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Report on the European Partnership for Alternative Approaches to Animal Testing (EPAA) "New Approach Methodology (NAMs) User Forum", 30–31 October 2024, Helsinki, Finland

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ABSTRACT

The European Partnership for Alternative Approaches to Animal Testing (EPAA) held the "New Approach Methodology (NAMs) User Forum" at the European Chemicals Agency, Helsinki, Finland on 30–October 31, 2024. The User Forum brought together stakeholders from regulatory agencies, industry, non-governmental organisations (NGOs) and academia, as well as European Union competent authorities. Lessons learned from applying NAMs for regulatory use were provided by the European Food Safety Authority (EFSA) and European Chemicals Agency (ECHA). Progress in the development of the developmental and neurotoxicity *in vitro* battery (DNT IVB) and Alternative Safety Profiling Algorithm (ASPA) were described, as well as five case studies describing uses of NAMs for chemical safety assessment. The presentations confirmed progress in NAMs and, in particular, the value of tiered testing strategies to bring together different lines of evidence. Specifically, tiered testing strategies for non-animal information are organised into three tiers, which may be relevant to hazard, exposure and toxicokinetic information. Progress into, and the needs for improvement of, the tiered strategies were discussed with a particular focus on the types of NAMs (*in silico* and *in vitro*) that may be required at each tier and the how confidence may be assigned to making a decision.

1. Introduction and aims to the workshop

This report summarises the presentations from, and the main findings of, the European Partnership for Alternative Approaches to Animal Testing's (EPAA's) "*New Approach Methodology (NAMs) User Forum*". The workshop was a hybrid event held at the European Chemicals Agency (ECHA) in Helsinki, Finland and on-line over two days (30–October 31, 2024). It was attended by approximately 50 participants representing regulatory agencies, industry, non-governmental organisations (NGOs) and academia, as well as European Union (EU) competent authorities.

The aim of the User Forum was to explore further and share experiences with the use of New Approach Methodologies (NAMs) in chemical safety assessment. The particular focus of the meeting was the ability to make decisions with regard to chemical safety assessment from NAMs' data. This was mostly in the context of the use of NAMs as part of tiered testing strategies. This User Forum followed on the User Forum Kick-Off Workshop held on the 7–8 December 2023 (Cronin et al., 2025a). With regard to definitions of NAMs in the User Forum, a similar context can be applied as with the 2023 User Forum, where it was stated "*NAMs were considered in a broad sense to include in silico, in chemico and*

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Abbreviations

ADME	Absorption, Distribution, Metabolism and Excretion
AOP	Adverse Outcome Pathway
APCRA	Accelerating the Pace of Chemical Risk Assessment
ASPA	Alternative Safety Profiling Algorithm
BER	Bioactivity-Exposure Ratio
CEP	Chemical Effect Predictor
Cmax	Maximum Concentration in Plasma
CRO	Clinical Research Organisation
CS	Case Study
DA	Defined Approach
DART	Developmental and Reproductive Toxicity
DEG	Diethylene Glycol
DNT	Developmental Neurotoxicity
DNT IVB	Developmental Neurotoxicity *in vitro* Battery
ECHA	European Chemicals Agency
EFSA	European Food Safety Authority
EPAA	European Partnership for Alternative Approaches to Animal Testing
EU	European Union
IATA	Integrated Approaches to Testing and Assessment
ISTNET	International STakeholder NETwork
IVIVE	*In vitro-In vivo* Extrapolation
KIC	Knowledge and Innovation Community
LOAEL	Lowest Observed Adverse Effect Level
MMP	Matched Molecular Pair
MOIE	Margin of Internal Exposure
MOS	Margin of Safety
NAM	New Approach Methodology
NGO	Non-Governmental Organisation
NGRA	Next Generation Risk Assessment
OECD	Organisation for Economic Cooperation and Development
PBK	Physiologically-Based Kinetic
PoD	Point of Departure
QAF	QSAR Assessment Framework
(Q)SAR	(Quantitative) Structure-Activity Relationship
RA	Retinoic Acid
qSIM	Quantifying Suitability of Analogues
SB	Sodium Benzoate
SCCS	Scientific Committee on Consumer Safety
STOT-RE	Specific Target Organ Toxicity - Repeated Exposure
TTC	Threshold of Toxicological Concern
VPA	Valproic Acid

in vitro approaches, -omics approaches or omic-enhanced in vivo studies combined as Defined Approaches (DAs) and/or Integrated Approaches to Testing and Assessment (IATA)" (Cronin et al., 2025a). With regard to the 2024 User Forum, tiered testing strategies were discussed more than IATA.

The purpose of this workshop report to summarise the presentations and case studies (Section 2) and key learnings from the presentations and discussion (Section 3). It is not intended to provide detailed minutes of the User Forum.

2. Summary of the presentations and case studies at the User Forum

The User Forum heard a number of oral presentations (in person and hybrid). Section 2 summarises the content and main findings from the presentations, Section 2.1 is a summary of updates from the European Food Safety Authority (EFSA) and ECHA, Section 2.2 is a summary of two on-going initiatives, Section 2.3 summarises the case studies presented.

2.1. Updates from the European Food Safety Authority and European Chemicals Agency

2.1.1. European Food Safety Authority (EFSA): Lessons from applying NAMs for regulatory use

An overview of the lessons learned from the application of NAMs from EFSA was given by Dr Sofia Batista Leite (EFSA). It was noted that EFSA works within many legal frameworks on EU Food Law, which require different information requirements. The EFSA Strategy 2027; EFSA, 2021) highlights EFSA's commitment to the development and integration of new scientific developments focusing on NAM-based methods. To achieve their commitment, EFSA has published a roadmap for action on NAMs and risk assessment (Escher et al., 2022) that helped with the prioritisation of EFSA's projects on NAMs.

