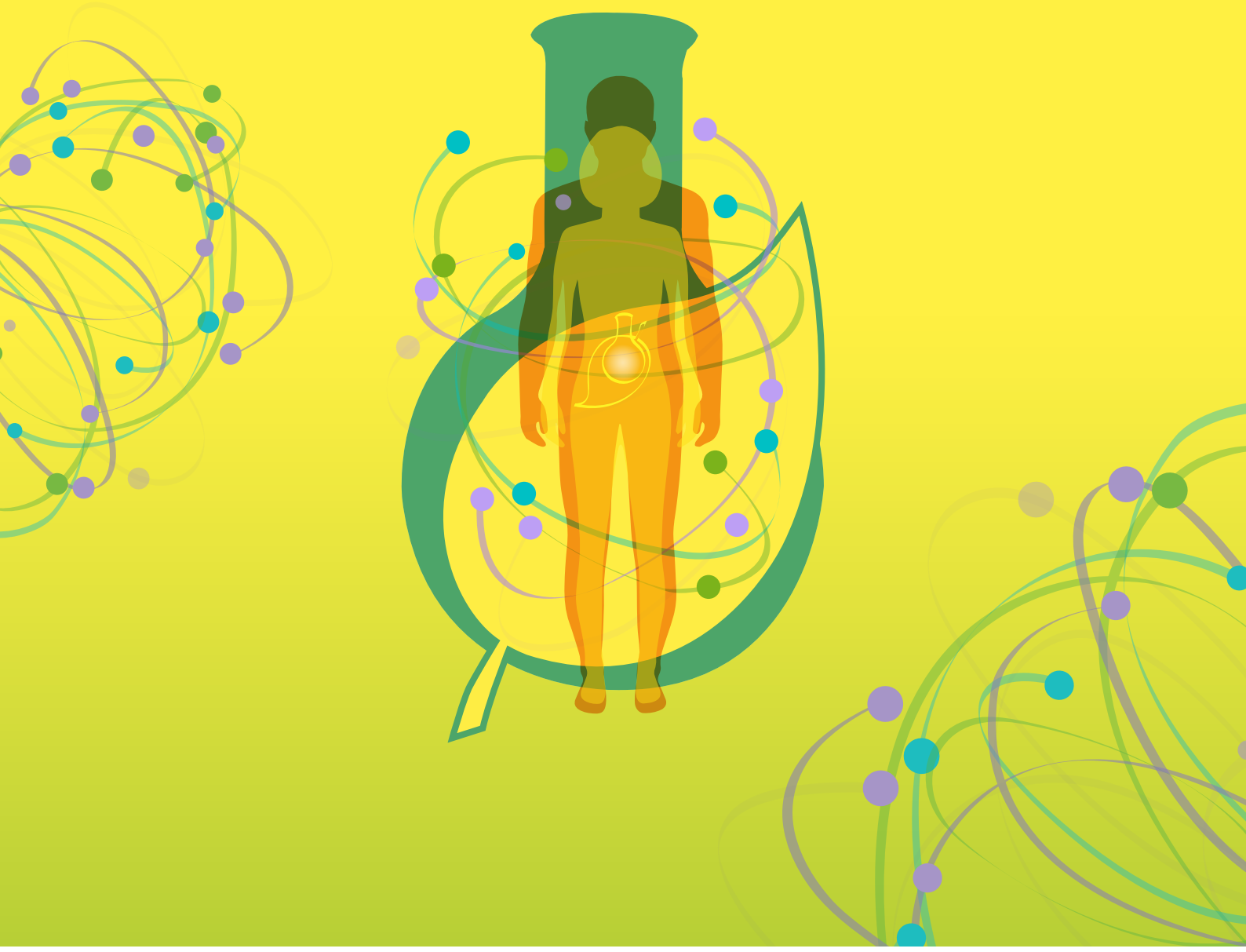
FOURTH ANNUAL FORUM ON ENDOCRINE DISRUPTORS

Exchanging Knowledge, Identifying Challenges, Building Synergies

21-22 September 2022

FORUM REPORT



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Opening session

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Virginijus Sinkevičius
European Commissioner for Environment, Oceans and Fisheries

Welcome to the Fourth Annual Forum on Endocrine Disruptors. If you are here, you know that these chemicals are a cause of concern. I myself am deeply concerned - not only as the Commissioner for the Environment, but also as a citizen and a father.

Endocrine disruptors affect us at critical moments, when the body is particularly vulnerable; during embryonic development or foetal development, during early childhood and puberty.

The problem is the way they mimic our body's hormones with lasting effects. Sometimes they are even passed on to the next generation. They disrupt critical body functions, and they can lead to diseases like diabetes, obesity, and cancer. They weaken our immunity, and cause great suffering.

Their economic effects are also considerable, as they effect the workforce and pose challenges for healthcare systems. Studies put the costs to European society as somewhere between 46 to 288 billion euros, every single year.

In the EU, we have always been committed to ensuring a high level of protection for citizens and their environment. We have been acting on this problem for 25 years, since the days they were first discovered. That long commitment has led to many actions, but now it is time to move into a different gear.

Therefore, we are delivering a breakthrough regulatory change in the two most important regulations on chemicals management: REACH and the CLP regulation on the classification, labelling and packaging of substances.

The draft Commission proposal for the CLP Delegated Act is being published this week. The idea is to introduce new hazard classes for endocrine disruptors. We consulted the relevant stakeholders and the competent authorities of the Member States at the drafting stage. Right now, we are consulting the public.

Our plan is to have the Commission adopt the proposal by the end of November 2022. After that, it goes to the Parliament and the Council for a scrutiny period of two months. If they have no objections, it will be published as adopted by all three institutions early next year.

That means that the EU will finally have horizontal criteria for endocrine disruptors. Some Member States have already published lists of endocrine disrupting chemicals. Those lists will help speed up our efforts at identification, classification and labelling.

But we will not stop there. In the Chemicals Strategy for Sustainability, we also aim to ban endocrine disrupting chemicals from consumer products. The only exceptions will be if their use in a product is essential for society. This will be made possible by the upcoming revision of REACH and also product-specific legislation.

And work continues on many more fronts. We are developing and validating test methods, funding Horizon research projects on the effects of endocrine disruptors, consolidating data, and improving data transparency with "one substance, one assessment".

When you put it all together, the EU clearly emerges as a driving global force on hazard and risk management of endocrine disruptors.

This Forum is important because it is genuinely international. It brings together our partners from around the globe, and their tremendous expertise.

We are also here to learn from each other, and to pave the way for other regions to adopt similar measures. We are here because it is a battle we share; because we all want a toxic-free environment - for society, for nature, and for the future health of our children.

The European Commission's Communication of 7 November 2018, "Towards a comprehensive European Union framework on endocrine disruptors", committed the Commission to organising an Annual Forum on Endocrine Disruptors, to bring together all interested parties to help guide policy.

The Fourth Annual Forum was co-organised by DG Environment and ANSES, the French National Agency for Food, Environmental and Occupational Health and Safety. The Forum was held on 21-22 September 2022 in the Charlemagne building in Brussels, and was also followed by a large online audience worldwide. It follows up a first segment of the event, which took place in Paris on 12 May 2022.

Photo: © European Commission

Matthieu Schuler
Managing general Director, Science for expertise division, French National Agency for Food, Environmental and Occupational Health Safety (ANSES)

It is an honour for France, and especially for me as a representative of ANSES, the French "One health agency" to take part in the organisation and introduction of this Fourth Endocrine Disruptors Forum.

At first glance, our involvement has been due to the French Presidency of the EU. But I feel that the main reason is that ANSES has played a key role in showing an early path from scientific evaluation of endocrine disruption to regulatory acts with protective measures, at national and European level, specifically for bisphenol A (BPA).

Endocrine regulation is essential for life. Hormones are part of our biology, from our early creation steps, through childhood to adulthood, and until the end of life. This regulation varies with the needs of our bodies, therefore leading to different susceptibility to endocrine active substances according to our age and biological status.

Our "biological clock" has evolved, in the long term with human evolution, to cope with endocrine active substances, partly generated internally and partly received as intakes - especially via food. If I dare use an image, the "software" of our organism is able to regulate an adequate level for a large number of endocrine active substances. But some substances overpass this regulation capacity, and lead to diseases. These are the so-called endocrine disruptors.

This overview explains why the WHO definition - which serves as the basis for the regulatory definitions of endocrine disrupting substances – can appear complex.

Moreover, it calls for a precise scientific evaluation to be able to determine the substances that are endocrine disruptors from among all the many endocrine active substances. There are nearly 1 000 substances, for instance, in the long list of "substances of interest" established by ANSES in 2021, requiring an evaluation with sufficient level of confidence.

One more aspect of endocrine disrupting substances' characteristics: some of them show, as usual toxic substances, their adverse effects at high levels of exposure - precisely when the intake overwhelms the regulating capacity, as is the case for resorcinol or cholecalciferol. But some may produce effects at chronic or low doses, such as BPA. This is why endocrine disruptors have to be considered as a distinct hazard class. In particular, it does not mean that endocrine disrupting effects occur for every substance at every dose - the usual distinction between hazard and risk applies.

Of course, these characteristics need scientific data at hand for the evaluators, like these now required for biocidal products or plant protection products along with their submission dossiers. The question is now raised, along with the coming addition to the CLP Regulation, what will be the requirements for these data in other regulated domains.

After having sharing with you some key characteristics of endocrine disruptors, I will conclude with keys for timely actions.

The number of substances for which assessments are needed represents a real challenge for health and safety agencies across the EU, requiring them to join forces to cope with the evaluation of substances toward endocrine disruption as a hazard class. It is important to speed up the "one substance, one assessment" principle and apply it for that purpose. Of course, this also requires adjustment in the regulatory requirements for data, and the development of the corresponding testing protocols.

Another key is the permanent need for precision, transparency and information exchanges at all stages of the substance's life cycle (from design to market) and, as a common backbone, a clear and widely understood regulatory framework.

I wish you all a fruitful Forum, an exceptional occasion for all stakeholders to exchange and gain mutual understanding of these both complex and concern raising questions.

"An endocrine disruptor is an exogenous substance or mixture that alters the function(s) of the endocrine system and consequently causes adverse effects in an intact organism, or its progeny, or (sub) populations."

Source: World Health Organisation / International Programme on Chemical Safety (WHO/IPCS) 2002.

Session 1:

Updates from the Commission and Agencies

The Chemicals Strategy for Sustainability: state of play

Photo: © European Commission

Cristina de Avila
Head of Unit, Safe and Sustainable Chemicals, DG Environment, European Commission

Cristina de Avila gave an overview of progress on the implementation of the Chemicals Strategy for Sustainability (CSS). She highlighted the Delegated Act to introduce new hazard classes for endocrine disruptors in the CLP (classification, labelling and packaging) Regulation, published on 20 September 2022, the day before this Forum.

The Strategy is an offspring of the European Green Deal. It aims to improve the legislative framework for chemicals in Europe to protect people, including the most vulnerable, and the environment. This will be achieved through a clear vision, objectives, and a concrete action plan to address present and future challenges.

The vision is to ensure that by 2030 there is a toxic-free environment where chemicals are produced and used in a way that maximises their benefits to society, while avoiding harm to people and the planet.

Key objectives are to: (i) strengthen legislation and promote innovation, to ensure all chemicals on the market are used safely and sustainably; (ii) promote and reward substitution of chemicals causing long-term adverse effects on humans and the environment; and (iii) phase out the most harmful chemicals in consumer products.

The Strategy has a specific place for endocrine disruptors, calling for a ban of them in consumer products, together with persistent and other harmful substances. There is also a place for specific initiatives, like the action plan for PFAS that will only allow their essential uses. In addition, the Strategy also addresses the combination effects of chemical mixtures. To reduce the risk posed by chemicals of concern, the Commission is implementing amendments to REACH and CLP, the two main horizontal pieces of chemicals legislation in Europe. This includes new hazard classes for endocrine disruptors.

REACH

In REACH, the Commission is updating the information requirements for the registration dossiers that companies have to provide for placing substances on the European market. This will ensure these dossiers have sufficient information to allow risk management of endocrine disruptors.

Specifically, the Commission is updating REACH Annexes I and VII to X to include new data requirements on endocrine disruption. This takes into consideration: (i) the OECD Conceptual Framework for Testing and Assessment of Endocrine Disrupting Chemicals; (ii) the update of biocides and plant protection products Annexes; and (iii) the tiered obligations under REACH requiring different levels of information depending on the volumes (tonnages) of the chemicals being registered.

A second important change in REACH is that endocrine disruptors will have their own place as Substances of Very High Concern (SVHC). This replaces the situation where endocrine disruptors are of concern within other categories.

Substance evaluation in REACH is the tool to confirm or clear a potential concern for a substance. The REACH amendment will streamline evaluation procedures. This includes clarifying how to establish concerns, what can be asked for, and streamlining the Community Rolling Action Plan (CoRAP); and also supporting chemical grouping, to move away from the more inefficient procedure of dealing with substances one-by-one.

The REACH revision will also extend the generic risk management approach (followed the example of CMRs) to implement restrictions on endocrine disruptors, as well as persistent, bioaccumulative and toxic (PBT) substances, very persistent and very bioaccumulative (vPvB) substances, immunotoxicants, neurotoxicants, respiratory sensitisers, and specific target organ toxicants (STOT). In particular, the CSS calls for the avoidance of all these chemicals in consumer products.

There is a further call to extend this generic risk approach to professional uses. All restrictions will be accompanied by the possibility of derogations for essential uses for society, the details of which are still to be decided.

In terms of indicative timing of action, the Commission has launched a number of supporting studies that are coming to an end. The draft proposal for the revision of REACH is expected to be adopted in the first quarter of 2023.

CLP

The Commission has committed to introducing new hazard classes for endocrine disruptors under the CLP Regulation, as the repository of hazard classes in the EU. The call comes not only from the CSS, but also the Commission's Communication of 2018 on endocrine disruptors that recognised the benefits of horizontal identification for a coherent regulatory response.

The Commission shared the draft hazard categories under the CLP Regulation on 20 September 2022 for public consultation. It included hazard classes for endocrine disruptors for human health and the environment within two categories (Cat 1 and Cat 2). There will also be hazard classes for PBT and vPvB substances, basically bringing all the criteria and expertise developed in REACH into the CLP Regulation for a more coherent approach. The endocrine disruptor hazard classes: (i) are based on the WHO definition; (ii) build on existing criteria for pesticides and biocides; (iii) are to be applied horizontally across all legislation; and (iv) are separated for human health and environment to aid their implementation in specific pieces of legislation.

Category 1 is for known or presumed endocrine disruption for human health. This will largely be based on evidence from human and/or animal data. The data should provide evidence that the substance meets the three criteria: (i) endocrine activity; (ii) an adverse effect in an intact organisms or its offspring or future generations; (iii) and a biologically plausible link between the endocrine activity and the adverse effect.

Category 2 is for suspected endocrine disruptors for human health. Again, this is largely based on evidence from human and/or animal data. The difference between Categories 1 and 2 is the level of evidence.

For human health, a new hazard statement will be printed on labels; either "may cause endocrine disruption in humans" (Cat 1) or "suspected of causing endocrine disruption in humans" (Cat 2).

