METHODOLOGY AND SCIENTIFIC SUPPORT UNIT

Workshop on the EFSA NAMs Project on Environmental Neurotoxicants

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WEB-conference

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Participants

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1. Welcome and apologies for absence

The Co-Chairs welcomed the participants.

2. Introduction to the EFSA Project on New Approach Methodologies (NAMs)

Although the legal framework for the use of New Approach Methodologies (NAMs) in regulatory assessments is well defined, their incorporation in risk assessment is still limited. In EFSA's remit information from animal studies is frequently available, but often with deficiencies that generate uncertainties when performing humans' and animals' health risk assessments. In this context, the EFSA NAMs project aims at promoting the implementation of NAMs to specifically address the identified data gaps through the incorporation of existing information and the generation of NAM-based data, combining exposure and hazard assessment. The EFSA Project on NAMs comprehends a series of NAMs case studies that represent real proof of concept cases covering different areas under EFSA's remit. The undertaken approach for the establishment of these case studies consists of a first co-design phase between researchers and risk assessors to define the strategy for the assessment, the development of Integrated Approaches to Testing and Assessment (IATAs), and real implementation phase followed by validation of results into the regulatory context.

3. Introduction to the EFSA Pilot Project on the use of NAMs for the risk assessment of the pesticide Tebufenpyrad

In 2016 the EFSA Panel on Plant Protection Products and their residues (PPR Panel) published the Scientific Opinion 'Investigation into experimental toxicological properties of plant protection products having a potential link to Parkinson's disease and childhood leukaemia' (EFSA PPR Panel, 2016[1]), which represented the starting point for the EFSA Pilot Project on the use of NAMs for the risk assessment of the pesticide Tebufenpyrad. Tebufenpyrad is an active substance used in Plant Protection Products (PPPs) approved in the European Union and currently under renewal process with France (ANSES) as Rapporteur Member State (RMS). As suggested by its structure, Tebufenpyrad is a mitochondrial complex I inhibitor potentially able to cross the blood brain barrier. Concerns regarding the neurotoxic potential of this active substance were therefore identified and linked to the possibility of association between Tebufenpyrad exposure and Parkinsonian motor deficiencies. Following a dialogue between EFSA and ANSES as RMS, Tebufenpyrad was included as a Case Study under the EFSA NAMs project to explore the capacity of NAMs for addressing this concern through the development of an IATA informed by the Adverse Outcome Pathway (AOP) developed by Terron at al., (2018)[2]. The final goal is to integrate the results obtained during the EFSA peer-review process. This case study is subdivided in two parts: Part 1, 'Development of physiologically-based kinetic (PBK) model coupled with pulmonary and dermal exposure' (GP/EFSA/SCER/2020/02), and Part 2, 'Hazard characterisation and identification of the Reference Point' (NP/EFSA/SCER/2020/02).

4. EFSA Pilot Project on NAMs for the risk assessment of the pesticide Tebufenpyrad: results, experience, and lesson learnt

4.1. Part 1, 'Development of physiologically-based kinetic model coupled with pulmonary and dermal exposure' (GP/EFSA/SCER/2020/02) - ANSES

The first part of the project was assigned to France, which is the RMS for the renewal of Tebufenpyrad, approved for the first time in 2009. Since 2009 potential issues for the Central Nervous System (CNS) were identified. There is existing evidence for mitochondrial toxicity via Electron Transport Chain (ETC) modes of action (MoA) (complexes I, II, III, IV inhibition and inhibition of ATP production), which

[1] EFSA PPR Panel (EFSA Panel on Plant Protection Products and their Residues), 2017. Scientific Opinion on the investigation into experimental toxicological properties of plant protection products having a potential link to Parkinson's disease and childhood leukaemia. EFSA Journal 2017; 15(3):4691, 325 pp. doi:10.2903/j.efsa.2017.4691

[2] Terron A, Bal-Price A, Paini A, Monnet-Tschudi F, Bennekou SH, Leist M and Schildknecht S, 2018. An adverse outcome pathway for parkinsonian motor deficits associated with mitochondrial complex I inhibition. Arch Toxicol, 92:41-82. doi: 10.1007/s00204-017-2133-4

however are not currently part of the regulatory mandatory requirement of the EU Pesticide Regulation.

