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Guideline

Hazard-based risk management of anthropogenic trace substances in drinking water to secure a long-term drinking water supply

Tamara Grummt, Thomas Braunbeck, Henner Hollert, Meike Kramer

TxBOX

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Foreword

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This work is dedicated to Tamara Grummt, who died on 26.01.2020 and left a painful gap on a personal and an expert level in our community.

In Germany, the requirements for drinking water quality are specified in the Drinking Water Ordinance (TrinkwV). According to § 1 of the TrinkwV, drinking water should be provided to the consumer in a state fit for consumption and as pure as possible. However, modern life leaves its traces even in well-protected water resources. There are, among other things, anthropogenic trace substances that can adversely affect drinking water and, therefore, human health. Although anthropogenic trace substances are often contained in drinking water only in minute concentrations (hence the term „trace substances“), these are undesirable in drinking water even at toxicological inconspicuousness.

Anthropogenic trace substances are currently not regulated by the TrinkwV; however, the so-called minimization requirement is anchored in § 6 (3), which makes sure „that the concentrations of chemical substances that contaminate the drinking water or adversely affect its nature are kept as low as possible in accordance with the generally accepted rules of technology and with justifiable effort and taking into account individual cases“.

The basis of all regulations is the “absence of a health concern” (according to § 4), i.e., whether damage to human health by the newly analyzed substance can be excluded. After the chemical identification of an anthropogenic trace substance in drinking water in concentrations above the general precautionary value (VWa) of 0.1 µg/L, an evaluation of the substance must be performed to ensure the high level of protection required for drinking water.

This evaluation has to be performed upon occurrence of the substance, even if toxicological data are incomplete. The German Environment Agency (UBA) has commented on this with the recommendation „Evaluation of the presence of partially or non-assessable substances in drinking water from a health point of view” (Umweltbundesamt, 2003), thus developing the theoretical concept the evaluation is based on.

To identify a substance, which potentially induces health effects, the endpoints geno-, neuro- and immunotoxicity as well as endocrine disruption are the modes of action considered relevant for evaluation. In turn, this means that substances with these modes of action are particularly undesirable in drinking water. This is a quality requirement that not only holds for the control of known and quantifiable potential threats, but also as a precautionary principle against such potential hazards. In fact, since there is only limited hope for progress in the reduction of adverse health effects associated with diseases such as cancer and neurodegeneration, the minimization of pollutants with such impact profiles is essential.

For the evaluation of anthropogenic trace substances in the field of drinking water, the concept of a Health-Related Indicator Value (HRIV) is applied. It is based on the four toxic modes of action cited above as relevant for drinking water evaluation (geno-, neuro- and immunotoxicity as well as endocrine disruption). The present guideline entitled „Risk-based risk management for anthropogenic trace substances to secure the drinking water supply“ is intended to form the base for a harmonized approach to experimentally record modes of action and to derive HRIVs accordingly. This should allow for a timely evaluation of trace substances in drinking water as well as a rapid determination of policy options and their review by monitoring programs.

For this end, endpoint-related test strategies are presented. The design of these strategies (i. e. test protocols and evaluation criteria) will be discussed in the text sections below for the modules genotoxicity, neurotoxicity and endocrine effects. The content of this guideline is restricted to characterizing those modes of action, which form the base of the HRIV concept.

For each endpoint, its significance for human health will be evaluated and current scientific and regulatory developments are summarized. The guide has been designed to provide a transparent decision basis of the UBA for deriving HRIVs for authorities, water suppliers and the interested public. This will increase the general acceptance of HRIV-based decisions not only with regard to the precautionary principle, but also in relation to a reasonable cost-benefit balance.

The present guideline is a result of the joint project Tox-Box (Funding Code: 02WRS1282A – 02WRS1282I) within the funding program „Risk Management of new pollutants and pathogens in the water cycle (RiSKWa). http://www.bmbf.riskwa.de/index.php.

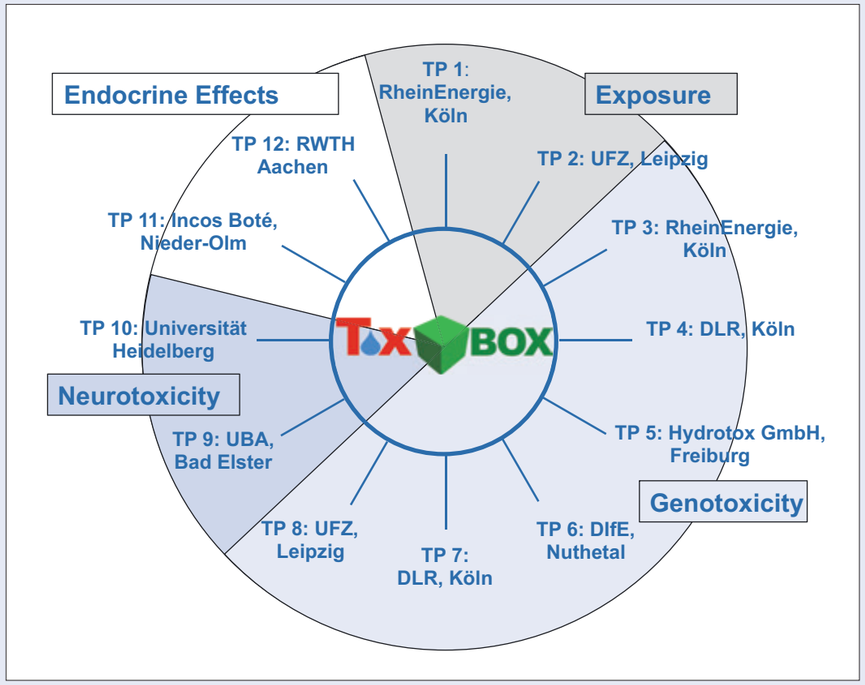

Figure 1: Tox-Box organization partners

Dr. Tamara Grummt,
coordinator of the joint research project Tox-Box.
German Environment Agency, Head of Sub-Department: “Toxicology of Drinking Water and Swimming Pool Water”

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1 Introduction

The number of “unregulated substances” in drinking water will continue to rise sharply with the increase of entries of anthropogenic trace substances and with further development of analytical techniques. Since for the majority of newly detected substances, no or only insufficient toxicological data are available, a complete toxicological evaluation in the traditional sense (presence of animal studies) is frequently not possible. Nevertheless, there is a need to evaluate these substances with respect to their health hazard potential. Since 2003, the UBA has, after a hearing of the Drinking Water Commission of the then Federal Ministry of Health and Social Security (BMGS), addressed this need with the recommendation for an „Evaluation of the presence of partially or non-assessable substances in the drinking water from a health point of view” (Umweltbundesamt, 2003). Colloquially the recommendation is referred to as the “Health-Related Indicator Value (HRIV) Concept“.

In practice, the HRIV concept has proven suitable as an evaluation approach and has been accepted by the different groups involved such as health authorities, water suppliers, or consumers. The HRIV is an instrument available for the event-related and timely estimation of immediate potential health hazards. With the action plan based on the HRIV concept, a safe drinking water supply can be assured. At the same time, the harmonized approach in the administrative area grants certainty for health authorities in cooperation with the water suppliers.

If the concentration of a substance under discussion is above the HRIV, measures have to be taken to improve the human toxicological evaluation. In parallel, health authorities in cooperation with water companies and other responsible authorities explore possibilities of resource conservation with the goal of minimizing or preventing the contamination of raw water through environmental contaminants; ultimately the procedure is designed to end up with drinking water after appropriate treatment.

As soon as can be foreseen that – based on an improved toxicological database – a guideline value for life-long exposure will not be met through resource conservation measures within the specified timeframe, suitable technical measures such as additional treatment or improvement in the distribution network have to be implemented by the water company. With the derivation of a guideline value, the HRIV is superseded. Although in most cases the final guideline value will turn out to be higher than the HRIV, the minimization rule is still valid from the point of drinking water hygiene in accordance with the generally accepted rules of technology.

1.1 Theoretical concept for derivation of Health-Related Indicator Values (HRIV; in German: Gesundheitlicher Orientierungswert (GOW))

The Health-Related Indicator Value (HRIV) concept is based on available substance-specific toxicological data for modes of action relevant for humans (including genotoxicity and neurotoxicity). The HRIV is a precautionary trigger value for the protection of human health; for this reason, it is always set low enough that increasing completion of the database will usually result in the same or a higher values, but never in lower guideline values (Dieter, 2014). For a detailed description of the theoretical HRIV concept, please refer to the UBA recommendation „Assessment of attendance partial or non-assessable substances in drinking water from health point of view“ (Umweltbundesamt, 2003).

Figure 2 illustrates the hierarchical definition of the HRIV. In the absence of sufficient toxicological data, an $HRIV_1$ of 0.1 µg/L is defined in accordance with the minimum requirements laid down in § 6 (1) TrinkwV 2001, postulating that even life-long consumption of potentially contaminated drinking water is no cause for human health concerns.

