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Meeting Report

Innovative *In Vitro* Strategies for Food and Environmental Safety

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Our diet and the environment are relevant sources of xenobiotic exposure for humans (Alloisio et al., 2019). In recent years, the scientific community has become increasingly interested in non-occupational exposure to contaminants and their mixtures and in their adverse effects. Attention has focused primarily on chemical agents but, more recently, also on biological and physical contaminants. Several bioassays have been proposed as tools to investigate the hazards of these unintentional exposures. *In vitro* models for food and environmental safety were discussed at the meeting "*In vitro* toxicology: Innovative strategies for food and environmental safety" organized by CELLTOX Italian Association of *in vitro* Toxicology in May 2020, which was presented as a webinar in compliance with the containment measures of SARS-CoV-2 infection. The meeting was chaired by Diego Baderna and Susanna Alloisio and attracted more than 100 participants.

Francesca Caloni and **Alessia Bertero**, Università degli Studi di Milano (ESP), presented an integrated approach for testing the emerging mycotoxins beauvericin (BEA) and enniatin B1 (ENNB1), natural food and feed contaminants of emerging concern (Caloni et al., 2020; Albonico et al., 2017; Prosperini et al., 2017), using an *in vitro* strategy based on the combination of human and species-specific *in vitro* models. Models for oral and topical exposure to emerging mycotoxins, alone or combined also with traditional mycotoxins, were applied, such as human intestinal barrier with Caco-2 cells differentiated on inserts in serum-free medium (Ferruzza et al., 2012), species-specific intestinal barrier with IPEC-J2 cells cultured on inserts (Zakrzewski et al., 2013), and human reconstructed skin and cornea (MatTek Corporation). The toxicological effects of the mycotoxins on trans-epithelial electrical resistance (TEER) and cytokine release in the intestinal barriers and on cell viability (MTT) in the human reconstructed skin and cornea models were evaluated. A novel integrated *in vitro* toxicological evaluation is proposed that combines human and species-specific models (i.e., bovine granulosa cell, swine intestinal models, human barriers, etc.).

Isabella De Angelis, **Valentina Prota** and **Olimpia Vincentini**, Istituto Superiore di Sanità (ISS), described a pilot study to develop an OECD Test Guideline on an *in vitro* approach for determining the gastrointestinal fate of ingested nanomaterials. Several parameters can influence the physical-chemical properties and bioavailability of nanomaterials (NMs) during their passage through the gastrointestinal tract. Caco-2 monoculture on inserts is an ideal system for rapid assessment of intestinal permeability,

however, it lacks mucus, microfold cells (M-cells), and immune cells from the gut-associated lymphoid tissue (GALT). Mucus has important defensive properties and strongly affects the mobility of nanoparticles, while M cells are involved in the absorption of nano-sized particulates (Beloqui et al., 2017). An advanced tri-culture model based on Caco-2 cells/HT29-MTX mucus secreting cells/lymphoblastoid Raji-B cells has been developed for qualitative/quantitative assessment of absorption and translocation of NMs (García-Rodríguez et al., 2018; Kämpfer et al., 2020). Conversion of Caco-2 cells into M-like cells, achieved by co-culturing Caco-2 with Raji B cells, has been assessed by electrophysiological, histological, biochemical and functional measurements. Different endpoints of M cell phenotype induction and mucus production in the tri-culture model, such as TEER decrease, ZO-1 protein expression, barrier permeability to FITC-dextran and fluorescent silica nanoparticles have been discussed. Current results highlight the need for further standardization of experimental protocols to increase robustness and data comparability.

Susanna Alloisio, **Cristina Scarone** and **Francesco Misurale** presented an integrated *in vitro*/*in vivo* approach applied to the evaluation of the effects of harmful algal blooms (HABs) on human and environmental health. HABs occur when algae, unicellular photosynthetic organisms that live in the sea and freshwater, grow out of control, producing toxins which are harmful to people, fish, shellfish, marine mammals and birds (Perovic et al., 2000; Ben-Gharbia et al., 2016). In the proposed approach, four dinoflagellates were selected: two toxic species (*Ostreopsis* cf. *ovata* and *Prorocentrum lima*); one species with uncertain toxicity (*Coolia monotis*); and a non-toxic species (*Scripsiella* cf. *trochoidea*). The main objectives were i) the optimization of the protocol for a fast and simple algal preparation that allows direct administration of the toxin mixture to mammalian cell culture, ii) the *in vitro* evaluation of skin and eye irritation potential of the algae using reconstructed human epidermis and corneal tissue, iii) the investigation of the neurotoxic potential of the algae by means of MTT-assay and MEA-based electrophysiological approach on cultured primary murine neuronal networks, and iv) the evaluation of algal ecotoxicity by means of a lethal toxicity test performed on *Artemia franciscana* nauplii.