The deadline for commenting on the draft hazard categories under the CLP Regulation is 18 October 2022. Discussions will also be held with experts, with a view to adoption in November 2022.

Finally, concluded Ms de Avila, the EU is setting an example globally, which is one of the building blocks of the CSS. The CPL Regulation is the implementation in the EU of the UN Globally Harmonised System of Classification and Labelling of Chemicals (GHS). The new hazard classes will be proposed for the GHS, starting in the discussions of the GHS Sub-Committee on 7-9 December 2022.

Q&A

Katrina Sichel (moderator) directed questions to Cristina de Avila from the audience in Brussels and from online participants via SLIDO.

Norbert Kaminski (Michigan State University):
Could you give an update on the second part of the CLP revision via the Ordinary Legislative Procedure?

Cristina de Avila: We are revising CLP with two proposals instead of just one. One was published yesterday (20 September 2022), which is a Delegated Act to amend the Annexes in CLP to introduce the new hazard classes. The other Act will amend the terms of the CLP itself through the Ordinary Legislative Procedure, which cannot be done in any other way. The idea is to have both acts adopted at the same time in November 2022, and then it will continue to the Council and the Parliament.

Norbert Kaminski (Michigan State University):
You mentioned criteria that were being proposed, and one of them was "essential use". Could you elaborate a bit on how that will be determined.

Cristina de Avila: We are talking about the concept of essential uses in existing processes that we have, in this case not only in REACH but also in other pieces of legislation, such as the Cosmetic Products Regulation and the Toys Regulation. In REACH we already have existing restriction and authorisation procedures, but what the Strategy is calling for is the introduction of essential uses in the case of restrictions based on generic approaches as the only way of derogating. So we need to find a way of operationalising this concept into our existing restrictions process. The final decision of our restriction lies with the Commission, via an amendment to an Annex of REACH. If we are talking about an authorisation, it is also the decision of the Commission, but addressed to the company that has asked for the authorisation to use this SVHC. We are now looking at what are the elements of essential uses for their criteria, and how we will apply them in practice, and who will oversee the governance.

Malik Duhaut (Covestro):
Is the Commission planning to apply generic approaches to endocrine disruptor category 1 (or both category 1 and 2)?

Cristina de Avila: I think this is a decision that will have to be taken for each piece of legislation. What the Strategy calls upon is an extension of the current system for endocrine disruptors.
In REACH we have generic approaches that apply to CMRs category 1.
In other pieces of legislation, that may be different. Generic approaches rely on a harmonised classification.

Caroline Bassoni (COSMED):
The definition of "biologically plausible link" is quite vague and we feel it is open to different interpretations. Is there a plan to define more criteria to allow a fairer assessment of this link?

Cristina de Avila: There is always a possibility of developing a guidance of what it is going to be, if it is not clear in practice. That would be for industry, who need to self-classify, but also for administrations, especially ECHA who are in the risk assessment committee taking steps for harmonised classification.

Helene Loonen (European Environmental Bureau):
When do you think that the new hazard classes will be operational? We are also concerned about the slow progress made on the REACH revision, especially calls from the European Parliament to postpone it, so what will the Commission do? Will it commit to deliver on the objectives of the Green Deal and Chemicals Strategy to rapidly phase out substances of concern including endocrine disruptors?

Cristina de Avila: The transitional period is maybe 18 months or longer. When you have hazard classes in place it is not only an option of the Commission, there is a scrutiny period with the Parliament and the Council. Once in law, in principle there is an obligation after the transition period to self-classify and we are free then as authorities to start harmonised classifications of endocrine disruptors. After that, it is up to Member States to introduce the new harmonised classifications. We are committed to deliver the REACH revision under this Commission. The calls from the Parliament are only from some sectors. Overall, we have full support from the European Parliament in an opinion adopted in June 2022.

ED and non-ED active substances for plant protection and biocidal products

Karin Nienstedt
Policy Officer, DG SANTE, European Commission

Karin Nienstedt provided an update on pesticides and biocides since 2018, as regards endocrine disruptors, in the context of the Biocidal Products Regulation (BPR) and the Plant Protection Products Regulation (PPPR).

New criteria for endocrine disruptors were adopted in the BPR and PPPR in 2018. This means that since 2018: (i) Member States, EFSA and ECHA have been applying the criteria for all active substances to be used for pesticides and biocides (dossiers submitted since 2018, and also dossiers that were pending at that time); (ii) a Joint EFSA/ECHA Guidance Document has been available to help apply the criteria; and (iii) training for risk assessors to help them apply the new criteria, e.g. Better Training for Safer Food (BTSF).

The new criteria for endocrine disruptors for biocides and pesticides are based on the three elements of the WHO/IPCS definition: endocrine mode of action, an adverse effect, and a plausible link. They identify known and presumed endocrine disruptors, and are fully equivalent with the two categories of the draft criteria just published for the CLP Regulation.

Beyond the criteria, new data requirements were also adopted under both pieces of legislation. In 2020, Annexes II and III of the BPR were amended, setting data requirements for determining on endocrine disruptors. Two Communications led to revisions in data requirements for the PPPR, listing the relevant guidance documents and test methods.

Biocides

In 2022, after four years of implementation for biocides, 42% of the Review Programme is completed (for a combination of active substances and product types). There are still 128 active substances in the Review Programme under assessment by the evaluating Member States or ECHA for endocrine disrupting properties. As regards ECHA opinions published since 2018, four active substances were identified as endocrine disruptors: cholecalciferol, DBNPA, cyanamide, and propiconazole. For cholecalciferol (vitamin D3), a derogation was approved allowing its use as a rodenticide.

Eight active substances were identified as clearly not being endocrine disruptors for human health or the environment. There have been 19 ECHA opinions where evaluations were inconclusive, and these are being looked at case-by-case.

What is important for biocidal products is that there is a provision to trigger an early review of approvals (BPR Article 15). For instance, an ongoing early review was triggered for three substances to check for endocrine disruptor criteria in the light of recent concerns: iodine, PVP-iodine and zineb.

Pesticides

The endocrine disruptor criteria has been applied to new and pending dossiers for pesticides since November 2018. For pending dossiers (submitted before November 2018) a 'stop-clock' has been implemented to enable time for conclusions to be reached. The criteria have also been applied to some MRLs (maximum residue level) processes, and updates of 90 substances for **ongoing MRL processes** are published by EFSA.

In a nutshell, processes have been initiated or finalised for 95 active substances used in pesticides: 40 finalised, 26 on 'stop-clock', 25 where 'stop-clock' has been resumed, while a few dossiers were withdrawn.

For the 40 dossiers where EFSA conclusions were finalised: 28 active substances were clearly identified as not being endocrine disruptors, 6 were identified as endocrine disruptors for human health and 3 for non-target organisms (environment). There were a few others where no conclusion could be reached and additional data is needed. In one case for human health, a substance was banned for other reasons.

Among the substances identified as endocrine disruptors, mancozeb has not been approved for use, a decision also taking into account other risks. Currently, three substances are still under discussion in the PAFF Committee (The European Commission's Standing Committee on Plants, Animals, Food and Feed): asulam-sodium, benthiavalicarb, and clofentezine.

Q&A

Katrina Sichel (moderator):
What is the timeline for the 'stop-clock'?

Karin Nienstedt: The 'stop-clock' takes 3-30 months. This is a flexible timing that depends on the assessment process of EFSA and relevant Member States. They should look at what is in the dossier, what other data is needed, and should give time to applications to generate this data. We put an upper limit of 30 months so as not to delay the process for years.

Highlights from the session on Endocrine Disruptors of the EFSA One conference

Photo: © Maria Arena

Maria Arena
Scientific Officer, European Food Safety Authority (EFSA)

Maria Arena presented the highlights of the session on endocrine disruptors from the **EFSA One conference** held in June 2022.

The four-day EFSA One (Health, Environment, Society) Conference 2022 was co-designed with its sister agencies: European Chemicals Agency (ECHA), European Centre for Disease Prevention and Control (ECDC), European Environment Agency (EEA), European Medicines Agency (EMA), and the Joint Research Centre (JRC).

A key aim was to foster engagement among experts, Member States and stakeholders from diverse backgrounds. The focus was on food safety in the context of sustainable food systems, developments in risk assessment and regulatory science, and the European Green Deal. It also marked EFSA's 20th anniversary.

Following the opening day's plenary sessions, days 2 and 3 were dedicated to thematic breakout sessions within four parallel tracks (One Society, One Life, One Planet, and Many Ways). The final day comprised the closing plenary sessions.

The endocrine disruptors session was in the 'Many Ways' track, focusing on what works and what needs improving in endocrine disruptor assessment. It began with a keynote presentation by Andreas Kortenkamp (Brunel University); then four posters were selected for special presentation; followed by six 'flash reports' from representatives of academia, the private sector, EU agencies, European Commission, NGOs and Member States. Finally, there was a moderated panel discussion and a wrap up session with closing remarks.

The main outcomes were: (i) agreement on the need for harmonised criteria across legislation for endocrine disruptor identification, though this is not always possible or warranted (e.g. low tonnage substances under REACH); (ii) hazard-based cut-off criteria are warranted in the vast majority of cases and across legislation, though in some cases a risk assessment approach might be an option; (iii) 'one substance, one assessment' is desirable, but requires mechanisms for sharing data across agencies so consistent conclusions can be reached.

Regarding New Approach Methodologies (NAMs): (i) alternative methodologies (e.g. *in vitro* test batteries) increase mechanistic understanding and move away from animal data; (ii) validation of NAMs is a key step for their regulatory acceptance; and (iii) procedures are needed to speed up their regulatory implementation.

Concerning human health, it was noted that the quantification of thyroid effects associated with the induction of peripheral effects is important. However, the impact of the thyroid hormone T4 on the brain in the sensitive population (i.e. foetus and new-borns) is still unknown, and models to define this impact represent a complex research task.

For environmental effects: (i) it was agreed that identifying endocrine disruption in the environment is challenging because of a lack of methods and knowledge; (ii) more should be invested for advancing knowledge and methods in ecotoxicology; (iii) mechanistic understanding should be increased to enable extrapolation between taxa; and (iv) the breaking of walls between mammalian toxicology and ecotoxicology should be encouraged, with data considered in a more holistic way.

Sharing data on endocrine disruptors for research and regulatory purposes: EASIS

Siegfried Morath
Scientific Officer, Joint Research Centre (JRC), European Commission

Siegfried Morath gave an overview of EASIS, the Endocrine Active Substances Information System. This freely-accessible web-based application was introduced a few years ago, but JRC have recently updated it with current technologies.

Currently, the EASIS database contains over 10 000 curated entries from peer-reviewed scientific publications for more than 600 substances. The data includes mechanistic and adverse effects information, relevant to disturbances of the endocrine system in humans and the environment.

Key features of EASIS include (i) support for the "one substance, one assessment" approach and the Chemical Strategy for Sustainability, with the aim of making better use of academic data in regulatory assessment; and (ii) IUCLID software, which is internationally used for collecting and exchanging data on hazards of chemicals, and is a reporting tool of REACH.

Additionally, EASIS follows FAIR (Findable, Accessible, Interoperable and Reusable) data principles, with the aim of improving the value of scientific data.

EASIS integrates different types of data. On one hand, human health and environmental effects data are obtained mainly using conventional approaches, like *in vivo* methods. On the other hand, mechanistic information for endocrine disruptors generated by New Approach Methodologies (NAMs) is captured via the OECD Harmonised Template 201 (OHT 201).

The insights gained can facilitate scientific development, for example by: (i) putting more standards into science (e.g. effect-based reporting data formats); (ii) associating mechanistic effects with adverse outcomes; (iii) supporting the prediction of effects from chemical structures; and (iv) enabling the use of Artificial Intelligence and Machine Learning tools.

Dr Morath invited participants in Brussels to try out an EASIS demonstration system on both days of the Forum. Anyone can access the **EASIS landing page** online.

Discussion and Q&A

Katrina Sichel moderated the discussion at the end of the first session, with questions selected from the floor and via SLIDO.

Q&A

Daniela Fruth (knoell Germany):
Does it mean that currently identified endocrine disrupting substances under BPR and PPPR are considered as Cat 1 under the draft CLP update for Human Health and Non-Target Organisms?

Karin Nienstedt (DG SANTE): From the scientific point-of-view, the criteria on biocides and pesticides are fully equivalent to Cat 1 under the draft proposal for CLP. However, from a regulatory point-of-view what has yet to be decided is that if a substance identified under biocides or pesticides legislation as an endocrine disruptor will then be fully taken over on CLP or if then a complete new dossier has to be submitted.

Q&A

'Luca':
Regarding assessment of biocidal non-active substances (co-formulants), are food flavourings/additives likely to not possess endocrine disruptor properties?

Maria Arena (EFSA): At this stage, the criteria in place apply only to pesticides and biocides. Food ingredients and flavourings do not have such criteria for the identification of endocrine disruptors. These substances are also used in low percentages. Though hazard-based criteria at the moment are just for pesticides and biocides, in CLP they could in practice be applied to other frameworks.

Q&A

Daniela Fruth (knoell Germany):
What exactly does peer-reviewed data mean - any authority review, or information from publicly available (active) substance dossiers included?

Siegfried Morath (JRC): It is primarily data from peer-reviewed scientific publications, after applying internal search terms and criteria for study selection and data extraction for our database.

Q&A

Yvonne Andersson (Swedish Chemicals Agency):
Could you elaborate, or give examples, for which endocrine disrupting substances a risk assessment approach might be an option?

Maria Arena (EFSA): During the EFSA conference the risk assessment approach was mentioned for mixtures, and it could be used for other situations, but there were no discussions of how specifically this could be done. We don't want to give the idea we are pushing for a risk assessment approach, because we have the hazard-based criteria, but there may be opportunities where risk assessment could be used.

Q&A

Sandra Jen (EDC-Free Europe):
You mention 9 substances identified with ED properties and 3 under discussion, what was the timeline for the others? What is coming next for substance where no conclusions could be reached?

Karin Nienstedt (DG SANTE):
To clarify, it is 6 substances for human health and 3 for environment, which are also included in human health, so in total it is 6 not 9. There are some conclusions of EFSA which are very recent, where we have not looked at the details for decision making. If no other risks are identified, these 4 will join 3 other substances where case-by-case discussions are ongoing, such as for possible derogations.

Q&A

Ninja Reineke (CHEM Trust):
At the EFSA conference, there was a sense of urgency during the endocrine disruptor session, we need to get quicker and more efficient in their identification. What can EFSA do to achieve this?

Maria Arena (EFSA): I think we can be faster based on regulations we have, and also using all available data in a holistic manner to conclude on them. When we cannot conclude, it is because there are no data to draw conclusion. There are many projects ongoing to improve the situation, such as those for AOPs and NAMs.

Session 2:

From bench to validated test guidelines: (pre)validation of test methods

The "integrated Fish Endocrine Disruptor Test" (iFEDT)

Photo: © European Commission

Lisa Baumann
Heidelberg University, Germany

Lisa Baumann talked about ecotoxicological work with zebrafish, mainly resulting from the EU tender project iFEDT ("integrated fish endocrine disruptor test"). This work is leading to the development of a new fish test method, for use by industry to test for environmental effects of endocrine disruptors.

