition, the European Parliament has recently prepared a resolution urging the European Commission to accelerate the transition to innovation without the use of animals in research, regulatory testing and education. At an international level, the Organisation for Economic Co-operation and Development (OECD) takes actions to include NAMs when developing new guidelines for the testing of chemicals, and runs the Integrated Approaches to Testing and Assessment (IATA) Case Studies project to provide experts with a platform to share experience on the use of NAMs in a regulatory context. An additional example of international cooperation is the government-to-government Accelerating the Pace of Chemical Risk Assessment (APCRA) initiative whose aim is to promote collaboration and dialogue on the scientific and regulatory needs for the application and acceptance of NAMs in regulatory decision making (Kavlock et al., 2018).

Most efforts on the implementation of NAMs in legislation have focused on cosmetics and generic chemical legislations, such as the Regulation on Registration, Evaluation, Authorisation and Restriction of Chemicals (REACH) (Regulation (EC) No 1907/2006) and the Classification, Labelling and Packaging (CLP) Regulation (Regulation (EC) No 1272/2008) (Ball et al., 2022; Zuang et al., 2022). The application of NAMs in food safety has commonalities with other areas, but requires more specific and tailored considerations (Blaauboer et al., 2016; EFSA, 2014). Following recent reviews on the requirements for food safety assessments in the US (Karmaus et al., 2020) and chemicals in general in the EU (Pistollato et al., 2021; Zuang et al., 2022), this review complements the scene, with ongoing EU developments in food and feed safety focusing on activities led by the European Food Safety Authority (EFSA).

EFSA is a European agency funded by the European Union that provides independent scientific advice on food safety to the European Commission, the European Parliament and EU Member States (Devos et al., 2022a,b). With its food safety assessments, EFSA, together with EU Member States, contributes to protect human, animal, plant and environmental health and animal welfare in the food chain from farm to fork. Regulated products falling in EFSA's remit include plant protection products (PPP), food and feed additives, nutrients, novel foods, and genetically modified organisms (GMOs). EFSA is also responsible for assessing food contaminants and updating risk assessment methodologies according to scientific and technical progress. In this respect, EFSA, together with its Scientific Committee (SC), develops cross-cutting guidance, applicable to all sectors across the food chain, while the specific EFSA Panels and Units develop the detailed sectoral guidance in line with regulatory requirements. Since current guidance for chemical risk assessments still focuses on apical effects measured in animal studies, opportunities for using NAMs have been highlighted in recent EFSA risk assessment guidelines (EFSA SC 2021a,b,c). In addition, EFSA's 2027 Strategy (EFSA, 2021) advocates a more systematic reliance of NAMs in support of food and feed safety assessments (EFSA, 2022).

This review provides an overview of the current EU regulatory requirements for food and feed safety and explores how NAMs can be included to meet (at least in part) such requirements. The review describes key EFSA projects and reflects on future expectations that aim to follow a more realistic approach where NAMs are integrated with available animal and human data and other *in vivo* methods. While the main focus of this review is on human health chemical risk assessments, progress and expectations in two related areas, protein toxicity/allergenicity and environmental risk assessment, are also included.

2. Current regulatory requirements for chemicals relevant for food and feed in the EU

The EU has developed a large and complementary regulatory frame for chemical substances, managed by different actors. Two EU Regulations, REACH and CLP, handled by the European Chemicals Agency (ECHA), represent the central pillar. Specific regulations for drugs, handled by the European Medicines Agency (EMA), and for chemicals in food, handled by EFSA, complement the legal frame. The legal frame includes several interactions; for example, for pesticides EFSA is responsible for the risk assessment, while ECHA sets the hazard-based classification under CLP. EFSA is also responsible for assessing the risk of residues in food of biocides and veterinary medicines covered under Regulation (EC) No 396/2005, while other aspects are covered by ECHA and EMA, respectively. Cosmetics have received large attention, but as the focus is on dermal exposure, they will not be considered further in this review. There is also the possibility that a same substance has different uses covered under different regulations, as a result of which it may be assessed under various pieces of legislation, by various actors and at different points in time. To ensure that such safety assessments are done in a coordinated, transparent and to the extent possible synchronised manner, a "*one substance, one assessment*" approach is followed, as prescribed by the new EU Chemicals Strategy for Sustainability (EC, 2020b). In addition, substances regulated under one or several sectoral processes, including those unauthorised in the EU, may require assessment as contaminants in food and feed.

This complex regulatory framework may lead to different information/data requirements across sectoral legislations, and thus the sources and methods available for generating/gathering the evidence. Table 1 compares the central pillar of the framework created by REACH and CLP with the sectoral regulations covering the food and feed area.

EU legislation promotes the implementation of the 3R principles, while bans animal testing for cosmetics (Arnesdotter et al., 2021). In addition to legislative changes, the integration of NAMs can be facilitated through specific recommendations in guidance documents that define risk assessment processes and methodologies. As summarised in Table 1, the EU regulatory frame is unharmonized, focusing on sectorial needs. However, harmonisation efforts have been introduced by EFSA in the development of the risk assessment methods. The approach for pesticides is closer to REACH, with an extensive list of data requirements involving animal studies; in contrast, the requirements are not linked to tonnage bounds, and have a tiered structure, i.e., some results would trigger additional animal testing. Moreover, the initial risk assessment is carried out by a EU Member State as a "rapporteur" (RMS) and not by applicants manufacturing and importing chemicals. For other regulated products, a 90-day toxicity study and the genotoxicity battery constitute the central pillar, unless the assessment can be based on human data (Vrolijk et al., 2020; Knight et al., 2021). Results may trigger further testing. Non-testing approaches, in particular read-across or comparative assessments, are relatively frequently applied, but based on human or animal studies. The possibility for excluding concerns using *in vitro*

Table 1
EU regulatory framework for data requirements.