Genotoxic & mechanism of relevance in humans ?	YES	NO	NO	NO	NO	NO
Genotoxic ?		YES / no data	NO	NO	NO	NO
Immuno- and/or neurotoxic ?			YES / no data	NO	NO	NO
Subchronic toxicity ?				YES / no data	NO	NO
Chronic toxicity ?					no data	NO
Axiom of concern					3.0 ($HRIV_4$)	> 3.0
				1.0 ($HRIV_3$)		
			0.3 ($HRIV_2$)			
		0.1 ($HRIV_1$)				
Health-Related Indicator Value [µg/L]	≤ 0.01 ($HRIV_0$)					
Precautionary principle						

Figure 2: Theoretical concept for deriving a HRIV (based on Dieter, personal communication, modified)

Since possible measures to minimize pollutants depend on the respective HRIV, which spans a concentration range from 0.01 to 3.0 µg/L, the supplementation of the theoretical concept by experimental test strategies not only broadens the scientific basis, but also takes into account the request for a risk management that should be based on the most reliable scientific database available.

Over the last two decades, considerable progress has been made in the field of *in vitro* toxicology. Yet, so far, this has made little, if any change to the evaluation process. This guideline will, therefore, also adapt the theoretical concept for deriving the HRIV, which is still based on conventional assessment strategies, to the scientific progress made in this field.

In the original HRIV concept, endocrine disruption as a relatively new endpoint is completely missing. Given current scientific developments and increasing knowledge as well as the high degree of societal concern, the research project underlying the present guideline also covers the implementation of endocrine effects into the HRIV concept.

Germ cell mutagenicity will be estimated by *in vitro* genotoxicity via evaluation of numerous databases that are almost consensus among professionals in the field of genotoxicity. Likewise, endocrine effects are integrated into the HRIV concept by an *in vitro* test battery. Figure 3 illustrates the integration and the numerical equalization with an $HRIV_0$, which is based on the impact profiles in the low-dose range typical of endo-

Endocrine effects (estrogen specific effect) ?	YES	NO	NO	NO	NO	NO
Genotoxic & mechanism of relevance in humans ?	YES	NO	NO	NO	NO	NO
Genotoxic ?		YES / no data	NO	NO	NO	NO
Immuno- and/or neurotoxic ?			YES / no data	NO	NO	NO
Subchronic toxicity ?				YES / no data	NO	NO
Chronic toxicity ?					no data	NO
Axiom of concern					3.0 ($HRIV_4$)	> 3.0
				1.0 ($HRIV_3$)		
			0.3 ($HRIV_2$)			
		0.1 ($HRIV_1$)				
Health-Related Indicator Value [µg/L]	≤ 0.01 ($HRIV_0$)					
Precautionary principle						

Figure 3: Theoretical concept for deriving an HRIV including endocrine effects (based on Dieter, personal communication, modified)

crine active substances. It should be mentioned that integration of endocrine disruption and neurotoxicity into the HRIV concept required the largest amount of development work and was only possible due to the flexibility of the HRIV concept.

1.2 Experimental design of the HRIV concept

Traditional toxicology allows for testing only a small number of substances that have to be assessed in costly long-term animal experiments. In addition, conventional strategies are often accompanied by inconsistent results with respect to human biology and pathophysiology. Last, not least there are ethical concerns associated with traditional animal testing. As a result of this dissatisfactory situation, a shift of paradigm has evolved in experimental toxicology as described in the „Toxicity Testing in the 21st Century (Tox 21)“ report (National Research Council, 2007).

Among other new technologies, the new strategy uses human cell-based *in vitro* test methods for the identification of toxicological key mechanisms that allow for a prediction of possible *in vivo* effects. In the toxicological scientific literature, this procedure is referred to as high-throughput screening (HTS). International research programs are on the way, having the following goals:

- identification of *in vitro* test methods, which indicate modes of action involved in the expression of adverse effects;
- recognition of signature patterns of *in vitro* test methods, which allow for better translation into and better predictability of *in vivo* effects;
- prioritization of substances for further toxicological investigation; and
- risk assessment regarding human health hazard by using toxicological pattern recognition in *in vitro* test procedures.

The implementation of this strategy for the detection of toxicological safety allows for toxicity testing beyond high-dose studies (toxicological risk), which are not relevant for the natural environment. Hitherto detected substance patterns of anthropogenic trace substances have consistently shown exposure in the low-dose range. Since a „zero burden“ cannot be achieved, not the toxicological risk of a substance, but its toxicological safety should be characterized when dealing with anthropogenic trace substances. For experimental work, this means that primary modes of action will have to be identified at the cellular level. In the following chapters, the *in vitro* test procedures for detection and evaluation of potential hazards for the endpoints relevant in the HRIV concept will be described as laid out in the final report of the joint project „Tox-Box“ (Grummt et al., 2013).

The starting point for the development work in Tox-Box was the assumption that effects can in principle be recorded *via* test batteries: By means of a small number of selected *in vitro* tests, it should be possible to characterize the effect with sufficient certainty to allow for a yes / no decision in the sense of the HRIV concept. Of course, such test batteries are built on the current level of knowledge, but generally should be regarded as flexible toolboxes (Grummt et al., 2018).

1.3 Perspectives

The application of a consensus-built evaluation strategy leads to a stronger collaboration of regulators and practical users. Moreover, it is increasingly important that water companies build up own competencies in risk assessment and perform toxicological investigations for the priority parameter genotoxicity in order to be able to assess the evaluation as such and to speed up evaluation processes as a whole (Kramer et al., 2010).

This is especially important against the background of potentially deduced costly countermeasures and trustful communication with consumers, politics and media. Simple bacteria-based test procedures with well-standardized short-term protocols can also be applied in drinking water laboratories. Since the progress in chemical analysis leads to the detection of more and more substances at ever-lower concentrations, the development of competences in the area of substance evaluation and risk assessment allows water companies the transition from reaction to action.

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2 Module Genotoxicity

Meike Kramer, Carsten Schmidt, Eva-Maria Prantl, Petra Rettberg, Elke Rabbow, Sebastian Feles, Stefan Gartiser, Martina Knauer, Hans-Rudolf Glatt, Walter Meinl, Christa Baumstark-Khan, Luis Spitta, Christine Hellweg, Eberhard Küster

2.1 Definition of the mechanism of action and basic scientific principles

Mutagens generally cause DNA damage, not mutations. DNA damage includes, for example, DNA strand breaks or DNA adducts (where a molecule is bound covalently to the DNA). These typically do not change the sequence or number of nucleotides in DNA. In contrast, mutations are changes in the sequence or number of nucleotides, and these are made by the cells when they either incorrectly repair the DNA damage or try to replicate past unrepaired DNA damage. Thus, mutagenesis is a cellular process, typically requiring DNA repair and replication to produce mutations, and it is the cell that makes the mutations. Genotoxicity refers to both DNA damage as well as to mutations. Thus, a genotoxic compound is identified as a compound that can induce DNA damage in an assay that detects DNA damage, such as a chromosome aberration assay or a micronucleus assay, and/or can induce mutations in an assay that detects mutations, such as the *Salmonella* (Ames) bacterial mutagenicity assay.

In the derivation of Health-Related indicator values (HRIV) for drinking water not evaluated previously for toxicological endpoints, the presence of genotoxic contaminating substances is one of the priority parameters that is considered (Umweltbundesamt, 2003). For most substances with genotoxic effects, a HRIV of 0.1 µg/L will be derived only for a few highly genotoxic substances (e.g., carcinogenic compounds), and this safety value is seen to be safe to a maximum of 10 years. For genotoxic substances with adequate metabolism, then a HRIV 0.01 µg/L applies.

Relevant for evaluation in the sense of the HRIV concept are the multitude of metabolites and transformation products and not the anthropogenic trace substances for which genotoxicity data are already existing (plant protection products, medicines, industrial chemicals, etc.). The metabolites and transformation products are contaminating the water cycle via sewage treatment plants or diffuse sources. Because of their polar properties they migrate into the raw water, drinking water or water treatment plant effluents via techniques like chlorination or ozonation.

2.2 Status of the regulation

For an adequate assessment of the genotoxic potential of a substance at least three different endpoints, namely the induction of gene mutations, changes in the chromosome structure (clastogenicity) and numerical chromosomal abnormalities (aneuploidy) have to be rated. Each of these events is involved in the development of cancer or hereditary diseases. Usually, various test methods are combined in a test battery and jointly evaluated because there is no single test which can map all relevant genotoxic endpoints. An internationally harmonized, hierarchical test strategy has been established in which the *in vitro* test procedure is the first evaluation step. If these findings are equivocal, the rating can be verified via subsequent *in vivo* tests. The selection of the *in vivo* test is then based on the specific endpoint. The tests recommended for the *in vitro* test battery differ only slightly in relevant international regulatory guidelines. The following list shows a test battery recommendation based on the example of respective WHO- criteria according to Eastmond et al. (2009):

- Gene mutations in bacteria
  - e.g. Ames test with five test strains according to the OECD guideline 471. Application of further test strains depends on the chemical structure and the substance class.

- Chromosome mutations in mammalian cells
  - e.g. Chromosomal aberration test
  - e.g. Micronucleus test

- Gene/chromosome mutations in mammalian cells
  - e.g. Mouse lymphoma thymidine kinase assay (detects both gene and chromosome mutations)
  - e.g. Hypoxanthine-guanine phosphoribosyltransferase assay (detects only gene mutations)

Depending on the regulatory context, a substance that shows no effects in the stage #1 (*in vitro* test battery) is classified as non- genotoxic (for example REACH, CLP, Cosmetics Directive). If, however, a higher or prolonged exposure level is expected for humans (e.g., most medicinal products for human use), even the substances that were not toxic in the stage 1 *in vitro* assays are then further screened with *in vivo* tests for additional safety (e.g. ICH for pharmaceuticals, VICH for veterinary pharmaceuticals).