Endocrine disruptor testing with aquatic species is challenging because: (i) many test guidelines for fish and amphibians are complex, long, expensive and use many animals; (ii) only a few tests currently cover all relevant life stages and include population-relevant apical endpoints; (iii) it can be difficult to distinguish endocrine disruption from general toxicity; and (iv) major gaps and weaknesses exist regarding the different EATS (Estrogen, Androgen, Thyroid, Steroidogenesis) modalities, for instance, currently EAS are mainly assessed in fish and T in amphibians, requiring the running of multiple tests to cover all modalities.

Therefore, DG Environment issued a call in 2018 asking for improved Test Guidelines for endocrine disruptors to address these issues. The iFEDT project resulted from this call.

The most popular Test Guideline (TG) for fish, OECD TG 229 (fish short term reproduction assay), uses adult fish, often zebrafish, exposed for 21 days to potential endocrine disruptors. Different endpoints are checked (e.g. vitellogenin biomarker, secondary sex characteristics, gonad histology, and reproduction).

In OECD TG 234 (fish sexual development test), fish are exposed from early embryo up to juvenile stage, and different endpoints are assessed that are potentially disrupted by endocrine disruptors, like growth, hatch, survival, vitellogenin, sex ratio, and gonad histology.

The iFEDT project team suggested merging the TG 229 and TG 234 protocols, starting with adult fish and going directly into the following generation. In total, for zebrafish such a test has a duration of 84 days (21+63 for the two parts of the merged test). This is considerably shorter than the existing medaka extended one-generation test (MEOGRT, TG 240) that runs over two generations, therefore reducing time taken and number of animals used.

The second key aspect of the merged test is the introduction of a thyroid modality endpoint, which currently does not exist in fish tests. The reason the thyroid is addressed in amphibians is that they undergo a clearly visible metamorphosis from tadpoles to frogs, but fish also undergo a metamorphosis with developmental changes regulated by thyroid hormones.

Research at the Universities of Heidelberg, Antwerp and Southern Denmark (and elsewhere) has shown a range of thyroid endpoints in fish sensitive to endocrine disruptor exposure. Eye development, swimbladder inflation, pigmentation, thyroid histopathology, and fin development are all regulated by thyroid hormones. The work in Heidelberg has focused on eye development, for which very specific endpoints have been defined.

The merged test - iFEDT - has new endpoints for adults (thyroid hormone levels and thyroid histopathology) and for developing embryos in the second phase (e.g. thyroid follicles, eye and swimbladder development, and hormone levels).

Therefore, iFEDT integrates all life stages and all relevant endpoints within one test.

To validate the iFEDT test, it was run with the model thyroid hormone axis disrup-

tor propylthiouracil (PTU), which inhibits thyroid hormone synthesis; and with the model estrogen disruptor ethinylestradiol (EE_2).

PTU exposure resulted in strong effects on endpoints, down into the sub-lethal range (ruling out general toxicity). Moreover, vitellogenin, used in many fish and amphibian guidelines to identify estrogen-related effects, disrupted F1 females, and acted as expected in male fish.

Thyroid histopathology clearly showed the effects of high PTU exposure, seen externally as a red outgrowth (goitre) due to proliferating thyroid follicles (trying to compensate for thyroid hormone synthesis inhibition).

The eye development endpoint (thickness of retinal layers) was also successfully validated. This endpoint is also important for population-level assessments, because fish with impaired vision will not survive in the environment. The iFEDT team produced a new **adverse outcome pathway** based on the action of PTU on eye development, proving the relationship between thyroid hormone synthesis inhibition, altered eye development, and behaviour that might lead to mortality.

Dr Baumann concluded by saying they are currently finalising the iFEDT test protocols. There is one remaining challenge, which is to improve the method for measuring thyroid hormone levels; this applies to all projects aiming to use measured TH levels as an endpoint in Test Guidelines for fish and amphibians. Two publications are currently been prepared that summarise all the iFEDT results. The final stage will be validation to make it an OECD TG.

Q&A

Mike Broderick (Health and Safety Authority, Ireland): ***The combination of the fish tests seems like a very good idea. However, are we sure that concentrating the ED tests on fish alone will protect amphibians?***

Lisa Baumann (Heidelberg University): We are not suggesting skipping the amphibian tests entirely, for exactly that reason. Amphibians are very vulnerable to many chemicals, and they are highly endangered, especially in Europe. For that we need amphibian tests, but we think they can be substantially reduced. Running two tests unnecessarily for fish and amphibians is something we want to avoid for 3Rs reasons.

Q&A

Andrew Turley (Chemical Watch): ***Where does this test fit into REACH and CLP? Where is the policy benefit?***

Lisa Baumann: I am talking as a biologist, not a regulator, so I would hope with the iFEDT test we can, for example, reduce in a step-wise approach the number of tests and animals used. By testing early you also get early alerts of problems.

Q&A

Cécile Michel-Caillet (ANSES): ***Is the eye's histopathology also usable for evaluating thyroid disruption in mammals?***

Lisa Baumanm: For our AOP development, we did not only collect evidence for fish, we also collected it for all other vertebrate classes, though for birds and reptiles it was limited. For mammals we know that thyroid hormones are very important for eye development. This is the concept we follow in the EURION project ERGO, to show that the effects we see in one species can be translated to others. We are developing adverse outcome pathways (AOPs) to show that what happens in a fish can also happen in a mammal or a human.

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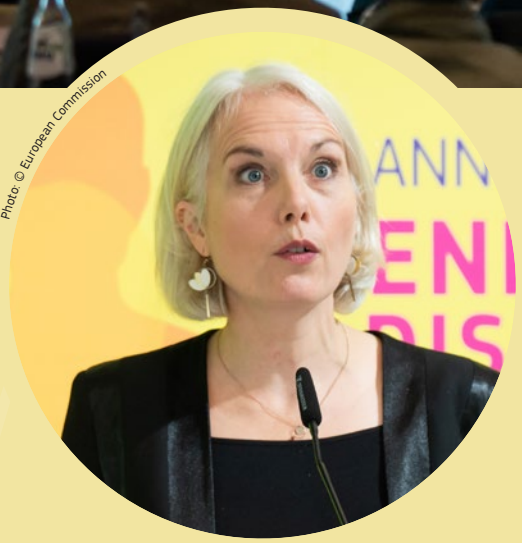

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Panel discussion

The panel discussion was moderated by **Cécile Michel-Caillet,** Head of the REACH-CLP-Endocrine Disruptors Unit, French Agency for Food, Environmental and Occupational Health & Safety (ANSES), Paris, France.

Panel members were:

Philippe Hubert, Director, Pepper Platform, France;
Anne Gourmelon, Principal Administrator, OECD Test Guidelines Programme;
Sharon Munn, Senior Scientific Officer, Joint Research Centre (JRC), European Commission; and **Lisa Baumann,** Heidelberg University, Germany.

Joint Research Centre (JRC)


Photo: © European Commission

Sharon Munn
(JRC)

The JRC is the knowledge service of the European Commission, supporting EU policies with independent evidence throughout the whole policy cycle. It has around 3 000 staff, almost 75% of them scientists, at research facilities across five EU Member States and at its headquarters in Brussels.

About forty staff work at JRC's Chemical Safety and Alternative Methods unit in Ispra, Italy. This unit contains the European Reference Laboratory for Alternatives to Animal Testing (EURL ECVAM), established under the Directive on the protection of animals for scientific purposes (2010/63), which focuses on the 3Rs: the Replacement, Reduction and Refinement of animal testing.

In relation to endocrine disruptors: (i) the JRC supports the updates to REACH and the CLP Regulation; (ii) the EURL ECVAM Thyroid Validation Study in assessing 18 methods; (iii) the newly-released EASIS (Endocrine Active Substances Information System) which provides structured data on endocrine activity and adverse effects; (iv) the eight EURION Cluster projects funded under H2020 on methods for identifying endocrine disruptors; and (v) it also supports OECD activities on endocrine disruptors.

Further information can be found in the **EURL ECVAM Status Report.**

OECD Test Guidelines Programme


Photo: © European Commission

Anne Gourmelon
(OECD)

OECD works with its 38 member countries to provide harmonised methodologies and tools for chemical safety assessments.

OECD's work on endocrine disruptors started in 1997, with the creation of a dedicated Task Force. Work has focused on the development of Test Guidelines (TGs) for the screening of endocrine disruptors. In 2002, the OECD developed a Conceptual Framework for Testing and Assessment of Endocrine Disrupters. This organised knowledge and data on endocrine disruptor testing and assessment, within 5 levels of increasing biological complexity.

In 2012, OECD published the first Guidance Document 150 on Standardised Test Guidelines for Evaluating Chemicals for Endocrine Disruption. A **Revised GD 150** was published in 2018.

In recent years, the Adverse Outcomes Pathway (AOP) concept has been developed to better understand modes of action and therefore to identify new testing methods.

OECD's work on endocrine disruptors involves collaborations with: (i) OECD member and partner countries; (ii) animal welfare and environmental NGOs; (iii) business and industry advisory committees; (iv) private and public laboratories (in member countries); (v) validation and evaluation bodies; (vi) private-public partnerships; and (vii) several Expert Groups and Advisory Bodies.


Photo: © European Commission

Pepper Platform, France

Philippe Hubert
(Pepper Platform)

Pepper (Public-private platform for the pre-validation of endocrine disruptor characterisation methods) is a non-profit public-private association established in 2020 to address the lack of pre-validated test methods for endocrine disruptors. In particular, it addresses the missing link between experimental design and validated methods; the aim is to fill this gap.

Pepper's tasks are to: (i) find mature methods that are pertinent to regulatory use; (ii) prepare procedures for validation, quantify experimental plans, and select labs; (iii) prove the transferability of the methods to other labs; (iv) test methods in blind ring trials with many substances to assess reliability, predictive capacity and applicability domains; and (v) organise submissions to OECD for their adoption as Test Guidelines.

Currently, Pepper is working on 6 methods, involving 17 labs. Submissions have started for 3 methods, the other 3 are in a transferability assessment phase. Work on a third series of methods starts later in 2022.

The private funding organisations involved in this private-public platform include BASF, BAYER, LVMH, and French councils (chemical industry, beauty, detergent, and hygiene products). There is a need to extend resources not only to obtain more financial support, but also to address the discrepancy that arises from most of the money coming from France; Pepper needs to be equilibrated.

Cécile Michel-Caillet (ANSES):
What does it mean in concrete terms to perform a validation of a test method?

Anne Gourmelon (OECD): 'The process by which the reliability and relevance of a particular approach, method, process or assessment is established for a defined purpose'. This is our definition of validation. Relevance relates to the biological and mechanistic meaning of the data. The reproducibility and reliability relates to the trust in the data, and the defined purpose relates to the regulatory relevance of the data.

Then comes the Modular Approach, the process by which you can achieve the validation of a method. This advocates for flexibility in undertaking a validation exercise, and depends on the pre-existing information available for a method.

There are different ways to demonstrate a method is valid: (i) prospective validation (lab work); (ii) performance-based validation (pre-existing standard and similar methods); and (iii) retrospective validation (collecting existing data). However, in all cases it is necessary to have a good protocol description, transparent reporting of results, and methods that are accessible to future users.

Lisa Baumann (Heidelberg University): iFEDT represents the first steps of developing a test method. We used known methods and endpoints, but did a lot of lab work to extend existing guidelines. As researchers, it was a new world to us, and knowing that our research will one day be an OECD TG changes a bit the way we approach the work and how we want to publish it.

Sharon Munn (JRC): Validation is a very demanding process. It does not always go to plan, and it takes time to introduce a new method into a naïve lab. It should also be kept in mind that there might be proprietary elements, and this should be sorted out at an early stage if the test method is to be used commercially.

Cécile Michel-Caillet (ANSES):
Is it easy to find methods mature enough to enter the validation process?

Philippe Hubert (Pepper Platform): The answer is no, to find mature methods there are a lot of things to consider.

At Pepper we have designed the **ReadEDTest self-assessment questionnaire** to help test developers know if their methods are mature enough. Factors asked about include a clear method description, the operational readiness of a laboratory, reproducibility and transferability, historical data, the presence of standard operating procedures (SOPs), the relevance of methods to regulatory needs, and if labs have sufficient time to do prevalidations.

Sharon Munn (JRC): During the thyroid validation study we found that the scientific literature was not detailed enough to enable implementation in the lab, for example, Standard Operating Procedures are often lacking. We think that the application of technical readiness criteria will be very helpful, and we have asked scientists within the EURION projects to go through such criteria themselves, to identify gaps in their own methods.

Cécile Michel-Caillet (ANSES):

What are the obstacles to the validation by the research labs themselves?

Lisa Baumann (Heidelberg University): Research labs run experiments for publications, the main goal is not to produce methods that can be run in lots of other labs. University scientists are under pressure to publish and discover novel things, making it difficult to allocate funding and space to run experiments already performed in several other labs, as is required to validate Test Guidelines.

Cécile Michel-Caillet (ANSES):

Can we take a bit more time to explain the differences between a validated method and a Test Guideline.

Sharon Munn (JRC): For a validated method there is a rigorous procedure behind it that shows the method is reproducible and it measures what it is supposed to measure. A validation process also involves an independent peer review, which puts a quality stamp on it. A Test Guideline is the next stage in the process, bringing in regulatory acceptance and consensus building.

Anne Gourmelon (OECD): A Test Guideline answers a regulatory question, and a context of use coming from a regulatory framework. There is a bit of a 'chicken and egg' question here: what comes first the validated method or the regulatory need? Regulatory frameworks have to be flexible to uptake innovations and new guidelines, to close the loop.

Cécile Michel-Caillet (ANSES):

What is the added value of validated test methods per se?

Sharon Munn (JRC): The most important things are the quality stamp; the reproducibility; understanding sensitivity, specificity and the limitations of the method; and the applicability domain.

Lisa Baumann (Heidelberg University): At last week's EURION meeting it was interesting to see the different perspectives. There is an idealistic value, to protect human health and the environment, and we want to have good methods to assess endocrine disruptors, but there are also regulatory and other pressures. I think there is added value in being very detailed in the lab regarding methods, it is sound science, and we should write everything we do down to ensure robust protocols.

Cécile Michel-Caillet (ANSES):

What would happen if industry or an authority relied on unvalidated methods?

Sharon Munn (JRC): Non-validated methods, such as academic data and the scientific literature are used for the identification of endocrine disruptors, especially when Test Guidelines are not there to base decisions on. The problem is that every time you have to assess if the method is good or if the results are as expected. With a validated method that has already been done for you, and you understand how to interpret the results.

Cécile Michel-Caillet (ANSES):

At OECD, there is also the concept of the mutual acceptance of data, can you tell us a little more about that?

Anne Gourmelon (OECD): The system of mutual acceptance of data relies very much on Test Guidelines and Good Laboratory Practice. The benefit is that "tested once, accepted everywhere" saves resources and avoids duplicate testing. So OECD member countries and partner countries are accepting data that has been generated in another country, if they have the same regulatory requirements.

