Meeting Report

Possible Applications of New Approach Methodologies (NAMs) in Ecotoxicology

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The IPAM annual meeting on "Possible applications of new approach methodologies (NAMs) in ecotoxicology" was held by the Italian Platform on Alternative Methods (IPAM)[1] on December 3, 2024 in collaboration with the Department of Veterinary Medicine and Animal Productions of the University of Naples Federico II. It was attended by about 100 researchers.

The meeting was opened by the President of IPAM, **Stefano Lorenzetti** (ISS, Italy), who introduced the importance of applying NAMs in ecotoxicology. The morning session, co-chaired by **Chiara Attanasio** (University of Naples Federico II, Italy) and **Francesca Caloni** (University of Milan, Italy), started with an opening lecture addressing the shift in ecotoxicology from animal testing toward NAMs, held by **ZhiChao Dang** (RIVM, The Netherlands) and entitled "*From OECD TGs to NAMs in ecotoxicology: Which perspectives?*" Significant efforts have been made to develop invertebrate test guidelines (TGs) as alternatives to vertebrate testing, including OECD TG 319 for bioaccumulation testing and an on-going project on detecting endocrine activity. Several NAM-based test guidelines for acute fish toxicity testing and endocrine activity detection have also been adopted (Dang et al., 2017). Next steps are to develop an integrated approach to testing and assessment (IATA) for acute fish toxicity, incorporate thyroid endpoints into existing fish TGs, and develop guidance on the use of a single solvent control in fish early-life stage toxicity tests. One of the opportunities to apply NAMs is the development of an integrated testing strategy to identify endocrine disruptors. Both the Amphibian Metamorphosis Assay (AMA, OECD TG 231) and the Larval Amphibian Growth and Development Assay (LAGDA, OECD TG 241) can detect thyroid-mediated modality and adverse outcome (Dang et al., 2022). The LAGDA is generally considered a follow-up to positive results from the AMA. However, based on an analysis of available data, this follow-up may not be necessary for agonists and most antagonists of the hypothalamic-pituitary-thyroid (HPT) axis. **Giovanni Libralato** (University of Naples Federico II, Italy) gave a talk about "*Risk assessment of natural and synthetic fibers in the aquatic environment*". Marine microplastics, including synthetic microfibers like nylon, polyester, polyethylene terephtalate, and polypropylene fibers, represent a potential environmental concern. Fibers originate mainly from textiles. There is still no clear understanding of their impact on aquatic organisms. Within the project BIOPLAST4SAFE[2], the ecological risks associated with both natural and synthetic fibers in aquatic ecosystems were assessed, focusing on toxicity data and their effects on different taxonomic groups (Albarano et al., 2024). To carry out species sensitivity distribution curves, a comprehensive analysis of scientific literature was conducted, collecting toxicity data related to various fibers. The potential ecological risks were evaluated by comparing measured concentrations in diverse aquatic environments with predicted no-effect concentration values. The calculation of risk quotients allowed to indicate areas where fiber abundance can pose potential threats to aquatic organisms, revealing that nylon fibers pose the highest toxicity risk, especially in the Atlantic and Pacific Ocean, Arabian Gulf, and Vietnam rivers. Mollusca emerged as particularly sensitive to different fiber types, likely due to their body structure and feeding habits facilitating the accumulation of microfibers. The research emphasizes the urgent need for further studies to acquire data for human health risk analysis and to develop comprehensive environmental management strategies to address the global issue of microfiber pollution. The talk "*Towards a green ecotoxicology: Innovative methods and invertebrate assays*", held by **Paolo Pastorino** (IZSPLV, Italy), addressed the growing ethical considerations in ecotoxicological research, highlighting the need for more sustainable approaches. The central focus of the lecture was the increasing use of invertebrate species as key model organisms in ecotoxicology. Invertebrates, due to their vast biological diversity, ecological importance, and lower ethical concerns compared to vertebrates, represent a powerful alternative that aligns with the 3R principles. Their adoption not only reduces reliance on vertebrate models but also enhances the ecological relevance of toxicological assessments, as invertebrates play crucial roles in both aquatic and terrestrial ecosystems. Alongside the use of invertebrates, the talk emphasized the importance of *in vitro* methods and *in silico* approaches based on computational models. These alternatives further contribute to minimizing animal testing while offering precise control over experimental conditions and reducing environmental impacts. As technological advancements continue and validation efforts expand, the integration of invertebrate-based assays with *in vitro* and *in silico* techniques is set to become a cornerstone of modern ecotoxicological research. **Anna Di Cosmo** (University of Naples Federico II, Italy) closed the first session with the talk "*Cognitive and developmental processes in marine invertebrates: Between ecotoxicology and ethics*", which focused on marine invertebrates frequently used as strategic model systems for studying neural networks and physiology due to their short life cycle, easy care and handling, and significantly more flexible alternative nervous system organization compared to that of vertebrates. Due to their relatively simple nervous systems and large neurons, both adult and planktonic larval stages of ecologically important benthic and pelagic species from the phyla Cnidaria, Mollusca, Crustacea, and Echinodermata have been extensively used in environmental risk assessments and in testing the toxic effects of