Nathalie Steimberg, University of Brescia, focused on the development of a battery of *in vitro* tests to assess the biological impact of wastewater. The characteristics of wastewater are studied primarily from a chemical-physical point of view, but biological tests and ecotoxicological tools are needed to better understand the environmental impact of these complex mixtures (Pedrazzani et al., 2020). While chemical analysis of the aqueous mixtures allows identification and quantification of known substances and their transformation products, biological tests assess the impact of the mixtures. The longstanding scientific collaboration within the Mistral interuniversity research center have allowed the development of a complex and multifaceted approach for the evaluation of the environmental footprint of wastewater and the efficacy of wastewater treatment plants (Bertanza et al., 2011, 2013; Papa et al., 2016; Pedrazzani et al., 2020). The biological endpoints investigated include: i) baseline toxicity: algal growth inhibition, Microtox, *Daphnia magna* motility assay, MTT and neutral red assays in mammalian cells, ii) specific target: genotoxicity/mutagenicity, tumor promotion, cell transformation, endocrine disrupting compounds (EDCs) activity. The overall approach combines physicochemical parameters, biological and ecotoxicological tests to assess the impact on human and wildlife health and to determine strategies for purification systems to break down pollutants.

Diego Baderna, Istituto di Ricerche Farmacologiche Mario Negri and Italian Institute for Planetary Health, focused on the use of *in vitro* models for the investigation of landfill leachate toxicity (Baderna et al., 2011, 2019). A recent review, covering 23 papers published in the period 2003-2018, compiled cell models that reflect potential targets of direct exposure to leachate (e.g., liver, skin and blood) and models for the study of specific mechanisms of action (e.g., breast cancer cells for the study of endocrine interference) covering the endpoints basal toxicity (viability, antiproliferative effects, cytotoxicity and apoptosis), genotoxicity, and endocrine interference. The studies showed that leachate is toxic, genotoxic, and can act as an estrogen-like compound and that the leachate from uncontrolled landfills tends to be more toxic than that from controlled landfills. It is clear that leachate can be a serious danger to human health and that a good management strategy must be adopted to avoid human exposure. The review highlighted how *in vitro* models were used not only for the study of leachate toxicity but also to evaluate the efficiency of purification and detoxification treatments. Studies showed that these treatments can reduce leachate toxicity, but not all of them are efficient in removing genotoxicity, probably due to the formation of highly reactive by-products.

The meeting highlighted the importance of multidisciplinary approaches to deal with common and emerging pollutants and their effects on humans and wildlife. In fact, the value of highly sensitive *in vitro* assays (both human-related and ecological) for assessing hazards and identifying modes of action is being increasingly recognized, not only for the study of toxicity, but also for environmental monitoring and the evaluation of detoxifying treatments. This has made biological assays indispensable tools alongside chemical analyses in this area.

References

Albonico, M., Schutz, L. F., Caloni, F. et al. (2017). In vitro effects of the Fusarium mycotoxins fumonisin B_1 and beauvericin on bovine granulosa cell proliferation and steroid production. *Toxicon 128*, 38-45. doi:10.1016/j.toxicon.2017.01.019

Alloisio, S., Gambardella, C., Giussani, V. et al. (2019). In vitro approaches to environmental pollutants: New models, endpoints, and strategies. *ALTEX 36*, 329-330. doi:10.14573/altex.1901181

Baderna, D., Maggioni, S., Boriani, E. et al. (2011). A combined approach to investigate the toxicity of an industrial landfill's leachate: Chemical analyses, risk assessment and in vitro assays. *Environ Res 111*, 603-613. doi:10.1016/j.envres.2011.01.015

Baderna, D., Caloni, F. and Benfenati, E. (2019). Investigating landfill leachate toxicity in vitro: A review of cell models and endpoints. *Environ Int 122*, 21-30. doi:10.1016/j.envint.2018.11.024

