Meeting Report

Air, Water and Soil: Which Alternatives? Alternative Models in Environmental Toxicology

doi:10.14573/altex.1802121

Environmental pollution from chemical, physical and biological agents is now a problem for the whole planet as a consequence of the growing world population and industrial activities. A pollutant is defined as any substance or energy derived from anthropogenic activities that alters physico-chemical characteristics of air, water and/or soil to the extent of producing harmful effects in living organisms and negatively affecting human and animal health. In compliance with European and international regulations, monitoring and evaluating environmental toxicological risk will increasingly have to adopt alternative strategies to the use of experimental animal models.

Alternative models in environmental toxicology were presented and discussed at the meeting "*Air, water and soil: Which alternatives?*" held in Brescia in May 2017, hosted by the University of Brescia and organized by MISTRAL (Integrated Models for Prevention and Protection in Environmental and Occupational Health) Research Centre of the University of Brescia, in collaboration with CELLTOX, Italian Association of *in vitro* Toxicology. The aim of the meeting was to give an overview of novel experimental models applied to different fields of environmental toxicology that are capable of reliably predicting the potential noxious effects of substances released in environmental matrices such as urban air, sea and freshwater, soil, sediments, municipal wastewater, etc. The meeting sessions were chaired by Francesca Caloni, President of CELLTOX, Giovanna Mazzoleni, Yula Sambuy and Chiara Urani.

Donatella Feretti, DSMC University of Brescia, presented a genotoxicity study of the effects of urban air on children utilizing *in vivo* and *in vitro* approaches. The MAPEC_LIFE (Monitoring Air Pollution Effects on Children for supporting public health policy) is a multicentre project founded by the EU Life+ Programme (LIFE12 ENV/IT/000614), which aimed to evaluate the association between air quality and early biological effects in children, considering also socio-demographic and lifestyle features (Feretti et al., 2014). The study was carried out on 1,149 6-8-year-old children living in five Italian towns (Brescia, Torino, Pisa, Perugia and Lecce) in two different seasons (winter and spring). Primary DNA damage (Comet test) and micronucleus frequency (MN) were investigated in buccal cells of children. Child exposure to air pollution was assessed analysing PM0.5 for chemical composition, *in vitro* toxicity (in the human pulmonary A549 cell line) and genotoxicity properties (in bacteria using the Ames test and in A549 cells by means of the Comet test and MN test), and collecting data on air quality during the study period. Details of socio-demographic and lifestyle features were collected using a questionnaire administered to the children's parents. The results, available at http://www.mapec-life.eu, confirmed that MN and Comet tests applied to childrens' mucosa buccal cells can detect early effects of air pollution exposure, which may be an indicator of possible future harmful effects on health (De Donno et al., 2016).

Giorgio Bertanza, DICATAM University of Brescia, presented an integrated chemical and biological assessment of waste water toxicity. Several processes have been proposed for removing trace pollutants from wastewaters, some of them being very effective. Nevertheless, unpredictable/unknown by-products with similar of even greater toxicological impact may be generated during treatment. Moreover, any additional treatment causes an increase of chemicals and/or energy consumption, which means new emissions to the environment. A multidisciplinary research group at the University of Brescia is working on methods for the biological characterisation of wastewater so as to measure the combined effect of hundreds of pollutants (by-products included) (Pedrazzani et al., 2018). Models for comparing the environmental impact on the hydrosphere and the atmosphere are also under development.

Susanna Alloisio, ETT SpA Genova, presented an innovative approach to detect neurotoxicity of microalgal biotoxins in seawater. Harmful dinoflagellate blooms of the genus Ostreopsis represent a significant and expanding threat to human health with a relevant socio-economic impact. However, the standard procedure to monitor toxic algae in bathing waters does not include toxicological or chemical analysis. Most existing toxicological data have so far been derived from an *in vivo* mouse assay and are only related to acute effects of the few known biotoxins, although European authorities encourage and facilitate the development of alternative or complementary methods (EU Regulation 15/2011). The aim of the presented study was to develop an *in vitro* approach to rapidly evaluate the toxicity of *Ostreopsis* cf. *ovata* (Alloisio et al, 2016) based on electrophysiological recordings measured on a rat neuronal network grown on multi-electrode arrays (MEA). Three algal treatments were tested: filtered and re-suspended cultured algal cells; filtered, re-suspended and sonicated cultured algal cells; sonicated al-

gal mixture (> 90% *Ostreopsis* cf. *ovata*) contained in a marine sample; the control was conditioned growth medium devoid of algal cells. The multi-parametric data analysis demonstrated that the system could be a useful method to evaluate the toxicity of marine algal samples within a few hours of collection.

Roberta Pedrazzani, DIMI University of Brescia, described the use of bioassays within the PEF/OEF (Product and Organisation Environmental Footprint) protocols that have been proposed to overcome the need to acquire a huge amount of chemical data. Object of the study were the liquid emissions of a municipal wastewater treatment plant. Chemical parameters (metals and organic compounds, such as polynuclear aromatic hydrocarbons and herbicides) were determined in order to calculate the plant's impact on human toxicity (non-cancer), human toxicity (cancer) and freshwater toxicity, by applying the standard protocol of PEF/OEF. In parallel, bioassays were carried out on different biological systems (bacteria and human cells, *Vibrio fischeri* and *Raphidocelis subcapitata*) in order to assess the effects of the whole liquid streams. The results of bioassays were then converted into biological equivalents of target reference substances and used in the PEF/OEF protocol. The adoption of biological equivalents showed a higher sensitivity with respect to the input of chemical parameters, also suggesting a great valence diversity among different bioassays (Pedrazzani et al., 2018).