There is general consensus that animal experiments cannot be completely renounced from general genotoxicity evaluation for reasons of evaluation certainty for the human health. The European Union nonetheless strives to increase the significance of the *in vitro* test battery to the extent that as few as possible *in vivo* follow-up examinations are required (EURL ECVAM, 2013).

To avoid false-positive effects, which would lead to unnecessary follow-up examinations, the screening should initially be based on a very limited number of tests. These tests should be well validated and informative at the same time (Kirkland et al., 2007).

In this regard Kirkland et al. (2011) postulated (on the basis an extensive literature evaluation) a basic test battery, which simply consists of two *in vitro* tests: the classic Ames test with five test strains (appropriate to the OECD Guideline 471) and the Micronucleus Test. Both Test systems would therefore collectively identify 73% of the examined rodent carcinogens and 78% of *in vivo* genotoxins. Although the Ames test is carried out with bacterial cells (prokaryotic system), the micronucleus test uses mammalian cells (eukaryotic system). Both test systems are standardized and have been used for many years and jointly cover all relevant *in vitro* toxicological endpoints. The Ames test identifies gene mutations, whereas the micronucleus test detects chromosomal mutations, including major structural damage to the chromosome (clastogenicity) and changes in the number of chromosomes (aneuploidies) (Kirkland et al., 2011; COM, 2011).

Additional *in vitro* tests are required to retrieve information about the responsible genotoxic mechanism of action and, at the same time, give indications about false-negative or false-positive results. A constructive way forward is the consideration of specific additive tests or test variants whose selection is based on the specific structural properties of the target substance (Kirkland et al., 2007; Reifferscheid & Buchinger, 2010). Thus the structural property analysis is very important. These are *in-silico* models that enable the prediction of the genotoxic properties due to certain substructures, the functional groups or toxicophores. Partially, databases may be used.

2.3 Test strategy for the evaluation of genotoxicity

To assess the genotoxic potential of drinking water contaminants within the framework of the HRIV concept, the fastest possible evaluation procedure is advisable. The specification of required test procedures for analysing the evaluation-relevant endpoints was an open point of the HRIV concept. The developed test battery now makes it possible to quickly and effectively evaluate the priority parameter genotoxicity. It focuses on the *in vitro* testing, but still provides a high level of relevance for human-relevant genotoxins (Figure 4). The associated speeding up of the evaluation process allows the participating water companies and health authorities to make timely decisions on whether to recommend further technical measures to reduce contamination. This facilitates communication with the public.

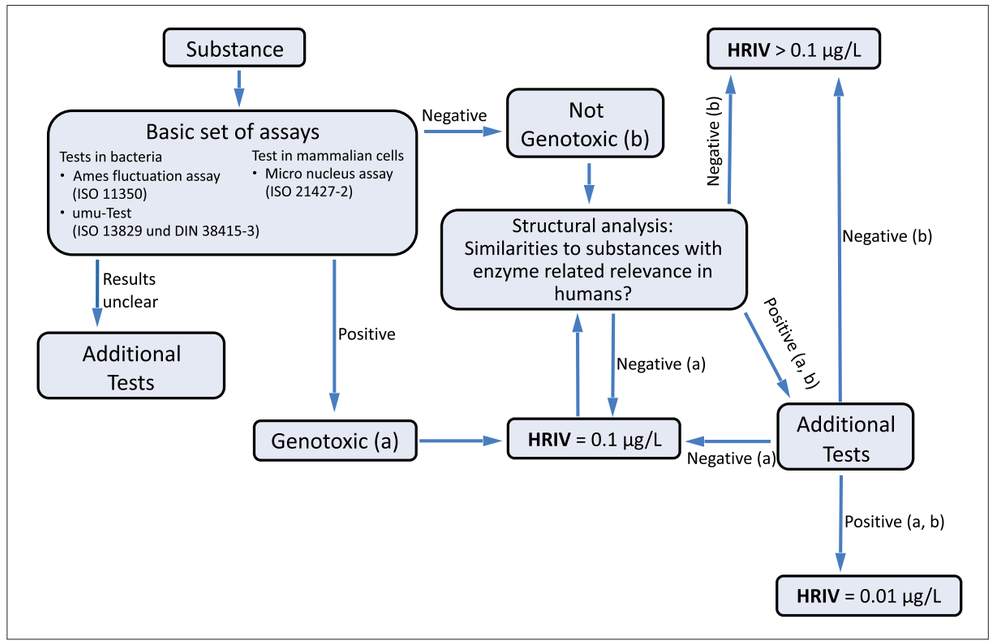


Figure 4: Schematic presentation of the test strategy for the parameter "genotoxicity". For a substance that is classified as "genotoxic" in the basic test battery, the following HRIV assessment yields different routes (a) than for a substance classified as "non-genotoxic" in the same test battery (b).

Using a detailed structural analysis, one can predict if the suspected test substance contains specific functional groups that can lead to a genotoxic effect requiring mammalian metabolism that might not be detected by the basic test battery. The basic test battery, which consists of a few highly standardized tests, then records all evaluation-relevant outcomes with a comparatively low risk of false-positive results (Figure 5). The use of additional highly specific test strains or cell lines (available for all procedures of the basic test battery) in the so called "extended test strategy" allows sensitive detection of the genotoxic effects of additional substance groups.

Basic test battery

The basic test battery was developed and validated with the help of selected individual substances that are relevant for drinking water production (Prantl et al., 2018).

Based on the findings of Kirkland et al. (2011), the Micronuclei and the Ames test were first selected for the basic test battery. However, data analysis done by Kirkland et al. (2011) reviewed only the results of the Ames test with five test strains that collectively detect different types of point mutations, as required by OECD Guideline 471. High levels of standardization

Ames Fluctuation Assay	Umu Test	Micronucleus Assay
ISO 11350: 2012	ISO 13829: 2000	ISO 21427-2: 2009
End point: Gene mutation in bacteria	End point: Induction of DNA Repair in bacteria	End point: Chromosome mutations in mammalian cells
Standard test strain:: *Salmonella typhimurium* TA98 *Salmonella typhimurium* TA100	Standard test strain:: *Salmonella typhimurium* TA1535/ psk 1002	Standard cell line: V79 cells (Chinese hamster fibroblasts)

Figure 5: Basic test battery consisting of three *in vitro* tests, which together represent the assessment-relevant endpoints "gene mutation" and "chromosome mutation".

have been achieved by the Ames Test Protocols of the ISO Standards for Water and Wastewater Samples (Ames Plate Incorporation Tests ISO 16240: 2005, Ames Fluctuation Tests ISO 11350: 2012), which provide detailed specifications for test performance and evaluation.

For the basic test battery described here, the Ames Fluctuation Test was selected, which ensures a higher evaluation reliability due to the underlying statistical procedure (Reifferscheid et al., 2011). As the study results from the Tox-Box project have shown, supplementing the Ames Fluctuation Test with the umu-test may increase its evaluation safety (Prantl et al., 2018). The advantage of the umu-test is that when using this single test strain, various types of DNA damage can be summarily detected because the umuC gene is involved in all major steps of bacterial mutagenesis (Oda et al., 1985; Rajagopalan et al, 1992; Little & Mount, 1982).

The *in vitro* methods selected for the basic test battery are characterized by a high degree of standardization, statistically validated evaluation criteria, relatively simple application with automation potential and low safety requirements for laboratory equipment (S1 laboratory).

The results of the individual tests of the basic test battery are evaluated together. Depending on the outcome, various options are available to achieve a satisfactory assessment at the *in vitro* level. Independently of the classification as "positive test result" or "negative test result", by using structural analysis, and possible toxicophoric groups are additionally screened (Figures 4 and 7).

■ Positive test result

a) If a positive test result is present only in the bacterial test, but not in the micronucleus assay with V79 cells, a bacterial specific genotoxic mechanism may be present in individual cases which, based on literature data, has been shown to have no human toxicological relevance. This for instance concerns substances like gyrase inhibitors (e.gg fluoroquinolones). They lead to genotoxic effects in the Ames test strains TA 98 and TA 100 and in the umu test, but not in the Ames strain TA 102 (Ciaravino et al., 1993; Clerch et al., 1992).

b) If a positive reaction is seen only in the micronucleus assay with V79 cells, but not in the bacterial test, and the structural analysis shows no evidence for toxicophoric groups, a follow-up with an alternative cell model system may be appropriate. The Chinese hamster V79 fibroblast cell line is on the one hand characterized by high proliferation rates, but on the other hand also by low metabolic competence and p53 deficiency. This favours

false-positive test results. For a verification, human p53-derived cells such as HepG2 (+ S9 mix) or HepaRG cells (rather higher metabolic competence, but few experience in routine use) from a human hepatocarcinoma are suited.

Negative test result

A negative result in all tests of the basic test battery is not sufficient for a classification as “non-genotoxic”. False-negative *in vitro* results may be due to inadequate metabolic activity or exogenous metabolic activation by the tested cell lines (Ku et al., 2007). If the structural analysis indicates toxicophoric groups, additional tests with specific adjunctive test strains must be performed to confirm the first findings of the basic test battery. Especially in the Ames test, the reliable detection of nitrosamines, bivalent metals, aldehydes, azo dyes and diazo compounds, alkaloids, allyl and nitro compounds is difficult without modification of the standard procedure (OECD 471). In addition to test strains which stand out by overexpression of individual extraneous metabolizing mammalian enzymes, the Ames fluctuation test may be complemented by other test strains which are recommended in the OECD Guideline 471. This holds especially true for strains which give notice of specific mutation patterns (usually GC point mutations). In particular, the test strains *S. typhimurium* TA 102 or *Escherichia coli* WP2 uvrA, which, unlike the other Ames strains, detect AT point mutations and are important for the detection of additional specific substance groups (e.g., hydrazines), may provide important additional information on a case-by-case basis. A scheme for evaluating the results is given in figure 6.