Ben-Gharbia, H., Yahia, O. K.-D., Amzil, Z. et al. (2016). Toxicity and growth assessments of three thermophilic benthic dinoflagellates (Ostreopsis cf. ovata, Prorocentrum lima and Coolia monotis) developing in the southern mediterranean basin. *Toxins 8*, 297. doi:10.3390/toxins8100297

Beloqui, A., Brayden, D. J., Artursson, P. et al. (2017). A human intestinal M-cell-like model for investigating particle, antigen and microorganism translocation. *Nat Protoc 12*, 1387-1399. doi:10.1038/nprot.2017.041

Bertanza, G., Pedrazzani, R., Dal Grande, M. et al. (2011). Effect of biological and chemical oxidation on the removal of estrogenic compounds (NP and BPA) from wastewater: An integrated assessment procedure. *Water Res 45*, 2473-2484. doi:10.1016/j.watres.2011.01.026

Bertanza, G., Papa, M., Pedrazzani, R. et al. (2013). EDCs, estrogenicity and genotoxicity reduction in a mixed (domestic + textile) secondary effluent by means of ozonation: A full-scale experience. *Sci Total Environ 458-460*, 160-168. doi:10.1016/j.scitotenv.2013.03.108

Caloni, F., Fossati, P., Anadón, A. et al. (2020). Beauvericin: The beauty and the beast. *Environ Toxicol Pharmacol 75*, 103349. doi:10.1016/j.etap.2020.103349

Ferruzza, S., Rossi, C., Scarino, M. L. et al. (2012). A protocol for differentiation of human intestinal Caco-2 cells in asymmetric serum-containing medium. *Toxicol In Vitro 26*, 1252-1255. doi:10.1016/j.tiv.2012.01.008

García-Rodríguez, A., Vila, L., Cortés, C. et al. (2018). Exploring the usefulness of the complex in vitro intestinal epithelial model Caco-2/HT29/Raji-B in nanotoxicology. *Food Chem Toxicol 113*, 162-170. doi:10.1016/j.fct.2018.01.042

Kämpfer, A., Busch, M. and Schins, R. (2020). Advanced in vitro testing strategies and models of the intestine for nanosafety research. *Chem Res Toxicol 33*, 1163-1178. doi:10.1021/acs.chemrestox.0c00079

Papa, M., Ceretti, E., Viola, G. C. et al. (2016). The assessment of WWTP performance: Towards a jigsaw puzzle evaluation? *Chemosphere 145*, 291-300. doi:10.1016/j.chemosphere.2015.11.054

Pedrazzani, R., Baroni, P., Feretti, D. et al. (2020). Methodological protocol for assessing the environmental footprint by means of ecotoxicological tools: Wastewater treatment plants as an example case. In K. Roy (ed.), *Ecotoxicological QSARs* (305-327). Methods in Pharmacology and Toxicology. New York, NY, USA: Humana. doi:10.1007/978-1-0716-0150-1_14

Perovic, S., Tretter, L., Brümmer, F. et al. (2000). Dinoflagellates from marine algal blooms produce neurotoxic compounds: Effects on free calcium levels in neuronal cells and synaptosomes. *Environ Toxicol Pharmacol 8*, 83-94. doi:10.1016/S1382-6689(99)00035-6

Prosperini, A., Berrada, H., Ruiz, M. J. et al. (2017). A review of the mycotoxin enniatin B. *Front Public Health 5*, 304. doi:10.3389/fpubh.2017.00304

Zakrzewski, S. S., Richter, J. F., Krug, S. M. et al. (2013). Improved cell line IPEC-J2, characterized as a model for porcine jejunal epithelium. *PLoS One 8*, e79643. doi:10.1371/journal.pone.0079643

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Meeting Report

R2N and the Use of Alternative Methods in COVID-19 Research

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The recent SARS-CoV-2 pandemic represents a global health emergency. In the urgent race to understand the molecular mechanisms of the disease and develop lifesaving treatments and vaccine candidates, many researchers are embracing *in vitro* and *ex vivo* approaches. Alternatives to animal experimentation are both time- and cost-efficient, and if human-derived cells are employed, experiments can be more human-predictive, thereby maximizing the quality and relevance of the investigations. In the battle against time, driven by the growing incidence of disease, the German research unit R2N (Replace and Reduce in Lower Saxony, https://r2n.eu/home-2) is ideally positioned to deliver a repertoire of physiologically-relevant alterna-