The aim of this first part of the project was to develop a PBK model implementing the EFSA Guidance on non-dietary exposure to pesticides (EFSA, 2014[3]). The model was intended to estimate from the external exposure the internal concentrations of Tebufenpyrad in human body (i.e. central compartment and target tissues (brain and neurons)) for different intended uses and target populations (i.e. operators, workers and residents). A dedicated report from the project will provide details on the experimental work conducted and analytical methods used for the *in silico* predictions and *in vitro results* of protein binding, intrinsic clearence, qIVIVE from hepatic systems, and PBK modelling. This first part of the project was then concluded with uncertainty analysis. The model and information developed were intended to support a NAM-based risk assessment for neurotoxicity using QIVIVE for the Reference Point derived *in vitro* from Part 2. In conclusion, the results obtained go beyond what is usually done in active substance exposure assessment and puts in practice "internal exposure" to support the AOP and the interpretation of neurological effects and NAM-mechanistic data. Results demonstrated that more advanced exposure assessments for CNS and risk characterisation in a regulatory context putting more relevance to human physiology are feasible. The presented approach has the potential to become routine for risk assessors in the short/midterm if supported by more data, more research on/within CNS distribution and by updated regulatory requirements. This Pilot Project is a case study that could be considered as an AOP-informed IATA which integrated existing information with *in silico* and *in vitro* technologies.

4.2. Part 2, 'Hazard characterisation and identification of the Reference Point' (NP/EFSA/SCER/2020/02) – University of Konstanz

The second part of the project was assigned to the University of Konstanz for conducting *in vitro* experiments in 2D and 3D human neuron systems to characterise and quantify the neurotoxicity potential of Tebufenpyrad as mitochondrial complex I inhibitor. The second part of the project demonstrated the ability of the test battery, in line with the AOP, to identify chemicals of concern for the proposed Adverse Outcome (AO) and to provide a PoD. The experimental work was specifically focused on reproducing *in vitro* specific conditions for neurons mimic conditions in humans. When *in vitro*, neurons can generate ATP from glucose and not from the mitochondria. If in the *in vitro* system glucose is substituted with galactose, the capacity to produce ATP is limited and the role of mitochondria become relevant. As identified by the AOP, Tebufenpyrad is a complex I inhibitor: a set of studies moving from the last Key Event (KE) close to the Adverse Outcome (AO) to the first KE (complex I inhibition) were performed to measure mitocondrial disfunction. The NeuriTox assay (KE4/AO) was used as starting point and was improved, since it appeared to be less sensitive than more proximal assays. A test variant, MitoMet was performed and showed higher Tebufenpyrad potency compared to NeuriTox results. Under MitoMet conditions (mitochondria-dependent cells) ATP levels were measured as endpoint for mitochondrial dysfunction. Tebufenpyrad is a molecule able to bind to plastic, proteins and lipids; many technical issues were taken into account to overcome the issue of plastic binding together with the difficulty in defining the (intra)cellular concentration of the substance. A lot of different studies were performed, and results were evaluated in line with the existing AOP for Parkinsonian-type neurotoxicity (Terron at al., 2018; https://aopwiki.org/aops/3). The resulting NAM-based concentration-response curves were used to derive an in vitro Reference Point to be used by ANSES to estimate concentration in the target tissue and in the brain with the QIVIVE.

