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ROMANIAN AQUATIC TOXICITY TESTING STRATEGY UNDER REACH

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Abstract. Regulation (EC) No 1907/2006 – Registration, Evaluation and Authorisation of Chemicals (REACH) is the most important European legislative regulation concerning chemicals and became applicable in Romania since June 2008. In the period 2008–2011, chemicals manufactures/importers and also eco-toxicological laboratories have made great efforts to comply with these requirements. The paper presents our contribution, as laboratory testing, to evaluate the aquatic toxicity of hazard substances and to provide information about environmental risks. Concerning REACH challenges, our laboratory has developed a toxicity testing methodology based on tests involving animals, but at the same time to provide the necessary data for the eco-toxicological characterisation of chemicals. As other countries, Romania has chemical pollution problems and in this context we performed a various aquatic toxicity studies for different substances, using an integrated eco-toxicological strategy, in an interdisciplinary collaboration. A reduced number of acute toxicity tests using fish were performed in our laboratory, but to obtain data it was necessary to evaluate the effects of the chemicals on the aquatic organisms, the LC_{50} bioassays were replaced with algae and crustacean EC_{50} tests. The fish bioassays were applied for fewer concentrations, the smallest between the EC_{50} obtained for algae and crustacean and only if the fish were more sensitive than algae and crustacean, acute fish tests were performed for lower concentrations. Our results led to predict the maximum allowable concentrations (MATC) in surface water and non observed effect concentration (NOEC) and also, to estimate the environmental risk coefficients. Our vision and assessment strategy are in continuous development in order to respond to future demands concerning toxicity on long-term, toxicity pathways, bioaccumulation, recalcitrant metabolites toxicity and inter-species effects extrapolations.

Keywords: ecotoxicity, chemicals, risk assessment, REACH.

AIMS AND BACKGROUND

The REACH legislation[1] is in force in Romania since June 2008. In the period 2008–2011, chemicals manufactures/importers and also ecotoxicological laboratories have made great efforts to comply the requirements of this Regulation. Like other countries, Romania also tries to ensure free circulation of the qualitative and environment friendly chemicals on the national and international markets, as well as the high level of human health and environment protection, as priorities of environmental and social politics.

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At national level 2 important frameworks act: Governmental Decision No 1408/2008 concerning the classification, labelling and packing of the dangerous chemicals and Governmental Decision No 351/21.04.2005 concerning the approval of the Program of disposal of the discharges, emissions and losses of the dangerous chemicals, designed to assure the environment health protection through dangerous and priority dangerous chemicals, the establishment of environment limits discharges and of specific risk phrases[2,3]. So, in present, Romania should respond to the all requests of REACH Regulation concerning the chemicals characterisation and assessment, according with the OECD guidelines[4]. As other countries, Romania has environmental pollution problems such as outdated environmental legislation, insufficient wastewater treatment, deficient monitoring programs, limited human resources and gaps in risk assessment of hazardous chemicals.

This paper presents the contributions of our research institute to evaluate the aquatic toxicity of hazard substances and to provide information about environmental risks, using an integrated toxicity strategy which combines chemistry, biology and biochemistry.

At international level, REACH operators try to implement different testing strategies for environmental toxicity under chemicals legislation, to provide risk assessment information in safety conditions, with low costs and low distress. In this context is intended to limit or decrease the number of testing animals or replace the conventional methods with alternative bioassays[5].

At this moment, in Romania, there are a limited number of manufacturers/ importers of chemicals requiring eco-toxicity testing, because these assessments are not included in environment monitoring programs, because exist some deficiencies in environmental protection norms implementation and the testing costs are quite high.

The basis of hazard aquatic chemicals identification is the toxicity effects assessment. Hazard classification is predicated on available toxicity data for fish, crustacean, and algae/aquatic plants, these taxa being generally accepted as representative organisms of aquatic fauna and flora for risk identification. To characterise the chemicals hazard on the aquatic environment and to assess the direct and complementary risks as well, it has to be ensured an integrated strategy for eco-toxicity evaluation based on experimental data obtained from standardised *in vitro* and *in vivo* tests performing, food chain covering, direct and indirect effects assessment and different scientific data sources.