The 7th Amendment to the EU Cosmetics Directive and EU REACH legislation have heightened the need for predictive *in vitro* ocular test methods. **Silvia Letasiova**, MatTek Bratislava, presented an eye irritation test (EIT), which utilizes a three-dimensional reconstructed human cornea-like epithelial (RhCE) tissue model, EpiOcular, based on normal human cells. The test has separate protocols for liquid chemicals and for solids and can discriminate between ocular irritant / corrosive materials (GHS Categories 1 and 2) and those that require no labelling (GHS No Category). Over 100 substances were tested during the development of the assay. Although the original eye irritation protocol was successfully pre-validated in an international, multicentre study sponsored by COLIPA (the predecessor to Cosmetics Europe), data from two larger studies (the ECVAM-COLIPA validation study (Pfannenbecker et al., 2013) and an independent in-house validation at BASF SE (Kolle et al., 2011), resulted in sensitivity for solid materials below the acceptance criteria set by the Validation Management Group (VMG) and indicated the need for improvement of the assay sensitivity for solids. By changing the exposure for solid materials from 2 to 6 hours, the EIT achieved 100% sensitivity, 68.4% specificity, and 84.6% overall accuracy, thereby meeting all the acceptance criteria set by the VMG. An independent validation study was performed with this protocol and extended shipping times in Japan, resulting in the concordant predictions of almost all chemicals that had been previously tested (Kaluzhny et al., 2011, 2015). This modified procedure together with the original liquid protocol has been approved as the new OECD TG 492.

Tommaso Sbrana, IVTech Lucca, presented a multi-organ *in vitro* model developed by IVTech (http://www.ivtech.it), to evaluate the toxicity of nanoparticles.

A roundtable was held at the end of the meeting. First, the special importance in environmental toxicology of considering the global effects of mixtures of contaminants, and the need for adequate experimental approaches to evaluate them was discussed. Mixtures are more frequent than single substances in environmental contaminations, and the study of their effects requires special attention. Integrated Testing Strategies (ITS) need to be more widely introduced in environmental toxicology in analogy with other toxicological fields, with the important contribution of *in silico* approaches. However, *in silico* approaches require the use of data acquired through standardized protocols, which are often lacking in the environmental field. Examples could come from the experience obtained in testing for human carcinogenesis. One interesting approach, already applied in the field of cosmetics toxicity testing, is the scoring system that distributes toxicants not in absolute terms but in ranges of toxicity, relative to the others. Especially important is also the integration of different disciplines to challenge new emerging problems in environmental toxicology. In some disciplines of toxicology, alternative experimental models are continuously being developed, improved and validated to replace experiments on animals. The experience accumulated in these fields should be shared with environmental toxicologists to promote the use of alternative models within this area. Meetings such as this, especially addressed to students and young researchers, can also contribute to these goals.

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Meeting Report

Giving Meaning to Alternative Methods to Animal Testing

doi:10.14573/altex.1802122

The 3rd edition of the advanced theoretical-training course "*Giving meaning to alternative methods to animal testing*" was held in Genoa on July 6-7, 2017. This course, chaired by Prof. Anna Maria Bassi (LARF-DIMES, University of Genoa, Italy) with the valuable support of the LARF Research Team, provided an update on new *in vitro* approaches. The welcome addresses were given by Prof. Marco Frascio, Prof. Adriana Voci, as Coordinators of Medicine and Surgery, and Biological Sciences degrees, respectively, of the University of Genoa, Italy, and by Dr Giuliano Grignaschi, head of the Animal Care Unit – Mario Negri Institute, Milano, Italy. In their welcome speech, all stressed the relevance of new *in vitro* technologies to improve the application of *in vitro* methods in toxicological research. The theoretical modules included talks by specialists from companies engaged in the field of advanced *in vitro* technologies, who offered participants the possibility to try out their technologies in the training modules.

The ethical issues associated with animal-based research were the topic of contributions by Prof. **Rosagemma Ciliberti**, **Ilaria Baldelli** (DISSAL, University of Genoa, Italy) and **Susanna Penco** (LARF-DIMES, University of Genoa, Italy). Their joint input focused on clinical evidence of the failure of animal-based models to predict effectiveness and safety of chemical agents in drug research. Dr **Eleanore Irvine** (Biogelx Inc., Scotland, UK) introduced new 2D/3D culture methods using self-assembling hydrogels made from di- or tri-peptide components that can be used as coating for 2D cultures and for embedding cells in 3D models. The stiffness of the gel can be regulated depending on a cell culture's requirements by changing the ratio of powder to water and the hydrogels can be functionalized with proteins and growth factors. Dr **Silvia Letasiova** (MatTek In Vitro Life Science Laboratories, Bratislava, Slovakia) introduced an *in vitro* alternative to the animal-based Draize Eye Irritation test. EpiOcular™ is a 3D reconstruction of human cornea developed by culturing normal, human-derived epidermal keratinocytes to form a stratified, squamous epithelium, which can be used for ocular irritation assessment of cosmetic, personal care and household products. Dr **Vladimir Mazurov** (acCELLerate GmbH, Hamburg, Germany) presented the acCELLerate *in*