In order to assist the harmonisation of approaches to the different legislations, Knowledge and Innovation Communities (KICs) have been initiated. The KICs are intended to be dynamic knowledge sharing and generating platforms, which aggregate information and discussion. One KIC focusses on NAMs, the aim of which is to harmonise activities and identify stakeholders in NAMs. The KIC on NAMs is also mapping the ongoing activities in Europe to allow for aggregation of activities such as working groups and the development of guidance and new tools. Currently, the EFSA funded projects on NAMs can be grouped in four areas: cutting edge development and implementation; advancing methodologies for toxicokinetics and toxicodynamics; protein safety assessment; and hazard identification and characterisation. Two ongoing EFSA-funded projects were described. The Developmental Neurotoxicity *in vitro* Battery (DNT IVB) is described in detail in Section 2.2.1 and its use illustrated in Case Study 5. The EFSA NAMS4NANO Project aims to integrate NAMs chemical risk assessments utilising information from case studies addressing nanoscale considerations. The work is organised in three lots: i) the development of a qualification system for NAMs; ii) the development of NAM-based case studies to fill data gaps in nanomaterial risk assessment; and iii) case studies to improve methodology.

At the time of the meeting, an interim report had been published providing an initial proposal for a "qualification system" for NAMs in food and the food sector, using nanomaterial risk assessment as example (Haase et al., 2024). EFSA recognise the implementation of approaches for nanoparticles risk assessment is urgent. NAMs are seen to play a vital role in the risk assessment of nanoparticles and offer a unique opportunity to fill data gaps and address toxicity. Qualification is viewed as a promising tool to assist the regulatory implementation of NAMs.

EFSA is contributing to the European Commission's roadmap for phasing out animal testing and chemical safety assessments (Cronin et al., 2025b). It is acknowledged that its implementation into the different legislations would be different as some follow data requirements that include animal testing (e.g., pesticides) whilst others do not (e.g., novel foods). Even if animal methods are still listed in the respective guidance, EFSA's guidance is straightforward to update with new recommendations. EFSA has identified a number of short-term actions that can support this work: phasing out of the use of animal studies that have shown redundancy or lack of relevant information (ongoing work regarding the use of dog for agrochemical risk assessment); better use of NAMs for absorption, distribution, metabolism and excretion (ADME) assessment; and to encourage advocacy and guidance of NAMs in EFSA panels period.

In summary, EFSA has a number of commitments to NAMs including the avoidance of redundant animal studies; increasing the acceptance and confidence in the use of NAMs; development of strategies to speed

up the acceptance of NAMs; and to collaborate with key partners. It also provides a number of resources including its journal (https://www.efsa.europa.eu/en/publications/corporate), and databases (https://www.efsa.europa.eu/en/applications/pesticides) and (https://www.efsa.europa.eu/en/data-report/chemical-hazardsdatabase-openfoodtox).

2.1.2. European Chemicals Agency (ECHA): Experience in developing and applying NAMs for regulatory use

An update on the experience of developing and applying NAMs for regulatory purposes was provided by Dr Tomasz Sobanski (ECHA). A number of challenges to the regulatory acceptance of NAMs were outlined. This includes the limitations of the current regulatory frameworks which may not yet incorporate the new methods. This means there is still a heavy reliance on *in vivo* testing, whilst there is policy and societal pressure for animal-free testing. There is also a need to build capacity in a number of areas of the use of NAMs as well as developing them further in emerging topics, e.g., polymers, nanomaterials, endocrine disruption, immunotoxicity and neurotoxicity, amongst others.

A three-step process for the use of NAMs for animal-free hazard assessment was described. Step 1 is to identify and address critical needs to enable the use of NAMs. It is essential to demonstrate that NAMs have applicability for a particular purpose. Firstly, NAM batteries must be demonstrated to be efficient for hazard identification for a given regulatory endpoint. Secondly, NAMs' ability to characterise hazard based on molecular and or cellular changes, as opposed to the currently used observed adversity at a higher level, is required. Thirdly, there is a need for reliable extrapolation to convert doses tested in the NAMs to the external equivalent dose or exposure. Existing *in vitro-in vivo* extrapolation (IVIVE) approaches are currently an area of high uncertainty and more reliable approaches are required.

Step 2 is to demonstrate and apply NAMs under the current regulatory systems to build experience and gain confidence. ECHA is currently focusing efforts in a number of areas where there is a significant potential for reduction of animal use. i) Wider application of *in silico* approaches such as (quantitative) structure-activity relationships ((Q)SARs) for less complex endpoints. The QSAR Assessment Framework (QAF), recently released by the Organisation for Economic Cooperation and Development (OECD), enables the evaluation of individual predictions for regulatory acceptance and will lead to broader acceptance of QSARs. ii) Improving the use of read-across and the better integration of NAMs such as -omics as bridging evidence. iii) Establishing robust protocols for Physiologically-Based Kinetic (PBK) and toxicokinetic *in vitro* measurements and modelling, with a better understanding how to optimise them to cover broad chemical space. iv) The better integration of -omics data in regulatory methods and gaining confidence in their use.

Step 3 is to consider the requirements for a new regulatory framework that incorporates NAMs. This includes the fact that a new framework may not rely on the same endpoints as currently used; the need to gain knowledge of how to derive Points of Departure (PoDs) from molecular data; calibration of NAM assays and data with well-defined protection goals; revision of Classification, Labelling and Packaging (CLP) criteria to comply with NAM data; performance, throughput and cost from a business perspective; and improving the validation system for *in vitro* tests. It is appreciated that communication is a key aspect to the implementation of NAMs, ECHA publishes an annual report on key areas of regulatory challenge (ECHA, 2024).

ECHA is supporting a number of projects relating to the use of NAMs for regulatory purposes. In addition to those noted above, there are efforts to encourage the sharing of data and knowledge including the evolution of IUCLID. ECHA supports several of these initiatives to develop NAM-based tools for hazard identification and characterisation through external contracts. ECHA is also an active partner in the initiatives associated with the Accelerating the Pace of Chemical Risk Assessment (APCRA). The APCRA case studies have demonstrated that NAMs can be used for conservative priority setting (Paul Friedman et al., 2020) as well as investigating the integration of NAMs assays for the assessment of data poor chemicals (Paul Friedman et al., 2025). The APCRA case study has demonstrated that PoDs from NAMs are not predictive of *in vivo* endpoints but may provide an empirical PoD indication for data poor substances which could be used alongside other techniques such as the threshold of toxicological concern (TTC) and QSAR. Other research by APCRA partners has demonstrated that there may be considerable uncertainty in exposure estimates which are required for Bioactivity-Exposure Ratio (BER) calculation.