The eco-toxicological characterisation of chemicals and also the compliance with the requirements for reducing the number of animals used in testing, in our research institute was developed a specialised laboratory (Bioassays – Biological Analyses Laboratory) focused on aquatic toxicity assessment of chemicals and environmental samples, with fish bioassays as conventional methodology and new alternative microbiotests with algae, crustacean and bacteria. Also, some ef-

ficient analytical methods for chemicals detection in test solutions were developed/ implemented.

We have performed acute, sub-acute and chronic toxicity tests using bacteria, green algae, crustacean and juvenile fish as test organisms.

Acute Toxicity Bioassays performed in our institute:

● Acute Toxicity Test with Fish – *Cyprinus carpio*/*Carassius gibelio* – OECD Test guideline 203;

● Acute Toxicity Test with luminescent bacteria – *Vibrio fischeri* sp. – MICROTOX according to standard method DIN EN ISO 11348-2;

● Growth Inhibition Test with Alga (micro-algae *Selenastrum capricornutum*) – ALGALTOXKIT F™ according to OECD Test guideline 201 and standard method ISO /DIS 8692;

● Acute Immobilisation Test with the freshwater crustaceans *Daphnia* sp. – DAPHTOXKIT F™ – according to OECD Test guideline 202 and standard method ISO 6341:1996;

● Microbial Assay for Risk Assessment (MARA) – a multi-species toxicity test based on responses of the 11 microorganisms (prokaryote and eukaryote bacteria) at toxic compounds.

According to the OECD methodology and guidelines (1998): '*Acute toxicity would normally be determined using a fish 96 h* LC_{50} (*OECD Test Guideline 203 or equivalent*), *a crustacean species 48 h* EC_{50} (*OECD Test Guideline 202 or equivalent*) *and/or an algal species 72 or 96 h EC50* (*OECD Test Guideline 201 or equivalent*)'.

To establish the sub-lethal effects of chemicals and to assess certain endpoints relating to growth, survival and development of aquatic organisms, sub-acute and chronic toxicity bioassays with common fresh water fish[6,7] and also, biochemical and histological tests were performed in our laboratory and physiological indicators were estimated[8]. Also, some biodegradation studies (i.e. *Activated Sludge*, *Respiration Inhibition Test – according to OECD Test guideline 209 and standard method SR EN ISO 8192/2007*) were applied to see the biodegradation behaviour of chemicals and the release of recalcitrant metabolites (Fig. 1).

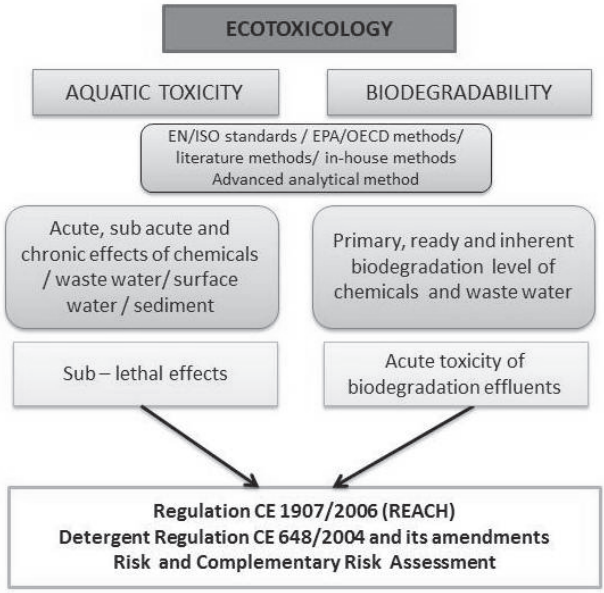

Fig. 1. Eco-toxicological research to assess aquatic risk of chemicals and environmental samples

EXPERIMENTAL

Because the present legislation require non-animal methods to provide environmental risk assessment data[9], we simplified the acute biotests, reducing the number of fish, the costs and testing efforts, through replacing of LC_{50} fish tests with algae, crustacean and bacteria EC_{50} tests, according to the other international strategies[10] and adapted to our conditions.

Acute testing.

