TCDD

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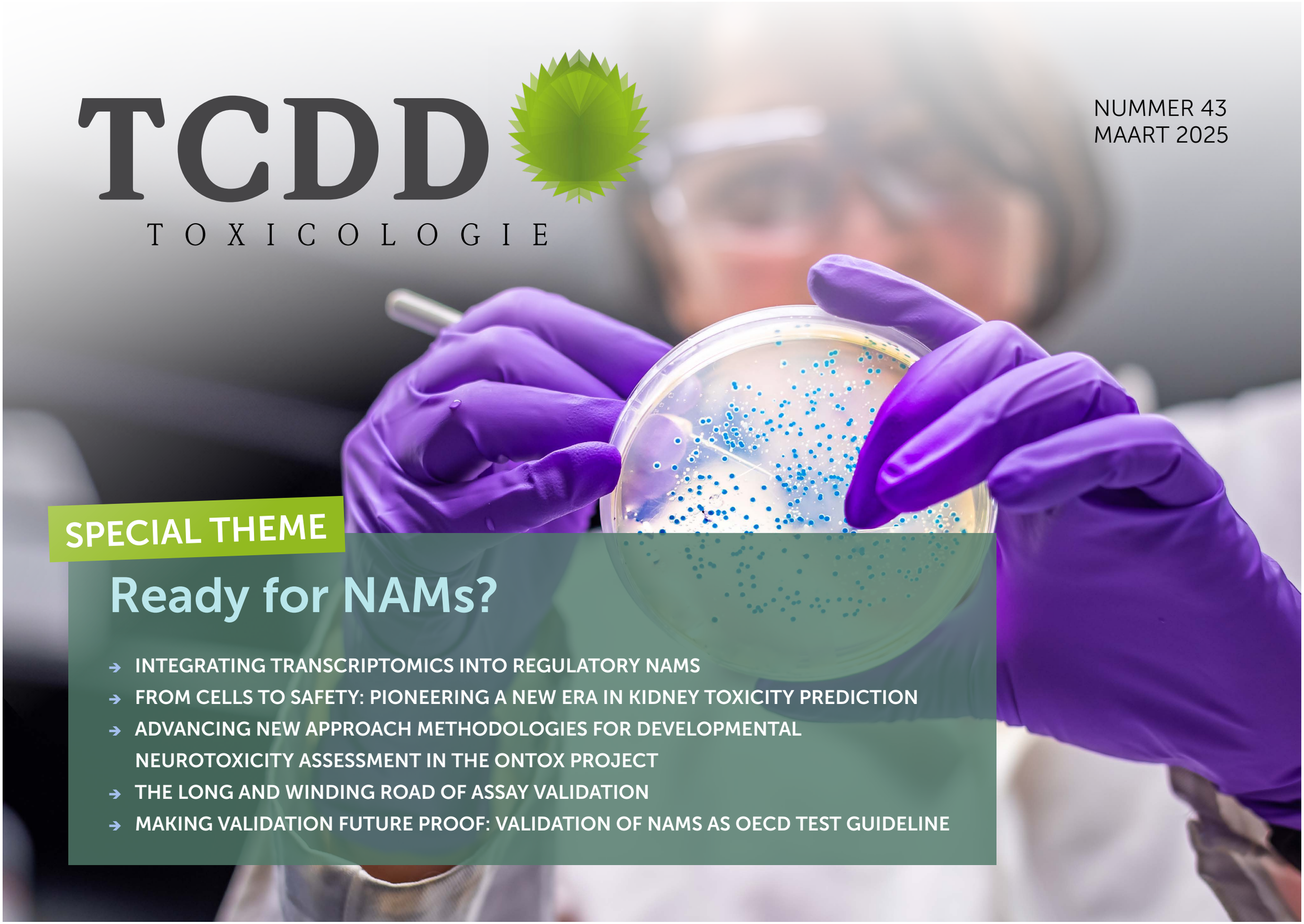

SPECIAL THEME

Ready for NAMs?

NEDERLANDSE VERENIGING VOOR TOXICOLOGIE

Colofon

Toxicologische Communicatie, Data en Documentatie (TCDD)

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Het lidmaatschap wordt automatisch verlengd tenzij de NVT-ledenadministratie vóór 1 december van het lopende jaar schriftelijk of per e-mail een opzegging heeft ontvangen. Hiervan ontvangt u een bevestiging.

Contributie NVT
Incl. abonnement TCDD 50,= euro
(extra kosten EEMS: 10,= euro)

Indien u ervoor kiest zelf uw contributie over te maken, dan worden € 5,- administratiekosten in rekening gebracht.

Sluitingsdata kopij 2025
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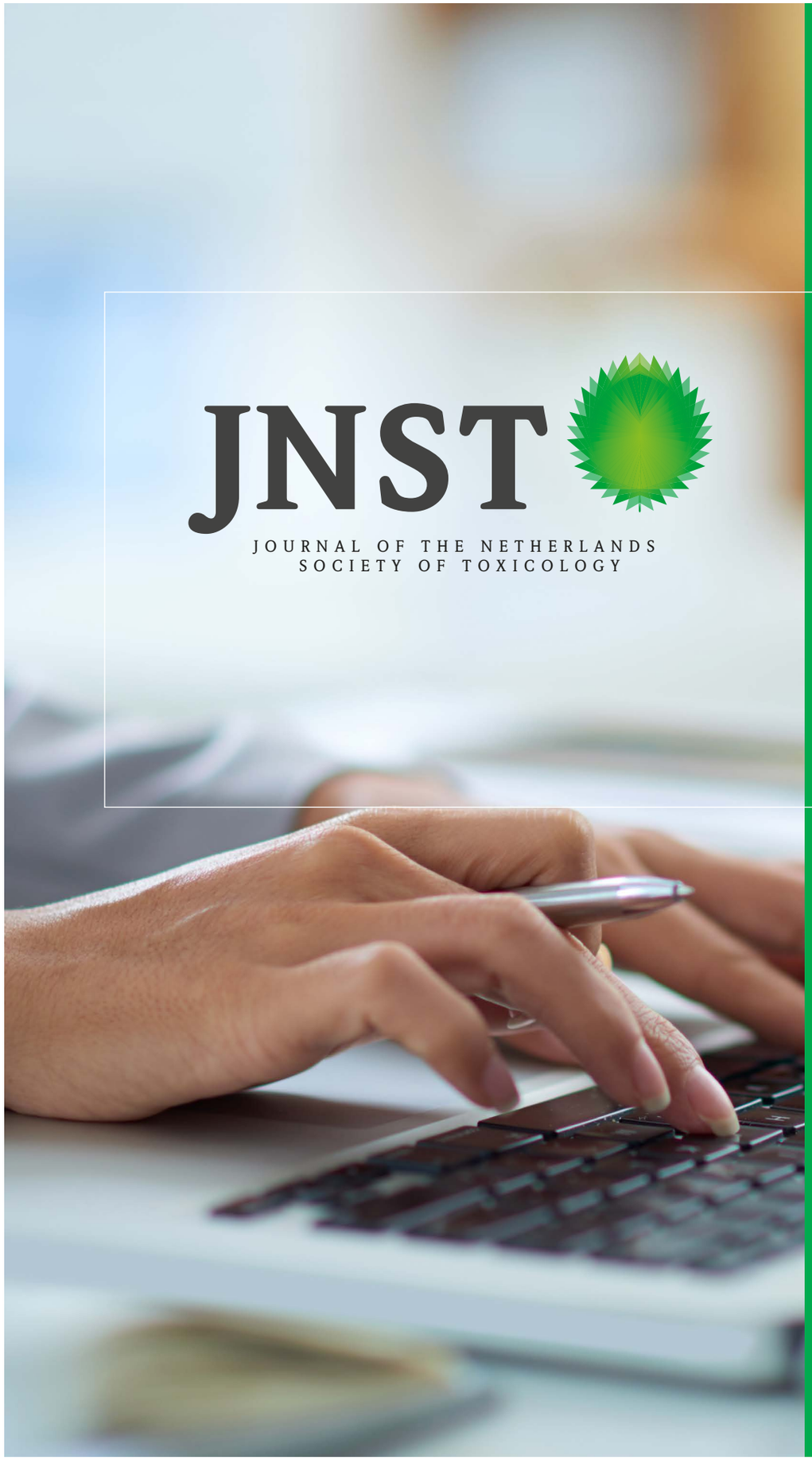
Inhoud



JNST

JOURNAL OF THE NETHERLANDS SOCIETY OF TOXICOLOGY

Submit your paper!

Call for submissions

to the Journal of the Netherlands Society of Toxicology

- Submissions can be made through ScienceOpen.
- A free account must be made with ScienceOpen prior to submission.
- Author guidelines can be found by following this link: Journal of the Netherlands Society of Toxicology – ScienceOpen.
- There is no deadline for submission.
- Once the submitted papers are accepted and have completed the peer review process, they will be available online and the journal entries will be appended to the TCDD.

TERUG NAAR INHOUD

Editorial

2025 is gearing up to be a year of change. The European Commission's 2025 Work Programme introduces major updates in order to simplify registration, evaluation, and authorisation processes for chemicals, modernize detergent market rules, and prioritize non-animal testing methods while enhancing safety and sustainability standards. Toxicologist the world over, including in the Netherlands, are actively working on alternatives to animal experiments or what's now referred to as New Approach Methodologies (NAMs).

Starting in 2025, the Dutch National Growth Fund (NGF) will invest €124.5 million in the new Centre for Animal-Free Biomedical Translation (CPBT, in Dutch: Centrum voor Proefdiervrije Biomedische Translatie) over a period from 2025 to 2034, to accelerate the development of safer, more effective treatments and reduce reliance on animal testing, with a total budget of €245 million supported by both public and private partners.

This issue's theme explores NAMs and is titled "Ready for NAMs?". Are we ready for NAMs? This question is tackled from various perspectives, covering transcriptomics, in vitro kidney models, developmental neurotoxicity NAMs, test method validation and more. As usual, we're also including an article from the Journal of the Netherlands Society of Toxicology (JNST), also about NAMs.

Have you written or thougth of writing in the TCDD or JNST? We're always open to new submissions from our members. Just shoot us an email at redactie@toxicologie.nl

Happy reading!

Sincerely,

Barae Jomaa

On behalf of the editorial team

Photo by Jess Bailey on Unsplash

News from the board

Spring is in the air! The season of renewal is here, bringing longer days, blooming flowers, and fresh opportunities. It's the perfect time to step outside, enjoy nature, and witness the beauty of new life and hope.

JOIN US AT THE NVT ANNUAL MEETING – JUNE 4-5, 2025!

We're thrilled to invite you to our NvT Annual Meeting, taking place on June 4-5, 2025, at the Reehorst Ede! This year's theme, "From Lab to Law: Bridging the Gap from Science to Policy," will dive into how scientific discoveries can shape impactful policies.

Both days will be dedicated to our entire toxicology community, featuring engaging discussions, networking opportunities, and insightful presentations. Please note that **both days are designed for all members** and will offer valuable insights. The General Assembly Meeting will take place on the second day. Don't miss out! Register and submit your abstracts by March 31!

CELEBRATING EXCELLENCE – JOEP VAN DEN BERCKEN PRIZE

We've received some outstanding PhD dissertations for this prestigious award, and we can't wait to dive into the evaluations! Interested in being part of the jury? Reach out to us - we'd love to have you on board!

DUTCH EVENING AT SOT 2025 – JOIN US IN ORLANDO!

Are you attending the Society of Toxicology (SOT) meeting in Orlando this March? We'll be organizing a Dutch evening for those joining the conference. If you'll be there, get in touch - we'd love to connect!

EXCITING COLLABORATION WITH GERMAN SOCIETY OF TOXICOLOGY (GT)

We're exploring a great opportunity to collaborate with GT for a joint meeting in March 2026 on overarching toxicology topics, possibly in Düsseldorf! There's also enthusiasm for cross-society interest groups, which could foster even greater collaboration and knowledge exchange. Stay tuned for updates!

Standing Together in Challenging Times

While spring brings hope, we recognize the challenges our colleagues in the USA are currently facing under the new administration. Many are struggling to keep their research going - even to keep their jobs. We stand in solidarity with them, hoping for resilience and better times ahead. Moreover, **diversity, equality, and inclusion (DEI)** which are at the heart of our community, are at risk. We strongly support the efforts of SOT's Special Interest Group "Out Toxicologists and Allies (OTA)," which champions LGBT+ scientists and strengthens inclusivity within toxicology. If you share commitment to diversity, consider getting involved!