	Are data requirements fixed by regulation or by guidance?	Are data requirements drafted in a way that *in vivo* data are mandatory?	Is it possible to conclude safety based on NAMs?	May external partners submit additional studies during the RA process?
General chemicals: REACH	REACH Regulation Annexes VII-X.	Yes, unless exceptions.	Yes, for read-across but not based on *in vitro* data only.	Only for some REACH processes.
General chemicals: CLP	No data requirements, based on existing information.	Not formally, but current criteria are based on information extracted from animal studies.	Not applicable, there is no classification in case of lack of information.	Yes, for harmonised classification, during the RAC process.
Pesticides	Commission Regulation (EU) No 283/2013 of 1 March 2013 setting out the data requirements for active substances, in accordance with Regulation (EC) No 1107/2009 of the European Parliament and of the Council concerning the placing of plant protection products on the market.	Yes, but methods are updated on regular basis.	No.	Yes, systematic literature review is a data requirement. Data can be submitted to the Rapporteur MS and also during EFSA consultation on the Draft Assessment.
Food additives	Regulation (EC) No 1333/2008 of the European Parliament and of the Council of 16 December 2008 on food additives. Commission Regulation (EU) No 257/2010 of 25 March 2010 setting up a programme for the re-evaluation of approved food additives in accordance with Regulation (EC) No 1333/2008 of the European Parliament and of the Council on food additives.	No, the regulation only specifies that 'Food additives must be safe when used'. No, the regulation stipulates that the information should be as comprehensive as possible to allow EFSA the re-evaluation and should be submitted following to the extent possible the applicable guidance on submissions for food additive evaluations.	No, minimum requirement includes a 90-day study.	Yes, if triggered by EFSA; the stop-the-clock procedure is in place to allow for additional data to be submitted.
Feed additives	COMMISSION REGULATION (EC) No 429/2008, complemented with EFSA guidance.[a]	Yes, for tolerance studies and for safety assessments.	No, minimum requirement includes a 90-day study; there are exceptions based on no exposure or substances already approved in foodstuff.	Yes, if triggered by EFSA; the, stop-the-clock procedure is in place to allow for additional data to be submitted.
Nutrients	EFSA guidance.[b]	No, human information is the most relevant source. If information is insufficient, requirements for animal studies are linked to novel foods and food additives.	Yes, human data can be complemented with mechanistic understanding using NAMs.	Yes, if triggered by call for data or stop-the-clock procedures in case of applications.
Novel foods	Regulation (EU) 2015/2283 of the European Parliament and of the Council of 25 November 2015 on novel foods, amending Regulation (EU) No 1169/2011 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, complemented with EFSA guidance.[c]	No, the regulation only specifies that 'the food does not, on the basis of the scientific evidence available, pose a safety risk to human health.'	In principle, No, minimum requirement includes a 90-day study, and triggers may lead to additional studies. However, considerations on history of safe use can be made.	If triggered by EFSA, stop-the-clock procedure in place to allow for additional data to be submitted.
GMOs	Commission Implementing Regulation (EU) No 503/2013 of 3 April 2013 on applications for authorisation of genetically modified food and feed in accordance with Regulation (EC) No 1829/2003 of the European Parliament and of the Council and amending Commission Regulations (EC) No 641/2004 and (EC) No 1981/2006complemented with EFSA guidance.[d]	Yes, but only for studies on the whole food and feed in rodents (single events).	Yes, in some areas (molecular characterisation; toxicity and allergenicity of newly expressed proteins).	Yes. if triggered by EFSA, the stop-the-clock procedure is in place to allow for additional data to be submitted.
Food contaminants	Council Regulation (EEC) No 315/93 of 8 February 1993 laying down Community procedures for contaminants in food. Commission Regulation (EC) No 1881/2006 of 19 December 2006 setting maximum levels for certain contaminants in foodstuffs.	No.	Yes, in principle.	Yes.

Comparison of EU general chemicals framework with legislations on chemical risk assessments in the food and feed area.

[a] See this link for details: https://www.efsa.europa.eu/en/applications/feedadditives/regulationsandguidance.

[b] See this link for details: https://www.efsa.europa.eu/en/applications/nutrition/regulationsandguidance.

[c] See this link for details: https://www.efsa.europa.eu/en/applications/novel-food-traditional-food/regulationsandguidance.

[d] See this link for details: https://www.efsa.europa.eu/en/applications/gmo/regulationsandguidance.

methods is basically limited to genotoxicity, and for assessing metabolites or degradation products. More recently, mandatory *in vitro* testing for comparative metabolism has been included in the EU information requirements for pesticides, opening the door for the application of further approaches based on NAMs.

In contrast to safety assessments of products regulated for their application in food or feed, the hazard characterisation of chemical contaminants in the food chain is based on available data and, as in the case of the CLP Regulation, does not include an application presented to EFSA, but relies on scientific information that is in the public domain. The two main pieces of legislation laying down the principles for the management of chemical food contaminants in the EU do not include any prescriptions on the type of data to be considered for the hazard characterisation of the assessed substances. It should be noted that some food contaminants are regulated under REACH or other legislative acts, linked to mandatory data requirements. The EFSA CONTAM Panel (EFSA Panel on Contaminants in the Food Chain) relies on the general WHO's Principles and Methods for the Risk Assessment of Chemicals in Food (IPCS, 2009) for the risk assessment of food contaminants. This framework takes into account all the relevant toxicological information available, including studies on experimental animals, humans, cell- and other systems (Alexander et al., 2012). While the classical *in vivo* toxicity studies in experimental animals still represent the most common evidence stream for the assessment of food contaminants, there is an increasing trend to integrate *in vivo* data with human data (considering the increasing availability of human biomarker data and physiologically-based kinetic modelling), mechanistically based *in vitro* assays, high throughput screening studies, and *in silico* approaches such as QSARs.

3. Current and future uses of NAMs in EFSA's human health chemical risk assessments

Much of the current legislation or where applicable, EFSA guidance documents, require *in vivo* animal testing. Yet, this does not exclude the possibility to use toxicity testing and risk assessment tools that do not rely on *in vivo* studies. Currently, NAMs on their own are not sufficient to mimic the real complexity of humans, even though IATAs combining NAMs with available information could better predict possible effects on human health. As the information provided by NAMs is usually related to early and intermediate effects, Adverse Outcome Pathways (AOPs) are needed to connect an effect with adverse apical effects (Carusi et al., 2022). A clear advantage of such an approach is the use of human relevant test systems (Arnesdotter et al., 2021). The mechanistic knowledge also facilitates science-based extrapolations, covering interactions and individual susceptibilities. This enables to advance the current risk assessment paradigm, even if significant efforts to reach regulatory acceptance for NAMs are still needed (de Boer et al., 2020; Westmoreland et al., 2022).

At present, the inclusion of NAMs data in regulatory market approval dossiers submitted by applicants to EFSA remains limited. Progress on NAM-based OECD guidelines has focused on: (a) eye/skin irritation and skin sensitisation; (b) fish acute toxicity; and (c) endocrine disruption. Within EFSA's remit, points (a) and (b) are relevant for feed additives and pesticides, while point (c) is broadly relevant, but with special focus on pesticides due to hazard identification regulatory requirements, supporting a joint guidance with ECHA that already highlights the use of NAMs for regulatory purposes (ECHA/EFSA et al., 2018). New provisions under the Transparency Regulation, and specifically the notification of studies together with the request for pre-submission advice, may facilitate the use of NAMs in a regulatory context in the future.

In 2014 and 2018, EFSA reviewed the new methods available at that moment (EFSA, 2014) and the state of the art of using omics in risk assessments (EFSA et al., 2018), respectively. More recently, the increased reliance on NAMs has been integrated as a strategic objective in EFSA's 2027 Strategy (EFSA, 2021), which in turn drove the development of EFSA's conceptualisation on NAM-based risk assessments (EFSA, 2022).

The first approach proposed by EFSA for avoiding additional animal testing focuses on the valorisation of existing data, using tools such as QSAR, read-across or thresholds of toxicological concern (TTCs). In most cases, these approaches extrapolate apical toxicity endpoints, enabling alignment with the current risk assessment paradigm. Successful uses of QSAR for EFSA's purposes include the prediction of genotoxicity from direct-acting mutagens (Benigni et al., 2019), as well as for certain minor metabolites of plant protection products (EFSA PPR Panel, 2016). Likewise for smoke flavourings, a substance may be considered to raise no concern for genotoxicity without the need for experimental genotoxicity testing, provided that *in silico* predictions of the genotoxicity endpoints are available, and that these endpoints are negative in a combination of independent and multiple QSAR models (EFSA SC, 2021d). The grouping of chemicals into chemically related groups and the application of read-across have been central to the approaches used for flavourings and used on an *ad-hoc* basis for food contact materials and some data-poor impurities. Read-across has also been proposed in the risk assessment of plant protection products' metabolites (EFSA PPR Panel, 2016) and for the assessment of the combined exposure to multiple chemicals, especially when dealing with (sufficiently) similar mixtures and to support the grouping of chemicals into assessment groups (EFSA SC, 2019; EFSA SC, 2021d). More recently, EFSA has initiated the development of a guidance on the use of the read-across approach in food safety assessment. The aim of this work is to develop a pragmatic guidance providing a harmonised approach for the use of read-across in the different sectors pertinent to EFSA's remit, but also to assess the impact of NAM data to decrease the uncertainty associated with chemical-only based read-across. Completion is expected by 2024. Similarly, when exposure to a chemical is very low, the TTC approach remains a useful non-testing approach. The basis to the TTC approach is that it provides a set of chemical structure-based thresholds that identify exposure levels below which the probability of an adverse health effects is considered low. The strength of the TTC approach lies in the fact that it can be applied to chemicals where there is no or very little information on their toxicological potential (EFSA SC, 2019).