[1] http://www.ipamitalia.org

[2] https://www.isprambiente.gov.it/en/projects/environment-and-health/bioplast4safe

pollutants, including neurotoxicity. Directive (EU) 2010/63 includes mollusk cephalopods used as animal models among sentient animals, requiring appropriate protocols. Cephalopods, smart active predators with advanced cognitive abilities and well-known for their unique camouflage system controlled by the nervous system, represent an emerging model in behavioral ecotoxicology, and the quantitative measurement of color change could be developed as a powerful endpoint for toxicological risk assessment. Currently, *Octopus vulgaris*, a key species in neurobiology research, is used as model organism at the Model Organisms Core – Octopus Core[3]. Studies ranging from adult neurogenesis to the development of neuronal cultures derived from lobes of the nervous system involved in memory and learning processes are performed (Maselli et al., 2018). Overall, cephalopods can contribute to a future in which ecotoxicological assessments are not only more predictive but also better aligned with ethical scientific practices.

The afternoon session, co-chaired by **Livia D'Angelo** (University of Naples Federico II, Italy) and **Stefano Lorenzetti** (ISS, Italy), was opened with a talk by **Francesca Caloni** (University of Milan, Italy) on "*Ecotoxicology and education: New approaches*". She underlined the importance of education in relation to NAMs and ecotoxicology. At the University of Milan, different courses on alternative methods commenced in 2010 (Caloni et al., 2011). They have been included in different curricula, and modules dedicated to replacement are available in master's degrees. Moreover, five editions of the International Summer School-Lake Como School of Advanced Studies[4] focused on NAMs, alternative methods and replacement have been held, with participants from different areas of science. In the curriculum Environmental Change and Global Sustainability, part of the course "Methods in Ecotoxicology" is dedicated to NAMs, and a module entitled "3Rs, Alternative Methods and New Approach Methodologies (NAMs) and Replacement" was included in the Environmental Sciences PhD course this academic year. Specific teaching paths on NAMs are emerging for the next generation of scientists with a new awareness and European vision (Caloni, 2023). **Michele Amorena** (University of Teramo, Italy) presented "Danio rerio *(zebrafish) as a model for the study of environmental contaminants*", highlighting zebrafish as an effective experimental model for studying environmental contaminants due to their high genetic homology with humans (around 70%), embryonic transparency, and rapid development. Zebrafish embryos can be exposed to contaminants through water or feed and morphological and behavioral changes can be observed in combination with histological staining with Alcian Blue and Oil Red O and the evaluation of morphometric parameters such as body length, swim bladder development, and head-trunk angle. Zebrafish embryos proved to be a suitable model for analysis of the toxicity of heavy metals, such as mercury, cadmium and lead, and endocrine disruptors, which may adversely affect embryonic development and neuro-behavioral functions (Vremere et al., 2022). Future prospects include the integration of omics technologies, such as transcriptomics and proteomics, to investigate the molecular mechanisms underlying environmental toxicity. The development of transgenic zebrafish will allow the effects of specific contaminants to be studied more precisely, while the analysis of interactions between multiple substances will provide a better understanding of the risks associated with combined exposure. Finally, the zebrafish embryos are also proposed as a model for investigating environmental stress resulting from climate change. An innovative *in vitro* approach was presented by **Paolo Roberto Saraceni** (ENEA Casaccia, Rome, Italy) on "*Teleost fish cell cultures as alternative models for ecotoxicological studies*". His research, carried out within the framework of the Pisces, Nanopisces, and Seed Talent projects, emphasized the value of teleost fish-derived cellular models for eco-toxicological applications (Miccoli et al., 2022; Saraceni et al., 2024). *Ex vivo* and *in vitro* experiments examining the effects of 20 nm and 80 nm polystyrene nanoplastics (PS-NPs) demonstrated the effectiveness of teleost cell models in evaluating nanoplastics toxicity. Specifically, the use