Wishing you a wonderful and inspiring spring! Let's continue working together for a bright and inclusive future in toxicology!

Warm regards, also on behalf of the board,

Frederike-Jan van Schooten

SECTIE ARBEIDSTOXICOLOGIE
EN SECTIE RISICOBEOORDELING

Een batterij aan nieuwe risico's?

Gevaren van lithium-ion batterijen op de werkvloer

Hierbij nodig ik je van harte uit voor de bijeenkomst die de Nederlandse Vereniging voor Toxicologie, sectie Arbeidstoxicologie en sectie Risicobeoordeling, organiseert in samenwerking met de Contactgroep Gezondheid en Chemie (CGC).

Donderdag 20 maart 2025
- Inloop fysiek 13:00 uur
- Inloggen online via TEAMS 13:20 uur
- start programma 13:30 uur
- sluiting 16:30 uur

Locatie: Goldon Tulip Hotel Central, Den Bosch (zie https://hotel-central.goldentulip.com/nl)

Let op! NVT-en CGC-leden moeten zich aanmelden via de link die ze via de e-mail toegezonden krijgen. Daarna wordt 1-2 dagen van tevoren de link naar de TEAMS meeting toegezonden. NB: dit is een persoonlijke link, dus niet doorsturen naar derden.

Een bewijs van deelname wordt alleen verstrekt aan deelnemers die óf lid zijn van de NVT óf lid zijn van de CGC, en na invullen van het evaluatieformulier.

Lithium-ion batterijen hebben zich in korte tijd ontwikkeld tot een onmisbare technologie in ons dagelijks leven en onze werkomgeving. Van elektrische voertuigen en mobiele apparaten tot energiesystemen.

Maar deze innovaties brengen ook risico's met zich mee. Lithium-ion batterijen staan bekend om hun potentiële ontvlambaarheid en de mogelijkheid van thermische runaway, wat kan leiden tot moeilijk beheersbare branden. Deze incidenten brengen niet alleen fysieke schade met zich mee, maar vormen ook een directe bedreiging voor de gezondheid van mensen die beroepsmatig aan deze risico's worden blootgesteld. Denk aan brandweerlieden en afvalverwerkers. Blootstelling aan giftige dampen, brandwonden en explosiegevaar zijn slechts enkele van de uitdagingen waarmee zij te maken hebben.

Tijdens dit symposium gaan we dieper in op de aard van deze risico's.

Over de sprekers

Henk Brans
Henk Brans werkt als onderzoeker-adviseur bij het NIPV en is opgeleid als experimenteel natuurkundige. Hij gebruikt zijn diepgaande kennis van natuurkundige, chemische en elektrische processen om complexe veiligheidsvraagstukken rondom de energietransitie te begrijpen en om te zetten in praktische inzichten, preventieve maatregelen en concrete handelingsperspectieven voor hulpverleners. De energietransitie brengt namelijk grote veranderingen in onze leefomgeving met zich mee en vraagt om een scherp inzicht in mogelijke veiligheidsrisico's.

Niels Veen
Niels van Veen (RIVM) is sinds 2019 projectleider van onderzoeken die betrekking hebben op meetvraagstukken of brandexperimenten.

Daarnaast werkt Niels parttime als adviseur gevaarlijke stoffen bij de brandweer, waarbij hij regelmatig vanuit praktische perspectief te maken krijgt met zijn onderzoeksonderwerpen.

Arjen Koppen
Arjen Koppen is als toxicoloog (ERT) werkzaam bij het Nationaal Vergiftigingen Informatie Centrum (NVIC) van het Universitair Medisch Centrum Utrecht. Binnen dit centrum ligt zijn focus op de toxicologie van industriële en huishoudelijke producten, CBRN en incidentbestrijding.

Naast zijn werk bij het NVIC heeft hij een piketfunctie bij het Crisis Expert Team milieu en drinkwater (CET-md) en is hij algemeen bestuurslid van de European Association of Poisons Centres and Clinical Toxicologists (EAPCCT). In 2024 is hij gestart met zijn opleiding tot Gezondheidskundig Adviseur Gevaarlijke Stoffen (GAGS) bij de Veiligheids-en- Gezondheidsregio Gelderland-Midden.

Angelique Allart
Ing. Angelique Allart is sinds 2015 betrokken bij de Vereniging Afvalbedrijven als secretaris van de Commissies Milieu en Arbo & Veiligheid en ondersteunt de leden van de vereniging bij diverse dossiers op dit vlak. Eén van de onderdelen waar zij zich mee bezighoudt binnen de brancheverenigingis het uitzetten en uitwerken van de brandenmonitor. Deze monitor wordt jaarlijks uitgezet onder de leden om inzicht te krijgen in de branden die plaatsvinden. Branden spelen (helaas) een grote rol binnen de afvalsector. Na haar studie Analytische Chemie in Delft heeft zij haar kennis opgedaan op het vlak van milieu en veiligheid in allerlei verschillende projecten door haar werk als projectleider bij een adviesbureau.

Programma

klik hier

SECTIONS PHARMACEUTICAL
TOXICOLOGY & RISK ASSESSMENT

Invitation Spring Symposium - 15 april 2025

Extractables and Leachables: a concern for our health?

Location
Campus Heyendaal Nijmegen:
Radboud university, Faculty of Science, Huygens building (**HG**) lecture hall: HG00.303
Heyendaalseweg 135, 6525 AJ Nijmegen

Abstract
For this joined spring symposium organised by the Dutch society of Toxicology the sections Pharmaceutical Toxicology and Risk assessment have combined their interest and knowledge on the topic Extrables and Leachables. During this symposium it is aimed to demonstrate multiple aspects and potential risks of extractables and leachables. With invited experts in the field of toxicity and risk assessment practical examples about pharmaceutical formulations and concerns for food or medical implants are going to be addressed.

Registration:
Deadline 1 April 2025. Due to a maximum capacity of 79 available seats, the registration is on a 'first-come-first served' basis. Registration can be done via:
Indicate your name and affiliation
There will be no charge for attending this symposium.
A certificate of attendance will be sent within a few weeks after the event.

Facilities Huygens building Faculty of Science:
Huygens building | Radboud University

Arrival by public transport:
There is a direct bus service from train station Nijmegen, towards bus stop: Huygensgebouw. **Line 10** (Departing approx. every 10 min.)
Train station Nijmegen Heyendaal is within walking distance towards the Huygens building.

Arrival by car:
There is a parking garage under the Huygens building: Car park P11 - Huygens | Radboud University

Other parking facilities can be found on the website: Parking spaces on campus | Radboud University

Restaurant:
Snack or lunch facilities are available in the lobby of the Huygens building**:**
Giga-Bite | Radboud University

Program

TIME	ACTIVITY	LOCATION
12:00 – 12:40	**Registration & Welcome**	**HG Registration desk**
12.15 - 12.45	*Business meeting – Pharmtox section members*	***HG** 00.303*
12:45 – 12:50	Opening *Daan Touw*	**HG** 00.303
12:50 – 13:25	**Presentation 1** *Tine Folkertsma and Angela Aalbers, Martini hospital, Groningen. Extractables and leachables from syringes*	**HG** 00.303
13:25 – 14:00	**Presentation 2** *Yolanda Ponstein, Pharming.* *Risk Assessment Manufacturing Related Leachables & Extractables – Example from Industry*	**HG** 00.303
14:00 – 14:30	Coffee & Tea break	**HG** Zuidstraat ground floor
14:30 – 15:05	**Presentation 3** *Bianca van de Ven, (Presentation in the field of Food; full title tba)*	**HG** 00.303
15:05 – 15:40	You only see it when you know it: effects of exposure to 'medical devices'. Prof. dr. Albert J. Feilzer, Em. Professor of General Dentistry	**HG** 00.303
15:40 – 15:55	Panel discussion *Marjolijn Woutersen*	**HG** 00.303
15:55 – 16:00	Closure	**HG** 00.303
16:00 – 17:30	Drinks	**HG** Zuidstraat ground floor

Integrating Transcriptomics into Regulatory NAMs

By *Marcha Verheijen*

Toxicology is undergoing a transformation driven by innovative, non-animal testing strategies. New Approach Methodologies (NAMs) have gained momentum as the field shifts towards reducing, refining, and replacing animal studies while improving human relevance. Among the array of NAMs, transcriptomics—an omics-based approach that analyzes gene expression patterns—has emerged as a powerful tool to assess toxicological responses at the molecular level.

Transcriptomics as a NAMs Tool

Transcriptomics offers a comprehensive snapshot of how genes respond to chemical exposures, capturing early biological changes that precede adverse effects. This technology has proven invaluable for identifying mechanistic pathways, defining toxicity signatures, and improving risk assessment strategies[1]. By measuring global gene expression changes in cells, tissues, or organoid models, transcriptomics provides insights into perturbations in biological networks that may indicate toxicity.

The key advantage of transcriptomics is its ability to move beyond traditional endpoint assays, which often rely on histopathological or biochemical changes that manifest late in toxicity processes. Instead, transcriptomic profiling detects early molecular disruptions, enabling predictive toxicology approaches[2]. This capacity aligns well with the goals of NAMs: to create robust, mechanistically driven, and human-relevant toxicological assessments.

Application in Predictive Toxicology

One of the most promising applications of transcriptomics in NAMs is its use in predictive toxicology. By employing gene expression signatures associated with specific toxic effects, transcriptomics enables the classification of chemicals based on their molecular impact rather than relying solely on phenotypic outcomes. For instance, the Tox21[3] and ToxCast[4] programs have integrated transcriptomics to screen thousands of environmental chemicals, identifying molecular signatures linked to toxicity pathways.

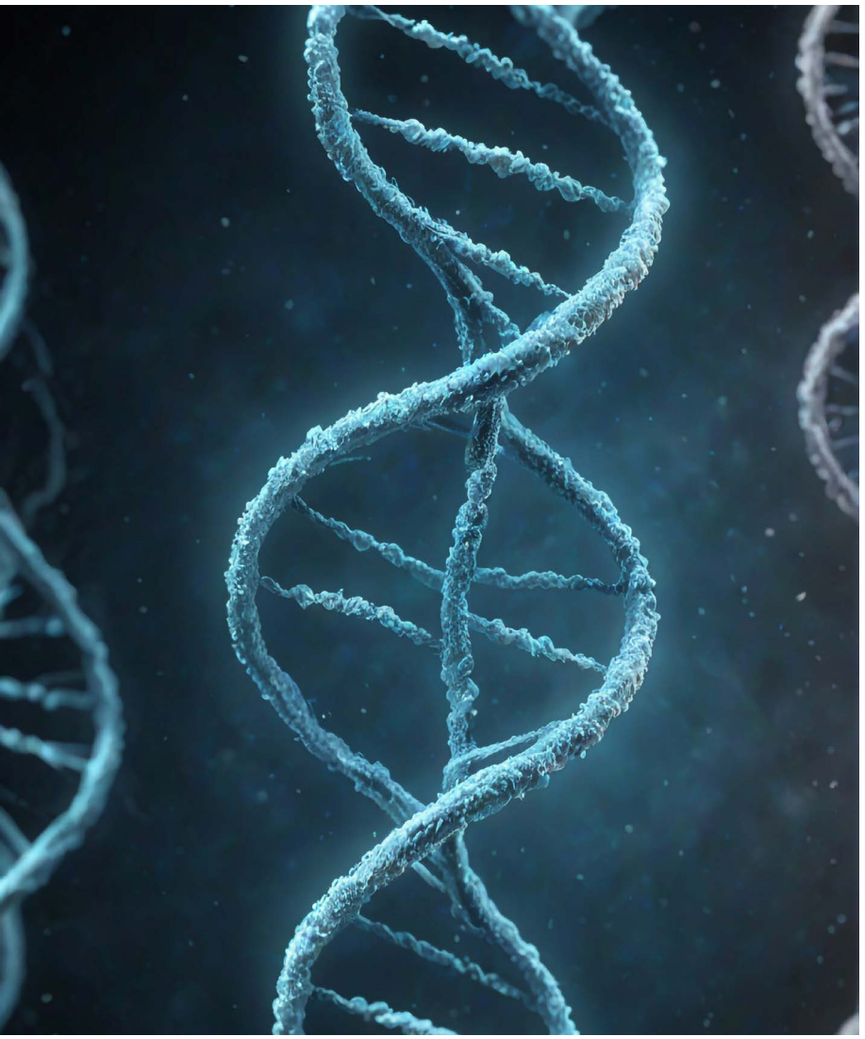
A notable advancement is the development of machine learning models trained on transcriptomic datasets to predict chemical hazards[5,6]. These models leverage large-scale gene expression data to classify compounds based on their likelihood of inducing toxic effects, improving the efficiency and accuracy of chemical safety assessments. The integration of transcriptomics into read-across frameworks further enhances chemical risk evaluation by allowing the comparison of unknown substances with well-characterized compounds based on gene expression similarities[7].