Philippe Hubert (Pepper Platform): Conversely, when people use non-validated methods, it can be very costly. Different methods (even when duly published) also do not always give the same results; you can start getting disagreements and controversies.

Cécile Michel-Caillet (ANSES):

How to optimise the existing process? Is it necessary to know who the method will be used by or what for to get a proper validation?

Sharon Munn (JRC): OECD 'Guidance Document 34 on the validation and international acceptance of new or updated test methods for hazard assessment' talks about validating a method for a particular purpose, so in that sense yes; though sometimes it is not that easy.

You should not start a validation if you don't know what you are aiming for. There should be an established regulatory need before finding assays to validate, though there is a bit of interplay between the two. This issue is dealt with by relevance committees in EURL ECVAM and Pepper.

Cécile Michel-Caillet (ANSES):

Do we have any words on how to optimise the process and mutualise the efforts?

Lisa Baumann (Heidelberg University): The key is the funding and also support while the methods are being developed. iFEDT and the EURION Cluster are good examples of that. In EURION projects to date, over 40 new endpoints and assays have been developed that will hopefully go into validation, including 15 for the T modality where there are a lot of gaps.

Sharon Munn (JRC): For EURION, to bring in prevalidation to get good method descriptions is really helpful. It would be good to take this forward in future research activities, such as PARC, the Partnership for the Assessment of Risks from Chemicals (launched on 1 May 2022).

Anne Gourmelon (OECD): There are ways to become more efficient. More key players (e.g. contract research organisations) need to play a role in validation. They will make money afterwards selling methods as services.

Philippe Hubert (Pepper Platform): I am a bit less optimistic, because if you develop a method from zero and fund all the validation yourself you will never have payback. That is a good thing per se in that it is an open system accessible to all; methods are not proprietary, but some proprietary elements are submitted to FRAND constraints. However, without external funding there will be nothing in the validation process.

Discussion and Q&A

Katrina Sichel (moderator) directed audience questions to the panellists.

Q&A

Erik Prochazka (Cruelty Free Europe):
With the new ED hazard criteria we are looking at a significant increase in animal testing. How can we speed up the validation process of new non-animal methods?

Ioana Bere (CHEM Trust):
How much time does it take, on average, to validate a method and also to develop a method?

Judith Giernoth (Covestro):
Reproducibility was mentioned as key for validation. How do you ensure reproducibility for academic studies, when they are included into assessments?

Maria Arena (EFSA):
I agree that eye-related endpoints are population relevant, but don't you think their reversibility can make the assessment challenging?

Lisa Baumann (Heidelberg University): I agree that the ability of animals to recover from the eye damage induced by endocrine disruptors is a challenge in those test methods. The younger life stages are more sensitive, though effects are for all ages, so I would say it is population relevance. What we advertise in iFEDT and ERGO is that we use a combination of endpoints that are related to thyroid, for example, the swimbladder is also population relevant and very sensitive to endocrine disruptors. For these combinations, we have to assess which tests are best for which endpoints.

Anne Gourmelon (OECD): It takes a few years to validate a method, but we have to recognise that when there is a clear need and a clear interest in validating a method, it can go quickly if resources are mobilised, and can be done in a couple of years. Also a test taking 3 days is different to one taking 90 days, we have to weigh that in.

Sharon Munn (JRC): For academic studies that are not validated, if several papers say the same thing there is a Weight of Evidence but reproducibility is not being covered. That is what validation is about – to show reproducibility of a method.

Philippe Hubert (Pepper Platform): The reason why we say that it is important to have validation is to have the proof of reproducibility. In a published paper you usually do not have data to show reproducibility. There are almost no papers on the methods themselves; the focus is on substances. Reviewers of papers are not so keen on pinning the issues on the methods.

Sharon Munn (JRC): Validation of new non-animal methods (NAMs) is a demanding process, but ways of speeding it up are being investigated and discussed. Usually we conclude that there are not many steps we can drop. There are some possible ways, such as the leaner Thyroid Validation Study. We could use retrospective data to help validation, to avoid big inter-laboratory reproducibility studies. We are constantly looking at how to innovate, so that NAMs can be used as soon as possible.

Cécile Michel-Caillet (ANSES): It should also be quicker to validate *in vitro* studies than chronic *in vivo* studies.

Anne Gourmelon (OECD): It is a matter of knowing when there is enough data, and to maybe not separate too much validation from prevalidation. Maybe it is sufficient to have prevalidation and some retrospective review of what's out there. We need to be innovative in how to validate methods that are really needed.

Q&A

Andrew Turley (Chemical Watch):
The main beneficiary of new validated test methods would seem to be the chemical industry, because it is test data that enables market access under systems like REACH or BPR. Do you agree, and is the chemical industry pulling its weight regarding funding? Does Pepper have experiences to share of obtaining industry funding for validation?

Philippe Hubert (Pepper Platform): It is not that obvious that the chemical industry is the one with the most interest in this issue. Where you have a problem is also in industries selling things to people - toys, food, medical devices. These industries are faced with customers who do not trust products. What we are trying to do in Pepper is to convince all those industries that they have to take part in the fight against endocrine disruptors.

Sharon Munn (JRC): Companies are involved with validation activities, for example, in support of the Thyroid Validation Study. There is also a business model called External Validation where a company can deliver a method and results to an independent body for peer review.

Anne Gourmelon (OECD): By being involved in validation, contract research organisations gain proficiency and competence in running the method, then have a competitive advance by participating in validation.

Q&A

Tine Vandenbrouck (Apeiron-Team NV):
How does combining two test methods like in iFEDT impact the validation? Is it a real added value?

Lisa Baumann (Heidelberg University): Regarding non-animal tests, there is high public pressure for good reasons to reduce animal use, so industry is facing pressure to use them. For combining existing validation Test Guidelines, the validation process should be shorter.

Anne Gourmelon (OECD): Hopefully we stop doing this dichotomy of prevalidation and then validation. We need to come back to the essentials of validation, the relevance and reproducibility, and take a decision when data is enough, and have trust due to adequate protocols.

Q&A

Norbert Kaminski (Michigan State University):
I agree that reproducibility is a big part of validation, but another important part is to convince the scientific community that endpoints or methods are actually measuring what you think they are measuring. That is why we use specific model compounds that have already been well characterised to validate a methodology.

Philippe Hubert (Pepper Platform): When we do validations, we are trying to find "positive" and "negative" substances. It is very important not to be limited to the old well-known ones, and to be open to other disciplines such as epidemiology or clinical results.

Q&A

Heather Patisaul (North Carolina State University):
Regarding reproducibility, one of the potential advantages of moving from animal testing to in vitro testing is you can capture biological variability that you cannot capture in animal models. But as you aim for perfect reproducibility – are you down to one genome, one individual, one sex, one age, one moment in time? As we move to in vitro testing, there is an enormous opportunity to capture biological variability.

Anne Gourmelon (OECD): This is not part of the validation itself, but there are research groups looking at biological variability, and upscaling our models to what it means in the population.

Philippe Hubert (Pepper Platform): My last sentence would be that we have to stop having to play hot potato, validation is something expensive, which has to be done and financed, so we need to stop discussing who should do it (industry or administration or researchers), and involve everyone; we are in this game at the moment.

Anne Gourmelon (OECD): I think the principles of validation, establishing relevance, and reproducibility are clear, but let's be more innovative in the process and approach so that we become more efficient.

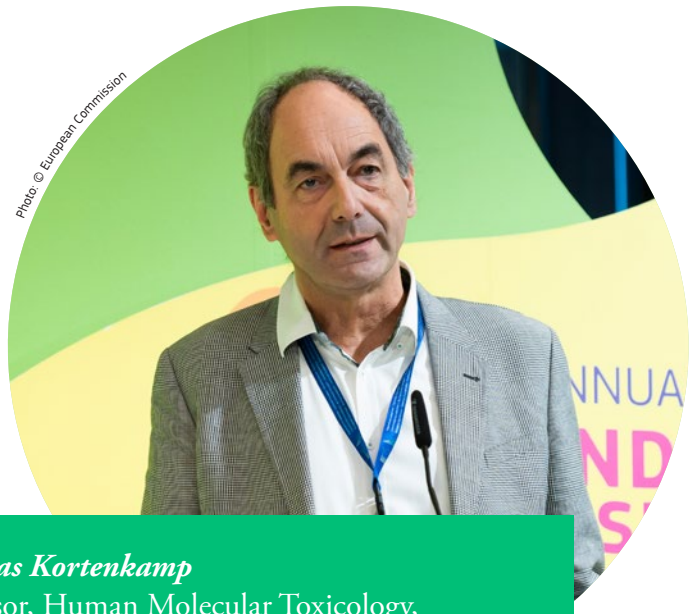
Sharon Munn (JRC): Validation and regulatory acceptance are interrelated but are different processes, so keep that in mind.

Lisa Baumann (Heidelberg University): I would repeat that funding is the central issue, to support researchers who develop the methods.

Session 3:
Focus on thyroid

European Cluster to Improve Identification of Endocrine Disruptors (EURION): Emerging results

Andreas Kortenkamp talked about the importance of the thyroid hormone system, its vulnerability to endocrine disruptors, and the work of EURION projects to develop tests for these harmful substances.



Andreas Kortenkamp
Professor, Human Molecular Toxicology,
Brunel University London, UK; EURION Cluster;
European Society of Endocrinology

A shift in cognitive abilities at population level, due to impaired brain development caused by endocrine disrupting chemicals, would massively impact the number of people with learning difficulties and intellectual disabilities, and also have considerable societal impacts.

Proper brain development depends on thyroid hormones, which are essential for the proliferation, migration and positioning of brain cells. In the 1990s, work by Gabriella Morreale de Escobar in Madrid showed how important thyroid hormones were to early brain development; previously, they were thought not to cross the placental barrier. Specialised transporters in the placenta are now known to enable these hormones to reach the foetus.

Thyroid hormones are thyroxine (T4) and triiodothyronine (T3), which differ only by one iodine atom. T3 is the active one in circulation, but the most crucial one is T4. Three essential processes for brain development are dependent on thyroid hormones: (i) radial cell migration (from base to brain cortex); (ii) GABA Switch (between excitatory and inhibitory); and (iii) interneuron differentiation leading to network formation (connecting cells via synapses). The effects of thyroid hormone on these early processes extends into adulthood.

Follicular thyroid cells make little internal globules, where thyroid hormone is synthesised. The process begins when iodide is imported into cells. Synthesis is driven by enzymes, most importantly TPO (thyroid pyroxidase). The newly-synthesised thyroid hormone is exported out of the cell. Iodide is recycled. Interfering with any of these stages can result in suboptimal thyroid hormone synthesis.

Foetal development depends on thyroid hormones crossing the placenta. Mothers with too high or too low thyroid hormones in pregnancy give birth to babies with a lower IQ. Another factor is iodide deficiency, requiring supplements to be taken during pregnancy. Even mild iodine deficiency can affect cognitive development (e.g. language).

In the ATHENA (EURION Cluster) project, these complex processes are being bought together in an Adverse Outcome Pathway (AOP) network. Several molecular initiating events lead to decreasing levels of T4 in the serum, impacting key events, eventually leading to adverse outcomes such as learning and memory impairment, and cognitive function declines. This AOP is being used to build rational testing strategies for thyroid hormone disruption, for example, which assays to deploy and in what sequence.

The project team cover several molecular initiating events using high throughput assays and screen tens of thousands of chemicals to build up a knowledge base, which feeds into work on quantitative structure-function relationships. A topological analysis of the AOP revealed nodal points, the most important being 'hippocampal gene expression altered' where up to nine pathways converge, followed by decreases in 'T3 in neuronal tissues', 'T4 in neuronal tissue', and 'T4 in serum'.

However, it was found that there are currently no validated analytical methods for measuring T4 or T3 in neuronal tissues. It is important to get information on T4

and T3 in neuronal tissues and hippocampal gene expression to interpret currently available assays, because there is no straightforward linear relationship between T4 in serum and T4 in tissues.

Key gaps in current OECD Test Guidelines and EU testing methods are: (i) no validated *in vitro* methods for these Molecular Initiating Events – ATHENA and other EURION projects intend to make a substantial contribution here; (ii) serum T4 changes are measured in various guidelines, but not in pregnant rats (where information is needed); (iii) thyroid histopathology is measured (less usefully); (iv) no methods for Adverse Outcomes in the brain; and (v) no consistent timing of serum T4 measurements across guidelines.

The thyroid gland features prominently in testing, but what is missing is the brain. One promising test approach could be based on ATHENA work at Denmark's Technical University on neuronal migration defects in the brain, and the clearly seen histology affects as a result of improper thyroid hormone action during development.

The EU endocrine disruptor criteria needs to show a mode of action (changes to serum T4), an adversity (change in thyroid gland histopathology), and a link between mode of action and adversity. For thyroid hormone disruption and brain functions, advancement of the methods will take years. The ATHENA team therefore propose a change in the criteria with respect to the thyroid hormone system, so that serum T4 and/or thyroid gland histopathology are considered as adverse.

Clinical endocrinologists regard changes in T4 as adverse, and in and of itself it triggers clinical interventions. In chemical regulation that is not the case, and we propose to bring the two in alignment until we know much more about the intervening key events in these adverse pathways, said Prof Kortenkamp. In addition, validation is proceeding too slowly, and it needs more funding at European level.

Q&A

'CamilaQM':
What do you think about the use of ToxCast high throughput essays for the investigation of thyroid MIE for substances already under evaluation?

Andreas Kortenkamp: I think that is a good idea. It is the reason why in ATHENA we do not cover *in vitro* assays for molecular initiating events that are well advanced already, we are covering the gaps. I would support that, with one caveat. In work at the Danish Technical University in ATHENA on TPO enzyme, some of these chemicals were surprisingly found not to be active in an animal. This means that there is a step missing, not only *in vitro* assays but also input from toxicokinetics to tell us if these chemicals are likely to accumulate or reach tissues at the right concentration to produce an effect.

Q&A

Andrew Turley (Chemical Watch):
You said that in a clinical setting serum T4 levels are indicative of adversity. Could you spell out what that means?

Andreas Kortenkamp: You will get medication, especially an expectant mother if a doctor finds T4 in blood serum is too low, with a drug that bumps up thyroid hormone levels.

Q&A

Martina Klaric (Huntsman):
If serum T4 has no linear and clear relation to T4 in the brain (and hence adverse effect), why would serum T4 level be considered as adversity?

Andreas Kortenkamp: Because, firstly, that is all we can do at the moment and, secondly, if T4 is definitely low in serum in the clinic that is a warning sign and will trigger an intervention. It is not good news if your serum T4 level is low, or too high.

Q&A

Sakina Mhaouty-Kodja (ANSES):
Changes in thyroid hormone levels can also affect gene expression. Is it something planned in the analysis of neuronal tissues?

Andreas Kortenkamp: That's again very tricky – we are measuring altered gene expression in neuronal tissues. These methods are far from validation, but they would be very important. You cannot properly regulate these substances just on the basis of knowledge of T4 levels in serum and histopathology. That is how it is currently set up, and it is totally inadequate.

Q&A

Cécile Michel-Caillet (ANSES):
To clarify, if we need to measure things in neuronal tissues, it means we need in vivo studies, or would 3D models be sufficient to answer this request?

