Standardised biological test methods for measuring toxicity of effluents and receiving waters in the Danube River Basin (DRB)

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Environmental measurements are required to determine the quality of ambient waters and the character of waste water effluents. Analytical methods are developed and evaluated to identify the concentration of chemical pollutants in drinking water, surface water, groundwater, waste water, sediment, sludge and solid waste. In addition to chemical analysis, biological test methods (biotests) are necessary to detect and quantify responses in aquatic organisms exposed to environmental stressors.

Biotests indicate a summarised response over adverse effects of all water constituents because waste water is a mixture of different chemical compounds. A set of standardised biotests with representatives of the aquatic ecosystem is available to measure acute and chronic toxicity and to use these methods in legal regulations. Some European countries (e.g. Germany, France) state that it is the national policy to enforce the prohibition to discharge toxic substances in toxic concentrations. The tests may be conducted in a central laboratory or in-site by the regulatory agency or an authorized person.

Standardisation requirements

A popular Chinese proverb says: *„Third-class companies assemble products; second-rate companies develop technologies and high-class companies set standards."* This puts “establishing standards” in perspective. Standardisation is an important and helpful requirement with benefits for products, governmental legislation and administration as well as scientific work. A sound method is the first step to „standardisation“ and good scientific practice. A formal standardisation of toxicity tests as it is accomplished, e.g., by the International Organization for Standardization (ISO) is a requirement for their implementation in legal acts and ordinances to carry out objective evaluations and proceed lawfully (ISO 2009). Standards for chemical analysis and biotests are associated with rules and procedures cited in international water directives (e.g., EU-Water Framework Directive) and national water and waste water acts/ordinances. General requirements for standardisation of toxicity tests (biotests) with respect to a regulatory framework of waste water effluents are given in *Table 1.*

Table 1. Requirements for standardisation of toxicity tests (biotests) for the evaluation of waste water

Requirement	Main objectives
evidence	representative status of applied organisms or clear recording of an effect
operational aspect	test result gives a direct and evident indication about objective and quality of a waste water treatment procedure
reproducibility	results must be reproducible in intra and inter laboratory trials
accuracy	small deviations between parallels, test of reference substances as validity criteria
legitimacy	determinations and definitions referring to a test and sampling procedure
legal security	intrinsic quality assurance, data for evaluation of measurement uncertainty
compatibility	compliance with national or EU-regulations; no conflict with other laws or directives

Toxicity of environmental samples

In general, toxicity means a harmful effect of chemicals on a biological system (cell organelles, cells, organisms). Such an effect is indicated by the reaction of the biological system, for example by death, changes in behaviour, and inhibition of growth, reproduction, or functional metabolic processes (photosynthesis, respiration, luminescence). Toxicity is not an intrinsic characteristic of a substance as bioavailability is a prerequisite. Toxic effects of chemicals to water organisms may depend, e.g., on water solubility, electrolytic dissociation, which may be affected by the pH, water temperature and, last but not least, concentration (hypothesis of Paracelsus: *Dosis facit venenum).* The benefit of a biotest performed with a complex mixture like waste water is to measure a summarised and integrated hazard effect. No detailed information about a particular constituent is necessary, irrespective of the availability of methods and resources for a chemical analysis.

Chemicals can affect the environment in different ways and at different levels. Basic criteria are persistence, bioaccumulation, acute and chronic toxicity, effects on reproduction, mutagenicity and carcinogenicity. There is a standard testing frame of OECD guidelines used, for example, in the context of REACH or other formal procedures to register or evaluate chemicals (OECD 2009). A set of standardised biotests is recommended to monitor the possible ecotoxicological effects on environmental samples. This scope, for example the investigation of waste water, is covered by national (German, DIN), European (EN) and International (ISO) standards *(Figure 1).* However, novel assays are necessary to monitor advanced toxic effects on biota with a particular focus on endocrine, immunotoxic or neurotoxic effects (Teodorovic 2008).

ISO member states decided to revise some of these standards developed by the Technical Committee 147 (Water Quality), e.g., algal growth inhi-