Regulatory Integration and Future Directions

Despite its potential, the regulatory acceptance of transcriptomics-based New Approach Methodologies (NAMs) is still evolving. One key challenge is the complexity

of transcriptomics processing pipelines, which involve multiple steps where variations can impact the final output. Therefore, ensuring a standardized and transparent data processing pipeline is essential, and its inclusion in reporting templates should be a priority[8].

Efforts such as the OECD's guidance on Omics Reporting Framework (OORF)[9], previously known as Transcriptomics Reporting Framework (TRF)[10], OECD's omics data integration and initiatives like the EPAA Transcriptomic Assessment Product (ETAP)[11] are working towards establishing transcriptomics as a standard tool in regulatory toxicology. A notable recent development is the OECD's evaluation of the R-ODAF (Reference Omics Data Analysis Framework) pipeline[12] for potential regulatory acceptance. Once validated, it will be designated as T-ROAM (Transcriptomics Reference Omics Analysis Method), providing a standardized workflow to ensure reproducibility, robustness, and regulatory compliance in toxicological assessments. This initiative is expected to enhance the credibility of NAMs and facilitate their adoption in regulatory decision-making.



Looking ahead, integrating transcriptomics with other omics disciplines—such as proteomics, metabolomics, and epigenomics—holds great promise for improving the predictive power of NAMs. Additionally, single-cell transcriptomics is emerging as a powerful tool for deciphering cell-type-specific responses, further refining toxicological assessments.

Conclusion

Transcriptomics is revolutionizing toxicology by providing high-resolution insights into molecular responses to chemical exposures. As part of the NAMs framework, it offers a predictive, mechanistic, and human-relevant alternative to traditional animal testing. Continued advancements in computational modeling, data integration, and regulatory acceptance—such as the OECD's work on T-ROAM—will further establish transcriptomics as a cornerstone of next-generation toxicology, ultimately leading to safer and more ethical chemical risk assessments.

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From Cells to Safety: Pioneering a New Era in Kidney Toxicity Prediction

By *Devon Barnes*

Kidney toxicity remains a significant challenge within drug development and environmental safety assessment. Our team at Utrecht University is at the forefront of developing an innovative in vitro test battery to revolutionise the detection and prediction of nephrotoxicity. This work is performed within the framework of ONTOX, the ontology-driven and artificial intelligence-based repeated dose toxicity testing of chemicals for next-generation risk assessment project, to develop an efficient, animal-free approach for human chemical risk assessment and address critical needs in toxicology (1).

The kidney is a major site of xenobiotic-induced toxicity, often manifesting during drug development, environmental exposures and clinical intervention. Current methods for predicting nephrotoxicity remain limited, often resulting in late-stage drug failures and potential patient harm. Our work aims to bridge this gap by creating a more predictive and efficient screening process. Recent years have seen significant progress in *in vitro* modelling systems toward an improved understanding of kidney biology and toxicology mechanisms (2). Our team is currently leveraging these advances, including the identification of novel biomarkers for kidney injury and development of human-based cell models to better reflect *in vivo* kidney function. Creating an effective *in vitro* test battery for nephrotoxicity requires a multi-faceted approach. Our work integrates varying aspects of cell model development, high-throughput screening, and multi-parametric analysis, to provide a battery that incorporates both traditional and novel biomarkers of kidney injury to offer a more sensitive and specific assessment of nephrotoxicity while focusing on two cases: tubular necrosis and crystallopathy. Furthermore, through the adapted high-throughput formats the assays established enable the rapid screening of large compound libraries.

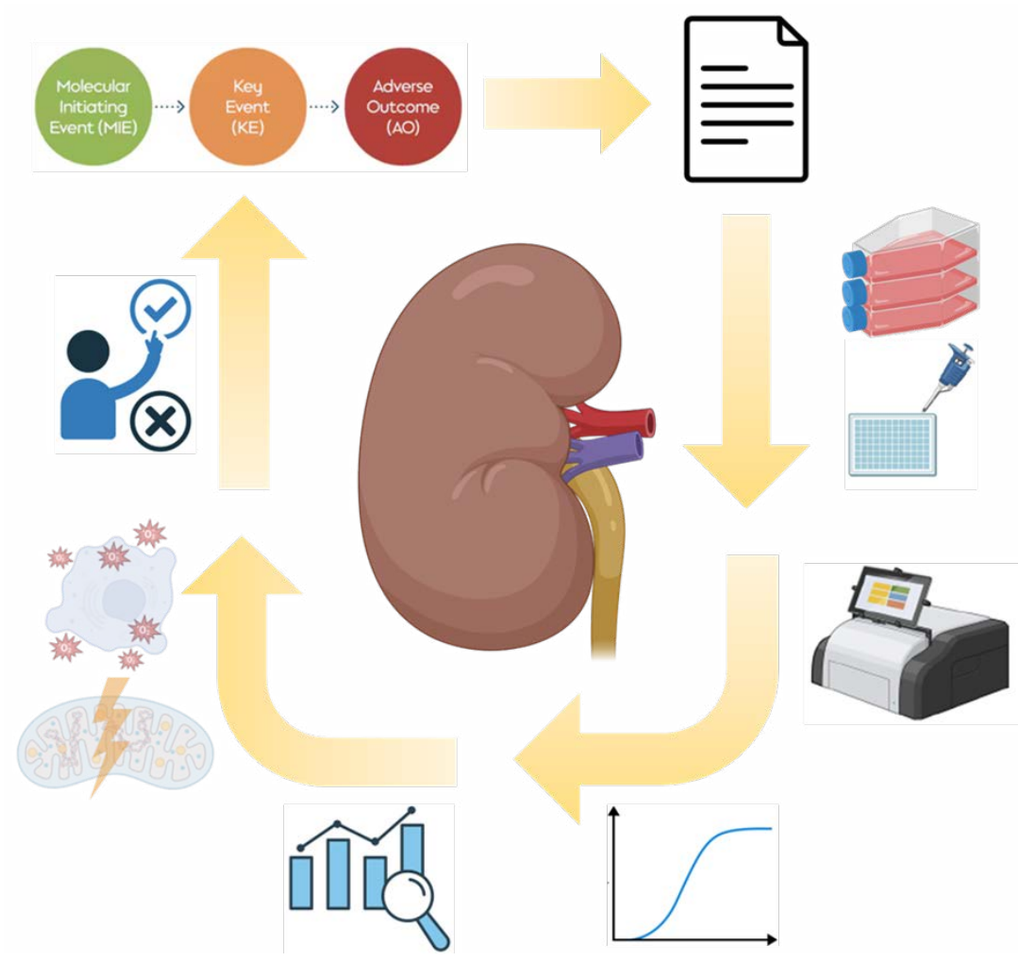

Figure. The workflow begins with a literature review to inform in vitro experimentation using cultured kidney cells. High-throughput screening methods generate dose-response data, which undergoes further analysis to identify key toxicity events. These events are used to construct or refine adverse outcome pathways (AOPs). The process then cycles back to the literature review stage, incorporating new findings to continually improve kidney toxicity prediction models and AOPs. This iterative approach ensures ongoing refinement of our understanding and assessment of nephrotoxicity.

This approach combines multiple endpoints, including cell viability, functional markers, and molecular readouts to establish a comprehensive toxicity profile.

As a part of the ONTOX project, we are incorporating the Adverse Outcome Pathway (AOP) framework as a new approach methodology for investigating nephrotoxicity (3). This framework allows us to systematically map the biological events that lead from initial chemical exposure to adverse health outcomes. By aligning our test battery with key events within nephrotoxicity AOPs, we seek to enhance the mechanistic relevance and predictive power of our assays (4). This approach not only improves our understanding of toxicity mechanisms but also provide a scientifically robust basis for regulatory decision-making. Achieving regulatory acceptance for this innovative test battery is our ultimate goal. We are addressing key factors to ensure widespread adoption, including data quality, reproducibility, and mechanistic relevance. Through rigorous quality control and comprehensive validation studies, we aim to enhance the predictive power of our assays to cover various mechanisms of nephrotoxicity to provide a more comprehensive assessment. As part of the ONTOX project, the nephrotoxicity case studies are developed in alignment with our projects partners developing *in vitro* batteries of hepatoxicity and neurotoxicity, whilst actively fostering collaboration with regulatory bodies, industry partners, and academic experts. These partnerships are crucial in aligning our approach with field requirements and regulatory standards. By integrating robust data, mechanistic insights, and cooperative development, we're not only advancing nephrotoxicity testing but also paving the way for seamless regulatory integration. This approach positions our work at the forefront of next-generation safety assessment tools, with potential impact on drug development and patient safety.

While significant progress has been made, there are challenges that remain. We continue to further refine the cell models implemented to better represent the diverse kidney cell types and functions, whilst simultaneously investigating and expanding the panel of biomarkers to capture a wider range of toxicity mechanisms toward further development and refinement of the AOP framework. Additionally, we seek to develop large-scale validation studies to demonstrate the battery's performance across a diverse range of compound classes. Our work at Utrecht University represents a significant step forward in nephrotoxicity testing. This comprehensive *in vitro* test battery stands to become a cornerstone in regulatory toxicology, offering a scientifically robust and ethically sound method for assessing drug safety and offer an alternative to traditional animal testing methods to ultimately improve patient outcomes. We invite collaboration and encourage engagement from the scientific community as we work towards a future where kidney toxicity can be more accurately predicted and prevented. By providing a more accurate and efficient method for such prediction, our research has the potential to pave the way for a paradigm shift in nephrotoxicity assessment, not only transforming pharmaceutical development, but also significantly enhancing environmental safety assessments.

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Advancing New Approach Methodologies for Developmental Neurotoxicity assessment in the ONTOX Project

By *Eliska Kuchovska, Oddvar Myhre and Ellen Fritsche*

Toxicology is undergoing a major shift away from traditional animal testing toward more human-relevant, efficient, and ethical alternatives known as New Approach Methodologies (NAMs). Within the ONTOX project[1], Work Package 9 (WP9) is at the forefront of developing innovative NAMs to assess developmental neurotoxicity (DNT). Our aim is to refine and implement advanced in vitro methods to predict the neurotoxic effects of chemicals on the developing brain.

Understanding the Challenge

The human brain develops through highly complex key neurodevelopmental processes (KNDPs), including neural progenitor cell proliferation, migration, differentiation, synaptogenesis, myelination, and neural network formation. Disruption of any of these processes due to chemical exposure can contribute to neurodevelopmental disorders and cognitive impairments (Aschner et al., 2017; Zhou et al., 2024). Traditional animal models have limitations due to species differences in brain development, which reduce their predictive power for human health (Paparella et al., 2020). These challenges highlight the need for human-relevant and more efficient mechanistic approaches. A key achievement in this effort is the OECD-supported Developmental Neurotoxicity In Vitro Battery (DNT IVB;

Figure 1: ONTOX WP9 team composed of scientists from two institutions – Leibniz Research Institute for Environmental Health (IUF, Düsseldorf, Germany) and Norwegian Institute of Public Health (NIPH, Oslo, Norway). From left bottom: Ellen Fritsche (WP9 leader), Graciela Lopez Soop (WP9 postdoctoral researcher), Eliska Kuchovska (WP9 postdoctoral researcher), Kristine Dolva (PARC PhD student), Oddvar Myhre (WP9 scientist), Malene Lislien (WP9 PhD student), Tim Hofer (WP9 scientist). Photo credits: Hubert Dirven (WP9 scientist).

OECD, 2023) a compilation of human-relevant test systems encompassing 17 *in vitro* assays designed to cover the critical KNDPs. To reach regulatory acceptance, DNT IVB set-up passed through a series of steps summarized recently (Blum et al., submitted). These steps can serve as a role model for NAM development in different areas and include: 1. Develop a test system and test methods based on robust human-relevant models, 2. Align it with regulatory needs, 3. Evaluate test method readiness, 4. Conduct scientific validation, 5. Obtain regulatory recognition (recommendation, guidance, guideline), and 6. Ensure public availability through Contract Research Organizations. By implementing this approach, WP9 aims to develop NAMs that are both scientifically robust and regulatory-ready, ultimately improving chemical safety assessment while reducing reliance on animal testing.