The second approach moves towards mechanistic understanding and includes scanning (such as high throughput screening, high content screening and omics) and advanced *in vitro* (e.g., multicellular, human induced pluripotent stem cells, organoids and organs-on-chip) methods, triggering the risk paradigm evolution. These approaches can be combined, e.g., through omics/mechanistic studies supported by read-across methods. Likewise, NAMs batteries can be combined into AOP/MoA-supported IATAs for hypothesis-driven Next Generation Risk Assessment (NGRA). The successful development of NAMs batteries based on mechanistic considerations is best illustrated by the projects on developmental neurotoxicity (DNT) and neurodegeneration. Moreover, technological developments are facilitating automation and data integration.

An area in which hypothesis-driven NGRA is already well advanced is the assessment of nanomaterials in food. EFSA's guidance on risk assessment of nanomaterials applied in the food and feed chain (EFSA SC, 2021a) recommends an exposure-driven approach and suggests mechanistic-based IATAs. This approach is already implemented by some applicants to integrate mechanistic *in vitro* information (EFSA NDA Panel, 2021).

4. EFSA's contributions for paving the way of NAMs in regulatory risk assessments

Over the last decade, EFSA has developed its chemical hazards database, OpenFoodTox, and explored the use of *in silico* tools such as QSAR models and biologically-based models such as physiologically-based (PB) toxicokinetic-dynamic (TKTD) models (EFSA, 2014; EFSA, 2018). Regarding *in vitro* methods, the first main project is linked to

pesticides neurodevelopmental toxicity and has been complemented with additional projects covering other areas and sectors. In 2022, EFSA has published the roadmap for NAMs (Escher et al., 2022) and started the implementation process launching three new projects.

Information on these activities is spread among a myriad of corporate documents, reports and scientific publications. The subsections below offer an up to date comprehensive summary.

4.1. OpenFoodTox database

Since its creation in 2002, EFSA has produced risk assessments for more than 5500 food and feed chemicals in over 2200 Scientific Opinions, Statements and Conclusions. The corresponding hazard data have been integrated into the OpenFoodTox database, which is open source and available for download (Bassan et al., 2018), and which can be used for data visualisation via EFSA MicroStrategy Tool; Dorne et al., 2017; Dorne et al., 2021).

Overall, the OpenFoodTox database contains the summary data for all substances evaluated by EFSA including substance characterisation, links to EFSA's outputs, applicable legislation. It has been structured using OECD harmonised templates (OHTs) for reporting chemical test summaries to optimise data sharing. Hazard data are available for plant protection products (PPP), food additives and flavourings, feed additives, food contact materials, vitamins, minerals, novel foods and food contaminants. For environmental risk assessment, the database provides data on key test species for pesticide active substances in terrestrial and aquatic organisms (e.g., fish, bees, insects, earth worms, algae etc), feed additives (e.g., coccidiostats), food and feed contaminants of anthropogenic origin and natural toxins produced by plants, fungi and other micro-organisms (Dorne et al., 2021; Astuto et al., 2022).

The OpenFoodTox database has provided a basis for the development of new QSAR models. Recent examples include QSAR models for predicting sub-chronic toxicity (90 days) of chemicals in rats using a large database (>1800 studies), acute toxicity of PPPs in bees, earthworms, trout and collembola and have been integrated within the open-source VEGA hub (e.g., VEGAHUB,; Benfenati et al., 2017; Carnesecchi et al., 2020; Gadaleta et al., 2021; Ghosh et al., 2020; Toropov et al., 2017). Structured data from OpenFoodTox have been integrated in existing modelling tools including OECD's QSAR Toolbox (as of version 4.4), which is an open source software (OECD, 2021a), and AMBIT-2, which is an industry funded (Cefic LRI) initiative for read-across/category formation (Saouter et al., 2018). OpenFoodTox data have also been used for the update of the threshold of toxicological concern (TTC) (Reilly et al., 2019) and the derivation of NAM-based point-of-departures (POD NAMs) based on high throughput predictions of bioactivity (Paul Friedman et al., 2020).

Current developments of OpenFoodTox 2.0 include the integration of new properties, such as physico-chemical properties, environmental fate, exposure data, toxicokinetic data as well the future integration of QSAR platforms such as VEGA or the OECD QSAR toolbox within the OpenFoodTox database to allow the prediction of physico-chemical, toxicokinetic or toxicological endpoints for data poor compounds. In this context, structured data covering different lines of evidence can be assembled, weighed and integrated using harmonised Weight of Evidence (WoE) approaches to support the use of NAMs in chemical risk assessment and the reduction of animal testing (Benfenati et al., 2017; EFSA SC, 2017b; Dorne et al., 2021). Finally, a number of QSAR models may be available for a given endpoint and predictions from such different models can be integrated within a WoE approach using a range of statistical methods which have also been recently reviewed (Benfenati et al., 2019).

4.2. Generic biologically-based models and TKPlate

Generic human physiologically-based kinetic (PBK) models and quantitative *in vitro in vivo* extrapolations (QIVIVE) models have recently been developed as well as pathway-related variability distributions in phase I (CYP2C9, CYP2C19, CYP2D6, CYP3A4, Paraoxonase 1, carboxyl esterases), phase II metabolism (UDP-gluruconysyl-transferases and glutathione-*s*-transferases) and transporters (P-glycoprotein, BCRP2, OATP, OAT) from bayesian meta-analysis of human kinetic data using available data from the scientific peer-reviewed literature on pharmaceuticals (Darney et al., 2019; 2020a,b; Kasteel et al., 2020; Lautz et al., 2020a,b; Quignot et al., 2021; Testai et al., 2021; Vichi et al., 2021). All individual kinetic data to generate such pathway-related variability are available in open access from the individual publications and in EFSA Zenodo, which is an open-access repository for datasets, documents and other research materials. In addition, isoform-specific metabolism data from *in vitro* studies in human cell systems have been generated for food chemicals including pesticides (i.e., triflumuron, chlorpyrifos, phosmet), natural toxins (e.g., microcystin variants, mycotoxins), food additives and polyphenols (i.e., resveratrol, tyrosol), as well as drugs (i.e., amiodarone) (Timoumi et al., 2019; Santori et al., 2020; Testai et al., 2021). The generic PBK and QIVIVE models combined with pathway-related variability and such isoform-specific *in vitro* data allowed to predict tissue residues in humans with satisfactory prediction results (Testai et al., 2021). In addition, human variability in toxicodynamic processes has also been explored for acetylcholinesterase activity, biomarkers of oxidative stress and other processes (Kasteel et al., 2020; Testai et al., 2021).