5. Introduction to the EFSA Project on Environmental Neurotoxicants

The large number of chemicals in the environment has overwhelmed the ability to determine their individual toxicity. Early life exposure to chemicals can have permanent consequences for neurodevelopment and for neurodegeneration in later life (Bellinger, 2013[4]). Toxic effects resulting

[3] EFSA (European Food Safety Authority), 2014. Guidance on the assessment of exposure of operators, workers, residents and bystanders in risk assessment for plant protection products. EFSA Journal 2014; 12(10):3874, 55 pp., doi:10.2903/j.efsa.2014.3874

[4] Bellinger, 2013. Prenatal Exposures to Environmental Chemicals and Children's Neurodevelopment: An Update. https://doi.org/10.5491/SHAW.2013.4.1.1

from chemical exposure can interact with other risk factors such as prenatal stress, and persistence of some chemicals in the brain and over time may result in cumulative toxicity. Because neurodevelopmental and neurodegenerative disorders, such as autism, attention-deficit hyperactivity disorder and Parkinson's disease cannot be fully explained by genetic risk factors alone, understanding the role of individual environmental chemical exposures is critical[5]. It is therefore crucial to understand how technologies can be used to identify and assess neurotoxic chemicals (DNT and NT) and/or identify risk factors. The strategy proposed by EFSA makes use of all available information and focuses on New Approach Methodologies (NAMs), that may allow previously unapproachable questions to be answered regarding toxicants and their effects on neurodevelopmental and neurodegenerative conditions. This issue has generated a lot of attention in recent years, including from the medical and research field, the media and the political arena. A particular concern is the recent recognition that Parkinson's disease is the fastest growing neurological condition in the world, and that environmental toxins such as pesticides may be contributing to this growth. Proposal to specifically screen the currently approved active substances for a possible association with Parkinson's disease (e.g. based on chemical structure and toxicological profile) were made. There is a need for conducting dedicated tests on specific toxic effects of chemicals in relation to Parkinson's disease which should include more than a mere screening for clinically detectable signs of parkinsonism in exposed animals, but also include dedicated post-mortem tests on the *substantia nigra,* including cell counts and measurements of cerebral dopamine (and metabolites) levels. Additionally, subsequent experiments should also consider the possibility that combined exposure to multiple pesticides lead to greater neurotoxicity and a further enhanced risk of developing Parkinson's disease. Since the initial EFSA commitment (2013) in Developmental Neurotoxicity (DNT) and Neurotoxicity (NT) several progresses have been made, culminating in several documents and databases. Nevertheless, only few pesticides active substances were assessed through this framework (AOP informed IATA), even if there is a recognition that the current dataset based on data requirement is not considered adequate for the assessment of DNT and specific forms of neurotoxicity (i.e. neurodegenerative diseases such as Parkinsonian Syndrome). AOP informed IATA, using NAMs and exposure models to fill the mechanistic data gaps, is the elected conceptual framework on which EFSA intends to move forward to conclude on hazard characterization and risk assessment in the area of environmental neurotoxicants. The aim is therefore to identify assays to be used in a screening programme (e.g. develop high throughput methods using molecular or cellular approaches), to prioritise chemicals to be tested (IATA case studies). Testing pesticides through these tools would represent a first step towards understanding the complexity of the mechanisms that regulate neurological functions, and, subsequently, towards developing regulatory action. EFSA would like to capitalise the experience so far and launch/sponsor a series of activities and provide more DNT and NT data based on NAMs and use them in the risk assessment of pesticides. The proposal is to implement NAMs methodologies in the regulatory risk assessment process providing empirical associations for causal relationships (quantitative AOP, computational modelling) strengthening the connection with EU projects (PARC and HBM4EU).

6. Presentation on the topic by key opinion leaders in the field of neurotoxicity

A series of presentations from key experts in the field of neurotoxicity were given.

6.1. Chemically induced neurotoxicity as cause of Parkinson's disease: evidence and limitations

Bas Bloem (Radboud University Medical Center, Nijmegen) provided a clinical overview of Parkinson's disease symptomatology and presented data relative to the globally fast increasing incidence and progression of the disease in the population. A focus on interaction between age, genetic factors and environment (especially considering exposure to pesticides) and the risk of developing Parkinson Disease was provided. Possible approaches and models for short- and long-term neurotoxicity testing, including different routes of exposure were presented. Bloem expressed a concern that currently used screening procedures offer insufficient insight into the neurotoxic effects of specific pesticides for the substantia nigra, and consequently, the possible risk of Parkinson's disease for humans, and called for