The ONTOX WP9 DNT team contributions to the implementation of NAMs

The WP9 DNT NAM development team (Figure 1), led by Prof. Ellen Fritsche, consists of researchers from IUF – Leibniz Research Institute for Environmental Medicine in Germany, the NIPH – Norwegian Institute of Public Health in Norway and, since recently, the SCAHT – Swiss Centre for Applied Human Toxicology. Our team works to develop new assays to refine the DNT IVB and close biological uncertainties in the current battery (Tal et al., 2024).
One of our key achievements was the development of a synaptogenesis assay using human iPSC-derived models, which was carried out by Oddvar Myhre, Malene Lislien and the team at NIPH. Additionally, the establishment of an astrocyte development assay using human primary and iPSC-derived cells is being led by Katharina Koch, Ellen Fritsche, Etta Zühr, and the team at IUF and was co-financed by cefic-LRI.

The regulatory alignment of NAMs is a critical aspect of our work. WP9 members actively engage with stakeholders by being members of the OECD DNT *in vitro* Expert Group and regularly exchanging with representatives of the European Food Safety Authority (EFSA) and the European Chemicals Agency (ECHA), ensuring that our methods align with regulatory needs. The work of ONTOX WP6, which is dedicated to stakeholder involvement, further strengthens this alignment (Diemar et al., 2024, 2025). Here, ONTOX acts in a concerted effort with the Horizon Europe project PARC and other EFSA-funded initiatives concerning NAMs for neurotoxicity. Regulatory assessment of chemicals can also be facilitated through the Adverse Outcome Pathway (AOP) framework (Bajard et al., 2023). In this regard, the WP9 team has compiled DNT-relevant AOPs, identifying biological and disease gaps (Jaylet et al., 2024). The team continues to refine the DNT AOP network to link them with neurodevelopmental disorders and physiological brain maps (Staumont et al., 2025) via KNDPs. This work led by Eliska Kuchovska (IUF) is being carried out in collaboration with the University of Liège, led by Prof. Liesbet Geris. Finally, the SCAHT team of Ellen Fritsche recently established an AOP HUB[2], a hands-on platform for information exchange and sharing to help the community in developing new AOPs, especially for DNT (Coerek et al. accepted).

The characterization of DNT *in vitro* assays is another priority. WP9 evaluates and describes its developed

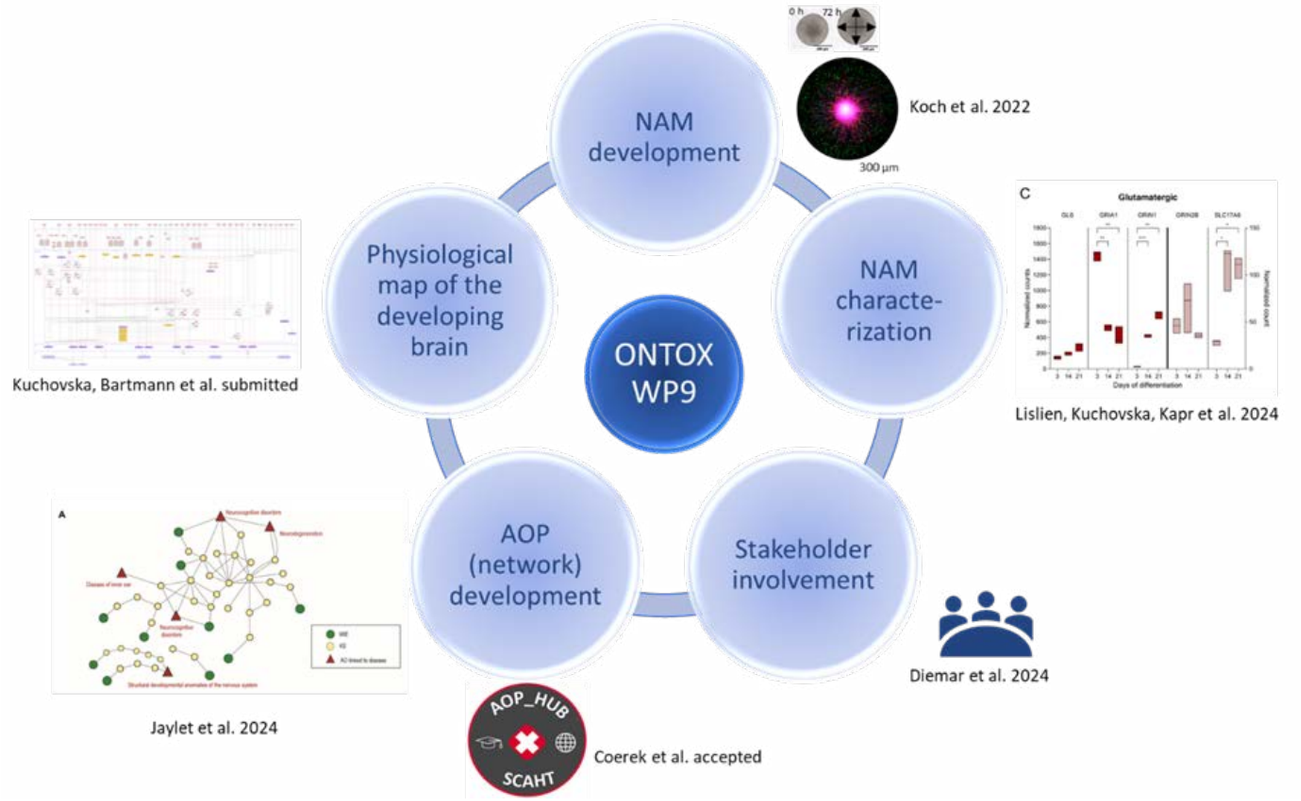

Figure 2: Graphical summary of WP9 activities.

methods using the ToxTemp method annotations (Krebs et al., 2019) which leads to increased regulatory confidence in the assays. A webinar on DNT ToxTemps was recently delivered by Eliska Kuchovska and is available on the ASPIS YouTube channel[3]. Assay characterization includes investigating metabolic activity, protein and lipid content (cooperation between IUF and Nynke Kramer's team from Wageningen University) necessary for the quantitative *in vitro* to *in vivo* extrapolation models and transcriptomic profiles of human iPSC-derived cell models (Lislien et al., 2025) (cooperation between NIPH and IUF). Additionally, scientific validation of the Neurosphere Assay (Koch et al., 2022), a key component of the DNT IVB (OECD, 2023), and the investigation of its biological applicability domain, was recently completed by the team at the IUF (Kuchovska, Bartmann, submitted). The final step in our framework is ensuring that NAMs are publicly available. WP9 NAMs will be accessible through the ONTOX Hub, a marketplace designed to promote ONTOX *in vitro*, laboratory, and consulting services while the DNTOX[4] company, a spin-off of the IUF, serves as a commercial provider of DNT safety assessment services for chemical risk evaluation.

Looking Forward

The progress made by WP9 (overview in Figure 2) represents a major advancement in DNT testing and the promotion of NAMs as regulatory alternatives. As ONTOX progresses, we look forward to implementing our first case study of probabilistic risk assessment[5], using our DNT NAMs and other innovative ONTOX tools. Our mission is to establish a future where chemical safety assessments rely on human-relevant, animal-free methodologies, ensuring better protection of public health and the environment. For more insights into our work, stay tuned for upcoming publications, presentations[6], videos[7], or podcasts[8] by the WP9 team.

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The long and winding road of assay validation

For decades, animal models have played a central role in toxicological research. Even today, in vivo testing remains a key component in the safety assessment of pharmaceuticals, chemicals, and consumer products. However, scientific advancements have paved the way for a diverse array of in vitro assays that offer promising alternatives to animal testing. Advanced cell culture systems, omics technologies, organ-on-a-chip platforms, and in silico approaches are rapidly expanding the toolkit for animal-free safety assessments.

By *Giel Hendriks, CEO at Toxys*

Despite the growing number of innovative in vitro models for toxicity testing, the regulatory adoption of these Novel Approach Methodologies (NAMs) remains limited. A major barrier to regulatory acceptance is the rigorous validation required to ensure robustness, reproducibility, and transferability. Multi-laboratory ring trials are often necessary to establish these criteria. A successful validation process is crucial for developing an OECD Test Guideline, which facilitates mutual acceptance of data and regulatory endorsement of a novel test.

The ToxTracker Assay: A Case Study in Validation

ToxTracker is an in vitro reporter assay designed to accurately predict in vivo genotoxicity. By monitoring the activation of six distinct reporter genes, the assay provides valuable insights into the mode of action of genotoxic compounds. Originally developed at Leiden University Medical Center and later commercialized by the biotech company Toxys, ToxTracker has been widely adopted by major pharmaceutical, chemical, and cosmetics companies for early-stage genotoxicity assessments. However, formal validation was required to support regulatory applications.

In 2016, an application for the validation of ToxTracker was submitted to the OECD and ECVAM. Following approval in 2017, an interlaboratory validation study commenced, involving seven expert laboratories under the supervision of an independent Validation Management Team. Each participating lab established proficiency in performing the assay before analyzing a carefully curated library of 64 genotoxic and non-genotoxic compounds. The study assessed within-laboratory and between-laboratory reproducibility, as well as the sensitivity and specificity of ToxTracker in predicting genotoxicity.

By 2022, the validation process was completed, demonstrating reproducibility rates of 80-90% and an impressive accuracy of 90% in correctly predicting genotoxicity. The findings were published in a recent study (Hendriks et al., 2024), further reinforcing the assay's credibility.

Lessons Learned and Future Directions

Although ToxTracker had already been in use for several years with strong scientific validation, the interlaboratory ring trial led to critical refinements in the assay protocol. Improvements included modifications to S9-based metabolization protocol, clearer guidelines for solubility and toxicity assessment, refined assay acceptance criteria, and optimized instrument settings. Additionally, an updated prediction model for data interpretation was developed based on validation test results.

While interlaboratory validation studies are time-consuming, costly, and labor-intensive, they are indispensable for the regulatory acceptance of NAMs. Rigorous validation is a cornerstone of broader in vitro adoption in toxicological research, ultimately advancing efforts to reduce or replace animal testing.

Recently, the Netherlands submitted a new SPSF application to the OECD to develop a formal Test Guideline for ToxTracker. This application will be reviewed during the OECD WNT meeting of national coordinators in April, marking a significant step toward regulatory recognition and broader implementation of this innovative assay.

Making validation future proof: validation of NAMs as OECD Test Guideline

By *Eva Streekstra, Betty Hakkert, Damiën van Berlo and Jelle Vriend*

Ongoing innovations towards advanced materials, new endpoints of concerns such as endocrine disruption and the growing need for non-animal testing require the development of new methods such as New Approach Methodologies (NAMs). Validation of new or updated methods is essential for their use in legislation. Awareness of its importance is increasing. In this article, we showcase a few examples that we are actively working on at the RIVM.

Before a new method can be adopted as an international guideline, it must undergo a validation process. Validation has different meanings depending on the context. It is crucial to know whether a method is reproducible (i.e., when repeating it in one or different labs the outcome is the same), robust (i.e., changes in experimental circumstances such as humidity or temperature do not affect the outcome), predictive (i.e., the result informs on expected effects in humans), which part of the biology it represents (the biological applicability domain) and for which types of chemicals it can be used (the chemical applicability domain). Such characteristics are essential to ensure that new products and substances are safe and to foster trust and confidence between stakeholders (e.g. industry, regulators, non-governmental organisations, contract laboratories). We therefore talk about method validation in accordance with the Organisation for Economic Co-operation and Development (OECD) Guidance Document 34 (GD34[1]). In OECD GD34, validation comes down to an evaluation of relevance (the extent to which the test correctly predicts the biological effect of interest in the target organism: usually humans) and reliability (it is reproducible within a lab and across different labs) of a method: these are important determinants for the regulatory acceptance of a method and can lead to development and adoption of an OECD Test Guideline.

The OECD Guidelines for the Testing of Chemicals form the core of EU regulations for safety testing. Via the OECD, test guidelines are developed to assess the safety of chemicals and their effects on humans and the environment. These guidelines ensure the generation of reliable and reproducible data accepted by all OECD member states, under Mutual Acceptance of Data (MAD) and Good Laboratory Practice (GLP). This saves costs (€309 million per year) and time, avoids replication of work and reduces the need for animal testing[2]. MAD ensures that results are accepted across OECD member states and is the single most important contributor to the reduction of animal use for regulatory testing in the last semicentury.