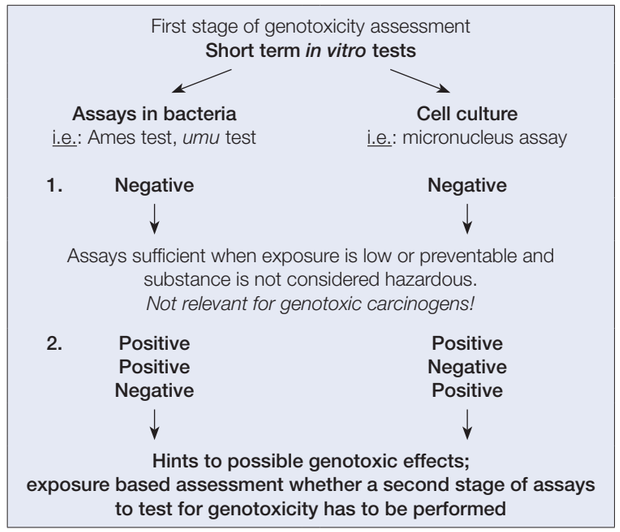


Figure 6: Test strategy for assessing genotoxicity

Extended test strategy

Through the targeted use of additional test strains or cell lines, which are available for all test procedures of the basic test battery, there is the possibility to expand the range of detectable genotoxins. In the sense of an extended test strategy and at the same time to obtain information on the responsible genotoxic mechanism the selection of the test strains is based on specific structural properties of the test substance.

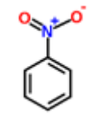
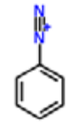
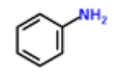
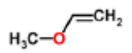
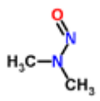
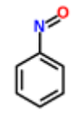
Structural analysis

In silico predictions on the genotoxicity of substances are based on empirical data and / or physico-chemical considerations. Structural elements that mediate or promote toxicological activity are termed “toxicophores” or “structural alerts” (Ashby, 1985; Miller and Miller, 1981). A direct genotoxic activity of a substance, in particular as far as it is based on an immediate interaction with the DNA, can thus be quite reliably estimated. Appropriate models were for example developed by the statistical comparison of specific molecular structures with positive effects in the Ames test (Benigni et al., 2008). In this context, Kazius and colleagues (2005) showed that most of the Ames-positive substances are represented by eight toxicophores and another 29 toxicophoric substructures (Figure 7), so that accurate predictions can be made with an error of only 15%.

The question of whether a substance can be converted into genotoxic metabolites and, if so, which enzymes could mediate activation (and inactivation) requires not only the enzyme class but also the critical enzyme form. The answer is very much possible for individual substance classes and in combination with molecular model calculations, but only if experimental data are available for a sufficient number of congeners. This becomes more difficult if arbitrarily selected substances are to be assessed. Simple established workflow procedures do not exist here, even if the problem of considering metabolism in toxicology is well known. Depending on the structure of a substance, it is advisable to consult a biotransformation expert and / or a computer-based system. You can choose between several freely available programs that have been developed in publicly funded projects, i.e. OECD QSAR Toolbox (https://www.qsartoolbox.org/) or UFZ ChemProp (http://www.ufz.de/index.php?en=34593) based on the EU project OSIRIS (http://osiris.simpple.com/OSIRIS ITS/itstool.do).

Selection of additional test strains or cell lines depending on substance properties

Genetically engineered cells that overexpress certain xenobiotic metabolizing enzymes from the mammalian metabolism are available for a variety of characteristic enzymes (e.g., cytochrome P450 (CYP), glutathione-S-transferase (GST), sulfotransferase (SULT) and N-acetyltransferase (NAT)). For the structure-de-

Name of toxixophore	Example	Name of toxixophore	Example
Aromatic nitro compound		Diazonium	
Aromatic amine		β- propiolactone	
Three membered heterocyclic compound		Unsubstituted, α,β-unsaturated aldehyde	
Nitroso compund		Aromatic nitroso compound	

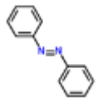
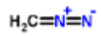
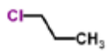
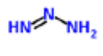
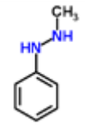
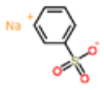
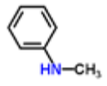
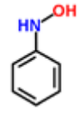
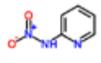
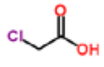
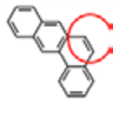
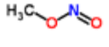
Name of toxixophore	Example	Name of toxixophore	Example
?		Azide	
Azo group		Diazo group	
Aliphatic halogenide		Triazene	
Polycyclic aromatic compund		1-Aryl-2-monoalkyl-hydrazine	
Carbon atom bound to sulfate		Aromatic methyl amine	
α, β-unsaturated aldehyde		Aromatic hydroxyl amine	
N-nitro compound		Carboxy halogenide	
Bay region		K region	
Alkyl nitrite			

Figure 7: Toxicophores according to Kazius et al. (2005), modified, structural formulas from http://www.chemspider.com

pendent selection of suitable additional strains, some recommendations are made below:

a) **Small molecules (Mr <150) and substituted biphenyls:** Recombinant *Salmonella typhimurium* strains and V79 lines in which human cytochrome P450 (e.g., CYP2E1) is expressed.

b) **Halogenated alkanes, in particular vicinal-dihalogenated alkanes**, should be investigated in the presence of glutathione transferase GSTT1. Glatt's research group at the German Institute of Human Nutrition has successfully expressed human GSTT1 in *S. typhimurium* TA1535 and in V79 cells (Meinl et al.). This enables an improved *in vitro* examination of the substances.

c) **Nitroalkanes** (such as 2-nitropropane) should be tested in cells expressing an appropriate sulfotransferase (SULT). Among others, *Salmonella* strains and V79 cells expressing the most important human SULT are available, also in combination with CYP1A2 or CYP2E1 in the V79 cells. HepaRG cells also express sufficient levels of SULT.

d) **Nitroaromatics** should be investigated with various transferases in the presence of a reductase. Suitable *Salmonella* strains are available for this purpose. Reliable testing of nitroaromatics is more difficult in mammalian cell systems as nitroreduction is not mediated by a single enzyme in mammals. The use of additional mammalian cell lines in the testing of nitroaromatics is therefore only useful if mechanistic investigations are the goal and if the willingness for a high experimental effort is existing.

e) **Aromatic amines** should also be investigated with various transferases in the presence of CYP. Transgenic bacterial strains are available for this purpose. When testing aromatic amines in mammalian cell systems, it is useful to use cells that co-express human CYP1A2 in combination with human NAT2, as well as cells that have human CYP1A2 and human SULT1A1.

f) **Benzylic and allylic alcohols** (and substances that can be metabolised) should be studied in SULT-proficient cells. We recommend testing these compounds not only in conventional cells from standard assays, but also in SULT-expressing bacterial strains or mammalian cell lines, giving priority to SULT1A1 because of its broad substrate tolerance and its high expression in many human tissues. HepaRG cells are also suitable.

2.4 Test protocols of the individual test procedures and evaluation of results

In the development and validation of the basic test battery described here (which was part of the research project Tox-Box) it was worked strictly according to ISO standards. The test protocols have reached a high degree of standardization, which guarantees a very good comparability of the results from different laboratories.

Ames fluctuation test according to ISO 11350 for the detection of point mutations

Test Principle: Due to a single point mutation in their genome, test bacteria lack the ability to synthesize a nutrient essential to their growth, the amino acid histidine. The bacteria are therefore dependent on the presence of histidine in the nutrient medium. Since mutations occur more frequently under the action of genotoxins, it is possible that the test bacteria can re-synthesize histidine due to a single point mutation and thus grow again in histidine-free medium. The test principle is based on the quantitative determination of such "revertants". The number of bacteria that grow after exposure to histidine-free medium serves as a measure of mutagenic activity. For the Ames test, a number of test strains are available that can display

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2 Module Genotoxicity

different mutation types depending on where in the histidine gene the original DNA defect is located. This gives indications of different damaging effects. The Ames fluctuation test, in contrast to the Ames plate incorporation test, is based on working with liquid cultures in microtiter plates (384 well plates), which allows significant material and time savings. The growth of revertants is visualized by adding a colour indicator.

Evaluation: The number of revertants is determined manually by counting the discoloured microtiter plate wells on a light plate. By photometric detection of the colour change (violet to yellow), the evaluation can be automated after experimental adaptation on a suitable plate reader.

Test organisms: The ISO test protocol recommends the use of the standard *Salmonella* strains TA98 and TA100 for detection of 2-base GC deletions and of GC base substitution mutations, respectively. These are two of the five test strains designated in the OECD Guideline 471.

Test protocol: The test is carried out according to the test protocol of ISO 11350. Supplementary note: Since ISO 11350 makes no precise specification as to when a growth inhibition should be considered cytotoxic, a value of 50% was established to ensure the comparability of the results.