ECHA concluded by summarising the lessons that have been learned in their investigation of the use of NAMs. These are discussed in more detail in the context of the whole User Forum in Section 3, but include appreciation that one-to-one replacement of *in vivo* tests will not be possible, solutions will be based around a combination of data-driven and knowledge driven approaches, the new approaches must demonstrate performance within the remit of realistic expectations, and for systemic toxicity it is essential to include toxicokinetics and metabolic activation, with the understanding that for industrial chemicals the current uncertainties associated with toxicokinetics are high.

2.2. On-going initiatives in NAMs

Invited presentations were made regarding two approaches to developing and implementing NAMs and tiered testing strategies.

2.2.1. The Developmental Neurotoxicity in vitro battery (DNT IVB))

An *in vitro* battery for developmental neurotoxicity (DNT) was described by Prof Ellen Fritsche (SCAHT - Swiss Centre for Applied Human Toxicology and DNTOX GmbH). The growth in neurodevelopmental disorders is recognised and prioritised internationally, however, only around 200 chemicals, mostly pesticides, have been tested for DNT. Current *in vivo* testing (OECD TG426 and TG443) is resource intensive with known uncertainties (Paparella et al., 2020). There is therefore an incentive for further DNT testing of chemicals and specifically the regulatory uptake of NAMs focusing on fit-for-purpose methods with high throughput and human relevance. Since 2005 there has been much effort in preparing acceptable NAMs for DNT (Smirnova et al., 2024). A particular turning point was a workshop which formulated an International STakeholder NETwork (ISTNET) to create a DNT *in vitro* testing road map (Bal-Price et al., 2015). The ISTNET brought together relevant stakeholders to agree how to move the tests forward as well as formulating the biology that controls the development of the human brain. The overarching processes of human brain development that, if perturbed, may result in an adverse outcome were identified allowing for a battery of eight endpoints covered by 17 assays to be defined – the so-called DNT *in vitro* battery (DNT IVB) (Aschner et al., 2017; Fritsche et al., 2018; Masjosthusmann et al., 2020). On-going case studies are assisting in the understanding of the confidence and applicability of the DNT IVB. The initial findings of the case studies and recommendations for guidance and interpretation of the information from them have been published by the OECD (OECD, 2023). The development of the assays within the DNT IVB requires demonstration of scientific validity to gain confidence in their biological relevance and predictivity, with an example being Koch et al. (2022). Performance was assessed against reference chemicals to determine sensitivity and specificity (Carstens et al., 2022; Blum et al., 2023). An important aspect to make the DNT IVB useable has been to ensure lab-to-lab transfer. To enable this, the DNT IVB is currently being transferred to a contract research organisation (CRO) "DNTOX" (www.dntox.de). Transferring assays to a CRO is an important process to demonstrate transferability and make the assays available. NAM availability through CROs is an important step on the path to their regulatory acceptance (Blum et al., 2025). An example of an IATA case study utilising the DNT IVB was performed by EFSA for the re-evaluation of the pesticide deltamethrin applying Adverse Outcome Pathway (AOP)--based knowledge to demonstrate altered oligodendrocyte differentiation

and neuronal network function (Hernández-Jerez et al., 2021). There are a number of on-going activities to gain more confidence in the DNT IVB, namely further compound testing to optimise the battery, assay refinement and development, and further AOP/IATA development. The DNT IVB has also been considered in the context of endocrine disruption with the possibility to extend it to include other nuclear receptor-guided pathways beyond thyroid hormone disruption (Koch et al., 2025).

The development of the DNT IVB demonstrates the lifecycle for sustainable regulatory application of NAMs. This starts with the available test systems, a roadmap that has consensus from different stakeholders on how to move forward, the requirement for test methods that are ready for use, reliable and relevant as well as OECD input for guidance, and lastly a CRO that makes the test method(s) available for use and ultimately into regulation (Blum et al., 2025). Using the DNT IVB as a role model, the approach has been extended to developmental and reproductive toxicity (DART), with an ISTNET – DART Meeting setting out a roadmap for this highly complex endpoint (Fritsche et al., 2024).

2.2.2. The Alternative Safety Profiling Algorithm (ASPA)

The Alternative Safety Profiling Algorithm (ASPA) was presented by Dr Andrew White (Unilever). ASPA has been developed within the European Union ASPIS Cluster of three projects (ONTOX, PrecisionTox and RISK-HUNT3R). ASPA intends to act as a workflow to implement and operationalise Next Generation Risk Assessment (NGRA) to support chemical safety assessment. It builds on existing tiered strategies for chemical safety assessment, including, but not limited to, workflows from SEURAT-1, US EPA, RISK21, ICCS, OECD guidance and those summarised by Browne et al. (2024). It is being developed and supported by case studies within the APSIS cluster, e.g., see the summaries of Case Studies 4 and 5 (Sections 2.3.4 and 2.3.5) in this report as well as Leist et al. (2025).

ASPA intends to support the assessment of systemic chronic health effects ensuring the protection of human health. Further, it is designed to be applicable to different regulations, being feasible, flexible and extendable to apply mechanistically-based NAMs. The aim is to provide an understandable and interpretable output demonstrating a degree of confidence for the user. As such, the ASPA workflow serves as a guide for data generation and interpretation for the assessment of systemic toxicity. The ASPA workflow intends to define, through a tiered approach, which tools and methods to use and how to evaluate data including an assessment of uncertainty. The workflow also provides context for the data in terms of a hazard or risk assessment scenario with multiple exit points at which a decision can be made. The case studies within the RISK-HUNT3R project (see Case Studies 4 (Section 2.3.4) and 5 (Section 2.3.5)) are using existing data to evaluate the workflow and thus to demonstrate its applicability, and to build confidence in determining human relevant protective doses.

The ASPA is modular and based around a series of options, questions and provides guidance on how to make a decision. It has three distinct elements (or columns) to determine hazard, exposure, and ADME properties for particular safety assessment scenarios. The outputs from these three elements feed into the risk assessment. The structure of the three elements is intended to be efficient in terms of resources, starting where possible with *in silico* approaches, going forward to experimental NAM data to increase confidence for a particular purpose. The APSA can be visualised as a decision tree using building blocks and decision points as the main elements. Each of the building blocks and decision points has a unique identifier and will be provided a link to dedicated guidance. The tiers within each of the three elements of the ASPA are described in more detail in Section 3.2.1 in the light of other similar strategies and discussion within the User Forum.