of rainbow trout *Oncorhynchus mykiss* RTG-2 and gilthead seabream *Sparus aurata* SAF-1 cell lines revealed that 20 nm PS-NPs induced greater cytotoxicity than 80 nm particles. Structural damage, including cell shrinkage, plasma membrane blebbing, and DNA fragmentation, was particularly evident in SAF-1 cells exposed to 20 nm PS-NPs. Furthermore, RNA sequencing (RNA-seq) analyses identified significant transcriptional alterations in critical biological pathways, such as "steroid biosynthesis", "TGF-beta signaling pathway", "ECM-receptor interaction", "focal adhesion", "regulation of actin cytoskeleton", and "protein processing in endoplasmic reticulum". Overall, teleost fish cell cultures were shown to be a valuable resource for ecotoxicological studies, supporting the 3Rs principle by providing an effective alternative to conventional animal models in environmental toxicology. Lastly, **Orazio Nicolotti** (University of Bari, Italy) closed the second session with a talk "*An* in silico *approach to assess the bioconcentration factor*" introducing computational methods and *in silico* modelling to bypass animal testing. Indeed, REACH (Regulation (EC) 1907/2006) and the Biocide Product Regulation (Regulation (EU) 528/2012) encourage non-animal testing for chemical risk assessment, increasing interest in quantitative structure-activity relationship (QSAR) models as regulatory alternatives (Nicolotti and Carotti, 2006). Evaluating the bioconcentration factor (BCF) is costly, time-consuming, and requires significant animal testing. QSAR models based on the ANTARES dataset, a large, verified collection of experimental BCF data (Gissi et al., 2014), were compared, and the best performing was a highly predictive nine-descriptor model. Trained on 608 chemicals, it was validated on 152 compounds and tested on a blind set of 76. The model's reliability was ensured through multiple validation strategies and a multi-step applicability domain approach. To enhance sensitivity, safety margins were applied, while biokinetic descriptors improved interpretability. The model demonstrated strong predictive power for external compounds, making it a viable alternative to *in vivo* testing. By offering a robust, ethical, and cost-effective solution, this model supports registrants in meeting regulatory requirements while reducing reliance on animal testing.

During the meeting, The IPAM Awards 2024 for the best Master's and PhD thesis were presented. This year, the award was dedicated to the memory of Prof. Antonia Lucisano, one of the IPAM founders. As remembered by **Maria Carmela Ferrante** and **Lorella**

[3] http://www.dipartimentodibiologia.unina.it/model-organisms/

[4] https://lakecomoschool.org/

Severino (University of Naples Federico II, Italy), Professor Antonia Lucisano was a full professor of Veterinary Toxicology at the Faculty of Veterinary Medicine, University of Naples Federico II. A strong innovative spirit and a forward-looking vision, with the courage to explore new paths, consistently drove her career. She was the first coordinator of the National PhD Program in Veterinary Pharmacology and Toxicology and then director of the Department of Pathology and Animal Health. Under her leadership, the first departmental document on workplace safety was drafted. This pioneering work laid the foundation for increasingly stringent guidelines. For many years, she served as the coordinator of the Erasmus program for international student mobility and as a member of the Advisory Committee for the assessment of veterinary drug technical requirements at the Ministry of Health. A scientific member of the Interuniversity Center for Bioethics, she was also a member of various scientific societies and associations, including IPAM. She was a cornerstone of the discipline of Veterinary Pharmacology and Toxicology. Most notably, she recognized the strategic importance of studies related to the toxicology of residues in food safety and environmental toxicology, with research on the impact of chemical pollutants on health from a One Health perspective. Her scientific work also significantly contributed to studies on the kinetics of drugs and pesticides in various animal species, as well as *in vitro* studies to evaluate mechanisms of action and toxic effects caused by numerous xenobiotics. She will be remembered for her welcoming spirit and the special attention she gave to young people, her generosity of heart, her inner strength, and her determination in achieving many professional goals.