For validation, different routes are possible which are described in the OECD Guidance for validation and international acceptance (OECD GD34). Coordination of the validation process can be on a method developer's own initiative by self-appointing a validation management team or via a validation centre like the EU Reference Laboratory for alternatives to animal testing (EURL ECVAM). EURL ECVAM has an important network to assess regulatory relevance and usefulness for end users, a network of reference laboratories (EU-NETVAL) that participate in validation studies and a network for peer-review (EURL ECVAM Scientific Advisory Committee or ESAC). Once the validation according to GD34 is completed, a test guideline proposal can be submitted to the OECD Working Party of National Coordinators of the Test Guidelines Programme (WNT). Once accepted in the test guideline program, the method can be implemented into the Test Method Regulation.

There are several misconceptions about validation, such as the belief that it is inherently slow and costly. Costs for validation range between €200,000-800,000, not fully

covering manpower, and, if a method was sufficiently optimized, validation takes one to three years[3,4] . Although validation demands significant resources, its costs and duration are comparable to, or even less than, those of the research and development phase for a test method. Despite the limited availability of funding for validation, investing in it is worthwhile, especially considering the potential cost savings in the context of MAD. Funding opportunities for validation are luckily gaining momentum.

Funding for validation in part can be applied for via public-private partnerships (e.g. PEPPER[5]) or partially via EURL ECVAM (co-ordination and peer review). However, most of the calls and EU-research projects do not focus on validation and the regulatory needs of NAMs. Moreover, the validation process often takes longer than the typical 4-year funding for EU-research projects. More awareness is needed and it is important that resources are made available for method optimization and ensuring that is already part of the development phase. The duration of validation is variable since sufficiently optimized methods and protocols take less time to validate. When looking at EURL ECVAM's 6Tracking System for Alternative methods (TSAR)[6], a database for alternative non-animal methods, most method developments stopped before going into validation. More importantly, once a method is validated, there is a high chance of successful adoption in test guidelines.

At RIVM we are involved in several projects to improve regulatory implementation of NAMs. To raise more attention to the importance of method validation and to make it future proof, we play a supporting role in the EU test method and validation strategy initiated by the Dutch Ministry of Infrastructure and Water Management together with their German equivalent. This strategy focusses on how to accelerate the availability of regulatory accepted methodologies for chemicals risk assessment, to stimulate participation of regulators and other stakeholders to address regulatory needs and to propose coordination of priority setting and funding.

In December we hosted a meeting of the OECD project group involved in the update of OECD GD 34 at RIVM. This meeting focused on updating the guidance document to streamline validation and international acceptance for new methodologies. The original document was drafted in 2005 and after 20 years it needs a revision to align it with current and new types of methods including NAMs, such as in vitro test batteries and computational methods. The revision focuses, among others, on how to better guide test method developers in the validation process. Together with the United States and the European Commission's Joint Research Centre (JRC) we lead the revision of the document. An important aspect of GD34 is the concept of readiness criteria. These criteria help the developer to make sure a test method is sufficiently optimized to allow efficient validation, saving time and costs, and greatly increasing the chance of successful validation.

Validation is an important step to achieve regulatory acceptance of methods. Interest in validation is growing including initiatives to fund validation. International guidance is revised to accommodate the validation process better to NAMs. However, challenges remain, particularly in assessing when a method is ready for validation and ensuring its regulatory relevance. In overcoming these challenges, a collaborative effort of relevant stakeholders such as regulators, academia and industry is key. A good start is to consider validation early in the research and development phase of new methods.

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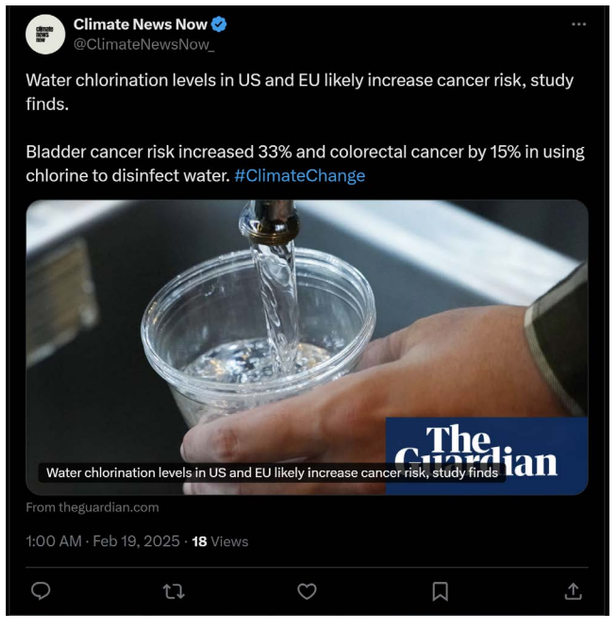
Exposure to Drinking Water Trihalomethanes and Cancer Risk

By *Barae Jomaa*



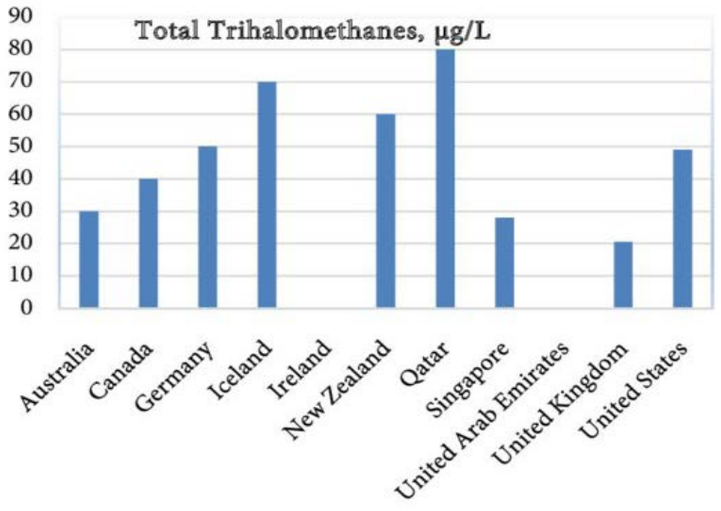
A recent study conducted by researchers from Karolinska Institutet and Uppsala University in Sweden has explored the potential link between trihalomethanes (THMs) in chlorinated drinking water and increased cancer risks1. Chlorination, a widely used method for disinfecting drinking water, introduces THMs as by-products, which are likely to be present in virtually all chlorinated public water systems (figure 1)2. Studies on animals and mechanistic research have established that three of the four most common trihalomethanes (THMs)—chloroform, bromoform, and bromodichloromethane—exhibit genotoxic properties. Moreover, all four THMs have been identified as carcinogens in rodent models.

Figure 1: Total trihalomethane (THM) levels in various countries. The United Arab Emirates and Ireland did not report THM levels. Reproduced from Karim et al., 2020 under CC BY 4.0.

The Swedish study, which systematically reviewed data from 30 studies involving over 90,000 participants, found limited-suggestive evidence that THM exposure may be associated with a 33% increased risk of bladder cancer and a 15% increased risk of colorectal cancer. The EPA reports that THM concentrations typically range from 40 to 60 parts per billion (ppb) – below the regulatory limits set by the US (80 ppb) and the EU (100 ppb).

While the study provides important insights, the authors emphasize the need for further high-quality research to confirm these associations and better understand the risks. Talking to The Guardian, Emilie Helte, the lead author, stressed the importance of continuing to drink municipal water and recommend using granulated activated carbon filtration systems at home to reduce THM levels[3].

Multiple news outlets have covered this study[3,4], highlighting its potential public health implications. The coverage underscored the delicate balance needed between effectively disinfecting drinking water and minimizing the potential health risks associated with chemical by-products. According to a study by Karim, Kaleh, Guha, and Beni (2020), sustaining reliable and contaminant-free drinking water is increasingly challenging worldwide due to human activity, industrial waste, and agricultural overuse. The



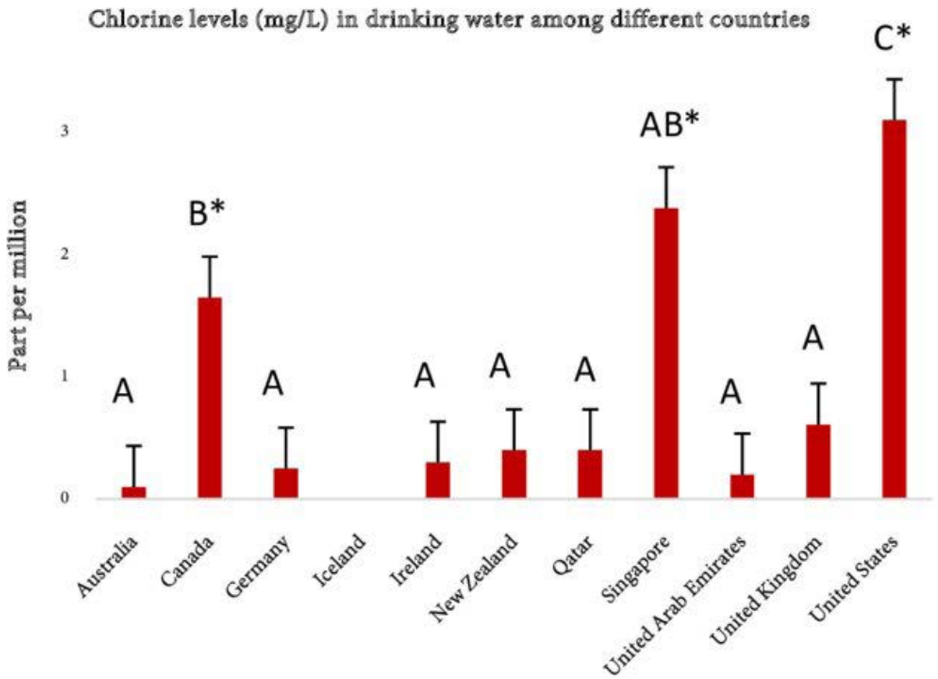


Figure 2: A statistical analysis was conducted to determine significant mean differences in chlorine levels (mg/L) among different countries. ANOVA statistical tests ($P < 0.05$, Tukey-adjusted ANOVA) revealed significant differences in chlorine levels in drinking water. The variations in chlorine levels across different countries are represented by letters A, B, and C, while statistical significance is indicated by an asterisk (*). Reproduced from Karim et al., 2020 under CC BY 4.0.

authors analyzed data on chlorine and THM levels in the European Union, the United States, Canada, the United Kingdom, Singapore, New Zealand, Australia, Qatar, and the United Arab Emirates. Chlorine levels in drinking water were found to show statistically significant variation among the countries studied (figure 1) whereas THMs were ubiquitous (figure 2)[2].

The Netherlands is one of the few countries that do not use chlorine for drinking water disinfection. Instead, it employs a multi-barrier treatment approach using the best available water sources, physical treatment processes like sedimentation, filtration, and UV-disinfection, while avoiding chlorine. This approach ensures microbial safety without introducing THMs into the water supply. The Dutch method emphasizes preventing contamination during distribution, producing biologically stable water, and monitoring the system to promptly detect any failures. Recent advancements in the Netherlands include implementing quantitative microbial risk assessment and researching biostability during water distribution[5].
Given the findings of the Swedish study, it might be worth considering whether more countries should adopt the Dutch approach to water disinfection. By employing a combination of advanced physical treatment processes and robust monitoring systems, other countries could potentially reduce the risk of harmful by-products like THMs while maintaining the microbial safety of their drinking water.
Nonetheless, it's unlikely that there will be a one-size-fits-all approach to providing safe and clean drinking water for all. Especially for developing countries, chlorine will remain the most effective, affordable, accessible, and convenient drinking water treatment method available. In many cases, the best approach to mitigate risks from THM exposure will be to set sufficiently protective regulatory limits.

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Is Your Drinking Water Safe? Study Suggests Chlorination Levels May Be Too High in the US

Experts say drinking water disinfection is essential, but current methods may expose people to potentially harmful compounds.

By **Stacey Leasca** | Published on February 21, 2025

PHOTO: FOOD & WINE / GETTY IMAGES

AIO toxafette - Houman Kahroba

In the toxafette, PhD-students working in the toxicology field get the chance to open up about their experiences in performing research. Every issue a new candidate answers a series of questions, and then pass the baton to a fellow PhD-student. This time Michele Davigo, from Maastricht University tells us about his project.

Can you introduce yourself?
Hi, I am Houman Kahroba, a dual PhD candidate in Translational Genomics at Maastricht University and Biomedical Science at Hasselt University. My academic journey started with a Bachelor's in Cellular and Molecular Biology, followed by a Master's in Human Molecular Genetics, and also joining to Molecular Medicine Department where I combined lab-based research with clinical applications. This unique background fueled my passion for understanding how environmental exposures, specifically air pollution, affect human health at a molecular level.