Other biologically-based models such as PBK models have been developed for farm animals (cattle, swine, sheep, chicken), fish (rainbow trout, zebra fish, fathead minnow, european stickleback) in the freeware R, calibrated and validated with case studies (Grech et al., 2017; Lautz et al., 2020a,b). For each model, open source databases reporting species-specific physiological data and R codes have also been published on EFSA knowledge junction and in the open access literature (Grech et al., 2019; Tebby et al., 2019; Lautz et al., 2020a,b). All these generic models have been implemented in a pilot platform: TKplate as a user-friendly graphical interface allowing modelling kinetic and dynamic processes for each species while reporting the results in an automated report (Bossier et al., 2020). This pilot platform will be published on EFSA's R4EU platform together with a user guide in 2023.

Finally, further development of such biologically-based models for humans and animal species are ongoing at EFSA to include other farm animal species (e.g., goat, turkey, salmon), test species (i.e., rat, rabbit, mice, dog) and subgroups of the human population (e.g., pregnant women, infants). When physiological data for animal species are lacking, modelling options include the use of allometric scaling and QSAR models as alternatives for modelling cross-species differences in kinetics and metabolic rates as well as sequence alignment to predict sensitivity to chemicals in animal species (Huang & Riviere, 2014; White et al., 2019; Trevaskis et al., 2020).

4.3. NAMs to fill data gaps in risk assessments

In 2019, EFSA started dedicated projects on NAMs to promote their implementation in regulatory risk assessment. Within EFSA's remit, information from animal studies is frequently available, but often with deficiencies that generate uncertainties when performing humans' and animals' health risk assessments. The EFSA NAMs projects specifically address identified data gaps and aim to investigate the possibility of filling them by generating new NAM-based information, instead of *in vivo* data.

To create real proof of concept cases, the projects implement a new targeted approach which consists of co-designing studies bringing together researchers and risk assessors. As a general practice, after the co-design effort in a dedicated workshop with experts in the field, EFSA finalises the technical specifications and launches an open call for a grant or procurement, with the ultimate goal of incorporating the generated data in EFSA risk assessments.

A list of the ongoing projects is provided below:

- EFSA Pilot Project on NAMs to explore the use of NAMs for addressing the neurotoxicity potential of the pesticide Tebufenpyrad. Results were published in January 2023 (Alimohammadi et al., 2023; Henri et al., 2023).
- EFSA Pilot Project on NAMs for the hazard assessment of nanofibers (GP/EFSA/SCER/2020/04).
- EFSA Pilot Project on the use of NAMs to explore the immunotoxicity of the contaminant PFAS (OC/EFSA/SCER/2021/13).
- EFSA Pilot Project on the use of NAMs to explore interspecies metabolic differences on essential oils as feed additives (OC/EFSA/SCER/2021/14).

All these pilot projects include the design and execution of new experimental NAM-based studies and the development of proposals for reporting the results of *in vitro* studies allowing the regulatory verification and the use of the results in chemical risk assessments. First experimental results of new NAM-based studies, together with proposals for reporting results of *in vitro* studies facilitating the regulatory use of NAMs tools and methods before the standardisation process, will start becoming available from 2023. Both EFSA Projects on NAMs for the hazard assessment of nanofibers and on the use of NAMs to explore the immunotoxicity of the contaminant PFAS have been selected as a Case Study led by EFSA under the international government-to-government initiative APCRA (Accelerating the Pace of Chemical Risk Assessment).

4.4. NAMs for developmental neurotoxicity testing

The current developmental neurotoxicity (DNT) testing paradigm is not fit-for-purpose for the assessment of many chemicals. Given the complexity of the developing nervous system and the availability of several non-animal methods to address DNT, integration of data from multiple studies is therefore necessary.

Over the last two decades, several scientific workshops and meetings have raised a concern that most chemicals released into the environment, to which children are potentially exposed, have not been assessed for DNT hazard and that the current testing, only based on *in vivo* apical endpoints, cannot compensate for the current gap in DNT testing. A scientific consensus has emerged that NAMs, and the integration of data derived from them, will facilitate the evaluation of chemicals regarding their potential to disrupt brain development. EFSA initiated a series of activities to address this need and, in collaboration with academic and regulatory organisations, proposed a DNT *in vitro* testing battery that is expected to be further harmonised in an international acceptance process. This will facilitate the use of the DNT *in vitro* testing battery for chemical screening and prioritisation, and hazard characterization. The fundamental underpinning scientific assumption paving the way for the new testing paradigm is that the assays included in the *in vitro* testing battery represent fundamental processes/key events (KE) in brain development and that the disruption of one or more of such processes/KEs could lead to a DNT adverse outcome. This resulted in the development and implementation of a battery of assays developed around the concept of designing phenotypic testing approaches for critical neurodevelopment processes. The approach was consolidated through a series of international meetings with scientists, regulators and stakeholders interested in DNT. The current assays included in the testing battery have been evaluated for their readiness. However, it is important to note that additional *in vitro* assays, which are not included in the list yet, can provide useful data and be included in the battery in the future.

A critical step in the project was the publication of EFSA's external report on the establishment of an *a priori* protocol for the implementation and interpretation of an *in vitro* testing battery (DNT-IVB) for the assessment of developmental neurotoxicity (Masjosthusmann et al., 2020). In this project, a human cell-based DNT *in vitro* testing strategy was set up and data generated therefrom. One hundred nineteen chemicals were tested and additional complementary data added by the US Environmental Protection Agency (US EPA). These efforts aim to support the development of an OECD guidance document on the interpretation and use of DNT-IVB data in regulatory decisions that is expected to be finalised in 2023.

Although there is no intention to substitute the *in vivo* Test Guidelines at present, there are several regulatory relevant scenarios for which data from the DNT *in vitro* test battery could be applied to inform decision-making. These scenarios will be captured as case studies in the OECD guidance to illustrate the applicability of the DNT-IVB. Examples of regulatory scenarios will include (a) follow up testing of biological activity when predictive computational models (including outcome from QSAR analyses and read-across) of DNT identify potentially active compounds, (b) screening for prioritization of large numbers of chemicals that lack or have limited data on DNT, (c) screening of small numbers of structure/class specific chemicals and (d) single chemical hazard assessments related to Weight of Evidence (WoE) analysis as part of e.g., a DNT tiered approach when no DNT data exist or is inconclusive, or when concern arises from new data on alternative species or from the literature. Moreover, it is envisioned that the AOP will be the basis of organising data and developing IATA. In this context, EFSA's PPR Panel, developed two case studies using an AOP informed IATA (EFSA PPR Panel, 2021a). The iterative process of the IATA included data produced using DNT-IVB, which provides a mechanistic support in the regulatory process of DNT hazard identification and characterisation. Moreover, the detailed analysis performed in the context of the IATA case studies, suggest that the *in vitro* testing alone would have been sufficiently protective for DNT hazard.

4.5. Comparative in vitro metabolism

Investigation of both metabolism and elimination of a chemical entity in the different animal species tested in the toxicological studies is pivotal to increase confidence in the use of data extrapolated from animal tests. The use of data to support the assessment of biological fate and systemic exposure of a xenobiotic is widely recommended in regulatory guidance. However, there are few explicit requirements in the European chemicals legislation for the generation of toxicokinetic data (i.e., *in vitro*, *in vivo* measurements or computational predictions).