Umu test according to ISO 13829 for the detection of DNA repair intensity

Test principle: The test principle is based on the fact that genotoxins activate the DNA repair system of a cell as a result of damage to the genetic material (DNA). The test organism for the umu assay was modified by the coupling of genes in the way that the DNA damage does not only induce a specific DNA repair system, but that also a sugar-cleaving enzyme is produced. This allows the detection by a colour change after addition of an artificial substrate which is cleaved by the newly introduced enzyme. Following the exposure of the bacterial cells to the test substance, the induction of the DNA repair gene can then be determined photometrically. The umu test is an indicator test that detects a wide range of different DNA damage. It shows a genotoxic effect, but not necessarily a mutagenic effect, since there is the theoretical possibility of a complete repair of the damage before the next cell division.

Evaluation: The evaluation is carried out photometrically by means of a microtiter plate photometer (required wavelengths: 600 nm, 420 nm).

Test organism: *Salmonella typhimurium* TA1535/pSK1002

Test protocol: The test is carried out according to the test protocol of ISO 13829.

Additional note: For the evaluation of the test results, the otherwise identical German DIN 38415-T3 makes stricter specifications (positive effects from an induction rate ≥ 1.5 instead of ≥ 1.3), which allow a higher evaluation reliability.

Additional comments on the test procedure

For the implementation of all *in vitro* methods of the basic test battery, higher-level specifications apply, which are explained below. These include, for example, the number of replicates, control samples and test concentrations and can be found in the relevant ISO standard.

■ Use of additional cell systems

The high degree of standardization of the test protocols of the basic test battery applies in particular in conjunction with the standard test strains or cell lines described in the respective ISO standard. For the use of additional cell systems in the extended test strategy, some experimental adjustments of the test protocol are required (e.g., incubation time, cell density, nutrient medium concentration, S9 fraction). It should also be considered that the cytotoxicity of a test substance may change in the presence of metabolic activation systems. Therefore, an accompanying cytotoxicity measurement should be performed for each test strain. In each case, the specific sensitivity of the additional cell systems should be checked with suitable positive substances and a positive control defined on this basis should be included.

Micronucleus test according to ISO 21427-2 for the detection of chromosome mutations

Test principle: The micronucleus test detects structural chromosomal damage and damage to the mitotic apparatus. Both chromosome aberrations and disturbances in the spindle apparatus lead to chromosomes or chromosome fragments which are then not incorporated into the cell nucleus of the daughter cell in the course of cell division. Chromatid fragments without a centromere do not move to the nucleus of the daughter cell during cell division and remain in the cytoplasm. All of these particles form the so called micronuclei in the cytoplasm. The cells are exposed for a defined period of time (24 hours without S9 mix, 4 hours with S9 mix) to different concentrations of a test substance. A significantly increased occurrence of cells with micronuclei compared to the spontaneous frequency in the negative controls indicates that the test substance can cause chromosome breaks or spindle disorders. From the frequency of occurrence of micronuclei, the micronucleus frequency is calculated. If it increases significantly (Chi^2 test) over the frequency of the untreated negative control, a genotoxic effect of the sample is to be assumed.

Evaluation: By default the evaluation is done microscopically. A less time-consuming and labour-intensive alternative is the evaluation with a flow cytometer (Avlasevich et al., 2011).

Test Organisms: V79 cells (Chinese hamster fibroblasts) have been proven in decades of *in vitro* genotoxicity testing with the endpoint of micronucleus induction due to their good cell culture capabilities, the rapid proliferation and high sensitivity to environmental chemicals. However, studies also show an increased frequency of false positives compared to *in vivo* genotoxicity (Kirkland, 2007; 2008, Fowler, 2011). The cause could be the lack of human metabolism, which is replaced by exogenous metabolic activation using the addition of rat liver homogenate (S9 mix). But this can only reflect the human metabolism to a limited extent. On the other hand, the limited p53 function as well as altered DNA repair mechanisms in the V79 cells have to be considered (Fowler, 2011). In individual cases, the use of additional V79 cell lines with modified metabolism or even human cells (for example HepG2 cells (+ S9 mix) or HepaRG cells) is recommended.

Test protocol: The test is carried out according to the test protocol of ISO 21427-2.

Supplementary note: As relevant information is missing in ISO 21427-2, the following maximum solvent concentrations in the test batches are recommended on the basis of experience in the Tox-Box project: max. 0.1% (v/v) DMSO; max. 0.8% (v/v) ethanol. Divergent from the information in ISO 21427-2, the test can also be evaluated by flow cytometry.

In contrast to the conventional microscopic evaluation, the flow cytometer allows a very high sample throughput and a coupling with the detection of further toxic effects. Using for example the “MicroFlowTM” test kit, Litron Laboratories Rochester, New York, the parameters vitality, apoptosis / necrosis, and cell proliferation can be determined simultaneously in addition to the micronuclei. Thus, the so-called “precursor” events (e.g. influencing the vitality and cell proliferation) are also recorded. In addition, the results of the micronuclei determination are validated under the aspect of

the induction of secondary genotoxicity due to cytotoxic effects (which can lead to an overestimation of the findings).

The *in vitro*-MicroFlow-test kit was developed for flow cytometric counting of micronuclei in mammalian cell cultures. It is an effective and fast process using a two-colour labelling technique. An advantage of the *in vitro* MicroFlow method over other automatic evaluation methods is the use of sequential staining. This allows the micronuclei to be distinguished from the chromatin of the apoptotic and necrotic cells. With this method, reliable micronucleus results are obtained even if a larger number of dead cells are present.

A key component in the kit is the DNA dye A (ethidium monoazide or EMA). The substance penetrates the damaged outer membrane of the apoptotic and necrotic cells. A special feature of this dye is also that it covalently binds to the DNA by photoactivation. After this step, the cells are washed and the cytoplasmic membranes are dissolved with detergents to release the nucleus and micronuclei. During the lysis step, the DNA dye B (SYTOX Green) is added, it binds to the entire chromatin. In this way a different colourisation of healthy and dead chromatin is achieved (Figure 8).

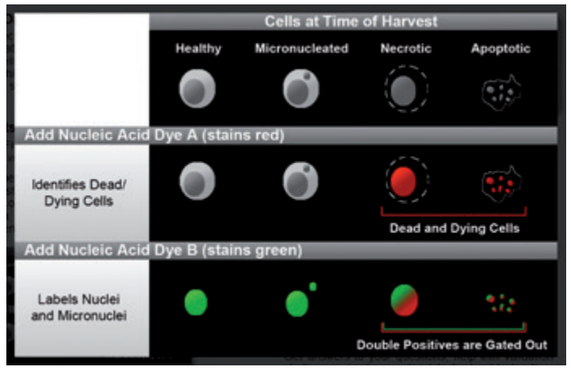

Figure 8: Principle of the MicroFlowTM measuring process
(Source: www.LitronLabs.com)

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Module Neurotoxicity 3

Tamara Grummt, Alexander Eckhardt, Rita Heinze, Ralf Junek, Thomas Braunbeck, Daniel Stengel

3.1 Definition of mechanisms of action and scientific background

The human brain is an extremely complex organ, maybe even the most complex structure in the body. Despite intensive research, especially since the turn of the millennium, "one does not even begin to understand the rules according to which the brain works" (Weber, 2014). The severe changes in personality that can be caused by brain disorders are illustrated by increasingly common diseases such as Alzheimer's and Parkinson's diseases, but also by less common diseases such as schizophrenia or depression. Because of their prevalence, the first two diseases in particular do not exclusively cause considerable suffering for those affected and their relatives, but represent a specific challenge to society as a whole.

Due to the complexity of the nervous system and a sometimes long latency period between exposure and effect, the investigation of potential neurotoxic effects must also attempt to detect precursor events in nerve cells. It is often not possible to detect these in the entire organism, because, e.g., the observation period would be too long or the frequency of events in the case of tumor formation is too low for a reliable detection. For this reason, similarly to studies into genotoxicity, the investigation of neurotoxicity increasingly uses *in vitro* test systems for the evaluation of toxic substances (Weber, 2014). On the other hand, due to the universal nature of neurotoxic principles, *in vivo* models based on lower vertebrates are increasingly being used for extrapolation to mammals and humans, especially for screening purposes (de Esch et al., 2012; Lee & Freeman, 2014; Ton et al., 2006).

The expression of neurotoxic effects can vary to a great extent: Some effects occur regardless of the age of the affected person, while others may only occur in a specific developmental phase or subsequent to chronic exposure. Acute toxic effects, e.g., by organophosphorus pesticides on acetylcholinesterase are age-independent. Sadly, chemical warfare agents such as sarin and tabun, which also belong to the group of organophosphates, have become infamous.

Two other known neurotoxins, on the other hand, show highly age-dependent or development-dependent toxicity: ethanol and lead. Whereas occasional moderate alcohol consumption is not a problem for a healthy adult, even a pregnant woman's one-off alcohol consumption can lead to massive developmental disorders in the fetus, especially in the first third of pregnancy. However, genetic aspects also play a role in adults: The metabolism of alcohol by alcohol dehydrogenase is genetically determined.

The increased sensitivity of infants and young children to lead exposure compared to adults was the reason for the step-wise reduction of the lead limit value in drinking water from 40 µg/L to 10 µg/L during the years 2003 to 2013. With regard to lead, children are more at risk than adults in two respects: firstly, they absorb about 50 % of the lead taken up *via* the digestive tract, while adults absorb only about 5 %. On the other hand, the nervous system of children is not yet fully developed, and the development of the nervous system is severely impaired by lead. The increased absorption rate leads to higher levels of lead in children's blood. This in turn can lead to developmental disorders such as hyperactivity, disorders of fine motor skills or even a reduction of intellectual capabilities (He et al., 2017).