ASPA and its implementation is ongoing and is considered to be a "living document". Whilst its implementation will be demonstrated through various case studies, a number of clear needs are already apparent. Amongst these are the requirement for the use of standard reporting formats, a greater and better appreciation of the role of uncertainty and how this informs the decision-making process and demonstration of how and where the APSA workflow could be applied within different regulatory contexts and for different industrial sectors. The workflow is currently being developed as a web-based tool and dashboard termed ASPA-*assist*.

2.3. Summary of the case studies

Five case studies (CS1-CS5), representing different endpoints and uses for NAMs were presented to the User Forum. The case studies were predominantly based on published material and are summarised, along with the relevant publication(s) below. The case studies were requested to provide specific comments, learnings and perspectives on topics such as the status of regulatory use of the described NAM, along with technical and performance aspects, as well as opportunities for future use and development. The learnings and insights from the case studies are compiled in Section 3.

2.3.1. Case study 1 - Using Next Generation Risk Assessment to make safety decisions for cosmetic ingredients under regulatory scrutiny

The objective of Case Study 1 (CS1), presented by Dr Sophie Cable (Unilever), was to demonstrate human safety assessment could be undertaken using NGRA. Specifically, NGRA for four case study chemicals was described, these were selected from the Scientific Committee on Consumer Safety (SCCS) priority list. NGRA was described as being exposure-led, hypothesis driven and designed to ensure the prevention of harm. *Ab initio* assessments were performed to benchmark the outputs from a NAM-based safety assessment. Previous case studies have illustrated the use of NGRA for coumarin (Baltazar et al., 2020) and benzophenone-4 (Baltazar et al., 2025). NGRA was based on a tiered framework incorporating *in vitro* data for hazard and exposure. Three tiers are applied, Tier 0 being problem formulation, *in silico* approaches and the application of TTC; Tier 1 being hazard and exposure (*in vitro*) data generation; and Tier 2 is the refinement of the assessment to increase decision certainty. Exit points exist within the three tiers if a safety decision can be made.

CS1 described in detail NGRA for climbazole in a use scenario of a preservative at 0.2 % in a face cream. The NGRA described in CS1 applies a systemic toolbox for early tier-testing. The toolbox is based on the determination of the PoD using transcriptomics and assays for cellular stress pathways for non-specific effects and *in vitro* pharmacological profiling assays for specific effects. *In silico* approaches such as QSARs and structural alerts provide leads to direct the specific testing. Exposure in the 0.2 % formulation was above TTC thresholds, and further information to inform risk assessment was required. Internal exposure was estimated through PBK modelling to provide a maximum concentration in plasma (Cmax). A BER distribution is calculated from the PoD and exposure estimate. The case study on climbazole was performed *ab initio*, on the assumption that there were no historic data on which to base a safety decision. *In silico* analysis indicated alerts for reproductive toxicity and carcinogenicity which informed the *in vitro* tests. NGRA demonstrated that it is possible to use NAM data from the systemic toolbox to make safety decisions protective of human health. *In silico* models such as PBK assessment could be over predictive and required refinement, this could be achieved with the inclusion of *in vitro* biokinetic data. With regard to determining hazard, the transcriptomics and cell stress assays covered most adverse effects, although there were concerns over the reliability of cellular effects and the metabolic competence in the minimal set of cell lines. More knowledge is required on the use of BER and associated variability and uncertainty in BER, with benchmarking of BER being a vital process to demonstrate its applicability.

2.3.2. Case study 2 - Improving efficiency and accuracy of NGRA for low toxicity substances – a case study with benzoic acid

The objective of Case Study 2 (CS2), presented by Dr Petra Kern (Procter and Gamble), was to demonstrate that a category could be created for substances with low toxicity to enable read-across to be performed to fill missing data gaps. Specifically, CS2 considered the quantitative assessment of the similarity of benzoic acid analogues using a variety of approaches. For the purposes of CS2, benzoic acid was the source substance with reliable toxicity data and a PoD of 500 mg/kg/d. Analogues were initially sought from the OECD QSAR Toolbox, however the profilers and similarity measures were not able to identify suitable analogues. Analogue identification was improved using a Matched Molecular Pair (MMP) approach that identifies molecules that differ only by a structural change at a single site or small portion of the molecule (Lester and Yan, 2021; Yan et al., 2023). It is well established that rating of analogues for read-across requires expert judgment (Lester et al., 2018). In order to optimise the process of analogue identification and reduce reliance on expert judgement, the "Quantifying Suitability of Analogues" (qSIM) approach has been developed (Lester et al., 2023). This incorporates information from metabolism, physico-chemical properties and structural alerts (coded as fingerprints) relating to reactivity and other toxicologically important properties.

In order to improve confidence in the use of read-across analogues, NAM data for *in vitro* metabolism were obtained. Confidence was also increased through the application of mechanistic NAM data, for instance existing ToxCast data as well as the generation of transcriptional and adapted pharmacological profiling (Burbank et al., 2024). A key aspect of the *in vitro* NAM testing was to set the highest concentration at a value consistent with exposure and the PoD in *in vivo* testing. Overall, CS2 demonstrated the need for better means to select analogues and that confidence could be achieved within a group of compounds associated with low toxicity when further, metabolically and mechanistically relevant, information was included.

2.3.3. Case study 3 - Read-across and New Approach Methodologies applied in a 10-step framework for cosmetic safety assessment – a case study with parabens

The objective of Case Study (CS3), presented by Dr Gladys Ouédraogo (L'Oréal), was to describe and illustrate the 10-step process for read-across in the context of NGRA. Full details of the 10-step process for read-across supported by NAMs have been published by Alexander-White et al. (2022).

The 10-step framework for read-across is organised into three tiers which are broadly associated with the ICCS principles for NGRA (Dent et al., 2018). Tier 0 includes steps 1–4 to identify the structure, supporting data and search for analogues. Assessment or estimate of exposure is key for Tier 0 from e.g., use scenarios, and can be defined in different ways which may be refined as further information is made available. If sufficient information is available at Tier 0 (or after Tier 1 or 2), a decision can be made. If insufficient information is available, the data collection proceeds to the next Tier. Tier 1 includes steps 5 and 6, which relate to ADME properties controlling bioavailability as well as data to inform on mode of action to better characterise the compounds. Tier 2 includes steps 7–10 and adds further refinement to the read-across through the collection of further information, e.g., through targeted use of NAMs testing or biokinetics, deriving a PoD, performing a Margin of Safety (MOS) evaluation and determining whether the level of confidence is acceptable. If sufficient information is not available, then the read-across will be ended.