Andreas Kortenkamp: Models may help as well. Going back to basics, it may be a bit premature to advocate routine blanket measurement of these hormones in neuronal tissues. I would say, at the present time, we need as a minimum a couple of chemicals to know how changes in serum T4 translate into hormonal levels in neuronal tissues. We know this for some chemicals but not enough, it is not routine testing but the results will enable us to come up with better testing strategies.

SCREENED project (EURION Cluster)


Photo: © Florian Caiment

Florian Caiment
Toxicogenomics department, Maastricht University

Florian Caiment presented an overview of SCREENED (Screening for Endocrine Disruptors), the smallest of the eight EURION Cluster projects.

SCREENED has three main objectives: (i) develop new 3D *in vitro* assays/models that mimic the complex structure and function of thyroid glands; (ii) gain understanding of adverse effects of endocrine disruptors on thyroid, by testing compounds in assays; and (iii) engage in dialogue with regulatory agencies, to ensure that the assays developed are fit for risk assessment.

The first *in vitro* 3D model developed (in Sabine Costagliola's lab at ULB) was an organoid, using murine embryonic stem cell-derived thyroid follicles; then human-derived stem calls to monitor cell proliferation, differentiation and organisation; and lastly using functional thyroid follicles from induced pluripotent stem cells.

The second 3D model being developed (by Roberto Toni's team at the University of Parma) is a decellurised thyroid. The aim is to obtain sex-specific thyroid lobe matrices from young adult rats, destroying the cell and keeping the surrounding structure, so that human cell material can be added surrounded by functional tissue.

The third 3D model being developed (by Lorenzo Moroni's team in Maastricht) is a bioprinted thyroid. The aim is to optimise bioprinting processing parameters (e.g. fibre size, type of hydrogel) and to develop a bioprinted perfusable vascular network. The functional bioprinted thyroid sits inside a hydrogel, enabling it to be exposed to test substances.

There has also been significant progress in developing other highly innovative technologies, in particular a microbioreactor that mimics the circulatory system in the body. Three sets of carrier module have been successfully developed, along with protocols for harvesting cells.

These new possibilities for a microbioreactor were demonstrated (in Stefan Giselbrecht's lab at Maastricht University), with different cavity shapes (flat carrier, tray, µ-cavities) and different membranous carriers for various cell models: (i) single flow (below or above cells); (ii) counter flow (different directions above and below cells), and (iii) cross-flow (same directions above and below cells).

All the models were challenged with selected endocrine disruptors, in this case 4 phthalates (used in the plastics industry), 4 organophosphate flame retardants (OPFRs), 4 polychlorinated biphenyls (PCBs), and 4 polycyclic aromatic hydrocarbons (PAHs). In the 3D models, these 16 compounds were screened over 24 hours. Most of the markers used were very highly expressed.

The project team looked at available omics data (e.g. genomics, proteomics) to identify gene and protein signatures for known classes of endocrine disruptors. Five compounds were selected for cytotoxic studies (DEHP, TPP, PCB-118, PCB-153, BaP), using a freely-available culture of human localised thyroid cells, in assays over 24 hours. Thyroglobulin was consistently detected in all samples with the selected substances, confirming it was a good thyroid model.

The next steps, said Dr Caiment, were setting up a pilot bioreactor inside an incubator, starting with different doses of substances flowing over mouse thyroid follicles in membranous carriers. Transcriptomics and proteomics are being measured (compared to a static petri dish system). The team are also working to get omics accepted in regulatory risk assessments. In addition, they will validate *in silico* models with experimental data for AOPs, perform *in vitro* testing of endocrine disruptors in the 3D models, and validate an integrated omics platform for endocrine disruptor screening.

Q&A

Katrina Sichel (moderator): ***SCREENED developing new assays for risk assessment using highly innovative technologies, but also engaged with regulators to ensure that assays developed are fit for risk assessment. How do you see regulators responding to these models?***

Florian Caiment: At first, we had ambitions to start validating the models against an *in vivo* rat model, to compare them. This is an important step to have them accepted by regulators, but it had to be removed from the project due to the difficulty of engaging with animal models.

The pesticide database on thyroid hormone disrupting system

Andrea Terron gave an overview of endocrine disruptor assessments conducted by EFSA up to September 2022. In total, 95 pesticide active substances were assessed for human health and/or non-target organisms in line with the ECHA-EFSA Guidance Document (2018).

Photo: © Andrea Terron

Andrea Terron
Scientific Officer, European Food Safety Authority (EFSA)

For human health, 24 substances were considered to have no endocrine disruptor activity; for 27 a waiver was considered (e.g. inert substances); for 32 substances additional data was requested; and 10 substances were considered to have endocrine disruption properties. Documentation on human health and non-target organisms are available on the **EFSA website.**

Of the 10 substances identified as meeting the criteria for endocrine disruptors, 7 affected the T (thyroid) modality (benthiavalicarb, mancozeb, metiram, clofentezine, asulam, metribuzin, and thiabendazole), with one each affecting the E, A, and S modalities.

The T modality has been evaluated for 95 substances so far. Additional data was requested for 6 of those substances, with the Comparative Thyroid Assay (CTA) requested in 5 cases (focusing on the sensitive population: the foetus and new-borns). For one substance, the OECD TG 443, with inclusion of the assessment of the thyroid hormone system and of the second generation, was requested.

In line with EFSA/ECHA guidance, the completeness of the available dataset for the T modality is generally not an issue for plant protection products; for both substances under renewal as well as for new active substances. In the current dataset, the tests available are: Thyroid histological evaluation (a standard requirement), thyroid weight (frequently assessed), and thyroid hormone (T3 and T4) and thyroid stimulating hormone (TSH) levels (generally included as additional information).

In the dataset of pesticides evaluated to date, there has been no case of a drop in T4 or increase in TSH without evidence of changes in the thyroid histology; though there have been perturbations in hormone levels with concomitant changes in thyroid histology.

There are several factors to consider regarding the database; including lack of robust data on modes of action (e.g. reliance on methods to measure thyroid hormone levels); a lack of data in the sensitive population (foetus, new-borns); the intrinsic limitations of the subjective thyroid histopathology evaluation and the possibility of false negatives for molecular initiating events that do not produce key event changes in TSH and/or thyroid histology.

Thyroid histology is considered a valid endpoint for hazard identification, and when positive it indicates that the TSH pathway is affected. The comparative thyroid assay (CTA) would likely represent the best study design for the assessment of thyroid histopathology and changes in thyroid hormones in the sensitive population.

Indeed, developmental neurotoxicity (DNT) is the real adverse outcome of concern for thyroid hormone system disruption. However, the sensitivity of DNT endpoints for changes in T4 is still unknown, and endpoints included in the DNT studies are not EATS mediated but EATS sensitive. Therefore, T modality conclusions would highly benefit from the inclusion of mechanistic data in the Weight of Evidence analysis.

In conclusion, looking at the outlook for the T modality, Dr Terron noted: (i) it is

important to investigate the molecular initiating events to better tailor the testing strategy, to minimise false negatives and help identify the mode of action; (ii) give priority to the CTA as a critical *in vivo* study and learn how to use both histology and thyroid hormones as T-mediated adverse endpoints in the sensitive population; and (iii) increase the sensitivity of DNT endpoints indicative of disruption of the thyroid hormone system to be applied in the regulatory setting; though, it is appreciated that this is a very challenging research task.

Katrina Sichel (moderator) selected audience questions to ask.

Q&A

Svenia Jasper (Henkel): There is no Test Guideline listed under the PPP/BP Guidance Document for T-related mechanisms for human health. Which test would you recommend?

Andrea Terron: The best scenario would be to determine if the molecular initiating events have been affected; then thyroid hormones are very critical because the histology will work only for some specific pathway. It is practically difficult to move away from thyroid histological evaluation, but the match between histology and hormones in the sensitive population remains a critical step forward. I hope that projects like ATHENA will give the possibility of measuring the thyroid hormone in the brain or identify biomarkers to be used as an endpoint in the overall Weight of Evidence.

Q&A

Natacha Cingotti (Health and Environment Alliance - HEAL): Renewal of suspected thyroid disruptors is paused due to data gaps (e.g. ziram, cyprodynil, spinosad). So data completeness is also an issue for renewals?

Andrea Terron: As a matter of principle, I do not remember any dataset for which histology was not conducted. Sometimes we have less data on organ weight, but in the majority of pivotal studies thyroid gland histology is assessed, and if positive this is sufficient, but we cannot exclude some false negatives. The dataset is frequently not adequate for non-target organisms (non-mammalian).

Q&A

Cécile Michel-Caillet (ANSES): You mentioned DNT endpoints coming from TG 443 or TG 426 studies. Have you views on the quality of data you get from these studies? Can you comment further on the limits of histopathology?

Andrea Terron: Yes, moving from the OECD TG 426 to the OECD TG 443, the quality of the study for the DNT is indeed an issue. We generally have many comments, for example, on how methods are described and how data is reported. We think that frequently more expertise or educational training are needed to conduct the DNT arm in the OECD TG 443. This issue should be resolved, because it concerns chemicals assessment and animal use.

On the intrinsic limitation of thyroid histopathology: The pathologist scores (e.g. minimal, mild, moderate, marked, or very marked) are subjective. This works well when the effect is evident, but the sensitivity of the eyes will never match the sensitivity of a morphometric analysis which is more objective and potentially able to capture effects at lower doses.

Methods for the identification of thyroid hormone disrupting substances, Update on JRC coordinated activities

Sharon Munn gave an overview of recent JRC activities on identifying endocrine disruptors affecting the thyroid hormone system.

Sharon Munn
Senior Scientific Officer, Joint Research Centre (JRC), European Commission

Thyroid disruption can result in metabolic disorders, problems with growth and development, learning deficiencies, hearing loss, and visual defects. The thyroid hormone (TH) signalling system can be perturbed in the hypothalamus, pituitary, thyroid glands (via interference with hormone synthesis), through metabolism of TH in the liver, transport of hormone via serum binding proteins, transport into cells and through interaction with thyroid receptor in the cells around the body. An **OECD Scoping Document** in 2014 describes the methods available to measure disruption to thyroid hormone activity, and their readiness for validation.

The OECD Conceptual Framework lists all the Test Guidelines (TGs) for measuring endocrine disruption. There is a big gap for Level 2 tests (*in vitro* mechanistic assays) for measuring effects on the thyroid pathway. Therefore, the JRC's European Union Reference Laboratory for Alternatives to Animal Testing (EURL ECVAM), with the help of 15 EU-NETVAL laboratories and 14 method developers, carried out validation studies for 18 methods to address this gap.

Methods were selected on the basis of: (i) level of readiness; (ii) suitability for implementation in a wide variety of laboratories (e.g. no complex equipment); (iii) confidence that test systems will be available in the future (e.g. accessible cell lines); (iv) where possible, human-based cells and high throughput systems; and (v) exclusion of human embryonic stem cells on ethical grounds.

Other issues considered included the training needs of EU-NETVAL laboratories, application of OECD's Good *In vitro* Method Practices (GIVIMP) Guidance Document, and using these methods in a Good Laboratory Practice (GLP)-compliant environment. In the move to non-animal methods, the substitution of animal-derived reagents for *in vitro* tests was also investigated and applied where practicable.

The 18 methods cover all 8 blocks of the OECD Scoping Document: higher level signalling from brain to thyroid gland (block 1); thyroid hormone synthesis (2); serum-binding proteins (3); metabolism and excretion by the liver (4); transport into cells (5); inside cell interaction with human thyroid hormone receptors (6); zebrafish embryo assay for T4 (7); and integrative cellular assays looking at consequences of over/under stimulation of TH receptors (8).

The validation study was divided into two stages. Firstly, the definition of the *in vitro* method (and a set of SOPs) and five valid runs with a limited number of control and reference chemicals to examine robustness and reliability. If successful, the method was tested with a wider range of chemicals – 30 blind-coded chemicals to achieve three valid runs per test chemical.

To select the 30 chemicals tested, experts were consulted. They considered existing evidence from *in vitro* and *in vivo* methods, clinical and epidemiology data. The final selection also considered distribution in chemical space, solubility, and commercial availability. At least two chemicals were selected for each mode of action tested. Progress can be monitored on the **TSAR website** (Tracking System for Alternative methods towards Regulatory acceptance) – search for 'thyroid'.

The aim is to bring these methods forward into regulatory use, as Test Guidelines. To this end, the OECD Thyroid Methods Disruption Expert Group was established, in June 2022, to coordinate the development of Test Guidelines for thyroid-related methods.

Ms Munn showed an image of an Adverse Outcome Pathway (AOP) network for thyroid hormone system disruption, depicting all the points a chemical may interact at the molecular level to impact thyroid hormone levels in serum and tissues, leading to an array of adverse effects in mammals (including humans), fish and amphibians. The selected methods in the validation study were located at different points on this AOP network, with the aim of building an integrated testing strategy to identify thyroid hormone system disruptors. This could include the use of toxicokinetic models to predict what concentrations reach the brain and tissues, compared to the effects shown *in vitro*.

In summary, Ms Munn said that to accelerate assessments for endocrine disruptors, mechanistic (mode of action) knowledge is needed. New technologies can provide this mechanistic information, but trust in their reliability and relevance is required. That is the reason for the EURL ECVAM validation study of methods to assess the disruption of the thyroid hormone signalling system, which will be finalised in April 2023.

Q&A

Andreas Natsch (Givaudan):
You have more than 10 tests and only 2 chemicals per "Mode of Action"/assay: So these assays will only be validated for robustness but not for predictivity, how to deal with this?

Sharon Munn: We had that balance to make and the idea was to test these 30 chemicals in all the assays. We were trying a 'lean' validation. Some aspects are missing, like transferability to other labs. BASF included extra chemicals, relevant to the particular mode of action. Data could also exist in the literature, so the OECD Expert Group is pulling this all together to identify remaining gaps and needs for further validation work.

How to differentiate general toxicity-related endocrine effects from endocrine disruption: the case study of carbon disulfide

Sakina Mhaouty-Kodja
Chairperson of the EDC Working Group, ANSES, France

Sakina Mhaouty-Kodja shared the experiences of the Endocrine Disrupting Chemicals Working Group at ANSES in identifying substances with endocrine disrupting activity.

The ANSES methodology asks: (Q1) what is a substance's adverse effect? (Q2) does it act through an endocrine mode of action (MOA)? and (Q3) does the endocrine MOA induce the adverse effect identified?

To answer these questions, data is needed from regulatory and academic studies. However, there are issues with appropriate analysis, statistics, information on hormonal levels, weight of hormone-sensitive organs, and (for females) oestrous stage at the day of tissue collection or analysis. A particular issue with regulatory studies is that huge doses of substances are often given, making it difficult to discriminate between endocrine and non-endocrine (general toxicity) modes of action on endocrine systems.

This can be illustrated by recent ANSES work on carbon disulfide (CS_2). It is a highly volatile liquid, used in the industrial manufacture of regenerated cellulose and viscose. It is also used as an intermediate in the manufacture of dithiocarbamate pesticides, and was considered a risk to workers, and to people and the environment near manufacturing facilities.