Ecotoxicology is also considered in terms of resource protection: Here, a considerable part of the studies that have been made available so far are limited to effects on fish, whereby most of the neurotoxic effects described above for mammals can also be observed in fish (de Esch et al., 2012; Lee & Freeman, 2014; Ton et al., 2006). However, since the protection goal of ecotoxicology is definitely not the individual performance, but the well-being of the population, neurotoxic effects in fish are mainly considered against the

background of behavioral changes (Selderslaghs et al., 2013; Tierney, 2011). These cannot only interfere with the interaction with potential predators and prey, but can ultimately also lead directly to a reduction of reproductive success. In recent years, especially fish embryos and larvae have been used extensively in screening programs for neurotoxic substances. In most cases, these have shown an excellent correlation between findings in fish and mammals when identifying neurotoxic substances.

As another special feature of fish, the lateral line system of fish is a sensory system which does not exist in this form in land-living vertebrates (including mammals); however, there are marked parallels to the inner ear of all other vertebrates, since the lateral line organ is innervated by a lateral branch of the statoacustic nerve, and the structure of the sensory cells in the lateral line system (neuromasts) is identical to that of the hair cells in the inner ear of terrestrial tetrapods. A special feature of fish, however, is the fact that, unlike the hair cells of the inner ear, the neuromasts can come into direct contact with environmental pollutants *via* the lateral openings of the lateral line system (Stengel et al., 2017).

Alternative methods for classical tests for neurotoxicity

For the investigation of neurotoxic effects, also with regard to developmental toxicology, there have been intensive efforts to find alternatives to animal experiments. When applying the current Developmental Neurotoxicity Assessment Directive (OECD, 2007; U.S. EPA, 1996) for a single substance, more than 1000 female animals have to be killed (Price et al., 1996). It is therefore necessary to establish a reliable battery of screening procedures on the basis of which further specific tests are carried out, if a substance has revealed toxic potentials (Crofton et al., 2011).

Due to the fact that neurotoxic effects can not only affect the nerve cells *per se*, but also the transmission of impulses by nerve cells, it is currently still necessary to carry out both *in vivo* and *in vitro* experiments for certain questions (Kais et al., 2015; Stengel et al., 2017). However, in the case of fish, embryos can already be used (see below).

A completely different approach to establishing alternative methods could be that behavior-based neurotoxicity screens with fish embryos and larvae, which have been developed in several laboratories, are used as a pre-screen to *in vivo* experiments with mammals (Tierney, 2011). This is in compliance with the requirements of, e.g., the German Animal Protection Act in two respects: Firstly, the law requires that corresponding experiments always be carried out with the least developed organism – and, in common understanding of phylogeny, fish resides clearly “under” mammals. On the other hand, in ontogenetic terms, fish embryos are not classified as protected until the onset of exogenous food intake according to the currently valid EU Animal Welfare Directive (EU, 2010); as a consequence, experiments with embryos of, e.g., zebrafish (*Danio rerio*) up to an age of 120 h do not require licensing by animal welfare authorities in many European countries (Strähle et al., 2012). Not least for this reason, the fish embryo test with zebrafish has been standardized as an OECD test guideline (OECD, 2013) and, thus, represents the first internationally standardized alternative method to a classical toxicity test with fish (Braunbeck et al., 2015; Strähle et al., 2012).

The approach of testing substances *in silico*, i.e. by computer models, which has been widely used especially in the field of drug development, has not yet been established in neurotoxicology. Although Cronin’s first approach in the mid-1990s to extrapolate from the structure of a substance, in this case organic solvents, through Quantitative Structure-Activity Relationships (QSAR) to its neurotoxic potential, has had only limited success (Cronin, 1996). Further investigations by Yazal et al. (2001) and Makhaeva et al. (2012) were restricted to serine esterase inhibitors

and do not allow generalization. This illustrates that neurotoxicity studies are still dependent on living cells and organisms.

3.2 Status of regulation

Although the assessment of the neurotoxic potential is possible on the basis of the Health-Related Indicator Value (HRIV) concept explained in the general introduction, there are limitations to this approach. As can be seen from the work by Kroes et al (2000), NOELs (No Observed Effect Levels) of drinking water-relevant substances, whose most sensitive endpoint is neurotoxicity, cover concentration ranges of at least 3 orders of magnitude. The HRIV concept reliably covers even the strongest neurotoxins relevant for drinking water. Therefore, the precise assessment of the neurotoxic potential may allow for short-term exceedances of a HRIV of 0.3 µg/L for neurotoxic substances in drinking water.

3.3 Test strategy for the *in vitro* assessment of neurotoxicity

In vitro testing for neurotoxicity is carried out using a three-step evaluation system (Figure 9). A crucial step is the distinction of neurotoxicity from cytotoxic effects. Therefore, in the first test stage, cytotoxicity studies are carried out. This also serves to determine the (maximum) test concentrations for subsequent specific neurotoxicity studies. This concept is a first important step in the evaluation of neurotoxicity and will be validated and, if necessary, modified in the follow-up project “NeuroBox” (BMBF funding code: 02WRS1419).

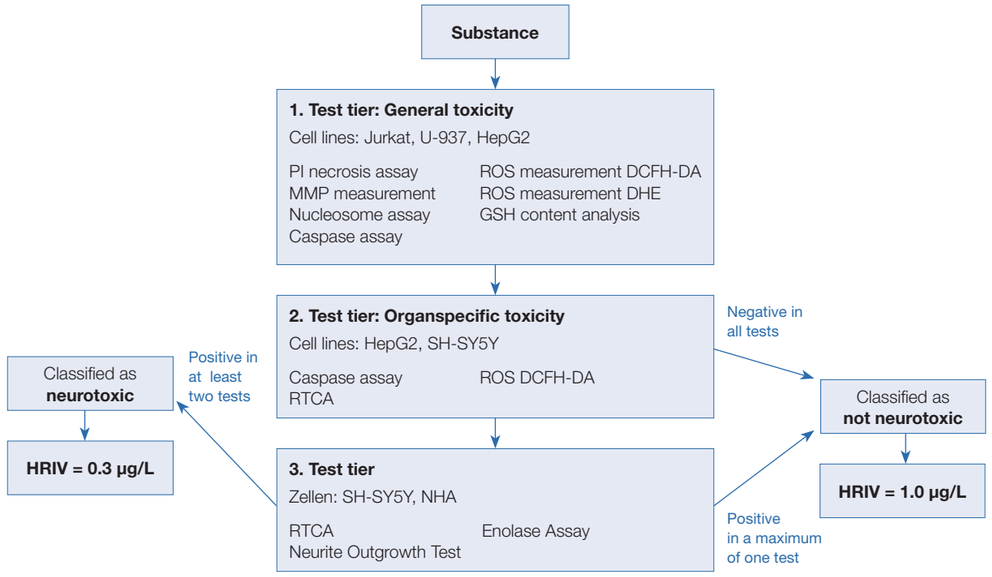


Figure 9: Scheme for *in vitro* testing for potential neurotoxicity

Cell culture studies are an important basis for rapid evaluation of neurotoxicity. For example, a comparison between the effects of a chemical on a liver cell line and the effects on a nerve cell line provides an initial indication of a possible specific neurotoxic potential. Established cell lines in this context are SH-SY5Y neuroblastoma cells and the human Ntera 2 cell line, which is capable of differentiation (Stern et al., 2014). For neurotoxicity testing in the context of this guidance, SH-SY5Y cells are used for screening and HepG2 cells serve for comparison. The use of SH-SY5Y cells saves the lengthy differentiation phase required for Ntera 2 cells, which allows a faster evaluation. Since both cell lines are of human origin, they are metabolically closer to humans than, for example, mouse or rat cell lines. The fact that primary cells would be metabolically more competent and, thus, better suited to mimic the original organ system seems less relevant for initial screening. However, as soon as neurotoxicity is suspected, further specific testing procedures must be utilized. For this end, primary human astrocytes (NHA, Normal Human Astrocytes) are used. NHA is not an immortalized cell line, but primary cells that are commercially available.

The influence of a substance on the proliferation of cells can be demonstrated with *in vitro* methods by means of the “Real Time Cell Analyzer” (RTCA™; Omni Life Science, Bremen, Germany). For this purpose, cells are seeded into a special cell culture plate and exposed to different concentrations of the test substance. Non-exposed cells serve as negative controls; a known neurotoxin serves as a positive control. The cell culture plates, so-called E-plates, have gold contacts by means of which the impedance of the cells can be measured. With increasing cell number and cell propagation, impedance increases and is thus a measure of proliferation. If the test substance reduces proliferation and, thus, impedance compared to the negative control, a toxic effect can be assumed. To determine the neurotoxic potential, effects on nerve cells are compared to effects on liver cells. In this specific approach, neuroblastoma cells (SH-SY5Y cells) are compared to hepatocarcinoma cells (HepG2 cells). A stronger effect on neuroblastoma cells indicates neurotoxic effects.