– *Acute tests with fish* (*Cyprinus carpio*) were performed with young juveniles 5 g in size for a period of 96 h. The observational endpoint in these tests was mortality and the acute toxicity was expressed as the concentration which was lethal to 50% of the test organisms (LC50);

– *Acute tests with crustaceans* (*Daphnia magna*) generally began with first instar juveniles and test duration of 48 h was used. The observational endpoint was mortality or immobilisation and the acute toxicity was expressed as the concentration which causes a measurable adverse effect to 50% of the test organisms (EC_{50});

– *The algal test* (*Selenastrum capricornutum*) was a short-term test and was performed to determine the effects of the chemicals on the growth of fresh water microalgae (green algae). Algae were cultured and exposed to the test substance

in a nutrient-enriched medium (in batch cultures) over a period of normally 72 h. Growth inhibitions were quantified from measurements of the algal biomass density depending on time. The test endpoint was inhibition of growth, expressed as logarithmic algal biomass increase (average growth rate) during the exposure period. From the average growth rates recorded in a series of test solutions the concentration which produces a specified 50% inhibition of growth was determined and expressed as the EC_{50}.

Chronic testing. Chronic toxicity, for purposes of chemicals classification, refers to the potential or actual properties of a substance to cause adverse effects to aquatic organisms during exposures which are determined in relation to the life-cycle of the organism. Such chronic effects usually include a range of sublethal endpoints and are generally expressed in terms of a Non Observable Effect Concentration (NOEC). Observable endpoints typically include survival, growth (length and weight changes) and/or reproduction. Chronic toxicity exposure durations can vary widely depending on test endpoint measured and test species used.

Our experimental results obtained after performing several parallel acute toxicity bioassays with fish, crustacean and bacteria led us to predict: the maximum-allowable concentrations of the test substance in surface water (MATC), Non Observed Effect Concentration (NOEC) and also, to estimate the environmental risk coefficients.

During the experiments was observed that the small organisms are more sensitive and based on result interpretation, was appreciated that the use of micro-biotests (with algae, crustacean and bacteria) are very practical and offer a good reproducibility and relevant results, but also is not recommended to renounce definitively to the conventional toxicity tests with vertebrate organisms (ex. fish) because these aquatic organisms represent the principal target of pollution. The toxicity tests with vertebrate have an important role in risk assessment, where can be used in combination with microbiotests. Using of the multi-species microbiotest battery, which can offer relevant answers concerning the chemicals/chemical products/ environmental samples risk, may lead to an economical and readily assessment of acute and chronic toxicity[11].

Considering the testing strategies of the European Commission for acute environmental toxicity assays we have implemented in our laboratory a *testing plan* which reduce the number of fish in acute toxicity tests and also decrease the assessment cost (Fig. 2).

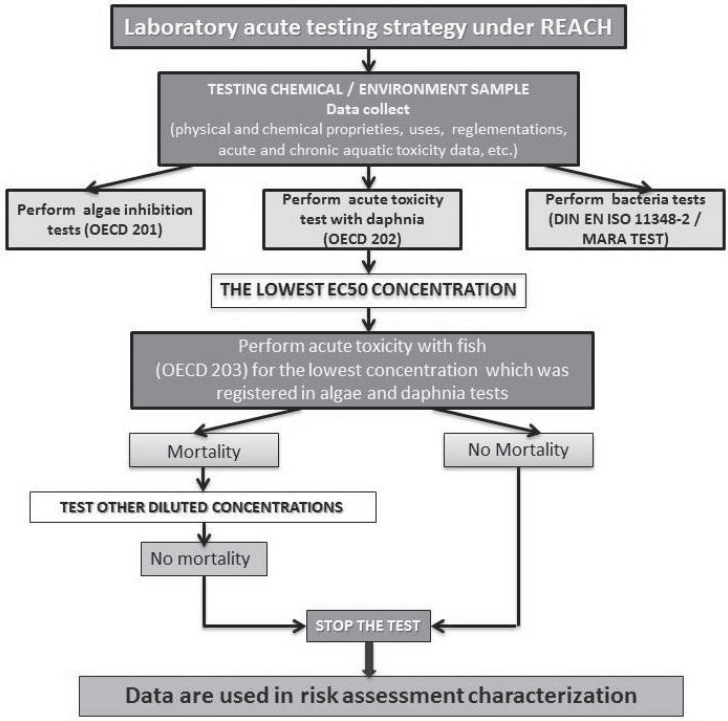

Fig. 2. Acute toxicity testing plan

First step is to collect all available data on intrinsic properties and characteristics of the chemical/ chemical product/environment sample to have a predicted vision about their toxicity in water. The data can be provided by safety data sheets (SDS) of chemical, literature data bases, different organisations records (OECD, EPA, ECHA website, etc.) or quantitative structure–activity relationships (QSAR model). All collected information is analysed in order to establish the need for future information and to establish what type of toxicity test should be performed – algae, daphnia and/or bacteria tests, to obtain the EC_{50} (effective concentration with inhibition/mortality/ immobilisation effect for 50% of tested organisms/upper threshold concentration – UTC). An acute toxicity test with fish is performed at the lowest experimental effective concentration (EC50) obtained in previous micro-biotests. The total number of fish used in testing is 10 (5 fish for test chemical solution and other 5 fish for the control solution). In case no mortality is recorded, the testing is stopped and the acute toxicity expressed as LC_{50} is reported greater than the UTC value. If case mortality is observed the testing is continued at low concentrations using a dilution factor until no mortality is recorded.