In the context of pesticide legislation, efforts were made to recognise the potential of NAMs in predicting toxicokinetics. Commission Regulation (EU) No. 283/2013, establishes that "comparative *in vitro* metabolism studies shall be performed on animal species (rat, mouse, dog, rabbit) to be used in pivotal studies and on human material in order to determine the relevance of toxicological animal data and to guide the interpretation of the findings and further define the *in vivo* testing strategy". The main aim of comparative *in vitro* metabolism studies on pesticide active substances is to evaluate whether all significant metabolites formed in the human *in vitro* test system are also present at comparable level in animal species tested in toxicological studies. This enables to assess whether the test species used for the toxicological assessment are protective enough to cover the potential toxicity of metabolites in humans.

The main limitation to use comparative *in vitro* metabolism studies for a regulated substance is that, currently, validated test methods are not available. In the context of the toxicological dataset submitted for the approval or renewal of pesticide active substance in the EU, these studies are either conducted using different experimental layouts or they are not provided in some cases. This may lead to high variability in the outcomes and consequently low confidence in the values used to predict the *in vivo* situation. Therefore, more standardised methods as well as specific test guidelines are needed to increase the quality, transparency and confidence in the interpretation of toxicokinetic results. This is foreseen as a pivotal step towards the increase regulatory acceptance and use of such methodologies to predict human relevance of toxicological studies.

EFSA's scientific opinion on the testing and interpretation of comparative *in vitro* metabolism studies (EFSA PPR Panel, 2021b)

provides a regulatory framework aimed to illustrate the testing strategy that should be applied to investigate interspecies comparative *in vitro* metabolism. Moreover, the opinion illustrates the minimum requirements that should be included in the testing protocol for the selected assays, and provides indications on how to interpret study results. The scientific opinion further reports future implications of this effort: (a) Km (Michaelis constant) and Vmax (Maximum velocity) data could be possibly evaluated in the comparative *in vitro* studies and may be useful for future application in toxicokinetic modelling; (b) improve the design of toxicological datasets gathered without or less animal testing; and (c) build generic PBK model to estimate the internal exposure to the parent compound and metabolites of concerns.

4.6. Artificial Intelligence to search, extract, harmonise, pre-validate and integrate NAMs data (AI4NAMs)

EFSA has recently launched the project "Exploring the Use of Artificial Intelligence (AI) for Extracting and Integrating Data obtained through New Approach Methodologies (NAMs) for Chemical Risk Assessment" (AI4NAMS). The main purpose of the project is to explore the use of AI-based tools and apply them to selected chemicals or chemical groups in EFSA's remit for searching, extracting, harmonising, pre-validating, and integrating data in AOP-like knowledge networks. The overarching goal of the project is to conduct an in-depth review of the available AI tools for the extraction, harmonisation and pre-validation and integration of NAMs-based data, followed by a real proof of concept approach testing and implementing combinations of chemicals and endpoints relevant for EFSA, that will then be integrated into EFSA's risk assessments.

As a first step, NAMs-related data searches from scientific sources through the use of AI tools should cover both structured data from databases and unstructured data from scientific literature. The AI-based/automated screening would benefit the implementation of structured and standardised reporting and a common ontology glossary, as suggested by the OECD Harmonised Templates (OHTs) and in particular OHT 201, for reporting of NAM study results in an internationally agreed format for mechanistic data. The harmonisation and pre-validation steps can be supported through the application of Risk of Bias Tools (RoB), validated critical appraisal tools (CAT) and cross-checking coherence among results from different studies and authors. The last step is the integration of retrieved NAMs data into AOPs-based knowledge networks, integrating information on molecular initiating events (MIE), and intermediate key events (KE), whose relationships are described by key event relationships (KER), that lead then to adverse outcomes (AO). The first tangible outcome is a review of available AI tools for searching, extracting and assessing NAMs data, to be published in 2023, followed by six case studies, chemical or endpoint centred, covering:

- Pyrethroids with a focus on Neurotoxicity;
- Phthalates with a focus on effects on reproductive health, metabolic health, (neuro) developmental health and the immune system;
- Bisphenols with a focus on effects on reproductive health, metabolic health, developmental health and nervous system;
- Dioxin and dioxin-like substances with a focus on (neuro-) developmental toxicity, reproductive toxicity, immunotoxicity;
- Endocrine-related hypothyroidism;
- (Cumulative) Liver toxicity.

4.7. EFSA's strategic NAMs roadmap for action

The recent amendments to the General Food Law introduced by the Transparency Regulation (Regulation (EU) 2019/1381) have prompted EFSA to integrate societal expectations for more transparency and openness in its risk assessment processes, and further invest in preparedness. Hence, future challenges must be anticipated to avoid the risk of becoming overtaken by new developments (Garcia-Vello et al., 2022; Devos et al., 2022a,b).

To this end, EFSA identifies scientific themes with relevant knowledge and/or data gaps or requiring dedicated efforts to translate research findings into regulatory science. The scientific themes are selected based on the priorities set in EFSA's 2027 Strategy (EFSA, 2021) as well as the objectives outlined in EU strategies such as the Farm to Fork Strategy (EC, 2020a) and the Chemical Strategy for Sustainability (EC, 2020b), while considering other international activities.

In this framework, EFSA initiated in 2021 the development of a strategic roadmap ("the NAMs roadmap") for action on the "NAMs scientific theme" (EFSA, 2022) for defining the EFSA priorities in the area of NAMs and outlining a multi-annual strategy which, in collaboration with other food safety actors, can facilitate the incorporation of NAM approaches in chemical food and feed risk assessments for human health and promote the use of mechanistic information for a paradigm shift. EFSA's vision, as outlined in its 2027 strategy and explored in the development of the NAMs roadmap is that by 2027, NAM-based integrated approaches will be the main approach used to address data gaps for human health food and feed risk assessments of chemicals thereby gradually reducing the need for animal studies in line with the "evolutionary approach" (Burgdorf et al., 2019). In the longer term, EFSA aims at transitioning to the "revolutionary approach" whereby mechanistic-based assessments using NAMs are the standard approaches in its regulatory framework, including not only the assessments for human health but also extending to animal health and the environment.

Work carried out in the context of the NAMs roadmap development through working with an external contractor (Escher et al., 2022) identified five different NAM research areas for which further scientific and regulatory efforts are needed:

1. Toxicodynamics (TD), making use of *in silico* tools and *in vitro* mechanistic and multi-omics data, high throughput screening, high content screening to identify Mode of Action (MoA) and inform AOPs;
2. Toxicokinetics (TK), using existing chemical data and modelling internal dosimetry, applying *in silico* tools, enhanced data models, TKTD modelling and QIVIVE, e.g., to be implemented in TK-plate;
3. Exposome data to inform exposure assessment, using epidemiological information and occupational or environmental human exposure including human inter-individual differences in metabolism and biomonitoring;
4. Susceptible human population, evolving the risk assessment paradigm through the integration of hazard and exposure drivers in mechanistically informed risk assessments for the identification of susceptible population groups;
5. Data implementation, templates and tools are needed to facilitate the implementation of NAM data and their reporting into risk assessment dossiers.

Considering the state of the art, conducting case studies of cross-cutting nature (with EFSA either leading, co-leading or simply collaborating with other relevant actors) specifically designed to build confidence into NAMs and thus improve the uptake and acceptance of such methodologies, is the recommended action for most identified NAM areas. EFSA aims to integrate the results of such studies to produce cross-cutting guidelines for the relevant EFSA domains and eventually enabling the definition of more specific (per sector) requirements. Other types of proposed actions include increasing the collaboration e.g., through the creation of an interactive platform and the development of a fit for purpose qualification system. This tool can be considered a type of "pre-validation system" (similar tools are used by other regulatory agencies in other sectors such as the EMA and the US FDA) that would allow NAM developers to submit proposals for "verification for regulatory use for specific uses" of e.g. new methods, tools or Standard Operating Procedures, in order to facilitate the regulatory use of results

from non-validated NAM methods and models.