The IPAM 2024 Master's Thesis award was given to **Marco Cellani** (San Raffaele Scientific Institute, Milan, Italy), whose degree in Biotechnology and Medical Biology was obtained with an experimental study about "*Development of new preclinical models for drug screening in chronic lymphocytic leukemia using 3D bioprinting*", and the IPAM 2024 PhD thesis award was given to **Chiara Pacelli** (University Sapienza of Rome, Italy), whose PhD in Biochemistry was obtained by an investigation entitled "In silico *design and evaluation of exon skipping-inducing antisense oligonucleotides for a potential therapeutic intervention in cancer*". A Best Poster award was given to **Chiara Ritarossi** (ISS, Italy), as presenter of the poster entitled "C. elegans *as a 3Rs-compliant model for the evaluation of micro- and nano-plastics biological effects*", co-authored by Ritarossi, C., Andreoli, C., Pannone, L., and Martinelli, S.

References

Albarano, L., Maggio, C., La Marca, A. et al. (2024). Risk assessment of natural and synthetic fibers in aquatic environment: A critical review. *Sci Total Environ 934*, 173398. doi:10.1016/j.scitotenv.2024.173398

Caloni, F., Alberizzi, V., Costa, A. et al. (2011). Master students' feedback on 3Rs education approach. 8th World Congress on Alternatives and Animal Use in the Life Science, 21-25 August 2011, Montreal, Canada. *ALTEX 28*, 210. doi:10.58847/ap.1201

Caloni, F. (2023). Advancing Three Rs uptake in university education through a European network. *ALTEX Proc 11*, 127. doi:10.58847/ap.2302

Dang, Z, van der Ven, L. T. M. and Kienhuis, A. S. (2017). Fish embryo toxicity test, threshold approach, and moribund as approaches to implement 3R principles to the acute fish toxicity test. *Chemosphere 186*, 677-685. doi:10.1016/j.chemosphere.2017.08.047

Dang, Z. (2022). Amphibian toxicity testing for identification of thyroid disrupting chemicals. *Environ Pollut 311*, 120006. doi:10.1016/j.envpol.2022.120006

Gissi, A., Gadaleta, D., Floris, M. et al. (2014). An alternative QSAR-based approach for predicting the bioconcentration factor for regulatory purposes. *ALTEX 31*, 23-36. doi:10.14573/altex.1305221

Maselli, V., Xu, F., Syed, N. I. et al. (2018). A novel approach to primary cell culture for octopus vulgaris neurons. *Front Physiol 9*, 220. doi:10.3389/fphys.2018.00220. Erratum in: *Front Physiol 9*, 1900 (2019). doi:10.3389/fphys.2018.01900

Miccoli, A., Mancini, E., Saraceni, P. R. et al. (2022). First evidence of in vitro cytotoxic effects of marine microlitter on Merluccius merluccius and Mullus barbatus, two Mediterranean commercial fish species. *Sci Total Environ 813*, 152618. doi:10.1016/j.scitotenv.2021.152618

Nicolotti, O. and Carotti, A. (2006). QSAR and QSPR studies of a highly structured physicochemical domain. *J Chem Inf Model 46*, 264-276. doi:10.1021/ci050293l

Saraceni, P. R., Miccoli, A., Bada, A. et al. (2024). Polystyrene nanoplastics as an ecotoxicological hazard: cellular and transcriptomic evidences on marine and freshwater in vitro teleost models. *Sci Total Environ 934*, 173159. doi:10.1016/j.scitotenv.2024.173159

Vremere, A., Merola, C., Fanti, F. et al. (2022). Oxysterols profiles in zebrafish (Danio rerio) embryos exposed to bisphenol A. *Food Chem Toxicol 165*, 113166. doi:10.1016/j.fct.2022.113166

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