In addition to proliferation, induction of apoptosis and necrosis is also compared. Upon cell destruction, apoptosis or necrosis may occur. Whereas apoptosis is programmed cell death, necrosis is a form of uncoordinated cell injury, which results in the premature death of cells in living tissue by autolysis. Although the result for individual cells is ultimately the same, this distinction plays an important role for the intact organism: Whereas apoptosis results in a significant uptake and recycling of cell components by the organism, necrosis results in uncontrolled cell destruction. Since during the latter process the contents of, e.g., lysosomes are released, there is a high incidence of inflammatory reactions, which may impair surrounding tissues. Such effects make a distinction between apoptotic and necrotic processes indispensable for the assessment of the toxic potential of a substance.

As a further step, the test battery for neurotoxicity determines the development of reactive oxygen species (ROS). Reactive oxygen species are considered as triggers for precursor events not only in the development of cancer, but also in the development of neurotoxic disorders. Due to its very high oxygen demands, the brain is particularly susceptible to ROS. In addition, brain tissues bear relatively low levels of endogenous antioxidants such as glutathione or vitamin C, and the polyunsaturated fatty acids of the nerve cells can easily be oxidized (Barnham et al., 2004; Mates, 2000). ROS is thought to play a crucial role in the development of neurodegenerative diseases such as Alzheimer´s or Parkinson´s disease as well as neurodevelopmental disorders such as autism and schizophrenia (Barnham et al., 2004).

The hierarchical structure of the test battery implies that tests specific for neurotoxicity are only carried out, if there is well-founded suspicion for neurotoxicity. The specific tests for neurotoxicity testing consist of (1) determination of proliferation of NHA cells using RTCA™, (2) the Neurite Outgrowth Assay with SH-SY5Y cells and (3) the neurite-specific enolase assay. The measurement of proliferation of primary NHA cells serves as a confirmation of results obtained with SH-SY5Y cells. The Neurite Outgrowth Assay is used to test whether exposure to the test substance leads to the formation of neurites. Although this test can be performed with various cell lines (Kim et al., 2007), this guidance only describes the use of SH-SY5Y cells, since these are of human origin and have therefore elevated human relevance. The neurites formed by the cells can be determined by comparison of the fluorescence surfaces of the cells in the different approaches. The larger the areas, the more neurites have been formed or the longer the neurites are. Reduction in neurite growth is a separate neurotoxic effect.

Since the mid-1980s, release of the enzyme neurite-specific enolase (NSE) into the extracellular space has been known and played a role in the evaluation of the severity of strokes and as a tumor marker (Al-Rawi and Atiyah, 2009; Dufour et al., 2006; Steinberg et al., 1984). If transferred to cell culture conditions, this means that nerve cells release increasing amounts of enolase into the medium due to damage to membrane integrity. The release of enolase is measured by ELISA in the fluorescence reader: the more enolase is released, the greater the damaging effect.

In the field of *in vivo* neurotoxicity studies, this guidance is restricted to studies that can be carried out on embryos of fish, a developmental stage not considered protected in the EU in terms of animal welfare. In addition to the classical measurement of acetylcholinesterase as a marker for disturbances in nerve transmission, effects on neuromasts in the lateral line system have proven to be a particularly sensitive marker; the neuromasts are regarded as a model for sensory hair cells in the inner ear of vertebrates, i.e. for a sensory organ of central importance.

If a substance can be classified as neurotoxic by *in vivo* studies, a HRIV of 0.3 µg/L is stipulated; if a substance is not neurotoxic, a HRIV of 1.0 µg/L is derived (see Figure 9).

3.4 Protocols for the *in vitro* test procedures and interpretation of results

The specific protocols for carrying out the individual tests can be obtained from responsible laboratories listed in chapter 8. The necessary equipment is also listed in the respective protocols. Citation of company names and brand marks is not meant as advertisement, but is exclusively based on the equipment used by specific laboratories. All other devices that can measure the required wavelengths are suitable for use. The same applies to the use of chemicals, test kits, etc.

Cell culture

In vitro testing stage 1: Basal (general) toxicity (cytotoxicity)

On this test stage, a first distinction is made between potential apoptosis and necrosis by means of cell cultures that do not originate from the nervous system. Tests are carried out with concentrations up to 1 g/L or maximum solubility. For substances, which are not cytotoxic up to a concentration of 1 g/L, potential cytotoxicity at higher concentrations is not assumed relevant for drinking water, since occurrence at such concentrations is regarded extremely unlikely. Test procedures require specific cell lines.

Necrosis is detected in a flow-cytometer using propidium iodide (PI): The cytotoxicity test is based on the inability of this fluorescent dye to penetrate intact cell membranes of viable cells. In contrast, the dye penetrates necrotic cells with damaged cell membranes and intercalates with DNA in the cell nucleus **(protocol 101**).

In contrast to necrosis, apoptotic cell death is characterized by a programmed sequence of complex processes. Therefore, application of several test procedures specific for individual steps during apoptosis is required. One indicator of apoptosis is the mitochondrial membrane potential (MMP): Only in intact cells, the cationic dye tetramethyl rhodamine ethyl ester (TMRE) accumulates within mitochondrial membranes. Therefore, cells with an intact MMP can be distinguished from cells with impaired MMP by their intensive red fluorescence in the flow cytometer and a decrease in fluorescence upon toxic exposure (protocol 102). As a second test method for apoptotic effects, DNA cleavage into nucleosomes is studied using a CDD^{+}-ELISA (cell death detection protocol 103). As characteristic fragments of genomic DNA, nucleosomes are DNA-histone complexes. The more the DNA is degraded, the more intensive the reaction

Table 1: Cell lines required for the recommended procedures

Cell line	Organ	Method	Origin	Protocol
Jurkat (suspension culture)	**Blood** T-lymphocytes T-cell leukemia	**Nekrosis** (PI) **ROS** (DHE) **Apoptosis** (MMP, TMRE)	Leibniz Institute, DSMZ, Brunswick (Germany), ACC-282	**Protocol 1**
U-937 (suspension cul-ture)	**Blood** Monocytes Histiocytic Lymphoma	**Apoptosis** (CDD^{+}-ELISA) (Caspases)	Leibniz Institute, DSMZ, Brunswick (Germany), ACC-5	**Protocol 2**
HepG2 (adherent cells)	**Liver** Hepatocellular carcinoma	**RTCA™** **ROS** (DCFH-DA) **Apoptosis** (MMP, TMRE)	Leibniz Institute, DSMZ, Brunswick (Germany), ACC -180	**Protocol 3**
SH-SY5Y (adherent cells)	**Nervous system** Neuroblastoma Subclone of the SK-N-SH cell line	**RTCA™** **ROS** (DCFH-DA) **Apoptosis** (CDD^{+}-ELISA, Caspases) **Neurite Outgrowth Assay** **Neuronspecific Enolase** **p38 MAP kinases**	Leibniz Institute, DSMZ, Brunswick (Germany), ACC -209	**Protocol 4**
Primary cells	**Organ**	**Detection method**	**Origin**	**Protocol**
Normal Human Astrocytes (NHA) (Clonetics™; adherent cells)	**Nervous system** Astrocytes	**RTCA™**	Lonza CC-2565	**Protocol 5**

with the fluorescent dye and the further apoptosis has progressed. In the course of apoptotic processes in the cells, a cascade of proteases known as caspases is activated. The measurement of caspase activities is a biochemical method to identify apoptosis (protocol 104): Strong caspase activity is an indicator of late stages of apoptosis.

In addition to necrosis and apoptosis, disturbances in the redox status of the cell are indicators of potential toxic effects. Both the formation of reactive oxygen species (ROS) and the consumption of cell-specific antioxidants such as glutathione (GSH) play an important role. Since ROS represent an entire group of substances, they are detected by two methods: (1) a test based on the dye DCFH-DA (protocol 105) and (2) a test with DHE (protocol 106). By this combination, a broader spectrum of ROS can be captured. The tripeptide glutathione (GSH) is used to neutralize ROS; it is present intracellularly at high concentrations. Changes in GSH content as visualized by the fluorescent dye monochlorobimane are therefore an important indicator of disturbances in the redox status (protocol 107).

As a result of these investigations, EC_{10} and EC_{50} values are determined, which serve as an initial maximum concentrations for subsequent studies. Since the first test stage does not use nerve cells, it is essential to carry out stage 2 tests, even if no toxic effects were found on stage 1, since it cannot be excluded that toxicity is specific of nerve cells.

***In vitro* testing stage 2: Organ-specific toxicity**

Test stage 2 has been designed to increase specificity with respect to neurotoxicity. Since the toxicity identified in stage 1 does not need to be neurotoxicity, comparative investigations with liver cells (hepatocarcinoma cell line HepG2) and nerve cells (neuroblastoma cell line SH-SY5Y) are performed in the second test tier. As in stage 1, the formation of ROS and the induction of apoptosis are investigated. In addition, changes in cell morphology and cell growth are recorded by means of a Real Time Cell Analyzer (RTCA™).

In stage 2 tests, apoptosis is detected by recording caspase activity **(protocol 104)**, and formation of ROS is measured with DCFH-DA (protocol 105). In the Real Time Cell Analyzer (RTCA™), impedance is measured to determine changes in cell morphology and/or number **(protocol 108)**. A reduction of impedance is an indicator of cell death or detachment of adherent cells from the bottom of the well.

If the comparison with hepatic cells indicates that nerve cells react more sensitively than liver cells in at least one test system, this is considered an indication of potential neurotoxicity, and studies on stage 3 will have to follow. If nerve cells do not react more sensitively than liver cells, no further testing is currently required out, and the substance is assumed not neurotoxic.