The 10-step read-across framework was applied to the safety assessment for the use of propyl paraben as a preservative at 0.18 % in cosmetics. A full description of the propyl paraben case study is available in Ouedraogo et al. (2022). Calculation of systemic exposure, which also included aggregate exposure, was above TTC, therefore the read-across assessment was initiated. The MMP approach (see CS2 (Section 2.3.2)) was applied and three significant analogues were identified on the basis of structural, reactivity and metabolic similarity. A variety of physico-chemical and *in silico* information was obtained for the target and source compounds including those associated with reproductive toxicity and endocrine disruption. Comparator molecules, with known activity, were also included to improve understanding of the *in silico* assessments. On the first attempt at read-across the MOS was too low, thus the systemic bioavailability was refined by the inclusion of further information, for instance for metabolism from studies in primary human hepatocytes, as well as a comparison of skin vs liver metabolism. Existing ToxCast and newly generated transcriptomics data (Naciff et al., 2022) were utilised to support mode of action. The NAM data confirmed the relationship between activity and alkyl chain, also that propyl paraben had lower activity than source compounds such as butyl paraben. This allowed for a refinement of internal exposure and bioactivity in Tier 2. Subsequent Tier 2 testing allowed further refinement and the use of Margin of Internal Exposure (MoiE).

The 10-step read-across framework provided a number of learnings with regard to the use of NAM data to support analogue selection and justification, as well as making risk assessment decisions. Read-across based on chemical similarity alone has limitations. However, the similarities and differences in toxicokinetics and toxicodynamics were informed by appropriate NAM assays which strengthened potency assessment and internal exposure estimates. The safety assessment decision was assisted by the use of the MoIE. Overall, NAM data were shown to make read-across more robust and assessment of the confidence was valuable.

2.3.4. Case study 4 - Prioritisation and screening: Which testing scope is sufficient?

The objective of Case Study 4 (CS4), presented by Matthias Wehr (Fraunhofer Institute for Toxicology and Experimental Medicine), was to evaluate an *in vitro* NAM assay battery for specific target organ toxicity - repeated exposure (STOT-RE) classification, within the context of the APSA workflow (see Section 2.2.2). The basis for the case study was an appreciation of the large number of chemicals which are used commercially but for which there are few, or no, toxicity data (EEA, 2019). CS4 focused specifically on the hazard element (column) within ASPA. Hazard identification in ASPA focuses on two steps, the first being the use of high throughput NAM assays for, e.g., prioritisation and screening, the second being to follow up on possible toxicological alerts or to reduce uncertainties with further mechanistic evidence based around the testing of AOPs. For prioritisation and screening, a key focus of CS4 was to determine the minimum *in vitro* testing approach to provide sufficient information to make a decision. Previous work has demonstrated for *in vivo* data that the Lowest Observed (Adverse) Effect Level (LO(A)EL) is driven by a relatively small number of main targets (Batke et al., 2013), therefore NAMs would not necessarily be required to cover every aspect of physiology and toxicology. The hypothesis is that assays for general signs of toxicity and effects on the main target organs could be sufficient for prioritisation and screening. A training set of about 30 toxic (STOT-RE1) and 30 low toxicity (no effect up to 1000 mg/kg bw/d) compounds was established.

Compounds were assessed in two tiers, the first using existing *in silico* tools and *in vitro* data, the second tier with an enriched test battery covering a broad biological space. Approximately three quarters of the compounds had ToxCast data – these showed good specificity but poorer sensitivity, and there were difficulties with when there were fewer data. Other information was obtained for about two thirds of the compounds from Chemical Effect Predictor (CEP) from DISGENET. CEP showed good sensitivity but poorer specificity (excluding data poor compounds). Further information was obtained from *in silico* predictions and alerts for liver metabolism and clearance.

As a second part to CS4, the data were included into a scheme to assign compounds to levels of concern based on activity and potential systemic availability (as defined by Berggren and Worth, 2023). In summary, the Tier 1 information applied through an ASPA workflow

was able to distinguish toxic from low toxicity compounds. As STOT-RE does not take account of mechanism of action, broad testing methods may be suitable to obtain a protective PoD. However, it is difficult to compare existing *in vitro* data with each other and between compounds, therefore Tier 2 testing was applied to enrich the biological coverage and information. This involved broad mechanistic testing using seven unique human liver reporter cell lines covering 31 reporter gene (Calux) assays as well as seven stress pathways, phenomics cell painting from HepG2 cells and whole transcriptome analysis in three different cell systems. The concordance of the different assays was analysed and for more than 50 % of the compounds the assays agreed and were consistent with the *in vivo* data. CS4 is on-going and intends to demonstrate how to best combine the information from the assays and use machine learning to identify the most discriminative approaches. This will assist in the identification of the minimal *in vitro* testing required in ASPA.

2.3.5. Case study 5 - Developmental neurotoxicity Classification Labelling and Packaging case study

The objective of Case Study 5 (CS5), presented by Dr Ellen Hessel (RIVM), was to evaluate the potential of the use of NAMs for CLP purposes. The particular focus of CS5 was to identify the barriers, gaps and challenges of using of NAMs for CLP of DNT within the APSA workflow (refer to Section 2.2.2). In this context, it was confirmed that CS5 intended to provide information regarding the intrinsic properties of a substance that are associated with its potential to cause harm, as stipulated by the criteria for classification. Thus, the exposure element (column) of the ASPA workflow was not considered in CS5, however, the ADME element will be considered to investigate if the compounds will enter the brain and cross barriers during pregnancy. Under CLP, DNT is currently considered under reproductive toxicity, mainly related to functional deficiency. The precedent in using *in vitro* NAMs for CLP, in the context of local toxicity (skin and eye irritation and skin sensitisation), through the use of defined approaches, was noted.