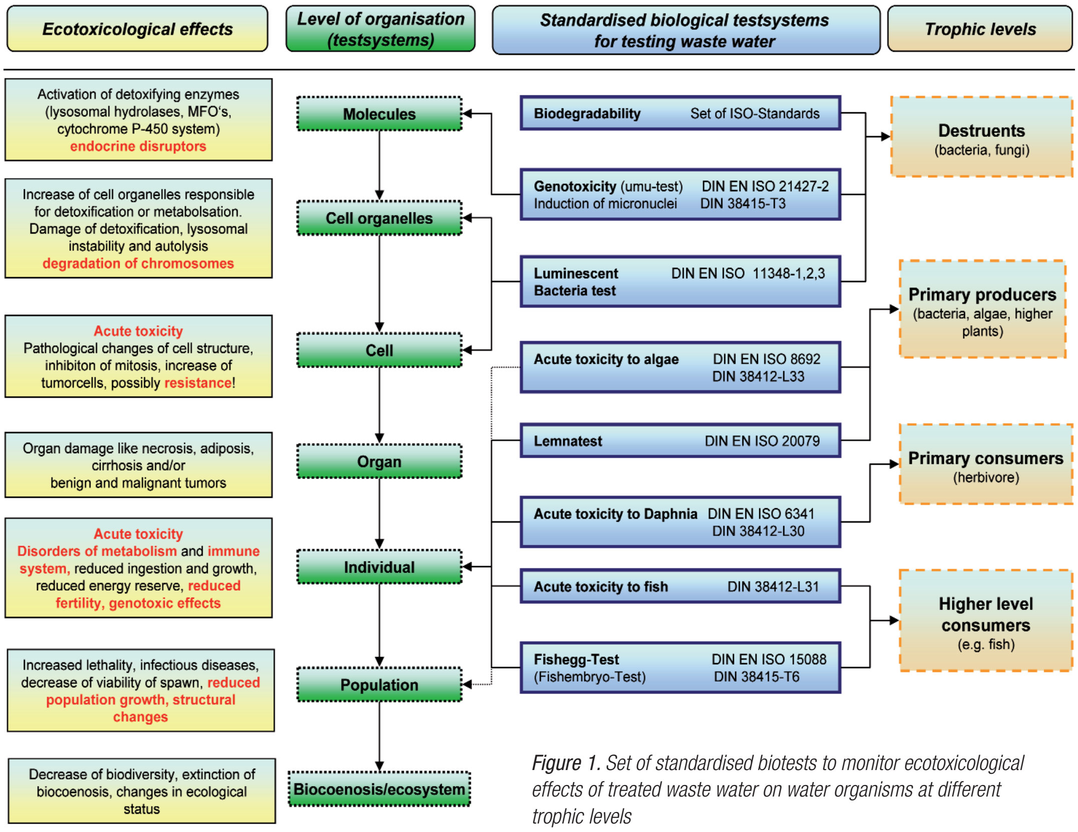

Figure 1. Set of standardised biotests to monitor ecotoxicological effects of treated waste water on water organisms at different trophic levels

bition test (ISO 8692) and acute toxicity to *Daphnia* (ISO 6341). The aim is to implement new techniques, for example to use microplates for the incubation of algae, to optimise the nutrient medium or to standardise *Daphnia* cultures.

Monitoring of industrial effluents

Aquatic toxicity tests are used worldwide to measure, predict and control the discharge of substances that might be harmful to aquatic life (US-EPA 1993). In Germany industrial effluents are periodically controlled by local authorities. Recognizing that no single test method or test organism can satisfy a comprehensive approach to environmental protection, a set of single species tests is used in Bavaria. The standardised toxicity tests referring to *Figure 1* are broadly accepted and measure toxic effects using organisms representing different trophic levels. In most cases any toxic effect indicated by one of the test systems is also shown by at least one of the other biotests. But the sensitivity varies between test systems and depends, for example, on the used organisms, the observed endpoint, the industrial branch, the constituents of the waste water tested, and the type of waste water treatment.

In the Bavarian part of the DRB the duckweed *(Lemna minor)* growth inhibition test according to ISO 20079 is applied. This test is presently used to evaluate the ecological effect of waste water originating from a flue gas scrubber of a Bavarian company. This specific effluent is treated by neutralisation and discharges into the Bavarian stretch of the River Danube. *Lemna minor* serves as a model organism for macrophytes. The plants are cultured for 7 days at different concentrations of an environmental sample, which are prepared by dilution. Industrial waste water may effect the vegetative growth of *Lemna minor*. This is indicated by a reduction of fronds (plant bodies) and/or biomass parameters like frond area, chlorophyll-a content or dry weight *(note: frond is a leaf-analogue part of a Lemna colony and a reproductive individual).* The number of fronds is counted via observation by eye. The frond area is detected by an image analyser (Medea-AV, D-91058 Erlangen). To quantify toxic effects on frond number and/or area the average specific growth rate is calculated for both parameters and for each dilution; then, the percentage of inhibition compared to a negative control is specified. From the two biomass parameters the most sensitive will be used to calculate the final test result given as