Three specific projects covering data integration, TK and AOP development/transcriptomics will be launched in 2022–2023. Considering that the recent EFSA "Nano Guidances" (EFSA SC, 2021a,b) already include NGRA approaches, nanomaterials have been selected as the driver for data integration. The project NAMS4NANO on integration of NAMs results in chemical risk assessments focusing on case studies addressing nanoscale considerations launched on June 15, 2022, aims to demonstrate that NAM-based IATAs could be not just equal, but even better for covering nanoscale considerations than animal studies. The project's objectives cover all regulated products under the EFSA remit and nanocontaminants in food, focusing on nanoplastics. Regarding TK, the project ADME4NGRA on the implementation of the EFSA NAMs Roadmap through Advancing Toxicokinetic Knowledge in Chemical Risk Assessment, also launched in June 2022, support through case studies the development of advanced *in vitro/in silico* ADME models to be used for QIVIVE through PBK models as well as advanced *in silico* models and open-access databases to depict ADME processes. The outcome will be a set of guidance and tools on the use of integrative QIVIVE and PBK models in human risk assessment. Finally, EFSA contributions to AOP developments, including the linking of transcriptomics data to AOP/AOP network(s) in a qualitative and quantitative way, will be addressed through a co-creation process based on proposals from EU Member States covering all aspects relevant for EFSA risk assessments.

The development and implementation of this strategic NAMs roadmap will enable the development of cross-cutting guidance and enable EFSA to effectively contribute towards the efforts for harmonisation at the European (e.g., PARC) (EFSA, 2021) and international (e.g., OECD, APCRA) levels for the wider use of NAMs (US EPA; OECD, 2020).

5. NAMs in support of food and feed protein toxicity and allergenicity assessments

Proteins can cause adverse effects in humans and animals, via a variety of mechanisms and in a variety of settings (Dang & Van Damme, 2015; EFSA GMO Panel, 2017; EFSA GMO Panel, 2022; Lucas et al., 2018). Toxic proteins, which can be produced by animals, bacteria and fungi, have been identified throughout the plant kingdom. Proteins can also cause adverse immune reactions, including life-threatening conditions (e.g., anaphylactic reaction) and chronic pathologies (e.g., celiac disease). Evaluating adverse immune reactions to proteins (hereafter referred to as "allergenicity") is a very challenging aspect in the protein safety assessment.

In the EU, the safety to humans, animals and the environment of (novel) proteins is routinely evaluated in various areas, summarised in Table 1. Dedicated tools and methodologies are deployed to assess the risks associated to toxicity and allergenicity of novel proteins; these are largely adapted from the chemical risk assessment area and include *in vivo* toxicological studies, as well as *in-silico* investigations, such as (bio)-chemical similarity searches with known toxins or allergens (e.g., EFSA GMO Panel, 2011; 2017, 2022).

The current methodology for the toxicological assessment of (novel) proteins shows various shortcomings: (a) it is based on the chemical risk assessment paradigm and tools (including animal studies) that are not fully fitting the purpose, considering the nature and complexity of proteins as compared to small molecules (Fernandez et al., 2019); (b) it may be not fully supported by specific guidance documents (e.g. on bioinformatic searches for similarity to known protein toxins); and (c) it does not take into account scientific and technological developments in protein science and related high-quality publicly available information. At the same time, protein science has significantly evolved in the last decades and the resulting wide-breadth scientific knowledge is well-structured and largely available to the public. 'Gold standard' databases (knowledge bases) offering expert-curated information are regularly updated and are available to the scientific community, industry and regulators. These offer a wide range of opportunities, ranging from the prediction of toxic proteins present in an organism of interest, to the provision of insights on molecular mechanism of action, to information on 'molecular signatures' relevant for toxicity (Negi et al., 2017; Palazzolo et al., 2020). Similarly, *in vitro* tools and strategies are significantly evolving, for example the behaviour and the fate of proteins can be explored in conditions mimicking environments relevant for the risk assessment, such as the digestive tract (Fernandez et al., 2019; EFSA GMO Panel, 2021).

Regarding allergenicity, no single test or parameter is currently available which provides sufficient predictive evidence. A weight-of-evidence approach is currently followed (Codex Alimentarius, 2003 – 2009; EFSA GMO Panel, 2011, 2017). In the current Codex Alimentarius paradigm, potential concerns on allergenicity are raised in cases such as: (a) reasonable evidence of IgE mediated oral, respiratory or contact allergy or non-IgE allergy is available on the source of the introduced protein or on the protein itself; (b) a newly expressed protein has sequence similarities to known allergens higher than 35%; and/or (c) highly stable proteins leading to resistant fragments following the classical pepsin resistance are identified. These principles were framed in in the late 90's; and have not been updated with current knowledge. EFSA's Panel on Genetically Modified Organisms (GMO Panel) has recently adopted a scientific opinion highlighting the need to review and clarify the main purpose of the allergenicity risk assessment overall and the vital role it plays in protecting consumers' health with existing food allergies and assessing the potential for foods and feeds to cause new allergies (EFSA GMO Panel, 2022).

NAM tools to support the toxicological and allergenicity assessment of (novel) proteins, potentially integrating and/or replacing *in vivo* studies are currently under evaluation by EFSA in the context of GMO risk assessments. The ultimate target is streamlining, modernising and strengthening the safety assessment of proteins; opportunities are summarised below.

5.1. NAMs developments in the field of protein toxicity

Three paths are under investigation: (a) *in silico* toxicity prediction; (b) *in vitro* toxicity testing; and (c) protein fate investigation during food and feed processing.

a) *In silico* bioinformatic searches are routinely conducted to investigate the similarity of proteins newly expressed in GMOs to known toxins (EFSA GMO Panel, 2011; Commission Regulation (EU) 503/2013). These searches are primarily based on protein sequence similarity analysis, and often make use of proprietary databases. There is no specific EFSA guidance supporting the execution of such searches, and their interpretation. A recent study commissioned by EFSA (Palazzolo et al., 2020) provided an up to date overview of publicly available high-quality knowledgebases (KBs) and, via an *in silico* pipeline, gathered from these a dataset of known toxic proteins, with information on their structure, function, mode of action and relevant toxicological information. EFSA recognises the opportunity to progress further in this field, and develop fit for purpose *in silico* methodologies to predict toxicity of novel proteins (see below). Noteworthy, possible enhancement in this field is also under the attention of other regulatory bodies, and industry (e.g., Bauman et al., 2022).
b) Predicting the function (toxicity) of a protein from its sequence alone is one of the long-lasting challenges in modern bioinformatics. Dozens of different tools were proposed in the last decades, as shown by the Critical Assessment of protein Function Annotation algorithms (CAFA) challenge now in its 4th edition (CAFA, 2020). Typically, these tools feature a system able to "learn" classification rules from a training dataset using statistics, machine learning, neural networks, deep learning or other approaches. The training dataset is most commonly a collection of known (experimentally validated) toxins, which is assembled from a public repository (such as UniProt, 2020).

Further work is needed to develop approaches based on machine learning and explore their performance compared to the so-called 'baseline methods', e.g., a simple BLAST search. Nevertheless, 'baseline methods' are only effective when a good reference is present and they lack the flexibility to 'learn' rules for *in silico* prediction which can limit their use. Recently, the University of Milan has been mandated by EFSA to develop *in silico* methodologies to predict the toxicity of novel proteins in the context of food and feed risk assessment. The identified methodologies can constitute preparatory work for the future development of a pipeline, architecture and software.