CS5 utilised five compounds and collected information from different *in silico* and *in vitro* NAMs. Valproic acid (VPA) and retinoic acid (RA) were chosen as positive control compounds due to their strong association with human DNT effects, consistent with findings reported by Aschner et al. (2017). 2-Ethylhexanoic acid showed DNT effects in mice and was therefore also included as a positive control. Diethylene glycol (DEG) and sodium benzoate (SB) were selected as negative control reference compounds (Blum et al., 2023). Key questions were addressed regarding the sufficiency of existing AOPs and AOP networks relating to the complexity of the human brain, which is the basis for many of the currently used and proposed NAMs. Other challenges identified included understanding the information required from NAMs assays and when there is sufficient information, as well as whether adversity can be measured *in vitro* and considerations of assay performance. Knowledge of the processes of brain development is available and is the basis of the DNT IVB (see Section 2.2.1 and Fritsche et al., 2018), in addition there is an AOP network for DNT (Spînu et al., 2019). However, whether these summaries of the main process in brain development are sufficient to describe it is essential and not yet known.

The hazard identification of DNT was performed using *in silico* alerts and QSAR predictions (at Tier 0 of the ASPA workflow) for DNT itself and MIE predictions. Tier 1 assessment used a variety of high-throughput *in vitro* assays including CALUX, cell painting, etc. The information from Tiers 0 and 1 will be combined to identify potential alerts to direct testing at Tier 2 – this process remains a clear challenge and will be informed by CS5. Tier 2 allows for hazard characterisation and is utilising the DNT *in vitro* battery (Crofton and Mundy, 2021; OECD, 2023) as well as complex assays within the RISK-HUNT3R project. Other key challenges include whether the complex Tier 2 assays cover all DNT effects, e.g., those associated with neurobehaviour and covering the complexity of the developing brain, and how this will relate to CLP for DNT. Other NAMs are investigating the use of systems biology networks and an *in silico* model for the closure of the neural tube.

The ADME element of the ASPA workflow was also considered. Whilst a PoD is not required for CLP, information was sought on whether compounds cross barriers as well as their bioavailability and metabolism. At Tier 1, toxicokinetics information from the literature will be utilised, in addition to knowledge of bioavailability and PBK modelling to the foetus. Tier 2 testing will include *in vitro* measurement of placental and blood-brain barrier passage. CS5 is on-going and will investigate further the use of the data, which are the most significant assays and how decisions can be made for CLP of DNT.

3. Summary of the learnings and insights from the NAMs User Forum

The User Forum illustrated the ongoing development and application of NAMs for chemical safety assessment and discussed in detail some of the practical aspects required for acceptance and decision making. There was a clear commitment to implementation of NAMs in chemical safety assessment from the participants in the User Forum, specifically from ECHA and EFSA.

This section summarises not only the main findings from the presentations and case studies, but also the discussion and comments submitted online and elsewhere. Where appropriate, reference is made to specific presentations or case studies. This section is organised around the needs to implement NAMs as well as their practical implementation.

3.1. Learnings from the development of the DNT In vitro battery (IVB)

The development of the DNT IVB (Section 2.2.1) represents significant progress in the development of NAMs for complex endpoints. It acknowledges that there will be no one-to-one replacement for complex *in vivo* endpoints. A number of significant aspects of the development of the DNT IVB could form a blueprint and be applied for further endpoints. These are summarised briefly according to Blum et al. (2025):

- There is a benefit to gain international agreement of biology, e.g., by one, or more, expert workshops that bring together relevant stakeholders to map the biological and physiological processes involved. The purpose here is to identify the key biological processes that result in adversity such that NAMs can be identified for them.
- Once the key biological processes have been identified, there is a need to evaluate currently available assays that cover these processes and which are adequate for use, as well as identifying gaps where further developed assays are required. For the DNT IVB this was again achieved gaining agreement from experts and stakeholders.
- There is a need to demonstrate reliability and relevance of NAM assays selected and benchmark against known activities. An assessment of performance of the test battery – determining false negatives and positives – is required.
- To demonstrate performance, there is a need for a reference set of chemicals and test results that cover recognised modes/mechanisms of action, as well as acknowledging which pathways are missing.
- Case studies are highly beneficial to investigate the performance of a test battery and build confidence. These will allow for the demonstration of the application of the test battery. CS5 is an example of such a case study that is ongoing that applies a tiered approach including DNT IVB data and additional more specific assays to follow up on mechanistic leads, that measure the functionality of the nervous system, to investigate whether NAMs can be used for hazard identification in CLP.
- Once developed, any NAM or battery of NAMs needs to demonstrate transferability, for instance from laboratory-to-laboratory. Such transferability goes beyond the development of the NAM assay itself and will require funding. In the case of the DNT IVB, EFSA has provided funds for this transfer. The transferability was enabled by the foundation of a bespoke CRO, although more than a single organisation may be necessary.

- o A tiered approach, including one or more Defined Approaches, is useful to make the IVB even more applicable. CS5 is demonstrating the use of NAMs, including data from the DNT-IVB, for CLP purposes within the ASPA framework.
- o It is essential to identify and characterise uncertainties in a test battery. This has been achieved for the DNT IVB where uncertainties are known and can be addressed. It is important that uncertainties of NAMs, such as those identified for the DNT IVB, do not hamper their application.

3.2. Application of NAMs in tiered strategies

A variety of tiered testing strategies to implement NAMs for chemical safety assessment were described at the User Forum. These attempt to combine information to allow decisions to be made with regard to, e.g., hazard identification or risk assessment. The tiered testing strategies frequently described three, or more, tiers as described in Section 3.2.1. The organisation of the tiered testing strategies is designed to have decision points when sufficient confidence can be placed to make a specific decision. Fundamental questions, which are expanded upon below, were:

- Is the coverage provided by the cell lines protective?
- Do NAMs provide the same level of protection?
- What is the extent of the biological coverage of the NAMs applied in the Tiers of the testing strategies?
- What are the protection goals of a particular tiered testing strategy?

The User Forum heard specific examples and learnings with the use of tiered testing strategies, which are summarised below.

