EU wide campaign exercise on bioassays and chemical mixture effects

Teresa Lettieri, Raquel N. Carvalho, Augustine Arukwe, Selim Ait-Aissa, Anne Bado-Nilles, Stefania Balzamo, Sabrina Barbizzi, Monica Buchetti, Anders Baun, Shimshon Belkin, et al.

EU wide campaign exercise on bioassays and chemical mixture effects

Teresa Lettieri[1], Raquel N. Carvalho[1], Augustine Arukwe[2], Selim Ait-Aissa[3], Anne Bado-Nilles[3], Stefania Balzamo[4], Sabrina Barbizzi[4], Monica Buchetti[4], Anders Baun[5], Shimshon Belkin[6], Maria Belli[4], Martin Beníšek[7], Ludek Blaha[7], Mirco dalla Bona[7], François Brion[3], Elisa Calabretta[4], Daniela Conti[4], Nicolas Creusot[3], Yona Essig[8], Valentina Ferrero[1], Vesna Flander-Putrle[9], Maria Fürhacker[10], Regina Grillari[10], Anne-Katrine Haldorsen[11], Christer Hogstrand[12], Chris Hopkins[8], Adam Jonáš[7], Bogdan Jug[10], Ramon Lavado[1], Robert Loos[1], Cristina Martone[4], Petr Masner[7], Carina Modig[13], Alena Nekvapilová[7], Per-Erik Olsson[13], Alessandra Pati[4], Smitha Pillai[14], Natasa Polak[8], Monica Potalivo[4], Marek Pípal[7], Nic R. Bury[12], Wilfred Sanchez[3], Andrea Schifferli[15], Sabine Schnell[12], Shirmer[14], Liv Søfteland[11], Stephen Sturzenbaum[8], Simona Tavazzi[1], Valentina Turk[9], Aldo Viarengo[16], Inge Werner[15], Sharon Yagur-Kroll[6], Radka Zounková[7]

[1]European Commission – DG Joint Research Centre, Institute for Environment and Sustainability, T.P. 270, Via E. Fermi 2749, 21027 Ispra (VA), Italy
[2]Norwegian University of Science & Technology, Trondheim, Norway
[3]National Institute for Environmental Technology and Hazards, Verneuil en Halatte, France
[4] Istituto Superiore per la Protezione e la Ricerca Ambientale (ISPRA), Rome, Italy
[5]University of Technical University of Denmark (Department of Environmental Engineering), Kgs Lyngby, Denmark
[6] The Hebrew University of Jerusalem, Israel
[7]Research Centre for Toxic Compounds in the Environment, Masaryk University, Brno, Czech Republic
[8]Analytical and Environmental Sciences Division, King's College London, United Kingdom
[9]Marine Biological Station Piran - National Institute of Biology, Slovenia
[10]University of Natural Resources and Life Sciences, Vien, Austria
[11]National Institute of Nutrition and Seafood Research, Bergen, Norway
[12]Diabetes and Nutritional Sciences Division, King's College London, United Kingdom
[13]Life Science Center, Örebro University, Sweden
[14]Swiss Federal Institute for Environmental Science and Technology / ETH, Switzerland
[15]Oekotoxzentrum/Eawag, Dübendorf, Switzerland
[16]Università del Piemonte Orientale, Alessandria, Italy
E-mail contact: teresa.lettieri@jrc.ec.europa.eu

---

1. Introduction

Thousands different chemicals are discharged into the environment from agriculture, industry, medical facilities, house-holds. Currently, there is an increasing concern for the environmental impact of mixture of compounds since the additive and eventual synergistic effects are unknown and could produce serious adverse effects. Indeed, it is virtually impossible to analyse, detect and quantify all chemicals present in the water bodies, including transformation products.

So far, the effects of mixture has been described in literature for combination of few compounds e.g. for Polyclic aromatic hydrocarbons PAHs, pesticides and only for few model organisms. Recently, a document from the European Commission on combination effects of chemicals highlighted the need to ensure that risks associated with chemical mixtures are properly understood and assessed.