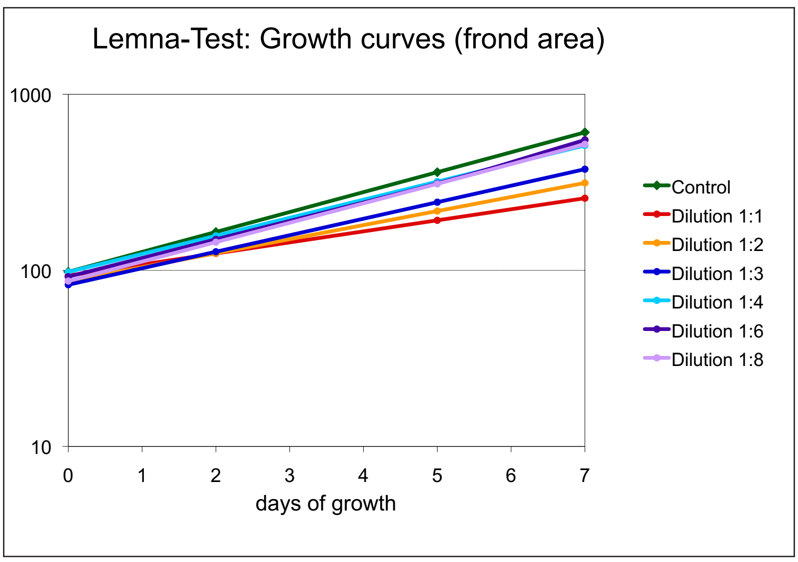

Figure 2. Effect of industrial waste water on growth of Lemna minor according to ISO 20079. Exponential growth curves at different dilutions of industrial waste water for the biomass parameter "frond area" (mm²)

EC(r)x value or according to annex B of the standard as LID (lowest ineffective dilution). The LID indicates the dilution at which an inhibition of < 10 % suggests a "no effect" concentration compared to the control. As shown in *Figure 2* the tested sample affects the growth of duckweed. The LID for water plants was calculated as dilution 1:4. Other test systems (see *Figure 1)* gave similar conclusions with a particular reference to an inexistent genotoxic effect.

From the perspective of emission control, the best available technique should be applied following the precautionary principle. Considering the huge dilution by the River Danube the predicted environmental risk caused by single discharge into the river may be negligible. If not, or if there are more discharges, an advanced treatment of waste water may be necessary or the discharge should be prohibited. This has to be stated in compliance with legal regulations as done in Germany, e.g., by the waste water ordinance and other national directives. However, national legal regulations on industrial effluents using biological test methods with regard to environmental protection as it is accepted in the upper DRB or in other European and North American countries are not yet state-of-the-art within the multinational DRB.

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Biomonitoring of aquatic pollution: from simple tradition to complex modern approaches

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Rationale of biomonitoring

Monitoring of aquatic systems refers to the systematic observation and surveillance of streams, lakes, groundwater, estuaries, coastal or marine waters. Monitoring represents a descriptive approach aiming to characterize the status and changes of aquatic systems being associated with or induced by stressors. Accordingly, monitoring programmes may aim to

- describe the condition of aquatic resources as well as spatial and/or temporal changes of the status (surveillance monitoring)
- determine the agreement of the resource condition with relevant regulations (compliance monitoring)
- evaluate associations between natural and/or anthropogenic pressures and the status of aquatic resources (impact monitoring)
- identify the causes of an impairment of the aquatic system (investigative monitoring)
- assess the effectiveness of measures enacted to improve an impaired status of the system (operational monitoring)

A major rationale for monitoring programmes is to survey pollution of aquatic systems and to evaluate the impact of pollution on aquatic resources. Approaches to monitor aquatic pollution include (1) chemical monitoring, which relies on chemical-analytical and bio-analytical tools to determine the nature and levels of chemical contaminants in water, sediments and biota, and (2) biomonitoring, which relies on the use of biological and ecological tools and parameters to characterize the quality status of an aquatic system and to assess exposure to and effects of environmental pollutants. Presence, condition and diversity of organisms respond to chemical – but also physical and biological – stressors, and biomonitoring exploits this responsiveness to indicate the cumulative response of biota. Biomonitoring is an essential foundation of ecological risk assessment as it directly addresses pollution-related biological and ecological status, while concentrations of pollutants inform only indirectly on biological and ecological effects. Advantages offered by biomonitoring include: (i) Biological elements respond to the mixture of all pollutants and to the cumulative impact, while chemical analytics – for reasons of efficiency and cost – has to focus on a sub-set of chemicals. Biological responses are also able to detect the environmental hazards caused by new emerging pollutants which may not yet be considered in chemical monitoring programmes. (ii) Biological elements provide a time-integrated response to pollutants. For instance, pesticides are often applied only during limited time periods so that chemical sampling performed at monthly or longer intervals may miss the transient presence of pesticides in the water body (particularly if the compounds are rapidly biodegraded). In contrast, a pesticide-induced change in, e.g., biological diversity, may be detectable for several