In vitro tools may strengthen the assessment of novel proteins introducing mechanistic information and become alternatives to animal models. Toxins act through different mechanisms and modes of action, affecting different organs and tissues. Gene Ontology (Gene Ontology, 2022) makes use of information on function and classifies toxins based on their molecular mechanism of toxicity (e.g., ion channel inhibition activity, metal ion binding), according to the triggered biological process (e.g., haemolysis, lipid catabolic process, defence response) or considering the cellular component affected. Information on toxin function/target tissue can serve as the basis to identify *in vitro* tools suitable to investigate protein toxicity. EFSA has recently launched a call contributing to further develop this opportunity.

c) The fate of proteins (e.g., protein degradation, protein denaturation) during the technological processing of food and feed can have an impact on their safety, and thus requires further consideration in safety assessments. For example, more efficient means to extract proteins from raw plant materials are under development. By processing less, the functional properties/quality of ingredients are maintained better (e.g., retention of fibre, micronutrients and natural microstructure). However, the lower degree of processing also implies that more residues and antinutritional factors may remain in foods, requiring further consideration in food safety assessments (Devos et al., 2022b). Furthermore, the fate of the protein in the gastrointestinal tract is considered an important element in the safety assessment of proteins. A refined *in vitro* protein digestion test that considers gastric and intestinal conditions clarifies the readout requirements, focuses on persistence/transience and abundance of stable fragments, and identifies cut-off values for the assessment has been proposed by EFSA (EFSA GMO Panel, 2017; 2022; Fernandez et al., 2019, 2021).

5.2. NAMs developments in the field of protein allergenicity

Proteins are large and complex biopolymers requiring specific considerations for immune-mediated adverse reactions (Fernandez et al., 2019). The EFSA GMO Panel has recently adopted a scientific opinion on development needs for the allergenicity assessment and protein safety assessment of food and feed products derived from biotechnology (EFSA GMO Panel, 2022).

Current Codex Alimentarius guidelines date back from 2003 and experience gained and new developments in the field call for modernization. Opportunities offered by NAMs include standardization on the use of available knowledge on the source of the gene and the protein itself, and modernization of *in silico* tools; and integration of *in vitro* methods informing on protein stability and digestion. This should trigger guidance update providing clarity on the use of the overall weight of evidence approach.

The pace of innovation will increasingly challenge the allergenicity risk assessment process. Setting clear safety objectives addressing new technologies are needed to ensure that allergenic risks are assessed in an appropriate, consistent and proportionate manner. A recent publication (EFSA GMO Panel, 2022) describes a roadmap to (re)define the allergenicity safety objectives and risk assessment where specific key questions for risk assessors and risk managers are posed, such as what is the purpose of the allergenicity risk assessment, what is to be assessed in the allergenicity assessment, what level of confidence is it needed for the predictions and what is considered an unacceptable or acceptable risk in the allergenicity risk assessment.

6. NAMs in support of regulatory environmental risk assessment (ERA)

EFSA is involved in the environmental risk assessment (ERA) of regulated products such as plant protection products, GMOs and feed additives, and can be consulted for the ERA of plant biostimulants and fertilisers. For ERA, the need to replace, refine and reduce animal testing focuses on vertebrates. EU legislation refers to cephalopods, but excludes non-independently feeding larval forms, triggering fish embryo testing (Sobanska et al., 2018). Moreover, ERA requires an extrapolation of potential adverse effects within species across different levels of biological organisation (that range from the molecular, individual, species to the population level), and between species/taxa. Since species operate in different receiving environments (in terms of pedo-climatic zones, agricultural systems, landscape structures, exposure to regulated products, and non-target organisms), such extrapolations also need to consider the heterogeneity and complexity of agro-ecosystems. Modelling and *in silico* tools hold great promise in this area (Astuto et al., 2022).

Significant progress has been achieved regarding the standardisation of *in vitro* methods for fish, including several OECD test guidelines adopted since 2013, such as TG-236: Fish Embryo Acute Toxicity (FET) Test (OECD, 2013) and the recent TG-249 Fish Cell Line Acute Toxicity - The RTgill-W1 cell line assay (OECD, 2021b). Considering that once an OECD guideline is available its regulatory implementation is clearly facilitated and that usually fish toxicity is included in a generic assessment for aquatic organisms, *in vitro* fish toxicity although not sufficient as replacement for *in vivo*, when complemented with additional information could be sufficient, in a weight of evidence approach, to cover fish testing in most occasions, with a main exception when fish are identified as the most susceptible group or there are concerns regarding long-term exposures.

The situation is different for birds, with limited developments regarding the development of *in vitro* methods, and also for mammals, were most development focuses on adding the *human* relevant component to the hazard assessment. Mechanistic understanding is relevant for both human health and environmental assessments, but for humans the focus is on effects of individuals, while for the ERA the focus is on population relevant effects. In fact, the advancement of NAMs for human health assessment may affect the information on mammalian toxicity currently used in ERA, and this require specific developments. NAMs may also provide solutions for covering amphibians and reptiles (EFSA PPR Panel, 2018).

Astuto et al. (2022) provides practical examples supporting the application of computational and modelling approaches for ERA of chemicals in real-world settings and different scenarios of resource availability. Future perspectives for addressing ERA in line with a "One Health" approach are also discussed. In this regard, the implementation of NAMs across different levels of biological organization (i.e., molecular, individual, population, landscape) represents a promising approach for enabling a mechanistic understanding of toxicity and the development of systems approach in ERA.

7. Discussion

While the number of alternative methods to animal testing is increasing continuously and many tools and frameworks are currently available, the uptake/acceptance of NAMs for regulatory risk assessments still needs significant efforts. A benefit associated with the use of NAMs is a better mechanistic understanding of the chemical-biological

interactions at different levels of biological organisation (Blaauboer et al., 2016; Karmaus et al., 2020). The development of an *in vitro* DNT battery represents an excellent example of using NAMs to inform decision-making (Blum et al., 2023). Immunotoxicity represents another promising area for which NAMs could reduce/replace animal testing. For those areas for which a well-established strategy integrating *in vitro* and *in vivo* methods is in place, mechanistic understanding can substantially reinforce the characterisation of risks and support decision-making, as exemplified in the area of genotoxicity (EFSA SC, 2021e).

NAMs can contribute to the current top-down approach (understanding the mechanisms associated to the observed apical effects). However, the most innovative part is to use NAMs as a bottom-up approach (Karmaus et al., 2020). Conceptually, the identification of the relevant effects at molecular or (sub)cellular levels, combined with validated AOPs and toxicokinetic data, could be sufficient for conducting safety assessments in certain cases, e.g., when the margin between human exposure and the levels triggering molecular and biological responses are sufficiently large to cover the additional uncertainty, and most frequently for supporting integrative approaches, e.g., NAMs to cover gaps observed in animal studies. The mechanistic information provided by NAMs results in more informative assessments. Two examples, which are very common in food safety, are mixture toxicity and the "confirmatory assessments" (confirmation of lack of toxicity or concern). Following EFSA's approach, the mixture assessment must be based on cumulative assessment groups, for which NAMs can provide the mechanistic information required to establish and validate such groups (EFSA SC, 2021d). In addition, NAMS are a powerful alternative for the analysis of relative potencies. Regarding confirmatory assessments, NAMs can be applied to confirm mechanistically that the point of departure selected from animal studies offers an adequate coverage of all relevant possible effects, i.e., identifying the molecular responses and intermediate events triggered by the chemical, and checking that the expected apical outcomes associated to these intermediate events are covered by the endpoints measured in the available animal studies; and indicating the additional endpoints to be considered if gaps are identified. For example, if intermediate events connected to neurotoxicity or immunotoxicity are triggered at low doses, the investigated apical endpoints should cover the expected neurological and immunological effects. Recent examples of EFSA proposals are: the IATA for nanoengineered nutrients and the proposal to cover Non-Monotonic Dose Responses (NMDRs) (EFSA SC, 2021a,c).