In EU regulations, carbon disulfide is classified as a suspected substance for reproductive toxicity (damaging fertility and unborn child development); and for severe health effects, in particular on the nervous and cardiovascular systems. That is why it has a current occupational exposure limit of only 15 mg/m^3. It is listed as a potential endocrine disruptor in two lists: the 'Database of endocrine disrupting chemicals and their toxicity profiles' (DEDuCT list), and 'The Endocrine Disruption Exchange' list (TEDX).

For the EDC Working Group evaluation of CS_2, all available information was gathered from Test Guidelines, the extended one-generation reproductive toxicity study (2019), the scientific literature, and reports of other agencies. This data was summarised on the ECHA website and in a recent **publication** (2022).

Looking at the adverse effects identified for CS_2 from available data, it can induce effects on male and female reproduction (e.g. reduce sperm count and embryonic development). Concerning the nervous system, it can reduce brain weight and change the thickness of some sub-regions of the brain associated with retinal atrophy. Behavioural effects from academic studies on developmental exposure, for lower doses than used in TG, showed induced delayed sensorimotor development and impaired locomotor activity. More data is available for adults (using rodents), which showed impaired motor activity, visual response and spatial learning and memory; as well as reduced brain weight, retinal atrophy, and axonal swelling of nerve fibres. Furthermore, human studies from workers exposed to CS_2 showed reduced nerve conduction velocity, impaired performance on psychomotor tests, altered autonomic nervous system, and retinopathy.

Data from a US EPA report did not show any effect of CS_2 on EATS modalities, but CS_2 is metabolised into thiourea which is active on thyroid peroxidase (TPO), an enzyme important in thyroid hormone synthesis, which may explain why exposure to CS_2 lowers T4 levels in rats (*in vivo* mechanistic studies) and in humans (occupational exposure). From this data, we can suggest that CS_2 fulfils the first two criteria for an endocrine disruptor, and has a possible adverse pathway via thiourea that impacts thyroid hormone levels through TPO disruption.

However, an analysis of all the data shows that CS_2 has several other modes of action. It can also: (i) interact with amino ac-

ids to form dithiocarbamate, these have been shown to trigger axonal swelling, and to reduce dopamine β-hydroxylase and noradrenaline levels associated with the behavioural impairments observed in experimental studies; (ii) reduce nitric oxide synthases activity, important in the production of the brain neurotransmitter nitric acid; and (iii) increase oxidative stress in the brain.

Therefore, there are several modes of action, working in parallel. To understand the most plausible mode of action (e.g. endocrine or non-endocrine) to trigger the thyroid and nervous system effects, the ANSES group used the European guidance criteria (temporality, specificity, essentiality...).

In summary: (i) neurotoxicity induced behaviour impairment but also reduced brain weight and axonal swelling; (ii) thyroid disruption due to endocrine activity was found at the same doses that trigger neurotoxicity; (iii) some of the neurotoxic effects preceded the reduced levels of T4; (iv) thyroid disruption did not appear as an initiating event; and (v) the high cytotoxic potential of the substance is not selective of endocrine cells. It was concluded, based on all the knowledge gathered, that CS_2 does not fulfil the WHO definition to be classified as an endocrine disruptor (as implemented in EU regulation).

Dr Mhaouty-Kodja said their conclusion was in contradiction with DEDuCT and TEDX lists. This highlights the need for thorough analysis using all available data and knowledge; and the relevance of Test Guidelines that can discriminate between endocrine versus non-endocrine modes of action, in particular TGs using doses that are lower than those inducing general or systemic toxicity. This is not just specific to the thyroid system, but can be applied to other endocrine systems.

Q&A

Svenia Jasper (Henkel):
In accordance with the horizontal approach, which T-related test is likely to be adopted into the PPP/BP Guidance Document? What is the proposed timeline?

Sharon Munn (JRC): T-related tests are used in *in vitro* tests. In that sense, REACH and the PPR and BPR regulations are in the process of updating data requirements, and the concern is that timelines do not really match. A placeholder was put into REACH information requirements for the *in vitro* thyroid methods, and we probably won't be in time for the next update, but we need to look at the possibilities for phasing in these tests when they become available.

Q&A

Andreas Kortenkamp (Brunel University):
It seems to me that CS_2 is a damn dangerous substance. You have produced data to show it may not be classed as an endocrine disruptor under EU law, the consequences may be that it is less restricted. Are we not dealing here with a crazy loophole in the whole regulatory arena? Is it not time to introduce tougher criteria for developmental neurotoxicants?

Cécile Michel-Caillet (ANSES):
The presentation focused on the analysis of all the data regarding the endocrine disruption endpoint. This case led us to question how to use ECHA/EFSA guidance, and to see the limits of data we have in hand to answer difficult questions such as secondary unspecific toxicity while working with highly toxic substances like CS_2. On the side of regulation, we have produced a Risk Management Option Analysis (RMOA), so we do not foresee any relaxing of the regulation at all.

Sakina Mhaouty-Kodja: To complete the answer, with the TG 443 we did not have enough data on developmental neurotoxicity. I heard Lisa Baumann's presentation about merging test guidelines and maybe we could consider this for other TGs, including neural toxicity which is lacking in data.

Q&A

Martina Klaric (Huntsman):
Is ECVAM planning to develop also the defined approaches out of the validated methods?

Sharon Munn: We have not made the decision to lead that activity at this point, we are just finishing off the validation, but we would be interested in being involved in such activities. We look forward to ATHENA and ERGO delivering some proposals for what a testing strategy might look like.

Q&A

Barnali James (FMC Corporation):
Is there any further information on why the NIS work stalled?

Sharon Munn: The NIS is not stalled now, it has started again. It was mainly stalled due to COVID and should be completed in April next year.

Session 4:

Developments on bisphenols

Keynote: Bisphenols Health Impacts on Children


Photo: © Anne-Simone Parent

Anne-Simone Parent talked about the effects of bisphenols on children's health.

Anne-Simone Parent
European Society for Paediatric Endocrinology; European Society of Endocrinology; Endocrine Society; University of Liège, Belgium

We are all exposed to dozens of endocrine disrupting chemicals, she said. Some of them are considered non-persistent, such as bisphenols that are rapidly eliminated from the body but we are exposed to them every day. Others are persistent, such as the PCBs or flame retardants that accumulate in the adipose tissue and can stay in the body for several decades.

There are two main windows of sensitivity to endocrine disruptors, the foetus and the child. This is when crucial developmental processes are set up, such as the neuronal connections that will control reproduction, energy balance or cognitive function. Hormones play a crucial role in those processes, and therefore the foetus and the child are extremely sensitive to very low doses of endocrine disrupting chemicals.

The risk is high because endocrine disruptors can cross the placenta; the foetus and children have high biological and metabolic sensitivity and are less able than adults to eliminate endocrine disruptors; and children use their mouths to discover the world, increasing the possibility of oral endocrine disruptor intake. There are two important concepts when discussing the effects of endocrine disruptors on children. One is the critical window of sensitivity, meaning that developing organisms are very sensitive to environmental factors. The other is the development origin of health and disease, whereby our environment during these sensitive early stages defines our health as adults.

Bisphenols are ubiquitous due to their use in plastics. They mimic estrogens, but can also disrupt thyroid hormone action. They are detected in amniotic fluid and cord blood, so can pass from mother to unborn baby. They are present in more than 90% of the population in the EU.

Data from the European Human Biomonitoring Initiative (HBM4EU) show that all adult humans across Europe are exposed to at least low levels of bisphenol A (BPA). BPA is detected in more 90% of the population on a given day. Importantly, replacement chemicals such as bisphenol S and bisphenol F are also now detected in 50% of the samples analysed under HBM4EU.

Studies have repeatedly shown that a few years after foetal exposure to BPA, children showed impaired working memory. Another study showed that exposure to bisphenol F and other replacement chemicals during foetal life was associated with impaired cognitive function in boys 7 years after exposure. It has also been repeatedly shown in laboratory studies that developmental exposure to BPA is associated with abnormal neuronal development.

BPA is also considered to be an obesogen. Development exposure is associated with increased weight gain in rodents, and lab studies have shown that exposure increased food intake and caused abnormal fat development. In epidemiological studies, with children exposed to four different levels of BPA, obesity prevalence more than doubled in children with the highest measured levels of urinary BPA.

Another important concept is that different tissues and organs have different sensitivities to given endocrine disruptors. This is the case for bisphenols. A dose of 300 000 µg/kg bw/day of BPA leads to an increase to the weight of the ovary, but much lower levels of exposure (down to 0.03 µg/kg bw/day), consistent with human exposure, are associated with disruption of genes in the hypothalamus in the brain, which controls puberty and reproduction. In more recent studies, exposure to such low doses for bisphenol S are involved in the disruption of genes in the placenta and in foetal growth.

A final concept that I would like to illustrate, said Prof Parent, is the BPA legacy. DNA has an epigenetic 3D organisation that is transmitted from one generation to the next. Several studies have shown that BPA modifies the genetic organisation of DNA. This can be transmitted to the next generation and, via exposure of the foetus' reproduction cells is also observed in the third and fourth generations, even though they have never seen the compound. Transgenerational effects of BPA have been shown for obesity, behaviour, and reproduction.

In conclusion: (i) developing organisms are extremely sensitive to endocrine disruption; (ii) different organs/systems have different sensitivities to endocrine disrupting chemicals; (iii) and in particular the brain is extremely sensitive to BPA at low levels; and (iv) replacement chemicals have similar effects at environmentally relevant levels.

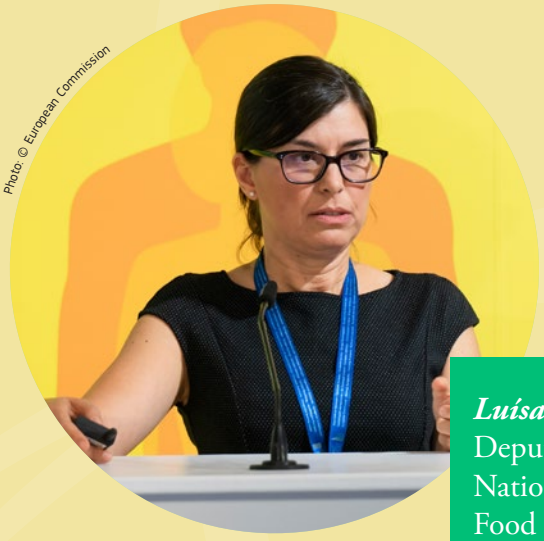
Q&A

Katrina Sichel (moderator):
Are Test Guidelines sufficient to capture the effects that you describe?

Anne-Simone Parent: I think that the tests don't cover all aspects, in particular they do not take into account the windows of sensitivity or transgenerational effects, or the different sensitivities of tissues and organs to a given compound. I would like to recommend the consensus statement that was published in **Nature Review Endocrinology** by a group of endocrinology experts who established 10 key characteristics of endocrine disruptors. If you look, only 3 of the 10 characteristics are covered by the current recommended tests; BPA corresponds to 9 of the key characteristics.

New approaches for regulatory toxicology: the bisphenols experience

This panel discussion on bisphenols was moderated by **Cécile Michel-Caillet** (ANSES). The panel members first gave presentations of their work.



CLARITY BPA: FDA/NCTR conclusions

Luísa Camacho
Deputy Director, Division of Biochemical Toxicology, National Center for Toxicological Research (NCTR), Food and Drug Administration (FDA), USA

Luísa Camacho, in her own opinion, talked about work in the National Center for Toxicological Research (NCTR) on bisphenol A (BPA). The NCTR is a part of the U.S. FDA located in Arkansas and, unlike the other Washington DC-based centers, it is focused on research rather than regulation. NCTR's mission is to conduct scientific research to support the decision-making of the FDA.

BPA is a high-production volume industrial chemical used in the production of many consumer products, including polycarbonate plastic food and drink containers, and epoxy resins used to line cans. BPA migration to foods results in widespread low-level human exposure, currently estimated to be lower than 0.5 µg/kg body weight/day.

In 2018, two reports from independent expert groups (NTP/CERHR and FDA/CFSAN) identified data gaps for BPA. The FDA/NCTR Research Program on BPA was set up to address these gaps, including toxicokinetics studies; PBPK (physiologically based pharmacokinetic) models; and a 90-day toxicity study that included perinatal exposure and internal dosimetry measurements. The group has produced numerous peer-reviewed publications.

The 90-day toxicity rat study identified several endpoints affected by high doses of BPA (resulting in a serum free BPA maximum concentration of 300 000 pM) and the synthetic estrogen (control) ethinylestradiol (EE_2). The BPA effects overlapped in most part with EE_2, supporting the view that at high levels BPA acts as a weak estrogen. No effects were detected for low doses of BPA (2.5 up to 2 700 µg/kg bw/day) or naïve (non-gavage) control.

CLARITY-BPA (Consortium Linking Academic and Regulatory Insights on Bisphenol A Toxicity) is a consortium of National Institute of Environmental Health Sciences (NIEHS)-funded grantees and federal scientists in the USA. It was a novel collaborative research model to bridge investigate and applied-regulatory science research. CLARITY-BPA had two key components: (i) NCTR core study (2-year chronic toxicity study), and (ii) grantee studies.

The CLARITY-BPA Grantee Studies evaluated endpoints (molecular, structural and functional) not typically assessed in guideline studies (brain and behaviour, cardiac, immune, mammary gland, ovary, penile function, prostate gland and urethra, testis and epididymis, metabolism and thyroid hormones, and uterus). The published peer-reviewed papers, to October 2021, are compiled in **National Toxicology Program (NTP) Research Report 18,** and all raw data is on NTP's **Chemical Effects on Biological Systems (CEBS)** database to ensure transparency.

Both NCTR and grantees used Sprague-Dawley rats from the same lit-

ters, housed and dosed together, to optimise animal use and data integration. Both also used specially-selected diets and housing to minimise background exposure to phytoestrogens and BPA. Background exposure to BPA was monitored: in diets it was 0.03-0.2 µg/kg bw/day, with no detectable exposure from housing.

There were two dosing arms: 'continuous dosing' (pregnant mothers, then directly from birth to 2 years); and 'stop dose' (dosing stopped at weaning on day 21 and animals kept for 2 years) to identify perinatal exposure induced effects later in life.

There were several exposure groups: vehicle control, 5 doses of BPA (2.5 up to 25 000 µg/kg bw/day) and two doses of estrogen control (EE_2 at 0.05 and 0.5 µg/kg bw/day). Endpoints assessed included body weight, survival, vaginal opening and cytology, litter parameters, 1-year sacrifice (e.g. organ weights, sperm analysis, histopathology), and 2-year sacrifice (histopathology).

EE_2 induced dose-dependent adverse effects, mainly in females and minimal effects in males. There were statistically significant effects at all BPA doses, but below 25 000 µg/kg bw/day they were unlikely to be treatment-specific because of a lack of dose-response, inconsistency across sacrifice times/dosing arms, and a lack of pattern across biologically-related endpoints and organ systems. However, adverse effects on female reproductive tissues and male pituitary in the 25 000 µg/kg bw/day group may be treatment related.

Overall, said Dr Camacho, our conclusion is that the data does not support "low dose" effects or non-monotonic dose-response by BPA. The main goal was to have these data integrated with all the other scientific literature on BPA, including grantee studies and other studies, across the world, to contribute to the ongoing safety assessment of BPA.