[5] National Academies of Sciences, Engineering, and Medicine. 2020. Environmental neuroscience: Advancing the understanding of how chemical exposures impact brain health and disease: Proceedings of a workshop. Washington, DC: The National Academies Press. https://doi.org/10.17226/25937

new experiments that should inform improved regulatory actions. Such experiments should include more than a mere screening for clinically detectable signs of parkinsonism in exposed animals, but also include dedicated post-mortem tests on the *substantia nigra,* including cell counts and measurements of cerebral dopamine (and metabolites) levels. When considering real exposure scenarios, it would be very relevant to screen for neurotoxicity for not just isolated pesticides, but to also specifically examine the risk of commonly occurring mixtures of pesticides, which may well exert cumulative and possibly even synergistic neurotoxic effects. In this context, a high need of developing reliable *in vitro* testing alternatives was identified that will eventually obviate the need for animal experiments.

6.2. Assessment of Neurotoxicity Associated with Repeated Chemical Exposure

Elizabeth Méndez (US EPA) from the US EPA's Office of Pesticide Programs showed a presentation on the licensing program regulating pesticide products in the United States using a risk-based approach. A list of neurotoxicity studies, endpoints and test guidelines (acute, sub chronic and developmental studies) for the derivation of Points of Departure, Chronic Reference Dose and Steady State Dietary Assessment was presented in the context of real pesticides assessments performed at US EPA. For the vast majority of regulated pesticides, the main outcome is that there could be more sensitive endpoints to be considered after repeated exposure than i.e., body weight, clinical chemistry, liver and kidney toxicity.

6.3. Health Canada Perspective on Testing for Neurotoxicity of Pesticides

Deborah Ramsingh (PMRA, HC) presented Health Canada's perspective on toxicological studies and neurotoxicity assessment risk-based approaches in use at the Pest Management Regulatory Agency (PMRA). A comprehensive list of toxicology studies required for pesticides' risk assessment was presented with a focus on neurotoxicity assessments, considering both the overall approach and specific testing conditions. Additional information might be provided by NAMs data (*in silico* and *in vitro* data part of the WoE) and mechanistic information, published scientific literature, incident reporting information and epidemiological studies.

6.4. Concepts of short- and long-term approaches for chronic neurotoxicity testing

Harm Heusinkveld (RIVM) provided an overview of the current regulatory requirements and assays for chronic- and short-term neurotoxicity assessments together with associated challenges and data gaps issues. RIVM published a report in 2021[6], considering pesticides and neurodegenerative disease and identifying from Epidemiology, Toxicology and Regulation information on which is the status of the current level of protection of the population. The report suggests both short term (i.e., OECD GD studies adaptation, additional endpoints in rodent studies) and long-term solutions to generate data. The second part of the presentation was oriented on future perspectives, highlighting the need for building ontologies based on human physiology, identifying key elements and build AOPs networks to define a testing strategy integrating NAMs data. A list of currently available models for Parkinson's disease (cell-based models, non-mammalian organisms and computational tools) was presented with a specific focus on the Zebrafish Parkinson Disease model.

6.5. AOP-based testing for neurotoxicity

Marcel Leist (University of Konstanz) provided an overview of AOP-based testing for neurotoxicity, which requires a broad battery of endpoints to represent multiple targets and effects, an interpretation strategy to translate effects from models to men and expert knowledge. Neurotoxicity concerns, with current requirements *in vivo* testing, could often be missed and methods do not present adequate level of sensitivity. Factors like age, genetics and environmental aspects are not captured in animal models, and may contribute to the mechanism of PD. In this context, NAMs represent a valid alternative. In the Tebufenpyrad case study, an AOP-informed IATA was used, a PoD was derived and integrated with PBPK model, used to predict target concentration and compared to the hazard

6 https://www.rivm.nl/publicaties/gewasbeschermingsmiddelen-en-neurodegeneratieve-ziekten-mogelijkheden-om

triggering concentration to derive a HBGV for the population. A quantitative AOP can be calibrated with reference substances and specific predictive dose-response curves of MIEs or KEs can be easily measured. A new strategy for neurotoxicity hazard evaluation can be designed establishing an *in vitro* test battery covering MIE/KE correlated to symptoms observed in humans through an AOP network.