Despite investments and progress, a comprehensive assessment relying on NAMs exclusively would still take time. However, a number of case studies confirm that NAMs capacity for integrating mechanistic understanding, as part of the Weight of Evidence, is achieving the readiness needed for regulatory assessments. This is specifically promising in the food and feed area, where in most cases, some toxicological information is available for the substance under assessment or related substances. Such assessments are frequently based on relatively old studies, triggering data gaps that could be filled by NAMs, instead of additional animal testing.

Most scientific publications and toxicological studies are currently based on *in silico* and *in vitro* approaches. Animal studies often incorporate omics and other mechanistic endpoints. This creates a dichotomy between the information generated by the academic community through research projects, and that incorporated in safety assessments. Complementing the mandatory guideline studies with a literature search is a good practice, which is mandatory in some regulatory areas (Dibusz & Vejvodova, 2020). EFSA and other regulatory agencies have developed risk assessment guidelines (EFSA, 2019) and are applying by its own initiative systematic literature reviews (EFSA, 2010; EFSA NDA Panel, 2022). For searches covering the last 10–20 years, the most retrieved studies are non-guideline NAM-based. Machine learning may facilitate the assessment (Wang, Bouzembrak, Lansink, & van der Fels-Klerx, 2022; Waspe et al., 2021). Therefore, EFSA is exploring this option in the AI4NAMs project. As information focuses on intermediate effects a re-thinking of the risk assessment paradigm is needed, specifically on intermediate effects that are not covered by AOPs. EFSA is proposing evidence-based methods integrated in AOP approaches (Hoffmann et al., 2022).

It should be noted that *in silico* and *in vitro* tools are already part of "traditional" risk assessment approaches. In the area of genotoxicity, a well-designed battery of negative *in vitro* studies covering gene mutation and structural and numerical chromosomal damage is sufficient to address the genotoxic potential without conducting animal studies (EFSA SC, 2017a; EFSA SC, 2021e). Even more, there is also general consensus that if available, not yet formally validated studies should be incorporated in regulatory safety assessments such as results from the *in vitro* comet assay as part of a battery of results on *in vitro* genotoxicity. Another example is the assessment of metabolites and degradation products, where *in silico* and *in vitro* methods are getting more relevance, and even becoming mandatory, such as the comparative *in vitro* metabolism study for pesticides (EFSA PPR Panel, 2021b). Instead of using NAMs in isolation, they are applied in combination with the available animal studies on the parent compounds.

This specific background has prompted EFSA's case studies and projects, which have the above-mentioned need of refining risk assessment strategies in common, using NAMs for facilitating the integration of available information, focusing on identified data gaps and areas where animal testing has clear limitations for covering human relevant endpoints. Good quality NAM data, generated from co-designed studies among risk assessors and researchers, may solve inconclusive assessments, avoiding additional animal testing. This can be complemented with a fit-for-purpose qualification system, already developed and implemented in pharmaceutical areas and explored by EFSA for food and feed in the NAMS4NANO project.

Collaborative efforts are essential. Excellent examples are the OECD projects (e.g., on DNT), the case studies under APCRA, or the more recent NAMs Working Group under EFSA's ILMERAC (International Liaison Group on Methods for Risk Assessment of Chemicals in Food). In the food and feed area, assessments often deal with substances already in the market, as such or in mixtures, or related to substances previously assessed. This situation facilitates the use of read-across and available data. The vision is to integrate NAMs data with animal studies, epidemiology, biomonitoring, TK data, etc., through the design and validation of IATA based case-studies. The experience gained will be the basis for updating EFSA's cross-cutting and sectoral guidance documents. In this context, the EFSA Roadmap for NAMs represents a comprehensive overview of: (a) the areas in which further scientific and regulatory efforts are needed, (b) the available tools, and (c) EFSA's priorities (Escher et al., 2022). It is expected that the majority of requests for additional data in the risk assessment procedure will be based on IATAs using NAMs.

8. Conclusions

In the EU, the mandatory reliance on animal tests for regulatory food and feed safety assessments is sector specific. For example, nutrients and contaminants are assessed based on existing information, as a result of which NAMs and human data may be sufficient to conclude the risk assessment. By contrast, pesticide regulations require risk assessments to be based on a large set of *in vivo* tests. In other cases, such as food additives, novel foods and some applications related to nutrition, an intermediate approach is followed, which rely on genotoxicity testing and a 90-day sub-chronic toxicity study. NAMs (*in silico* studies) are used in the assessment of (new) proteins, horizontal to various regulated and non-regulated food/feed areas (e.g. GMO, novel foods), generally complementing animal studies or human data. Even though the possibility to include NAMs in a WoE approach is open to all of EFSA's risk assessments, the current level of implementation is limited mostly to screening purposes and complementary assessments (e.g. metabolites and

degradation products).

In line with its strategic objectives, EFSA is conducting and/or commissioning a set of projects to facilitate the integration of NAMs for regulatory risk assessment and ease their regulatory uptake/acceptance. Some of these projects are linked to limitations of animal models and generically referable to all food and feed safety areas falling in EFSA's remit, such as DNT, immunotoxicity, allergenicity or TKTD modelling supporting QIVIVE. Others are more specific to the food sector, such as mechanistic homeostatic understanding of nutrients and nutrient sources. For cases with inconclusive assessments, EFSA's ambition is to rely on NAM-based IATA instead of requiring animal studies. In addition, using the results of the ongoing projects, EFSA will develop a cross-cutting guidance on the inclusion of NAM-based results in its safety assessments. This guidance will cover both standardised studies, and non-guideline mechanistic studies in regulatory risk assessment. The inclusion of intermediate endpoints measured in non-animal models will trigger a shift in the risk assessment paradigm and complement a parallel discussion on the use of intermediate effects observed in animal studies, which is also very relevant, but out of the scope of this review.

Author contributions

Tarazona J.V.- Conceptualization; Cattaneo I. - Project administration; Tarazona J.V., Liem A.K.D. - Supervision; Tarazona J.V., Cattaneo I. - Visualization; 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., Lanzoni A., Panzarea M., Paraskevopulos K., Parra Morte J.M., Tarazona J.V., Terron A. - Writing - original draft; 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., Lanzoni A., Liem A.K.D., Panzarea M., Paraskevopulos K., Parra Morte J.M., Tarazona J.V., Terron A. - Writing - review & editing.

Employment

This article is published under the sole responsibility of the authors who are currently employed with the European Food Safety Authority (EFSA), and may not be considered as an EFSA scientific output. The positions and opinions (if any) presented in this article are those of the authors, and do not necessarily represent the views of EFSA.

Funding

No specific funding has been obtained for this publication.

Declaration of competing interest

The authors declare that they have no conflict of interest.

Data availability

No data was used for the research described in the article.

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