3.2.1. Tiered strategies, frameworks and approaches will be utilised for safety assessment: an increase in understanding of their use is required

Various examples of tiered frameworks were presented at the User Forum (e.g., CS1, ASPA). Whilst there are differences between the tiered frameworks, they have the same structure (Tiers 0–2 and decision points). The ASPA framework was described in detail with illustrative case studies (see Section 2.2.2)

There was broad consensus in how the Tiers in a framework are organised, as illustrated by the ASPA, DNT IVB and case studies:

- Tier 0 involves the problem formulation, collection of existing information and data, for instance on hazard and exposure. Techniques such as TTC may be applicable. *In silico* methods such as QSARs, structural alerts, read-across can provide pointers for effects to follow up at higher tiers (these can also be applied at Tier 1).
- Tier 1 generally comprises a broad set of general *in vitro* or molecular biological NAM assays.
- Tier 2 generally comprises more specific assays to follow up on mechanistic leads. This should increase confidence in the decision being made.

There was agreement that the application of NAMs in tiered strategies can be used to make safety decisions. Associated with this is a need to combine data-driven and knowledge driven (NAM) approaches with performance demonstrated, or benchmarked, against a reference test set. An example of the need for, and utility of, reference test sets was provided by the DNT IVB.

Decision points within and between Tiers are critical. Should sufficient confidence in the data be apparent, the decision can be made and testing stopped. If there is insufficient confidence, then further information is required, for instance by passing to the subsequent Tier. The User Forum agreed that there is a need for more information on when to go to a higher tier or exit the tiered strategy. An example could be the types of *in silico* or *in vitro* alerts that would trigger moving to a higher Tier.

Currently the definition of the scope of a protective NAM battery of tests (at Tier 1) is limited. In addition, how can tiered strategies, such as ASPA, be applied to different industrial sectors should be investigated. To achieve such goals, case studies were seen as being useful to demonstrate the utility of tiered testing strategies, as well as address the on-going questions such as decision points, sufficiency of information etc.

3.2.2. Consensus on which NAMs and tools to use in a tiered strategy

In the descriptions of tiered testing strategies (e.g., in the case studies) the User Forum was presented with a variety of types of NAMs for different endpoints and purposes. There was no attempt to reach agreement or consensus in the User Forum as to which are appropriate. There is a recognised challenge to make NAMs applicable across all legislations.

There was agreement that regardless of which NAMs are used, there should be consideration of whether they are relevant for the context of use and the issue(s) being addressed, protective, sensitive etc. To ensure NAMs within tiered strategies are protective, benchmarks for NAMs and the tiered strategies should be considered (analogous to the benchmarking of the NAMs themselves). As part of the benchmarking process, the conservatism in NAMs to enable a decision to be made should be considered. The implementation of NAMs should find a balance such that they are not overprotective.

There was also agreement for the need to identify commonalities, confidence and limitations (uncertainties) of NAMs for use in tiered strategies. It is likely that a number of NAMs will be applied, machine learning may be able to identify the optimum combination in terms of efficiency, i.e., minimum data required to make a decision (see CS4). There is a need to demonstrate a baseline set of NAM assays that, within which, if nothing was observed, then no adverse effects would be expected *in vivo*.

The biological coverage of NAMs is largely unknown and needs to be defined and described. It was acknowledged that NAMs cannot have universal coverage and for successful and appropriate application their applicability domain should be defined. Specifically, further knowledge is required on whether NAMs (e.g., transcriptomics and cell stress assays, e.g., CS1), cover most/all adverse effects. One suggestion to assess the utility of NAMs and tiered testing strategies was to consider repeated-dose toxicity where there are data for many chemicals with a broad coverage of chemical space.

Some other specific recommendations and needs were identified:

- There is a need for compound selection in tiered strategy that will cover relevant mechanisms.
- There is a requirement for better understanding of NAM data, with regard to their capability to identify adversity as opposed to (bio) activity or adaptation.
- There is a requirement, for instance at Tier 2, that the NAMs cover the complexity of the endpoint being modelled. As example is the DNT IVB which needs to cover the complexity of the brain to a sufficient level to identify adverse effects.
- The lack of consistent NAM data for the existing assays is perceived to be a problem. There is a need for consistent data and to be able to identify where more are required to fill data gaps (CS4).
- The maximum *in vitro* concentration to be tested (that may be used to demonstrate no activity) is not consistent and will require more consideration (CS4).
- The metabolic competence of NAMs is not known. Many are performed without a metabolic component and the significance of this should be considered, also whether this should be part of Tiers 1 or 2 of a tiered strategy.
- More information may come from the APCRA studies and EPAA Designathon in terms of how to refine the information that is available from NAMs.

3.2.3. ASPA – an Alternative Safety Profiling Algorithm

The APSA is an example of a tiered testing strategy to implement a NGRA workflow (Leist et al., 2025). Progress on the APSA is on-going with the purpose to enable various decisions for chemical safety assessment. ASPA builds up evidence as defined in Tier 0–2 (Section 3.2.1). There are three main elements (columns) to ASPA: hazard, ADME and exposure leading to risk assessment. These can be adapted to specific purposes, e.g., for CLP purposes, hazard identification is key and does not require exposure (CS5). It is designed to have a standard reporting approach.

The ASPA is designed with a number of decision/exit points. When there is sufficient confidence in the information, a decision may be made. The identification and characterisation of uncertainty is essential and vital aspect to make a decision – this should be documented adequately. ASPA is designed to reduce uncertainty within the tiered approach, allowing for a conservative assessment of hazard and exposure. An essential challenge is how to make a decision and when there is sufficient information – to answer this question needs the input of regulators and PARC. In addition, the ASPA is designed to be flexible, adaptable and updateable.

A number of ASPA case studies are being conducted in the RISK-HUNT3R Project. Case studies are valuable to demonstrate the ASPA, how it can be applied to make decisions and develop it further. It is intended that the ASPA will be provided with guidance and a digital version (ASPA-*assist*) to implement it. Other recommendations included evaluating the ASPA to determine which parts could be applicable for regulatory use and how to promote consensus building within the ASPA.

3.3. On-going needs identified for the implementation of NAMs

The User Forum recognised that there is still considerable development needed in some areas of NAMs. Various needs for the development of NAMs that have been previously stated through EPAA workshops (Westmoreland et al., 2022) and User Forums (Cronin et al., 2025a) are not repeated here. However, some clear additional needs were identified in the User Forum, particularly with regard to regulatory implementation. These are summarised below.