RESULTS AND DISCUSSION

Acute toxicity testing plan has been implemented in our laboratory practice starting with 2008, and it was applied for research studies and also for economical environment needs. In Table 1 are presented the most significant compounds which were tested for aquatic toxicity in the last 5 years, when bioassays with fish, and acute toxicity tests with daphnia, algae and bacteria were performed in our laboratory to comply the international requirements of eco-toxicity assessment strategies. In addition, we have a great data base concerning the toxicity of heavy metals, organic compounds (aromatic amines, nitro-derivates, pesticides, organic-chlorinated substances) and also different chemical products (mineral oils, water-soluble paints and varnishes, detergents and other degreasers/disinfectants/cleaning products with surfactants or dangerous substances, etc.).

In our opinion, the use of multi-species biotests battery permits relevant answers concerning the acute or chronic effects of chemicals on aquatic organisms and micro-biotests with small organisms could be considered as alternative toxicity methodology because: may offer rapid and good results comparative with conventional tests with fish; the time of toxicity test is significantly reduced; may be applied for toxicity assessment of different samples: liquid or solid, pure compound, waste water, surface water, sewage water, industrial effluents, sediments and sludge; the reagents and samples volume is reduced; relatively low cost of tests and organisms maintenance[11].

According to Ref. 11, one of the most effective mechanisms to reduce the vertebrate used as biological material for toxicity tests, is data sharing through data bases with free access for chemical industry and authorities, avoiding duplication of testing efforts. In this context, our data are periodically disseminated in national and international meetings and in scientific articles.

At international level only 60% of chemicals were registered and for the rest of 40% of substances no match could be found, because were rare on the market, or have been substituted by less hazardous substances or has not been yet registered. European Chemicals Agency (ECHA) propose that new chemicals Safety Data Sheets to be public through IT data bases, such as REACH-IT, IUCLID 5 or CHESAR (Chemical Safety Assessment and Reporting tool), start with autumn 2012 (Ref. 12). On the internet are some relevant data bases of existing toxicity data which are useful to reduce studies duplication (Ecotoxycology Database – ECOTOX, Pesticide Ecotoxicity Database, Toxic Substance Control Act Test Submission Database – TSCATS, High Production Volume Information System (HPVIS), INCHEM, Environmental Health Criteria Monographs, Hazardous Substances Data Bank – HSDB, TOXNET, European Chemical Bureau, and other specific databases, etc.)

Table 1. Chemicals tested according to acute toxicity laboratory strategy

Name of chemicals	Species/LC_{50}/EC_{50} (mg/l)			
	Cyprinus carpio / Carrasius auratus	*Daphnia magna*	*Selenastrum capricornutum*	*Vibrio fischeri*
1	2	3	4	5
Organics				
Surfactants				
Benzenthonium chloride	4.57	0.39	0.56	1.2
Dialkyl hydroxyethyl ammonium metasulphate	22.90	4.78	3.48	2.89
Cocamidopropyl betaine	6.16	9.54	5.55	>100
Farmaceuticals				
Diclofenac	109.64	53.70	–	17.37
Ibuprofen	158.48	104.71	–	39.89
Acetaminophen	245.47	12.02	–	6.02
Naproxen	269.15	46.72	–	19.95
Ketoprophen	64.56	43.65	–	16.21
Indomethacin	79.43	22.38	–	7.94
Caffeine	229.08	162.18		77.62
Carbamazepine	43.65	21.87	–	51.28
Anilines				
o-Nitroaniline	15.49	6.46		
4-Chloraniline	15.13	0.11	4.1	28
Nitroderivates				
1-Ethyl-4 nitrobenzene	53.7	12.6	–	–
o-Nitrotoluene	44.9	46.6	–	–
2,4-Dinitrotoluene	9.9	36.3	–	–
4-Chloronitrobenzene	16.6	25.1	–	–
Nitrobenzene	58.48	0.68	–	–
1,2,4-Richlorbenzene	30	7.34	–	–
Other organics			–	–
Bisphenol	10.2	8.1	–	–
Dichloropropane	122.2	158.5	–	–
Ethylene chlorhidrine	478.6	251.2	–	–
Monochloroacetic acid	316.2	38	–	–
α-Amino-isobutyl-nitrite	15.85	3.8	–	–
Dichloroacetic acid	295	100	–	–
Oxalic acid	116.85	28.18	–	–
β-Naphthol	4.78	0.4		
o-Toluidine	72.94	7.08	–	–
Pentachlorphenol	1.52	0.31	–	–