6.6. NAM-based Testing for Chronic Neurotoxicity

Helena Hogberg (NIH) presented how NAM-based approaches could be relevant in the context of testing chronic neurotoxicity, specifically considering *in vitro* systems to assess long-term exposure, extrapolate from acute to chronic toxicity and introduce relevant elements as aging and chronic environment. An overview of NAMs models to be used for PD research considering application examples, advantages, limitations and future possibilities was presented (i.e., LUHMES cell models, iPSC-derived organoids, organ-on-chips and micro physiological systems). Priority mechanisms relevant in PD were identified together with examples of factors affecting chronicity.

7. Plenary discussion and brainstorming focusing to design the tender specifications of the upcoming EFSA NAMs Case Study on Environmental Neurotoxicants

Overall, there was broad consensus that the currently existing procedures, that are part of existing regulatory actions, are likely to give us an inadequate insight into the actual neurotoxic actions of specific pesticides for the substantia nigra, and consequently, offer an inadequate assessment of the risk of developing Parkinson's disease in case of human exposure. Additionally, there are clear ideas on how to perform experiments that will inform an improved screening procedure; this involves both improved in vivo experiments and the search for reliable *in vitro* alternatives.

Participants were invited to provide inputs on a series of proposed key question that will facilitate the design of the tender specifications of the upcoming EFSA NAMs Case Study on Environmental Neurotoxicants.

Which are the available assays (NAMs and/or non-NAMs) for assessing critical KEs in PD and PS?

The participants discussed on which test assays should be included in a test battery for the assessment of both Parkinson's Disease and Parkinson Syndrome, considering *in vitro* studies and TK analysis. Based on the general neuronal AOP network (that includes both Neurotoxicity and Developmental Neurotoxicity), the most relevant assays to PD would be the ones relative to KE that measure mitochondrial disfunctions, neuron inflammation and dopaminergic degeneration. More specialised assays could be then added (i.e., dopamine receptor signalling, disturbance of the signalling system). In general, a test battery including neurodegeneration and mitocondrial toxicity assays could be a potential screen one tier to identify chemicals of concern. Nevertheless, it should be considered that the test battery to be used closely depends on the KE considered and in the case of PD, focusing on mitocondrial toxicity might miss part of the pathways relevant for the AOP.

The availability of *in vivo* data could support PBPK modelling as part of the IATA, helping to reduce uncertainty in the model and to validate the order of magnitude calculated. *In vivo* experiments may help to predict concentrations in other compartments and could give relevance to the metrics calculated. *In vivo* data could give relevance to the metrics calculated and make comparison between KEs in AOPs. From a NAMs perspective kinetics point of view, it could be helpful to validate the model with a single intravenous dose and other routes to obtain the plasma profile of the analyte of interest and to measure tissues concentration in the animal to be combined with hazard assessments.

Which are the essential elements to be considered for the IATA e.g., PB-K, intracellular concentration, issues for reporting results in OHT 201?

Having information on the (intra)cellular concentrations of the chemical under assessment would be an extremely helpful information for the IATA. In particular, for those chemicals presenting high hydrophobicity and high concentrations losses (for more than 95%), to roughly estimate where the compound is distributed within the cell might represent a challenge. Performing a scoping project with representative sets of compounds to define alerts and flags in molecules with simple experiments could be an interesting proposal.

Which chemicals should be prioritised for testing (i.e. pesticides)?

Testing pesticide would be very relevant for validating the IATA. Nevertheless, to validate the whole methodology, it would be helpful to consider other well-known chemicals (i.e., anti-dopaminergic substances) as positive controls. Testing the method to gain confidence and knowledge should be the priority; the definition of testing prioritisation rules could follow (based on i.e., exposome, human exposure, chemical production volume…). The selection of chemicals that do not exhibit important systemic toxicity is relevant to address specifically neurotoxicity.