My current research focuses on how black carbon particles from air pollution impact pregnancy and fetal development. By working with the ENVIRONAGE cohort, I use cutting-edge tools to detect these particles in the placenta and fetal brain and investigate how they disrupt critical developmental pathways. With my PhD spanning two universities, two disciplines, and two research cultures, I thrive in interdisciplinary science and enjoy tackling complex environmental health challenges.

How would you explain the subject of your research to a layperson?
Imagine your body as a bustling city, where tiny "delivery trucks" (extracellular vesicles) transport important messages between cells. My research investigates how air pollution, specifically black carbon particles from car exhaust, hijacks these delivery trucks. These particles can travel from the mother's lungs to the placenta and even reach the developing fetal brain.
Think of it like unwanted packages sneaking into a secure building, pollutants bypass natural defenses and interfere with crucial communication between cells during pregnancy. This can impact fetal growth and brain development. By studying this process, I aim to uncover how pollution affects the earliest stages of life and find ways to better protect expecting mothers and their babies.

How is your research related to the field of toxicology, and why did you choose this subject?
My research bridges environmental toxicology, molecular toxicology, and developmental toxicology:

- Environmental Toxicology → Studies how pollutants, like black carbon, enter the body.
- Molecular Toxicology → Investigates how these pollutants damage cells and disrupt gene expression.
- Developmental Toxicology → Examines how pollution exposure during pregnancy impacts fetal growth and long-term health.

By directly detecting black carbon in the placenta and fetal brain, I contribute to chemical risk assessments and provide scientific evidence for stronger air quality regulations. My clinical genetics background also allows me to explore how genetic differences influence susceptibility to pollution exposure, an important step towards personalized environmental health.

I chose this field because air pollution is an invisible yet major public health crisis. We often think of pollution as an outdoor issue, but my research shows that it enters our bodies and affects the most vulnerable, unborn children.

What was your motivation for starting a PhD program?
I've always been fascinated by how our genes interact with the environment. During my master's, I became deeply interested in conditions like preterm birth and autism, where genetic and environmental factors collide, but clinical solutions remain unclear.

Air pollution stood out to me as a widespread yet understudied exposure that could be a hidden driver of developmental issues. Joining the ENVIRONAGE cohort, a pioneering study on pollution and placental biology, gave me the perfect platform to combine molecular genetics, environmental toxicology, and public health. The dual PhD structure also allowed me to leverage expertise from both Hasselt and Maastricht Universities, giving me a truly interdisciplinary research experience.

How do you see the future of your research topic? (Follow-up research / Social impact)

Short-term Goals:

- Improve real-time tracking of black carbon in extracellular vesicles, allowing earlier detection of pollution-related risks in pregnancy.
- Develop biomarkers that clinicians could use in prenatal screenings to identify high-risk pregnancies.

Long-term Vision:

- Translate findings into personalized interventions for pregnant women in high-pollution areas.
- Influence public health policies, for example, advocating for low-emission zones near schools and maternity hospitals.

Beyond science, I hope my research raises awareness. When people understand that pollution can reach unborn babies, they care more about air quality. Imagine a future where prenatal care includes pollution risk scores, just like we screen for genetic disorders. This is the kind of real-world impact I strive for.

Can you explain how different subjects relate to your research, and why this connection matters?

My work sits at the crossroads of multiple fields:

- Environmental Toxicology → Tracks how pollutants infiltrate the placenta.
- Molecular Biology → Examines how pollutants alter gene expression and cellular communication.
- Developmental Biology → Identifies critical windows of vulnerability during pregnancy.
- Nanotechnology → Uses cutting-edge imaging to detect nanoparticles of pollution inside fetal tissues.

A single-discipline approach won't solve environmental health problems. By integrating these fields, we can turn scientific discoveries into tangible public health solutions.

How do you expect society will benefit from your PhD research? (Social impact)

- Policy & Regulation → Provides scientific evidence for stricter emission standards.
- Healthcare → Develops biomarkers for early detection of pollution-related pregnancy risks.
- Public Awareness → Translates complex science into practical advice for expecting parents.
- Global Health → Adapts detection methods for use in low-income, high-pollution regions.

Bottom line: My research bridges molecular science with real-world action, ensuring cleaner air isn't just an environmental issue, it's a maternal and child health priority.

How do you balance your PhD with your personal life? (Work-life balance)

Doing a dual PhD across two universities is exciting but demanding. To stay balanced:

- I use a sprint-and-recover approach, intensive lab weeks followed by writing/analysis periods for mental recovery.
- I delegate tasks between my teams in Maastricht and Hasselt to avoid burnout.
- I set strict work boundaries, like avoiding emails past midnight.
- I make time for volleyball, squash, and dinners with friends and family, which keep me grounded.

Work-life balance is a constant adjustment, but sustainable productivity requires respecting both science and self.

How do you handle unexpected or questionable data? (Data management)

When I face unexpected results, I follow a structured troubleshooting process:

1. Replicate the experiment to rule out technical errors.
2. Check with instrument manufacturers, sometimes the problem is a software bug or calibration issue.
3. Consult external researchers on ResearchGate, collaboration often reveals hidden insights.
4. Bring all findings to meetings with my supervisors, ensuring transparency and new perspectives.

Instead of discarding anomalies, I treat them as opportunities, some of my most interesting discoveries started with "wrong" results!

What are your career goals after your PhD? Inside or outside academia? Would you go abroad?

I want to stay in academia and establish my own research line in close collaboration with my colleagues and promotors. Rather than focusing on methods and materials, I want to work with ideas, shaping innovative frameworks that advance our understanding of environmental toxicology and genomics.

I am also open to international collaborations, especially in high-pollution countries where my research can make a direct impact.

What do you like most about doing your PhD?

The thrill of connecting dots across disciplines! Seeing a pollution particle under a microscope, linking it to gene expression changes in fetal cells, and realizing it could explain rising neurodevelopmental disorders, that's what excites me.

Also, the human side of research, mentoring students, discussing ideas with peers, and translating science into real-world impact, makes every challenge worth it.

NVT Annual Meeting 2025

From Lab 2 Law: Bridging the Gap from Science to Policy

Dear NVT members,

The 46th Annual Meeting of the Dutch Society of Toxicology (NVT) will take place on **June 4 and 5**, 2025, at De Reehorst in Ede. Registration opens on February 14, 2025, and abstracts can be submitted until **March 31**, 2025.

The theme of this years' meeting is 'From Lab 2 Law: Bridging the Gap from Science to Policy. Toxicology plays a crucial role in shaping policies that protect human and environmental health, yet translating scientific findings into regulatory decisions remains a challenge. As scientific advancements continue to refine our understanding of toxicity, the question remains: How do we ensure that research findings effectively inform policy and legislation?

This meeting will explore the dynamic relationships between toxicology and regulation. Through keynote lectures, interactive workshops, and poster sessions, we will highlight how scientific evidence contributes to risk assessment, regulatory frameworks and decision-making. Experts from academia, industry, and government will share insights on navigating the complexities of policy development ensuring that toxicological research leads to meaningful impact.

The program is already available on: https://meeting2025.toxicologie.nl/
Curious? Stay tuned as we craft an engaging program tailored to your interests.
Mark your calendars for the 46th NVT Annual Meeting: NVT Annual Meeting 2025.ics

Kind regards,
NVT organizing committee

Damian Roelofsen, Filippo di Tillio, Imke Bruns, Olivia Klatt, Kirsten Lassing, Roxana Mirzarbandi, Frederik-Jan van Schooten, Hans Bouwmeester, Joanne Salverda, Laura Hondebrink, Paul Jenning

P.S. We will be active on LinkedIn this year to announce updates of the annual meeting. Be sure to follow our LinkedIn page: www.linkedin.com/in/nvt-meeting-78b52329a

59th Congress of the European Societies of Toxicology (EUROTOX 2025)

Dear Colleagues,

It is with a great pleasure and enthusiasm that we warmly invite you to the 59th Congress of the European Societies of Toxicology.

The EUROTOX 2025 Congress will take place on 14-17 September 2025, in Athens, Greece.

The real risks society faces, render necessary an approach that integrates scientific and technological advances, ensuring new technologies do not endanger health or the environment. Toxicology must evolve to address these challenges, developing sustainable solutions for long-term public health. The theme of Eurotox 2025, *"Toxicology addresses Society's real-life risks for sustainable health and well-being,"* addresses this need.

Eurotox 2025 will take place at the impressive Megaron Concert Hall and aims to be the key platform so as to advance this approach and develop practical programs that safeguard health and sustainability.

Our 2025 programme will focus on cutting-edge scientific breakthroughs and interdisciplinary collaboration. Emerging technologies and methodologies in toxicology will be highlighted, while fostering discussions on public health safety and environmental sustainability. We are thrilled to host keynote speakers from leading institutions and offer ample opportunities for young scientists to showcase their work through oral and poster presentations.

Athens is one of the world's oldest cities. It was a centre for democracy, the arts, education and philosophy, thus regarded as the cradle of Western civilization. The city's ancient landmarks such as the Parthenon, the Akropolis and Plaka reflect its exciting history and culture. The mild Mediterranean climate, the unique combination of glorious history with modern, urban innovation, the high standard hotel accommodation, the vibrant atmosphere, the world-renowned cuisine, feature Athens as the ideal all year round destination. The rich social program will complement your EUROTOX 2025 experience!

We look forward to welcoming you to Athens for a memorable and inspiring EUROTOX 2025!

Warm Regards,

Prof. Aristides M. Tsatsakis
EUROTOX 2025 Congress President

Dr. Thomas Weiser
EUROTOX President

EUROTOX
ATHENS GREECE 14th–17th September 2025

Congratulations to the TCDD Christmas Puzzle Contest Winner!

We are thrilled to announce that Peter van Kessel, Counsellor Chemical Compound at Environmental Governmental Service Flevoland, Gooi and Vechtstreek, is the winner of this year's TCDD Christmas Puzzle Contest! Peter's sharp problem-solving skills have yet again earned him this well-deserved recognition.

Kudos to Peter!

REGISTRATIE CIE

Inschrijving register

Voorletters	Achternaam	Datum inschrijving	Datum afloop registratie
J.	Vriend	18-02-2025	18-02-2030
L.	Zheng	18-02-2025	18-02-2030
J.	Chen	18-02-2025	18-02-2030
Q.	Ren	18-02-2025	18-02-2030
L.S.	Gerber	18-02-2025	18-02-2030
J.	Wang	18-02-2025	18-02-2030

Inschrijving TiO

Voorletters	Achternaam	Opleider	Datum inschrijving
M.H.F.	Graumans	Dr.ir. P.T.J. Scheepers	10-02-2025
C.	Xing	Prof.dr. R. Masereeuw	10-02-2025

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REVIEW

Barae Jomaa

Endpoints Lacking Animal-Free Alternatives Under REACH and the Remaining Challenges

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Review

Endpoints Lacking Animal-Free Alternatives Under REACH and the Remaining Challenges

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Abstract
The European Union's REACH regulation mandates comprehensive chemical testing to ensure human and environmental safety, prioritizing non-animal methods whenever possible. Despite significant advancements in alternative testing strategies, certain endpoints still require animal studies due to the lack of validated in vitro, ex vivo or in silico models. This paper examines these gaps by tonnage band, highlighting key areas where non-animal alternatives remain insufficient. Acute oral, dermal, and inhalation toxicity tests, repeated-dose toxicity studies as well as reproductive and developmental toxicity studies are among the endpoints that are still reliant on animal testing. While progress has been made in developing alternatives such as Defined Approaches (DAs) that may integrate physiologically based kinetic (PBK) modeling, these methods are not yet fully validated for regulatory acceptance. Continued investment in research, validation and international collaboration is essential to accelerate the validation and implementation of animal-free alternatives.