CLARITY BPA experience

Norbert Kaminski
Director, Center for Research on Ingredient Safety, Michigan State University, USA

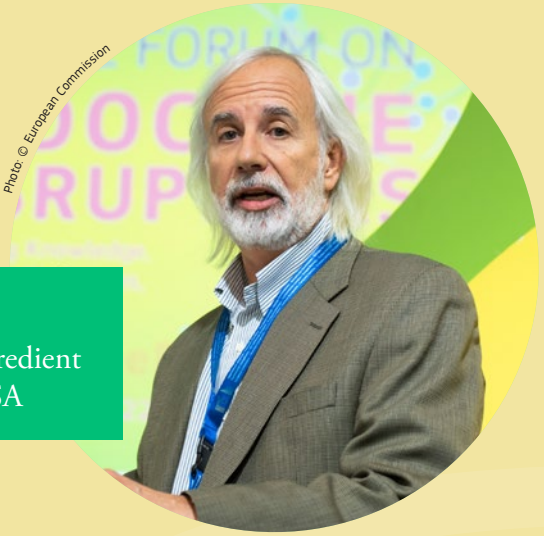


Photo: © European Commission

Norbert Kaminski, in his own opinion, gave a grantee's perspective of the CLARITY BPA study. He identified the contribution of academic investigators in labs across the US, provided an academic's perspective on the major strengths of the CLARITY-BPA Program, and gave an overview of the immunotoxicological evaluation of BPA.

Academic laboratories were included in CLARITY-BPA to provide input beyond that typically found in guideline studies that assess chemical safety. Most academic labs focus on mechanism, and that was a major contribution. In terms of the immunotoxicology evaluation at Michigan State, for instance, it was a combination of guideline/core and non-core experimental approaches.

Among the strengths of CLARITY-BPA: (i) it addressed one of the biggest challenges of working with BPA, its ubiquitous nature, with a protocol that went to great lengths to minimise and monitor background exposure, something most academic labs are unable to do; (ii) the sharing of animals minimised their use (though a lot of animals were used) and enabled tissues to be traced back to a particular animal; (iii) tissues received by grantees were blinded, so investigators did not know which tissues they were receiving (which is important in light of the **AMGEN paper** showing that when studies were blinded they were more likely to be reproduced by another laboratory); and (iv) the results and detailed methodologies are in a publicly-accessible centralised database, for anyone to further analyse.

A paper by **Heindel *et al.* (2020)** summarised work on data integration, analysis, and interpretation conducted by eight academic (grantee) CLARITY-BPA studies.

On the immunotoxicological evaluation of BPA, it should be noted that there is no single assay as the immune system comprises many different cell types with different functions. Our main interest was in estrogen-related receptors, said Prof Kaminski, due to several recent Japanese papers suggesting that BPA had a higher binding affinity to estrogen-related receptors (ERR) than the estrogen receptor (ER).

Starting on day 21, the Michigan lab evaluated leukocyte composition of thymus and spleen, for different cell types in terms of their proportionality in the two organs. They measured 21 different cellular phenotypes. On day 90, then 6 months and 1 year, they continued characterising leukocytes cell types in the spleen, but in addition quantified various leukocyte functions in these spleen subpopulations. 116 different functional endpoints were measured per spleen, totalling 137 endpoints per animal. So over 4 years, every time the lab received tissues from NCTR, an assembly line was immediately formed, with people assaying different endpoints. To ensure cell viability, the NCTR harvested spleens late in the afternoon for shipping overnight, for work to begin by noon the next day.

For the immunological characterisation, the team measured lymphocyte B cells and T cells (and two subpopulations); NK (natural killer) T cells (and two subpopulations); and assessed several endpoints for innate immune cells (myeloid and granulocytes). In terms of function, measure-

ments were made for lymphocyte B cells (e.g. antibody production of IgM), T cells, myeloid activation, and NK activation.

For the immunological characterisation, for all cell types, there was little effect on innate immune cells. In terms of myeloid cells, the one interesting finding was for the classical dendritic cells, where the same effects occurred with estradiol, the positive control and BPA. There were no effects for antibody production by B cells. For activation of T cell responses, modest effects occurred with estradiol but not BPA.

In conclusion, Prof Kaminski said that a trend was seen beginning at day 21, which became significant at 90 days, in splenic classical dendritic cell numbers was observed in female rats. However, no sustained effects were observed on either leukocyte cell subpopulations, for either total cell number or function, with either estradiol or BPA treatment. The results were published in companion papers in *Toxicology* in 2018: **Part 1** and **Part 2.**

Q&A

Cécile Michel-Caillet (moderator) invited two further members of the CLARTY-BPA study, **Heather Patisaul** and **Gale Prins,** to contribute as part of this Q&A session, and also asked the first question:

How many animals were used in the study?

Luísa Camacho (CLARITY-BPA): We have large animal rooms at NCTR and still we used all our animal space. That is why, although grantees thought it important to also run the estrogen control, initially not included in the study plan, we were only able to accommodate the EE_2 'continuous dosing' but not the 'stop dose' arm.

Heather Patisaul (CLARITY-BPA grantee, North Carolina State University), a neuroscientist, talked about other aspects of CLARITY-BPA.

The study made very efficient use of animals and aligns with the 3Rs. It leveraged the unique strengths of guideline and academic studies. There is a need to expand toxicity testing, and the core study did not have any brain endpoints. This is something as a grantee I and Cheryl Rosenfeld (University of Missouri) could bring to the table. Between us we published seven different studies, looking at behaviour, neuroanatomy, gene expression, brain development, and epigenetics. We did a lot of this work at NCTR.

Another unique aspect of CLARITY-BPA was that the tissues for diverse endpoint studies come from the same animals. This permits a systems level analysis of all the data, including data that can be difficult to interpret in isolation. When this cohesive systems-level analysis was done, what CLARITY-BPA showed was that there were very concordant effects on the organs that you would predict would be sensitive to BPA, namely the brain, ovary, prostate, and mammary gland. These results were consistent with the literature and human data. So I think there is a lot of lessons learnt that can benefit others interested in pursuing this type of endeavour.

Gale Prins (CLARITY-BPA grantee, University of Illinois at Chicago) introduced her work as principal investigator of academic studies on the prostate gland.

In CLARITY-BPA, we were able to repeat our previous studies that showed significant carcinogenic effects on the prostate gland with very low doses of BPA with additional estrogen exposure. A major strength of CLARITY-BPA is that it allowed evaluation of multiple organ endpoints, by sharing tissues from the same animals. This also enabled the integration of the statistically significant data from academic scientists, and permitted the expansion of a study to a systems biology level. A group of us published our findings on this in the Heindel *et al.* (2020) paper.

The findings reveal that BPA exposures simultaneously affect numerous organs in individual rats, a strong indi-

cation that the data are not spurious nor random events. Importantly, this approach confirmed that below 2.5 µg/kg bw/day, dose effects of BPA were observed in multiple organs and tissues at once, thus shedding light on the interconnectedness of these low dose effects across biological systems.

To summarise, the data indicates that BPA effects in one tissue were correlated with effects in another tissue, including immune cells, thus providing strong evidence that the data are revealing true effects at a low BPA dose in this guideline Good Laboratory Practice (GLP)-compliant study. It also shows that these low dose effects lead to systemic health consequences. Importantly, the only way to obtain this information was by using a CLARITY-like design, and I strongly recommend that others use this approach in the future.

Cecile Michel-Caillet (ANSES):
You have mentioned that it is really important that we now have data for multiple systems in one single animal. Are data published animal-by-animal?

Gail Prins: This was published in Reproductive Toxicology (Heindel *et al.*, 2020), where all the integration was done.

Katrina Sichel (moderator) addressed audience questions to the panel.

Laura Vandenberg (University of Massachusetts):
Can you speak about reproducibility? 17a-EE was included in CLARITY and a prior NTP study but numerous outcomes had opposite effects between these studies?

Luísa Camacho (CLARITY-BPA): We did a ***90-day study*** before CLARITY-BPA and in both studies we included a reference estrogen control. The EE_2 doses in the CLARITY study were ten-fold lower than in the previous study to really understand the sensitivity of the animal model to estrogenic activity. In addition to these studies, we also conducted NTP-funded multi-generational studies using the same animal model and animal diet, but there was a major difference in study design between the previous NTP multi-generational studies with EE_2 and the CLARITY-BPA study. In the previous NTP studies we used dietary dosing, in which the exposure during the perinatal period was through the dam/mother, while in the 90-day and CLARITY-BPA studies we directly dosed the pups rather than rely on milk transfer. The different routes of exposure resulted in very different internal dosimetry to EE_2 hence it is not surprising that the effects across studies were not 100% aligned.

Robert Thomas Zoeller (University of Massachusetts):
What kind of variability is imposed by the pattern of analysis, and how can you could measure it?

Norbert Kaminski: Surprisingly (or maybe not) the variability in general was not much, mainly because most of the endpoints we measured were by flow cytology, so you are evaluating probably about 10 and 30 000 cells per endpoint. In the two publications from our lab, we show graphically those endpoints with some effects or changes, giving an idea of the level of variability, which is very small.

Ninja Reineke (CHEM Trust):
I understand one of the main aims of CLARITY was to compare academic and guideline studies. Why didn't the FDA publish an integrated report to extract even more lessons from this innovative approach?

Luísa Camacho: We published our data not only in the ***NTP Research Report 9,*** but also as a peer-reviewed publication. In that manuscript we did try to integrate our data with the grantees' data published in the meantime. Overall, I think the consortium initially intended to publish a final report that integrated all the streams of data, but after 10 years of working together and since the regulators will reanalyse and reinterpret the data for their needs, I think there was a general consensus to do instead a compendium of all the peer-reviewed publications (NTP Research Report 18). All the raw data is available for further analysis by others. So, the publication of the NTP compendium was the end of the consortium.

Malik Duhaut (Covestro):

I see quite a discrepancy between your results and the results of EFSA. Very strong immunotoxin effects were detected by EFSA in their conclusion. How do you explain this discrepancy?

Norbert Kaminski: First of all, we did not evaluate the subpopulation of T cells called TH17 used in the EFSA study. The reason I think the EFSA conclusion is curious is that what they tried to do was link TH17 cells (which secrete a pro-inflammatory cytokine) with epidemiology studies on allergic respiratory airway disease. There is no scientific data cited in the EFSA report, or any in the literature, that show that TH17 calls have an effect on allergic airway responses, which are mediated by TH2 cells (so there is no plausible link). In addition, the epidemiology studies they try to link with the TH17 cells I suggest are very weak in terms of exposure, often having single data points, either for urine or blood, for BPA exposure. Finally, there are as many epidemiological studies showing a link to allergic respiratory disease with exposure to BPA as there are those that do not. Therefore, I am puzzled by the findings and conclusions of the EFSA report.

Ana Soto (Tufts University School of Medicine):

What statistical criteria do you use to assess non-monotonic dose responses (NMDR) and do these criteria ever detect NMDR?

Luísa Camacho: Test Guideline statistical studies usually don't look for non-monotonic dose responses, they assume a linear response. In our study, we did run additional statistical tests despite the risk of maximising the number of statistical findings that are not necessarily related to treatment. For instance, for the pathology data, in addition to the usual tests we also ran two additional tests, one being the relative treatment effect (RTE) method that does not assume a linear response and would probably be sensitive to a non-monotonic dose response.

Heather Patisaul: I just want to correct something that Luísa mentioned earlier, she said there was consensus on not producing an integrated study and that is inaccurate. Eight of the ten grantees took it upon themselves to do such a published study (i.e. Heindel *et al.*, 2020). I think that in a consortium study like this, there needs to be accountability from start to finish to ensure data get integrated at the end. A weakness of the CLARITY study was that we did not have our FDA partners with us when we integrated the data.

Andreas Kortenkamp (Brunel University):

To an outsider the contrast between the guideline studies and the grantee studies is considerable. Do you have any idea why you don't see an effect and the grantees do?

Luísa Camacho: The endpoints are different, the interpretations of data are different, so all together that comes to different findings for the same data.

An EU Member State experience on the grouping of bisphenols

Christian Unkelbach gave a regulatory overview on bisphenol A (BPA) with a focus on REACH, and an update on the German restriction proposal on bisphenols. His organisation, BAuA, is the competent authority for REACH in Germany, working in close collaboration with the German Environment Agency.

Christian Unkelbach
Federal Institute for Occupational Safety and Health (BAuA), Germany

In 2012, BPA was included in the first Community Rolling Action Plan (CoRAP) for substance evaluation, which started its assessment in Germany for endocrine disruption and other concerns. This substance evaluation was concluded in late 2016, and published in 2017.

The conclusion of this substance evaluation was that for human health no further regulatory measures were necessary, though there were some risk characterisation observations for specific categories in worst-case assumptions. However, for the environment the information was sufficient to conclude that BPA was an endocrine disruptor, and also a Substance of Very High Concern (SVHC) under REACH; therefore requiring measures to minimise emissions to the environment.

This substance evaluation procedure initiated a German Regulatory Management Option Analysis (RMOA), an informal process for directing regulatory action against BPA. In 2017, BAuA started a two-month consultation with stakeholders and met with commenting parties, leading to the publication of the RMOA conclusion document on ECHA's website (PACT section). This concluded the necessity to clarify the endocrine disruptor properties for the environment of BPA at the EU level via the SVHC identification process under REACH, as a first step, followed by a restriction to minimise exposure in the environment.

French colleagues have also worked on BPA and human health, and they identified BPA's reprotoxic and endocrine disruptor properties for human health in 2016. Germany added the label of endocrine disruptor for the environment to the candidate list entry for BPA under REACH in late 2017.

A place on the candidate list requires ECHA to recommend substances, at least biannually, for inclusion in Annex XIV of REACH. These are the substances undergoing authorisation. ECHA's recommendation is based on a points system using three criteria: hazard, tonnage, and uses (BPA scores quite highly on this prioritisation scale). ECHA forwarded the list including BPA to the Commission in 2019. However, the German RMOA concluded that inclusion in Annex XIV was the second-best regulatory measure for BPA, after a tailored restriction to reduce emissions to the environment.

Restrictions are included in Annex XVII of REACH. Restrictions may be tailored for the placing on the market of substances in mixtures, articles or on their own.

A preliminary wording of the planned restrictions on bisphenols (2021) centred on BPA, but can encompass other bisphenols of concern, if they fulfil structural requirements, and are identified as endocrine disruptors for the environment.

A core set of bisphenols will be addressed for restriction: (i) BPA and bisphenol B (already identified as endocrine disruptors for the environment); (ii) bisphenol S (proposed for identification); and (iii) bisphenol F and bisphenol AF (and its salts) (special assessment under REACH by the Member State Committee). Additionally, BAuA adopts a semi-dynamic mechanism, to enable the future inclusion of other substances in the bisphenol family with endocrine disruptor properties into the restriction.

Therefore, concluded Mr Unkelbach, this is not a wholesale ban on bisphenols, or a ban on bisphenols solely based on structural similarity to BPA. To qualify for restrictions, the bisphenol structure has to be linked with endocrine disrupting properties in the environment for which EU-wide consensus has to be established.