In 2012, European Commission JRC launched an EU-wide Exercise to test the effects of chemical mixture using existing and innovative bioassay applied to aquatic environment. The chemical Mixtures included pesticides, pharmaceuticals, heavy metals and polyaromatic hydrocarbons. The mixtures included each compound at Equivalent Quality Standard (EQS)[1,2] value, the safety limit concentration allowed by the European Water Framework Directive, (WFD) and distributed to 16 laboratories to test on bioassays routinely in use in each laboratory.

2. Materials and methods

Chemicals: Reference materials for Mix14 and Mix19 have been prepared as 1000-fold concentrated mixtures, with the organic solution (in methanol) prepared separately from the inorganic (in 2% nitric acid in water). Additional reference materials were prepared for Mix14 as 10,000-fold concentrated solutions, to allow the assessment of effects at a wider range of concentrations. The chemicals used for the preparation of the reference mixtures were of the highest purity (≥ 96%).
Bioassays were performed according to each laboratory protocol, some of them according to the OECD guidelines or ISO standards.

3. Results and discussion

The mixtures were analysed for their stability up to four months to -20ºC before being distributed. The bioassays were *in vivo*, *in vitro* assay including reporter gene assays and Estrogen Receptor binding and activity assays for the detection of endocrine disruptor chemicals.

Bioassay (*in vivo*)	**Bioassay *(in vitro*)**	**Endpoints (measured in more than one bioassay)**
Marine Microcosm (bacteria /phytoplakton)	Zebrafish embryo	Mortality
Bacteria	Frog embryo	Reproduction
Green algae	Fish cell lines	Development
Diatom	Mammalian cell lines	Teratogenicity
Yeast	Yeast (reporter genes)	Photosynthesis
Nematode	Bacteria (reporter genes)	Motility
Amoeba	Estrogen Receptor binding and activity assay	Immune response
Crustacean		Hormone interference
Fish		Zona radiata protein
Frog		CYP1A
		Stress response

Table 1: List of the test in vivo and in vitro performed in the 16 laboratories

3.1. Low trophic level

Mix14 and Mix19 were exposed to the marine bacterial/plykton community, three algae (two freshwater and one marine) and the curstacean Dapnia magna to test the effects on photosynthesis, growth and motility respectively. The mixture could influence the microcosm at very low concentration impairing the composiiton of the communiy (bacteria>>>phytoplakton). The three algae showed a growth inhibition following exposure for 24h with a slight sensitivity for the marine diatom *Thalassiosira pseudonana*. The acute immobilisation assay in *Daphnia* was significant and comparable among the laboratories showing an effect at 10XEQS for the Mix14.

3.2. Higher trophic level

Going up in the evolution scale, we analysed the effects on fish and frog embryo development while other assays on nematode, fish, mammalian and fish cell lines were performed for endpoints e.g. growth, biomarker expression, immunotoxicity. At 1X EQS value Mix14 and Mix19 showed an effect during the development in both fish and frog.

3.3 Reporter gene system

In our exercise, reporter gene systems were tested in nematode, bacteria, yeast and mammalian cell lines for metal, stress and DNA damage response, among others. Among them, four bioassays were dedicated to the detection of endocrine disruptor chemicals. There was good agreement among the laboratories performing the EDC assays, and Mix19 showed higher responses in these assays, as expected from the presence of additional endocrine disruptor chemicals.

4. Conclusions

We showed that the exposure to mixtures of chemical with dissimilar mode of actions and at the annual average environmental quality standard (AA-EQS), induced effects in both chronic and acute toxicity tests, affecting not only single organisms but population and potentially the entire ecosystem.

5. References

[1] European Directive 2008/105/EC on environmental quality standards in the field of water policy; Official Journal of the European Union, L384/84; 24.12.2008.

[2] European Directive 2013/39/EU amending Directives 2000/60/EC and 2008/105/EC as regards priority substances in the field of water policy; Official Journal of the European Union, L226/1, 24.8.2013.

Acknowledgement - The authors thank Marina Ricci (JRC-IRMM, Geel, Belgium) for the preparation of the reference mixtures. Robert Kase (Oekotoxzentrum; Swiss Centre for Applied Ecotoxicology; Eawag/EPFL; Dübendorf, CH) for the environmental quality standards for the emerging compounds. Joaquin Pinto grande for the technical assistance.