3.3.1. Appreciation of uncertainty in data and decision making

The appreciation of uncertainty in all aspects of the use of NAMs and their application in tiered testing strategies and NGRA is crucial. This is often a neglected and underdeveloped topic that requires further understanding. Specifically, there is a need to determine the acceptable level of uncertainty in NGRA, for instance with the use of NAMs in a tiered strategy. Assessment of uncertainty is recognised as being a vital component in the decision making process within strategies such as the ASPA framework.

Assessment and understanding of uncertainty is crucial for all the data inputs into chemical safety assessment. There was discussion in the User Forum regarding uncertainty in *in vivo* data. This is important because *in vivo* data are currently required under many legislations, as well as being the benchmark for the performance of many NAMs. It was acknowledged that uncertainty in *in vivo* data may be large and is often undefined.

The uncertainty associated with *in vitro* NAM data should be characterised. Given the possible high uncertainty in *in vivo* data, *in vitro* NAMs should not be expected to have lower uncertainty than the *in vivo* data. Currently there may also be high uncertainty in toxicokinetic data. High uncertainty in toxicokinetic data is not acceptable as it will propagate through the safety decision making process, e.g., as part of the BER. Various strategies to reduce uncertainty were presented, including the inclusion of NAMs data into ADME and exposure estimates (CS1) (see also Section 3.4).

3.3.2. There is a need to set goals and performance standards for NAMs

The importance of the validation of NAMs as part of their regulatory acceptance, and the challenges associated with that, are well acknowledged, for instance the discussion from a previous EPAA User Forum (Cronin et al., 2025a). However, the current User Forum acknowledged that a clear definition of success with regard to the use of NAMs is required. For instance, there could be an agreement of realistic goals and performance metrics for individual NAMs or groups of NAMs, such as specificity and balanced accuracy.

"Success criteria" for NAMs could be defined *a priori*. Once verified against these criteria, NAMs could be applied. This would support the easier development of tiered strategies and frameworks for chemical safety assessment. Clearly defined success criteria will allow the research community to understand what is required and expected when NAMs are being developed. There is also a need to benchmark the performance of NAMs/tiered strategies against the previous information requirements and decisions made. This should define and take account of the limitations of the new systems.

3.3.3. Further development of the Bioactivity-Exposure Ratio (BER)

The calculation of BER, or Margin of Exposure, is vital to apply NAMs in NGRA and within tiered testing strategies. This has been discussed previously in an EPAA User Forum (Cronin et al., 2025a). A variety of approaches to the application of BER were presented (e.g., CS1). However, no consensus was sought or reached in the User Forum as to how BER should be applied, the uncertainty in it and how it can be used to make a decision or be utilised in tiered testing strategy. The needs to benchmark BER to ensure it is protective, and better understand its uncertainty, were acknowledged.

3.4. NAMs to improve exposure assessment

The User Forum acknowledged that understanding exposure to chemicals is fundamental to the implementation of NAMs and application of tiered testing frameworks (e.g., CS1, CS3). There are a number of aspects to this relating to estimates of internal exposure, relevance of doses in NAMs assays through to aggregate exposure.

Exposure is fundamental for the application of NGRA, with several examples given in the User Forum (e.g., CS1, CS3). It is also one of the key elements within the ASPA. Further, knowledge of (internal and external) exposure is crucial to support the application of NAMs and tiered strategies for safety assessment. However, concerns were raised regarding the quality of the information relating to exposure and the possible high levels of uncertainties, e.g., in TK data (Section 3.3.1). As a fundamental part of NGRA, uncertainty in exposure assessment should be low, where possible.

It was observed that *in vitro* NAM data and information help improve exposure estimates and improve confidence. There is a definite need to reduce uncertainty in exposure assessment in NGRA. This may assist in refining the exposure estimates as there is progression from Tier 0 to 1 to 2. Key NAM data for improving confidence in PBK models include hepatic clearance, fraction unbound and blood-plasma data. PBK models were calibrated against human clinical data (CS1).

Overall, there is a need to determine the best use of exposure information in NGRA and gain greater certainty in exposure estimates. For systemic toxicity, all cases should incorporate toxicokinetic and/or ADME information. Various approaches using PBK modelling to determine exposure were presented (e.g., CS1) although there is no consensus in their use. There is also a need to map exposure scenarios across industrial sectors and uses of chemicals.

3.5. Progress in in silico and other NAMs: Read-across, -omics data and category formation

A number of other NAMs were described in the User Forum. A key *in silico* NAM is read-across, however read-across based on chemical structure and/or similarity alone was found to be limited. Structural similarity-based read-across may have too much uncertainty to be able

to make a decision. There is value in combining a variety of metabolic, physico-chemical and reactivity data to improve confidence in analogue selection whereby similarity can be quantified by considering multiple streams of data. The use of profilers with the OECD QSAR Toolbox was not sufficient to identify meaningful analogues, approaches such as MMP were found to be more sophisticated (CS2). A variety of NAM data (e.g., -omics and ToxCast data) to support read-across were presented, based on both toxicodynamics and toxicokinetics. Transcriptional profiling assisted in identifying analogues with similar mechanisms of action. It is recognised that ToxCast data are incomplete and their use is challenging, it is preferable (where possible) to consider only data from shared assays, although this reduces the number of data to be considered.

Read-across/category formation can be used to group low toxicity substances, this is well supported by NAM data and can assist in addressing low toxicity substances. There is still debate on how to provide confidence in confirming an assessment of "low toxicity". Extending the application of read-across, the Cosmetics Europe 10-step read-across strategy is a tiered approach which incorporates elements of NGRA. It covers parts of Tiers 0–2 as described in Section 3.2.1. It has different decision/exit points. This read-across strategy also allows for the inclusion of NAM data to support read-across and increase confidence (CS3).

4. Conclusions

The NAMs User Forum provided an opportunity to share learnings and experiences from a variety of stakeholders applying NAM data in NGRA. A variety of presentations were made which described the development and application of NAMs, typically within tiered testing strategies. A focus of the User Forum was determining the ability to make decisions from NAMs. Whilst some areas have made significant progress, e.g., DNT, for many areas of hazard identification and risk assessment further effort is required. The User Forum has provided an opportunity to identify areas where progress in implementing NAMs, through the use of tiered testing strategies, is required and essential to demonstrate the implementation of NGRA into practice.

Disclaimer

The views and opinions expressed in this manuscript are those of the authors and contributors to the workshop, they do not represent those of the European Commission, the European Chemicals Agency and the European Food Safety Authority.

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