The 'Planned Restriction on Bisphenols' will proceed: (i) via two additional informal 'Calls for Evidence' (launched November 2020 and October 2021); (ii) a public consultation process for bisphenol F and bisphenol AF for the environment, which will inform Member State opinion making; (iii) the Annex XV restriction dossier was to be submitted by 7 October 2022; and (iv) a formal 6-month public consultation will be launched after the dossier has been approved by ECHA's scientific committees (RAC risk assessment committee and SEAC Committee for Socio-economic Analysis), which is expected to happen at the end of 2022 or in early 2023.

European Chemicals Agency work on bisphenols

Francesca Pellizzato presented work that ECHA has done on the bisphenol group with some Member States, in particularly Germany, Belgium, France and Sweden.

Francesca Pellizzato
Scientific Officer, European Chemicals Agency (ECHA), Helsinki, Finland

ECHA's Integrated Regulatory Strategy is a process under which authorities identify and address substances of concern. There are different interconnected regulatory processes, but work on groups is central to the overall reiterative process. It is a transparent process, as ECHA publishes on its website the Annual Report, the Chemical Universe mapping of all REACH registered substances to the respective regulatory action pool, and the Assessments of Regulatory Needs (e.g. for bisphenols).

By grouping substances we: (i) screen groups of substances with a focus on those with suspected priority hazards and highest potential for exposure; (ii) bring consistency in how similar substances are treated; (iii) target the right substances at the right time; (iv) pool information to enable faster action; (v) increase predictability of authorities' actions; and (vi) support informed substitutions, and avoid regrettable substitutions.

The bisphenol group focuses on 148 bisphenols which share the same backbone, but differ in the bridge between the two phenyl rings, or have different substituents, or where phenolic hydroxyls could be derivatised. There is an upper cut-off value for Molecular Weight of 600. The bisphenols list comprises BPA and its derivatives (half of the 148); BPS and its derivatives; BPF and its derivatives; BPAF and its salts; and two heterogeneous subgroups.

In terms of hazard, the focus is on endocrine disrupting properties for both human health and environment, but other hazards are also covered (e.g. reprotoxicity, skin sensitisation). The methods used include finding patterns across groups, QSAR modelling, and data on mammals to inform environmental considerations (holistic approach). The information sources included REACH registration dossiers, other REACH and CLP information, and previous assessments under EU legislation.

Exposure information came from REACH registration dossiers (though the Chemical Safety Reports were not necessarily looked at), and information on uses is used as proxy for human exposure and releases to the environment. The potential for exposure was considered high for substances with widespread uses.

Grouping for regulatory action divided the 148 bisphenols into four groups: (i) based on 'Need for EU regulatory action' (34 bisphenols); (ii) 'Inconclusive - no hypothesis yet' (22); (iii) 'Currently no need for action' (26); and (iv) 'Currently no action – not possible to conclude' (66).

Of the 34 substances with a need for action, hazards were confirmed for 3 (including BPA); information is available to confirm hazards for 9 (actions are ongoing); and data for another 9 bisphenols may be required to confirm hazards.

The proposed actions are: (i) a group restriction has been identified as the best way to manage the risks of 34 bisphenols; (ii) German authorities are already preparing a proposal to restrict use of BPA and other bisphenols with environmental endocrine disruptor properties; (iii) once it is clearer which bisphenols the German proposal covers, the Commission will consider any further needs for regulatory action; (iv) extending the restriction of BPA in thermal paper; and (vi) as BPAF and its salt are also defined as PFAS they will be considered in the planned PFAS restriction.

Ms Pellizzato concluded that assessing chemicals in groups has been a successful approach. It makes it faster to identify which chemicals need regulatory action or more data, and those chemicals for which no further action is needed. The case of bisphenols has proved that working with groups of substances improves consistency and coherence of regulatory work, helps to avoid regrettable substitutions, and helps identifying early on substances registered for intermediate uses only, or currently not registered but which could be potential substitutes for known substances of concern.

Further information can be found on ECHA's website, including the **assessment of regulatory needs for bisphenols** and the **Integrated Regulatory Strategy.**

Q&A

Katrina Sichel (moderator):
Can you tell us a bit more about the semi-dynamic extension mechanism?

Christian Unkelbach: We did not want the restriction to just be buried in Annex XVII and stay there. We wanted to give the Commission an opportunity to add further bisphenols as they are identified as endocrine disruptors. There is a precedent for CMR (carcinogenic, mutagenic, or toxic for reproduction) substances in consumer products, where the Commission is able to add further substances.

Q&A

Andreas Natsch (Givaudan):
Does the restriction in the environment consider the rapid biodegradation of BPA and hence low exposure?

Christian Unkelbach: The restriction does consider degradation data in BPA in the dossier. The second part alludes to a potential lack of risk, but for us even low exposures of environmental endocrine disruptors are a cause of concern, and would give rise to this restriction. Therefore, biodegradation of BPA is not a reason to exonerate this substance, which is ubiquitous in the environment.

Q&A

Andrew Turley (Chemical Watch):
Regarding the restriction proposal and this dynamic mechanism, are you envisaging that the inclusion of new bisphenols would be an automatic thing, through a harmonised classification, or would there be a procedural mechanism that the Commission would follow with some sort of consultation process?

Christian Unkelbach: In short, it is the second option you proposed, that is where the semi-dynamic comes in. The inclusion should not only be based on the hazard identification, which would occur for example under the CLH (harmonised classification and labelling) procedure. There would be some additional kind of consideration with regard, for example, to transition periods for specific uses.

Q&A

Judith Giernoth (Covestro):
You did not consider the REACH registration Chemical Safety Reports of the substances. Could you explain why?

Francesca Pellizzato: This is a screening exercise, so we don't have time to go through the Chemicals Safety Report included in each substance dossier. We take uses as proxy for exposure information.

Cecile Michel-Caillet (ANSES): For the two groups of 26 and 66 substances, which data did you consider, those in the dossiers or those available in the literature?

Francesca Pellizzato: As it is a screening exercise, ECHA is not generally fishing for information outside of the registration dossiers. For bisphenols, Member States did alert us to relevant information out there useful for assessments, but as a general rule we stick to the information in the registration dossier.

Q&A

Natacha Cingotti (HEAL):
Why not already initiate a single restriction on all 34 bisphenols of concern to ECHA instead of duplicating efforts (with the limited German restriction for now)?

Francesca Pellizzato: As I said, it would be preferable to have a SVHC identification in REACH or a classification in the CLP Regulation as a first step, before proceeding with a restriction. I understand the urgency to act on these substances, but unfortunately we do not have the basis for acting on all of them immediately, although I think all of them would fulfil the definition of environmental endocrine disruptors, so they could go into the restriction from Germany.

Christian Unkelbach: This was considered as one of the many imaginable options, but data is lacking to really extend the restriction to all of the bisphenols identified at the screening level by ECHA.

Cecile Michel-Caillet (ANSES): For what was presented by Norbert Kaminski, I think there is one point we should probably highlight. It was said that based on the AMGEN paper, using blinded samples increases reproducibility of the data produced by others. We need to have validated Test Guidelines, but if we can have reproducible results it's already a good point when considering the Weight of Evidence approach.

Q&A

Sandra Jen (EDC-Free Europe coalition):
It is great we are having these scientific discussions on the effects of endocrine disruptors on the foetus and children across generations, but maybe we are missing a bit the human rights perspective. There has been lots of developments on this front lately, especially on children's rights, and the right to a toxic-free environment. I think we could imagine that affected EU citizens start envisaging legal actions for breach of these human rights, like the right not to be exposed to hundreds of chemicals before even being born. Is this something that regulators are starting to consider?

Christian Unkelbach: We are proposing to restrict bisphenols with a risk for the environment, but there will obviously be an impact on humans via the environment, so there will be a reduction of bisphenol exposure to humans if the restriction proceeds as we intend it to. With regards to potential legal action that is not something we have deeply considered.

Cecile Michel-Caillet (ANSES): I hope you see that we are trying to speed up our work using the grouping approach, with the aim of protecting the environment now and for our children.

Q&A

Gale Prins (University of Illinois at Chicago):
Samples were not only blinded when they came to us, but we had to upload all data into a CEBs database where it was locked down and no-one had access to it to change anything once quality control was done; it became a read only format. They did not release the results back to us until all the investigators locked down their data. Once everyone had finished, the data was decoded. It was tightly controlled in terms of making sure no-one was talking to each other behind the scenes about what they found in different groups. I see that as beneficial, it provides confidence and transparency.

Erik Prochazka (Cruelty Free Europe):
The regulatory action on bisphenols took a long time – did conflicting results from animal studies contribute to this, and could it be resolved using NAMs?

Adama Traore (Givaudan):
EU vs USA strategies: might harmonisation be helpful for the industry, particularly for BPA.

Norbert Kaminski (Michigan State University): I think harmonisation is very important, especially from the private sector perspective because it is very difficult to make specialised products that are just for certain countries, or certain parts of the world. I would also, regarding data not being consistent across studies, say that the results are still a little unsettled, and I think a very important aspect is exposure levels. One also needs to consider what levels people are exposed to, because every chemical at some dose is going to be toxic.

Luísa Camacho: To frame CLARITY-BPA data in terms of FDA vs grantees, I don't think that is a correct dichotomy. I think there is space for discussion in how to interpret the data and that is part of the scientific process. I think our primary role as researchers is to generate data of sufficiently good quality for regulators to use for decision making, and I think we were very successful in CLARITY-BPA in that respect. FDA is also interested in NAMs and reducing animal use whenever possible, and efforts are ongoing in the US to improve reproducibility and other aspects of NAMs.

Francesca Pellizzato: I don't think that conflicting results is the issue for why it takes so long to regulate substances. With regard to NAMs we have tried QSARs for the 148 bisphenols to identify degradation products or metabolites. This exercise did not give very good results for the BPA derivatives. However, for the subgroup of TBBPA and its derivatives results were very encouraging and in the Assessment Report we based our conclusion a lot on QSARs, but we then need further data to confirm what NAMs are predicting.

Christian Unkelbach: With current legislation in place, NAMs can inform on a potential concern but it is then necessary to have other methods to come to a robust conclusion, and that is the framework we are working with right now.

Cecile Michel-Caillet (ANSES): NAMs are really useful for endocrine activity, but we have three parts in our definition, and it is more difficult for adverse effects. It did not prevent France from identifying bisphenol B, but animal data are still needed.

Luísa Camacho: Metabolism is important when defining toxicity and sometimes NAMs fail there; it is important to keep this in mind.

Q&A

Andreas Przybilla:
If ECHA acknowledges the risk of bisphenols as endocrine disruptors, but lacks data from registration dossiers, wouldn't it be necessary to create the data themselves, for example, with direct requests to laboratories?

Francesca Pellizzato: ECHA is not currently asking laboratories to perform tests. I think there is some discussion at Commission level to funds regulators to test some substances, but at the moment there is nothing of this sort in REACH.

Cecile Michel-Caillet (ANSES): Maybe we could mention the PARC initiative that goes in that direction, to help us create data that are missing.

Q&A

Judith Giernoth (Covestro):
You mentioned that in the CLARITY study you found effects that were maybe treatment related in the highest dose group, in 25 000 µg/kg bw/day. Could you also say whether the effects could be considered as adverse?

Luísa Camacho: In the 90-day study the highest two doses were 100 000 and 300 000 µg/kg bw/day of BPA, continuous doses. Those two doses, especially the highest one, induced several consistent adverse effects in related endpoints, so we are very confident those doses of BPA induced adverse effects. In the CLARITY-BPA chronic study, the highest BPA dose was 25 000 µg/kg bw/day. There were some signals in that dose group that may be interpreted as treatment effects, but they were subtle, for example, in the male pituitary and uterus in continuous dose, but not as robust as in the 90-day study.

Conclusions and next steps

In the form of a conversation with moderator **Katrina Sichel, Cristina de Avila** summed up some of the Forum's key outcomes and looked ahead to the next steps.

Cristina de Avila
Head of Unit, Safe and Sustainable Chemicals, DG Environment, European Commission

What were the key takeaways for you? What particularly resonated for you and for the Commission?

In the Forum, we focused on test methods and research. There are some 40 test methods for endocrine disruptors in the pipeline, but validation is crucial and this is a process that needs widespread cooperation and considerable funding going forward. The early integration of the regulatory approach was also highlighted.

We heard a very important and concerning message, which is the very specific effects of endocrine disruption on the thyroid and the link with declining IQ-levels. This is alarming news for our society, and it is extremely important that we make the right decisions now.

We organised the Forum to listen to different perspectives, exchange information, and to seek synergies. We also learn from other regions, and I would like to thank in particular our US speakers for having joined us for this exchange.

What do you think are the main takeaways to underline for the audience?

Endocrine disruptors are a very specific form of hazard and they are probably the most complex.

The first segment of this year's Forum in Paris was very informative on how to communicate on endocrine disruptors. The general public has been made aware, and is rightfully concerned about these hazards. It is our responsibility as a public authority to not only raise awareness, but also take the necessary regulatory actions.

As an EU institution we feel it is beneficial to continue working closely with the Member States that are willing to do communication and awareness raising, to better communicate with European citizens.

The European Commission is very committed to continue working intensively to regulate endocrine disruptors. We will soon have a breakthrough regulation on the identification of endocrine disruptors, with the proposed new hazard classes to be adopted by the Commission before the end of this year. We hope the European Parliament and Council will have no objections, so the Act can enter into force according to plan in early 2023.

Where would you like to see more efforts focused in the coming year or two?

We heard today that investing in and funding validation of test methods is crucially important – since my institution, the European Commission, is one of the possible funders, we will be certain to pass on that message to our colleagues that distribute the funds.

Validated test methods are the basis of the process, to be able to identify endocrine disrupting substances. We are focusing a lot on their classification into hazard classes.

Only once they are identified and classified, can we regulate them. And our plan is to ban endocrine disruptors in consumer products – apart from where their use is essential for society, as we announced in the Chemicals Strategy for Sustainability.

Yesterday and today there was a lot of focus on "the criminals", but with the Chemicals Strategy we also want to give a message of hope and stimulate innovation and the development of chemicals that are "safe and sustainable by design", for which criteria will be coming out in the coming months.

Of what are you especially proud and optimistic?

Very broadly, I am proud of this Commission's pioneering European Green Deal, because it generated the Chemicals Strategy for Sustainability which is really ambitious for the better protection of health and the environment.

For the implementation, we are delivering one action after the other but we are under extreme time pressure to make regulatory proposals in the first half of next year, notably the REACH revision. I want to be optimistic that we will make it because we must make it.

I am also proud that the EU is a worldwide frontrunner in regulating endocrine disruptors.

This Forum has made it clear once again that we all have much work ahead of us, but we are highly motivated and ambitious to carry on.

I would also like to look ahead to the 5th Annual Forum on Endocrine Disruptors next year. This annual event will keep us on our toes, to keep presenting the latest developments and progress in a transparent way, possibly together with the Spanish presidency at that time. It would also be nice to continue an outreach to and an exchange with non-EU countries and regions.

For now, I would like to thank all our speakers, the participants here in the room and the online audience for their active interest and contributions.

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