Keywords
REACH Regulation, Animal Testing, Non-Animal Alternatives, Toxicity Assessment, In Vitro Methods, Regulatory Gaps, NAMs

1. Introduction

The need to test chemicals for their safety pre-dates the modern era. In ancient times, poisons were tested directly on humans, especially less fortunate ones. Mithradates, king of Pontus in northern Anatolia, and Attalus III, king of Pergamon, both engaged in early forms of chemical safety testing (1). Attalus III, ruling from 138 BC to 133 BC, tested antidotes for poisons, including venom from various creatures, exclusively on condemned criminals. Mithradates VI, ruling from 120 BC to 63 BC, famously developed an immunity to poisons through what was later called ″Mithridatism,″ the regular ingestion of sub-lethal doses. He also tested poisons and antidotes on condemned criminals, continuing a practice of human testing that is famously illustrated in Alexandre Cabanel's 1887 painting titled "Cleopatra Testing Poisons on Condemned Prisoners". Unethical testing on human subjects was mostly abolished when the atrocities of Nazi human experiments came to light and the Nuremberg Code was drafted. The code not only requires consent from the human subjects but also states that such experiments should be based on the results of animal experiments. Animal experiments remained the gold standard of chemical safety testing for the most part of the 20th century. The shift towards animal testing free alternatives began in the 1980's with the development of a Reconstructed human Epidermis (RhE) skin model (2) which would form the basis for in vitro skin irritation tests(3). The development of this alternative test was largely in response to public outcry over the testing of cosmetics on the eyes and skin of animals – in many cases causing excessive suffering (4).
The EU regulation regulating the Registration, Evaluation, Authorisation and Restriction of Chemicals (REACH) requires comprehensive testing of chemicals in order to identify and manage the potential risks to humans and the environment. The testing requirements are organized by annual tonnage bands at which a chemical is placed on the market – starting from 1 metric tonne and ending with > 1,000 metric tonnes. The higher the annual tonnage the more demanding the testing required in terms of both time and cost. Importantly, the regulation mandates the utilization of animal testing only as a last resort. In many cases an analogous substance or group of substances can serve as a surrogate for new testing (5). This read-across approach must be founded on a clear hypothesis that connects similarities, such as common functional groups, common precursors, common degradation products, or consistent potency trends, to a predicted outcome. Supporting evidence is essential and can include quantitative structure-activity relationships (QSARs), information on toxicokinetics and experimental data from in vitro or in in vivo (bridging) studies. This is explained in detail as part of ECHA′s Read-Across Assessment Framework (RAAF), which offers a systematic framework to assess the scientific validity of a read-across (6). Adherence to the principles of RAAF makes it more likely that a read-across will be accepted by regulators. Nonetheless, when such a read-across cannot be scientifically justified, and when suitable validated non-animal methods are not available for a given endpoint, regulators may still require animal tests to be conducted.
A read-across can also be used as part of an Integrated Testing Strategy (ITS), now more commonly referred to as an Integrated Approach to Testing and Assessment (IATA), to fulfil an information requirement while minimizing the use of animal testing. IATA combines data from multiple sources, such

as in vitro assays, computational models, Defined Approaches (DAs) and read-across, to create more comprehensive, human-relevant predictions. IATAs employ a flexible, weight-of-evidence approach and expert judgment to assess chemical safety, often eliminating the need for animal testing. In contrast, DAs, rely on fixed data sources and interpretation procedures and can be incorporated within an IATA or used independently to address specific hazard information requirements. As they are based on fixed data sources, DAs, unlike IATAs, can be validated and incorporated into OECD test guidelines. Such guidelines benefit substantially from the Mutual Acceptance of Data (MAD), whereby experimental data generated in compliance with OECD Test Guidelines and Good Laboratory Practice (GLP) are accepted by all member countries for regulatory purposes (7).

Endpoint	REACH Annex	OECD Test Guideline No.	Test Animal
Acute toxicity: oral	VII	420, 423, 425	Rats [a]
Acute toxicity: dermal	VIII	402	Rats [a]
Acute toxicity: inhalation	VIII	403, 433, 436	Rats [a]
Sub-acute (28-day) repeated dose toxicity: oral	VIII	407, 422	Rats [a]
Sub-acute (28-day) repeated dose toxicity: dermal		410	Rats, rabbits or guinea pigs [a]
Sub-acute (28-day) repeated dose toxicity: inhalation		412	Rats [a]
Screening for reproductive/developmental toxicity	VIII	421, 422	Rats [a]
Short-term toxicity testing on fish	VIII	203	Fish
Genotoxicity in vivo: Mammalian Erythrocyte Pig-a Gene Mutation Assay	VII-X [b]	470	Rats or mice
Genotoxicity in vivo: Mammalian erythrocyte micro-nucleus test	VII-X [b]	474	Rats or mice [a]
Genotoxicity in vivo: Mammalian bone marrow chromo-some aberration test	VII-X [b]	475	Rats or mice [a]
Genotoxicity in vivo: Dominant lethal (DL) test	VII-X [b]	478	Mice [a]
Genotoxicity in vivo: Mammalian spermatogonial chromo-somal aberration test	VII-X [b]	483	Mice [a]
Genotoxicity in vivo: Transgenic Rodent Somatic and Germ Cell Gene Mutation Assays	VII-X [b]	488	Rats or mice [a]
Genotoxicity in vivo: Mammalian alkaline comet assay	VII-X [b]	489	Rats [a]
Extended one-generation reproductive toxicity (EOGRT)	IX	443	Rats [a]
Pre-natal developmental toxicity	IX and X [c]	414	Rats or rabbits [a]
Sub-chronic (90-day) repeated dose toxicity: oral	IX	408, 409	Rats [a], non-rodents e.g. dog, respectively
Sub-chronic (90-day) repeated dose toxicity: dermal	IX	411	Rats, rabbits or guinea pigs [a]
Sub-chronic (90-day) repeated dose toxicity: inhalation	IX	413	Rats [a]
Long term fish toxicity: Fish, Early-Life Stage Toxicity Test	IX	210	Fish
Long term fish toxicity: Fish, Juvenile Growth Test	IX	215	Fish
Bioaccumulation in fish, aquatic (assume dietary test)	IX	305	Fish
Chronic toxicity	X	452 or 453	Rats [a]
Carcinogenicity	X	451 or 453	Rats [a]
Long term or reproductive toxicity tests on birds	X	206	Birds

Despite the advancements in animal testing free methods and approaches, ECHA still requires animal testing for certain endpoints. This paper will focus on the gaps that still exist for the full replacement of animal tests within the framework of REACH, by tonnage band (Table 1).

[a] preferred species, justification needed if another species is used.

[b] Annex VII triggers Annex VIII in vitro mutagenicity testing and if any of in vitro mutagenicity studies are positive, relevant in vivo tests are required, unless it can be shown that the substance is not expected to have systemic availability.

[c] Required in one species under Annex IX and two species under Annex X information requirements.

Table 1: Animal testing, within the scope of EU Directive 2010/63/EU, that is required under REACH and with no validated in vitro alternatives that can act as a full replacement

2. 1-10 tonnes per annum (REACH Annex VII)

The first tonnage band of REACH requires substantial physicochemical testing but only a handful of tests covering human health. These include tests for skin irritation/corrosion, eye irritation/damage, skin sensitization, in vitro mutagenicity and acute oral toxicity. From these tests, only acute oral toxicity does not currently have a validated non-animal method. As will be discussed later in more detail, positive results from in vitro genotoxicity studies trigger the need to test in vivo.

2.1 Acute oral toxicity

In vitro tests for acute oral toxicity are challenging due to the complex nature of a whole organism. While standalone tests using isolated cells or tissue constructs can replicate the effect of chemicals on the epidermis and corneal epithelium, these tests can't replicate the intricate processes of a whole organism, such as metabolism, absorption, and distribution. Additionally, organ-specific toxicity poses another challenge, as different organs can react differently to chemicals, making it hard to predict overall acute oral toxicity using a standalone in vitro method. It is more likely that a replacement would have to include a test battery supplemented by Physiologically based kinetic (PBK) modelling.

Correlations between LD50 values in animals and IC50 cytotoxicity values in vitro have been explored as part of a NICEATM/ECVAM validation study of the Neutral Red Uptake (NRU) Cytotoxicity assay. The results showed that the ability of this test to predict GHS acute oral toxicity categories was only 29-31%, depending on the cells used. Nonetheless, ECVAM conducted a follow-up study that found potential use of the NRU Cytotoxicity assay for predicting substances with an LD50 greater than 2,000 mg/kg bw. The results indicated high sensitivity of 92-96% but specificity of only 40-44%, indicating a tendency to produce false positives. The conclusion was that this test may be used as part of a weight of evidence approach but may underpredict chemicals that first require biotransformation or exert their action via specific mechanisms of action (8).

2.2 Mutagenicity

Under REACH Annex VII, the bacterial reverse mutation assay (Ames test, OECD 471) is required as an initial screening tool for gene mutation potential. This test is widely used due to its high sensitivity in detecting mutagenic carcinogens. However, because bacterial systems lack the metabolic complexity of mammalian cells, a positive result in the Ames test necessitates further investigation following tests typically mandated for Annex VIII, in order to

determine whether the mutagenic potential extends to eukaryotic systems. This is needed because the Ames test may produce false-positive results due to fundamental differences between bacterial and mammalian cells in metabolism and DNA repair. Additionally, the rat liver post-mitochondrial fraction (S9) used in the Ames test differs from human liver metabolism and lacks the barrier functions present in intact hepatocytes. (9). As with most standalone tests, the absorption, distribution, metabolism, elimination (ADME) that occurs in a whole organism is also lacking – potentially leading to overexposure. While the Annex VIII tests are also in vitro, positive results in those tests may trigger the need to test in vivo.

3. 10-100 tonnes per annum (REACH Annex VIII)

3.1 Mutagenicity – further testing

In vitro mutagenicity tests required under annex VIII include in vitro mammalian chromosome aberration or micronucleus formation (OECD TG 473 or 487) and, if negative and the Ames test is also negative, an in vitro gene mutation test in mammalian cells (OECD TG 476 or 490). Any positive result in vitro, requires in vivo mutagenicity testing (OECD TG 470, 474, 475, 483, 488 or 489), unless it can be shown that the substance is not expected to have systemic availability. Positive results in in vitro tests need to be confirmed in vivo since, as with the Ames test, Annex VIII mutagenicity tests lack ADME and suffer from differences between human liver metabolism and the rodent S9 fractions to simulate metabolic activation. The selection of an appropriate in vivo genotoxicity assay depends on the nature of the positive in vitro response and must be determined on a case-by-case basis (10). For substances that have tested positive for chromosomal aberrations in vitro (using OECD TG 473 clastogenicity or OECD TG 487 for both aneugenicity and clastogenicity), follow-up in vivo somatic cell tests should be selected to investigate structural (clastogenic) or numerical (aneugenic) chromosome abberations. For in vitro clastogens, follow-up testing with an in vivo CA test or the in vivo rodent micronucleus (MN) test assay is acceptable, whereas for in vitro aneugens, the in vivo MN test is required since it is the only validated assay for measuring aneugenicity. For substances inducing gene mutations in vitro, in vivo transgenic rodent (TGR) assays are preferred but a comet or Pig-a test may also be conducted. If an in vivo somatic cell study is positive, the potential for germ cell mutagenicity should be evaluated based on all available data, including toxicokinetics, with further in vivo germ cell studies considered if necessary. These include the rodent dominant lethal (DL) test, TGR or the Mammalian Spermatogonial Chromosomal Aberration Test (Table 1). In order to avoid unnecessary animal testing, if the TGR assay is conducted on somatic tissues, germ cell samples should be collected, frozen, and later analyzed for mutagenicity if the result of the somatic test is positive.

3.2 Acute inhalation and dermal toxicity

Various in vitro models of the respiratory tract are available but they have not yet been validated and therefore also lack an OECD test guideline (11). 3D models of the skin are available and in wide use for skin irritation and sensitization testing – endpoints with OECD test guidelines. Moreover, in vitro dermal penetration can be conducted using OECD TG 428 (12). The in vivo test for acute dermal toxicity (OECD TG 402) is designed to assess the potential health hazards of a substance following a single, short-term dermal exposure. This type of study evaluates both local effects, like skin irritation, and systemic effects resulting from the absorption of the substance through the skin (13). Overall, while skin irritation and penetration testing in vitro can provide information on certain aspects of the dermal toxicity test in vivo, they are unable to cover more complex systemic effects that can be tissue specific and may be influenced by ADME. Additionally, these assays do not account for interactions involving the immune system, endocrine system, or the interplay between different tissues and organs.

3.3 Sub-acute (28-day) repeated dose toxicity

Replacing repeated dose toxicity tests conducted in vivo with in vitro alternatives for chemicals is challenging due to several factors. In vitro systems must replicate the complex interactions and environments found in living organisms. The concentration-effect relationships of chemicals are influenced by their activity, target sensitivity, and distribution within the in vitro system. In repeated dosing the challenge to develop in vitro replacements is even greater. For instance, in a paper outlining results from the EU FP7 Predict-IV project, lipophilic chemicals were found to have a tendency to bind to plastic labware, while some chemicals bind to cell-attachment matrices (14). Additionally, certain chemicals, can accumulate in cells over time, leading to increased toxicity with repeated dosing. Nanomaterials present further challenges since they can remain suspended in the culture medium, resulting in limited direct exposure to cells, or settle, increasing local concentrations at the cell surface and potentially enhancing cellular uptake (15,16). Current in vivo studies, such as OECD 407 and OECD 408, assess various endpoints, including body weight changes, over extended periods (28 or 90 days). While it is conceivable to have, in the future, a DA combining a test battery with PBK modelling, there are currently no existing methods to predict in vivo chemical effects on mammalian growth based on in vitro studies.