to be continued

Continuation of Table 1

1	2	3	4	5
Heavy metals				
Antimony ($SbCl_5$)	758	148	–	–
Titanium (TiO_2)	0.28	5.56	–	–
Zirconium ($ZrCl_4$)	0.31	91.20	–	–
Nickel ($NiSO_4$)	65.77	–	–	–
Zinc ($ZnSO_4$)	12.23	–	–	–
Copper ($CuSO_4$)	2.17	–	–	–
Cadmium ($CdCl_2$)/($CdSO_4$)	0.16/ –	– / 0.14	–/–	–/3.4
Arsenic (As_2O_3)	0.4	–	–	–
Chromium ($K_2Cr_2O_7$)	30.1	–	–	–
Lead ($Pb(NO_3)_2$)	119.9	–	–	–
Pesticides				
Tiuram	0.335	–	–	–
Ziram	4.07	–	–	–
Dazomet	2.499	–	–	–
Leguzin	37.58	1.52	–	–
Captan	0.609	15.13	–	–
Mancozeb	8.61	1.52	–	–
Dichlorvos	151.3	0.012	–	–
Faltan	3.16	0.89	–	–
Carbetox	13.2	0.048	–	–
Sinoratox	703.1	0.3	–	–
Carbaryl	3.77	0.0085	–	–
Naphthaline	18.20	18.62	–	–
Mecloran	11.8	2.24	–	–
Metoben	17	0.4	–	–
Atrasine	12.59	–	–	–
Mevinphos	0.047	–	–	–
Trichlorphon	60.26	–	–	–
Monolinuron	57.54	–	–	–
Cypermetrin	0.16	0.15	–	35.25

In this direction we are in permanent contact with national authorities with rights in environmental decisions and control, providing them our research information concerning chemicals aquatic toxicity effects useful to establish the maximum allowed concentrations of substances in WWTP effluents and surface waters. We have interdisciplinary collaboration with national and international research institutes and universities in order to offer a wide range of solutions in environmental protection area. Also we have organised trainings for manufactures and importers about REACH requirements in order to respond easily and efficiently.

The REACH legislation is flexible and can incorporate new expertise and advanced testing method and in this area our vision and assessment strategy are in continue development in order to respond to future demands concerning long-term toxicity, toxicity pathways, bioaccumulation and recalcitrant metabolites toxicity and inter species effects extrapolations. Another concern of our laboratory is to develop the terrestrial and marine toxicity methodology and to obtain more information concerning the pollutants effects at different trophic levels. Concerning the testing methodology are still unresolved ecotoxicological problems regarding the complex mixture of toxicants, interactions in complex matrices, bioavailability of toxicants and extrapolation from laboratory to ecosystem scale in the field.

CONCLUSIONS

Eco-toxicology assessment in context of REACH implementation in Romania is found at the beginning due to social-economic and politic factors. The new requirements of REACH, as modern approach to environmental risk assessment, are a stimulant of national eco-toxicological researches in the field of chemicals.

To be in trend of risk assessment, scientific community of Romania should develop infrastructure, training of personnel, interdisciplinary collaboration and accessing funds in order to be prepared to respond to the new challenges of environmental protections programs.

Our paper proposed a specific toxicity strategy for Romanian laboratories, which integrate different type of data into the decision-testing process of chemicals. In addition, the present testing strategy predicts toxicity endpoints and hazard assessment in the final risk assessment for a substance, offers an environmental better protection, reduces the number of animal used in tests, testing time and the costs. In perspective we will continue to improve and develop our eco-toxicological activities with new testing methodologies in laboratory and in the field to have a great contribution in environmental protection of Romania.

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