3.4 Screening for reproductive/developmental toxicity

Efforts to find alternatives to in vivo screening for reproductive and developmental toxicity have included the use of zebrafish embryos, which are not protected under EU Directive 2010/63/EU (17) on the protection of animals used for scientific purposes. The directive applies to live non-human vertebrate animals, including independently feeding larval forms and foetal forms of mammals as from the last third of their normal development, as well as live cephalopods (18). Zebrafish embryos were used to evaluate developmental toxicity, leading to the development of a General Developmental Score (GDS) system. This system extends the existing General Morphology Score (GMS) by incorporating additional parameters affected by toxic compounds. The GDS system, which includes both developmental morphologies and dysmorphologies, proved effective in detecting developmental toxicity and may be useful in integrated testing strategies to reduce, refine, and potentially replace animal testing (19).

However, besides the official EU consensus (20), ethical concerns may persist since this approach may not be unanimously accepted as animal-free (21), highlighting the need for continued research and development to address these issues. Moreover, interspecies differences further complicate the translation of in vitro results to humans, as biological responses can vary significantly between species.

In vitro tests that do away with whole organisms, regardless of their developmental stage, and instead focus on alternatives such as cell-based, tissue-based or organotypic models, will face similar challenges to what has been described. This includes, but is not limited to, lack of ADME, and the overall complexity of trying to mimic an entire organism and its tissue specific responses, in vitro. These challenges underscore the necessity for ongoing advancements in this field.

3.5 Short-term toxicity testing on fish

While there isn't a standalone replacement for short-term toxicity testing on fish, the RTgill-W1 cell line assay, described in OECD Test Guideline 249, offers a promising alternative to traditional animal testing for acute toxicity. This assay uses a permanent cell line from rainbow trout gill (RTgill-W1) to assess cell viability after 24 hours of exposure to test chemicals. The assay generates concentration-response curves to determine the effective concentration causing 50% loss in cell viability (EC50). In this manner, it can serve as a range-finding tool before a fish acute toxicity test or as part of an IATA combining approaches such as QSARs, read-across and non-guideline methods (22). The Fish Embryo Acute Toxicity (FET) test (OECD TG 436) can also be used to reduce the number of fish used in acute aquatic toxicity tests (23) or as part of an IATA.

4. 100-1,000 tonnes per annum (REACH Annex IX)

4.1 Sub-chronic (90 days) toxicity

While a sub-acute (28-day) study already encompasses aspects that are difficult to capture in vitro, such as metabolic transformation and distribution across multiple organ systems, the sub-chronic (90-day) toxicity study remains essential under REACH. This is because it also captures cumulative and delayed effects that a shorter exposure period may miss. Over an extended period, chemicals can induce progressive changes in organ function, lead to histopathological alterations (such as fibrosis, which takes time to develop), or trigger compensatory biological responses that only emerge after prolonged dosing. Although alternative methods to animal testing can be developed to simulate key metabolic and distribution processes, replicating the dynamic, integrated responses of a whole organism over extended periods remains a significant challenge.

In an effort to address this challenge, researchers from the European Joint Research Centre (JRC) developed a method to predict chronic toxicity by analyzing time-concentration-response relationships in vitro (24). This method introduces a chronicity index to measure cumulative chemical effects over time, improving the applicability of in vitro methods for long-term toxicity assessments. By examining how concentration and exposure time jointly influence toxic responses, this approach aims to estimate in vivo chronic toxicity based on in vitro testing. Nonetheless, limitations remain and include potential variability in cell responses and the challenge of accurately extrapolating in vitro results to in vivo scenarios.

4.2 Pre-natal developmental toxicity

The OECD TG 414 study assesses the effects of chemical exposure on pregnant animals and their fetuses (25). Developing in vitro alternatives for this study presents significant challenges, primarily due to the complexity of maternal-fetal interactions. The placenta plays a critical role in nutrient exchange, chemical metabolism, and immune regulation, influencing fetal susceptibility to toxicants. While placenta-on-a-chip models (26) and trophoblast cell cultures (27) can simulate some placental functions, they cannot fully replicate dynamic maternal metabolism, placental transport kinetics, or endocrine signaling over an extended period.

Another major limitation is the time-dependent nature of organogenesis. In vitro assays, such as embryonic stem cell tests (ESTs), provide valuable mechanistic insights (28) but lack the ability to model late-stage fetal development (29) and are limited by the absence of maternal metabolism.

4.3 Extended One-Generation Reproductive Toxicity (EOGRT)

The OECD TG 443 study evaluates the effects of chemical exposure on fertility, in utero development, postnatal growth, neurotoxicity and immunotoxicity in offspring (30). This study provides information on transgenerational effects, making its replacement with in vitro alternatives particularly challenging. This transgenerational aspect of EOGRT studies, which assesses how parental exposure affects subsequent generations, would require the evaluation of epigenetic modifications, germline effects as well as endocrine-mediated development. Additionally, neurobehavioral toxicity endpoints rely on complex nervous system interactions that push the boundaries of current in vitro methods (31). Until validated in vitro approaches can reliably predict these complex effects, OECD TG 443 remains necessary under REACH.

4.4 Long-term toxicity testing on fish

4.4.1 Fish early-life stage (FELS) toxicity

The Fish Early-Life Stage (FELS) Toxicity Test, as defined by OECD TG 210, is designed to assess the effects of chemical exposure on fish development from the embryonic phase through early larval stages. This long-term study evaluates critical endpoints such as hatching success, developmental abnormalities, and survival, which are indicative of the overall developmental toxicity. Developing in vitro alternatives for this test presents significant challenges because early-life stage development is governed by intricate, time-sensitive processes that involve coordinated morphogenesis and endocrine signaling. Although in vitro methods—such as fish embryo toxicity (FET) assays—offer useful mechanistic insights, they are generally limited to short-term exposure scenarios and do not fully capture the cumulative and delayed effects on growth and developmental progression. Moreover, in vitro systems cannot readily replicate whole-organism interactions, including nutrient transfer and compensatory developmental mechanisms, which are essential for a comprehensive assessment of early-life stage toxicity. Consequently, achieving regulatory acceptance of alternative methods that fully predict the in vivo outcomes of the FELS test remains a formidable challenge.

4.4.2 Fish, juvenile growth

The Fish, Juvenile Growth Test (OECD TG 215) is designed to assess the effects of chemical exposure on growth parameters over an extended period, integrating endpoints such as survival, weight gain, as well as abnormal appearance and behavior (32). Developing in vitro alternatives to this test is challenging because growth in fish results from complex, multi-organ interactions and endocrine regulation that cannot be easily replicated in isolated cell systems. In vitro models, such as fish cell lines or organoids, can provide useful mechanistic insights into cytotoxicity and metabolic responses; however, they lack the physiological context necessary to mimic the integrated processes of energy metabolism, tissue development, and endocrine control that underlie growth over time. Moreover, long-term exposure studies require the simulation of chronic exposure conditions, including the gradual accumulation of effects that influence organismal growth—a scenario that is difficult to capture using current in vitro systems. Consequently, despite advances in alternative methods, the complexity of in vivo growth processes makes it challenging to fully replace the Fish, Juvenile Growth Test with non-animal approaches.

4.5 Bioaccumulation in aquatic species, preferably fish

Bioaccumulation data is crucial for understanding the environmental behavior of substances and is utilized across various regulations, including REACH and BPR. This data is essential for PBT (Persistent, Bioaccumulative, and Toxic) assessments, hazard classification, and chemical safety assessments, including food chain exposure modeling. Highly bioaccumulative substances pose significant risks as they can transfer through the food web, potentially leading to biomagnification.

The n-octanol/water partition coefficient (Log Kow) is widely used as a preliminary indicator of bioaccumulation potential. However, for a more comprehensive assessment, higher-tier data often necessitates in vivo fish testing following the OECD 305 Test Guideline. Alternative methods, such as the freshwater amphipod Hyalella azteca bioconcentration test (HYBIT, OECD TG 321), have been developed to provide more ethical and efficient bioaccumulation assessments that avoid the use of vertebrate animals. In October 2024, the EU Member State Committee (MSC) unanimously approved HYBIT as the standard method for bioaccumulation studies under the REACH regulation, marking a significant shift towards reducing vertebrate testing. However, it's important to note that HYBIT is currently validated for organic substances where aqueous exposure is feasible; for substances not amenable to aqueous exposure or for inorganic substances, traditional fish-based studies, such as OECD TG 305, remain applicable (33). To overcome limitations caused by substances that are not amenable to aqueous exposure, exposure is conducted via feed.

5. 1,000 or more tonnes per annum (REACH Annex X)

5.1 Chronic toxicity

Chronic toxicity testing under REACH is still required to capture cumulative and delayed effects over extended exposures (10). This is conducted using OECD TG 452, which involves a chronic toxicity testing phase typically

conducted over 12 months, or OECD TG 453, which includes a 12-month chronic toxicity testing phase followed by a 24-month carcinogenicity testing phase. As discussed in the sub-acute and sub-chronic toxicity sections, replicating whole-organism responses in vitro remains a significant challenge. While promising approaches like in vitro time-concentration-response modeling are emerging, they have yet to achieve regulatory acceptance as complete replacements for animal testing.

5.2 Carcinogenicity

Carcinogenicity testing may be required, under REACH Annex X, even after in vitro and in vivo mutagenicity studies have been conducted, particularly for substances with widespread dispersive use or frequent and long-term human exposure (10). This testing can be conducted using OECD TG 451 or OECD TG 453 (Table 1). If a substance is classified as a germ cell mutagen (Category 2) or if repeated-dose toxicity studies indicate hyperplasia or pre-neoplastic lesions, further carcinogenicity studies may be proposed by the registrant or required by the European Chemicals Agency (ECHA). However, for substances classified as germ cell mutagens (Category 1A or 1B), a genotoxic mechanism for carcinogenicity is presumed, and additional testing is typically not necessary. Although mutagenicity is an important indicator of potential carcinogenicity, it does not fully predict tumorigenic potential, as carcinogenesis involves multiple biological processes beyond mutation induction (34). Specifically, differences in metabolic activation, DNA repair mechanisms, and non-genotoxic pathways such as chronic tissue irritation or receptor-mediated effects can contribute to tumor formation (35–37). Therefore, while mutagenicity testing is a crucial step in chemical safety assessment, carcinogenicity studies remain necessary in certain cases.

5.3 Long-term or reproductive toxicity to birds

Long-term or reproductive toxicity to birds may be required, under Annex X, when there is evidence suggesting that a substance could have long-term environmental effects, particularly to predators exposed through the food chain. Despite the species differences, it is recommended to first consider the mammalian toxicity data available. Moreover, in ECHA's Endpoint specific guidance, it is stated that avian toxicity testing will typically not be necessary and that such tests are more likely to be carried out to comply with the requirements of the biocidal products regulation (BPR) or for approval of Plant Protection products Regulation (PPPs) (38). OECD Test Guideline 206 (Avian Reproduction Test) is the standard method to assess the effects on egg production, hatchability, chick survival, and effects on young birds (39). There are no validated in vitro alternatives available or being developed (38).

6. Conclusion

The REACH regulation is a significant piece of legislation in the European Union aimed at ensuring a high level of protection for human health and the environment from the risks that can be posed by chemicals. To achieve this, REACH requires comprehensive testing of chemicals to identify and manage potential risks. While there have been significant advances in alternative testing methods, certain tests still rely on the use of animals due to the complexity of the biological processes involved.

The REACH regulation mandates the utilization of animal testing as a last resort, thereby promoting alternative methodologies whenever feasible. Over the past two decades, the European Union has invested more than 1 billion euros into researching and developing non-animal testing methods, demonstrating a significant commitment to ethical scientific practices (40). Nevertheless, fulfilling certain REACH information requirements still relies on in vivo tests due to the absence of effective, validated in vitro alternatives.

Phasing out animal testing is both a scientific and regulatory challenge. As the scientific community advances, it is crucial to address the current limitations and gaps in non-animal testing methods. Sustained research, funding, and international cooperation are essential for developing reliable and effective alternatives to in vivo testing.

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