***In vitro* testing stage 3: Further investigation of neurotoxicity**

The third stage aims at evaluating the neurotoxic potential with reference to the HRIV concept. As in step 2, RTCA™ is used to record possible effects of the test substance on cell number and morphology. For improved specificity, however, primary human astrocytes are used in stage 3, since these are more sensitive to toxic chemicals than neuroblastoma cells **(protocol 108)**.

Another component of stage 3 testing is the determination of neuron-specific enolase **(NSE; protocol 109)**. Enolases are enzymes of glucose metabolism, with γ-enolase being specific of nerve cells. An increase in NSE concentrations both intracellularly and in the cell culture supernatant is an indicator of disturbances in neuronal cell metabolism.

The third component of stage 3 testing is the detection of neurite differentiation by the so-called Neurite Outgrowth Assay, which analyzes whether exposure

of SH-SY5Y cells to the test substance results in differentiation of the cells and thus leads to the formation of neurites **(protocol 110)**. Changes in the degree of differentiation are indicators of the neurotoxic risk potential of the test substance.

If the test substance fails to give any indication of neurotoxicity in stage 2 testing, it is currently classified as non-neurotoxic and stage 3 testing is not required. If, however, stage 2 testing provides at least one indication of neurotoxicity, stage 3 testing is performed. If at least two stage 3 assays provide evidence of neurotoxic effects, the substance is classified as neurotoxic. In order to obtain a result as robust as possible, all three assays of stage 3 have to be carried out.

According to the HRIV concept, a substance classified as neurotoxic has a HRIV of 0.3 µg/L.

In vivo investigations in the neurotoxicity module

The *in vivo* part of the neurotoxicity test battery is performed in embryos of the zebrafish (*Danio rerio*), which are not classified as protected life-stages in terms of animal welfare under EU Directive 2010/63/EU (Braunbeck et al., 2015; EU, 2010; Strähle et al., 2012). For testing, zebrafish embryos are exposed according to OECD test guideline no. 236 **(OECD 2013; Protocol 111)** and then analyzed for changes in the two following endpoints:

(1) alterations of acetylcholinesterase activity at cholinergic synapses **(protocol 112)**; and
(2) changes of the neuromasts in the lateral line system of the fish **(protocol 113)**.

As further endpoints, exposure-related changes in olfactory epithelia and retina were investigated (Figure 10). However, in the sublethal range of EC_{10} values, effects in these sensory organs proved not to be sensitive enough to demonstrate a harmful effect in concentrations relevant for drinking water.

Base level of *in vivo* neurotoxicity testing: Fish embryo test

As in the *in vitro* tests for the detection of neurotoxic effects (see above), it is of crucial importance that primary neurotoxic effects are separated from changes that result as secondary effects in the course of general toxicity. For this purpose, EC_{10} values for both acute toxic and sublethal effects (see Table 2) are determined from the fish embryo test according to OECD TG 236 (Protocol 111). The EC_{10} values are the maximum concentrations tested in all subsequent *in vivo* neurotoxicity tests.

In vivo detection of specific neurotoxic effects I: Acetylcholinesterase assay

The inhibition of acetylcholinesterase after exposure to insecticides from the class of phosphoric acid esters and carbamates is a classical enzyme assay for neurotoxic effects in animal organisms, which was optimized for the embryos of the zebrafish (Kais et al., 2015; Küster and Altenburger, 2006; **protocol 112**). The EC10 value determined from the fish embryo test is used as the maximum test concentration (Figure 11); higher concentrations should not be used, since non-specific toxic effects might pretend specific neurotoxic effects. The activity of acetylcholinesterase can be determined photometrically in microtiter plates over a longer period with high precision using the method described in protocol 112 (Figure 12).

In vivo detection of specific neurotoxic effects II: Neuromast assay

The lateral line organ of aquatic vertebrates consists of neuromasts, secondary sensory cells corresponding to the hair cells in the human inner ear (Stengel et al., 2017); therefore, changes in the neuromasts of the lateral line organ are also used as indicators of damage to hearing in mammals and humans. Neuromasts can be visualized *in vivo* with simple fluorescent dyes in 72 h old embryos of the zebrafish (Figure 13): DASPEI ([2-[p-(dimethylamino)styryl]ethyl-pyridinium iodide) is used to stain mitochondria, DAPI (4′,6-diamidino-2-phenylindole) is used to stain the nuclei of the neuromasts. Damage to the neuromasts after exposure to neurotoxic substances can be visualized by a decrease in staining intensity (Figure 14; **Protocol 113**).

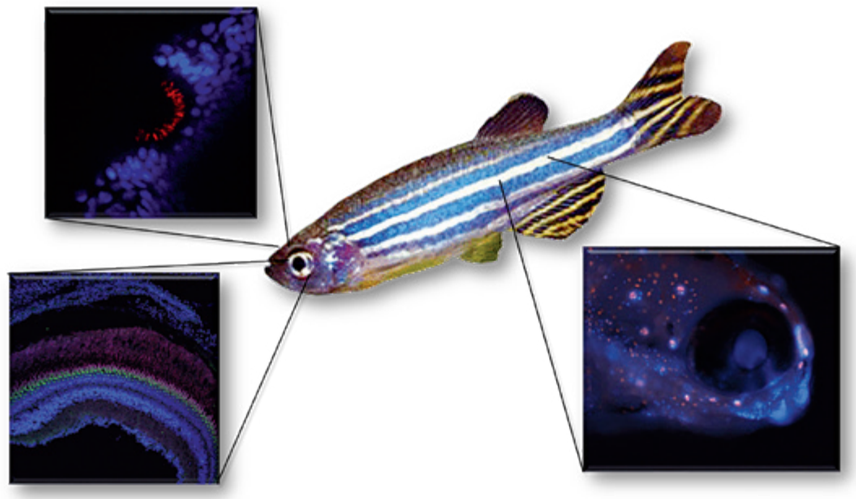


Figure 10: Detection of specific neurotoxic effects in the olfactory epithelium (top left), the retina (bottom left) and the lateral line organ (neuromast assay) in zebrafish (Danio rerio; Stengel et al., 2017).

Table 2: Toxicological endpoints for acute and sublethal toxicity in the fish embryo test with zebrafish (*Danio rerio*) according to OECD TG 236 (compiled from Ensenbach, 1998; Nagel, 2002 and Braunbeck et al., 2015).

	Toxicological endpoints	24 h	48 h	72 h	96 h
Acute Toxicity (OECD TG 236)	Coagulation of embryos	◆	◆	◆	◆
	Lack of somite formation	◆	◆	◆	◆
	Non-detachment of the tail bud	◆	◆	◆	◆
	Lack of heartbeat		◆	◆	◆
Sublethal endpoints (teratogenicity)	Lack of blood circulation		○	○	○
	Decrease in blood circulation		○	○	○
	Cardiac malformation		○	○	○
	Head malformation		○	○	○
	Malformation/absence of eyes	○	○	○	○
	Malformation of sacculi/otoliths	○	○	○	○
	Malformation of somites	○	○	○	○
	Malformation of the tail	○	○	○	○
	Shortening of the tail	○	○	○	○
	Spinal malformations (scoliosis, lordosis)	○	○	○	○
	General developmental delay	○	○	○	○
	Formation of edemata	○	○	○	○
	Changes/absence of pigmentation		○	○	○
	Deformation of the yolk sac	○	○	○	○
	Lack of spontaneous movement	○	○	○	◆

◆ Lethal criteria for the determination of acute toxicity (LC values)
○ Sublethal changes used to determine EC values

Within the framework of the Tox-Box project, an atlas for substance-specific changes of the neuromasts was prepared, with effects being quantified using a four-stage scoring (Stengel et al. 2017; Table 3; Figure 15).

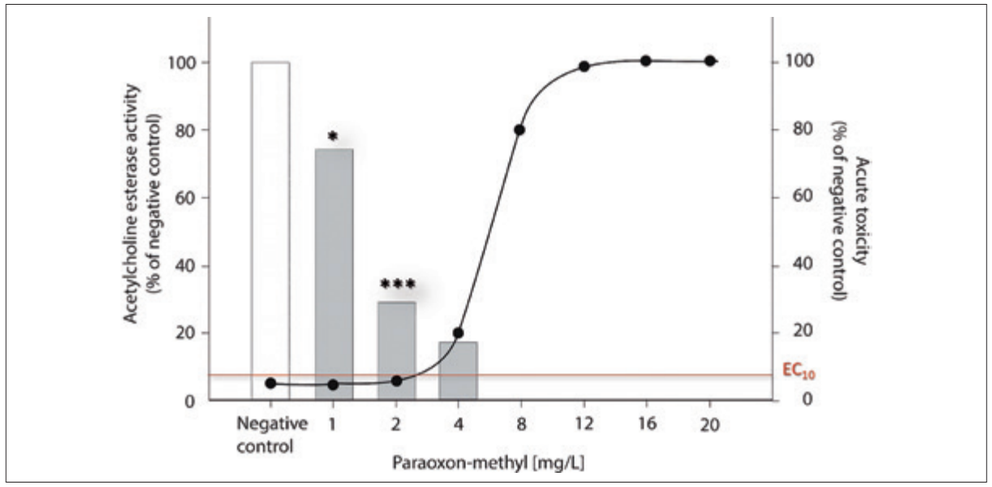


Figure 11: Specific acetylcholinesterase activities and acute and sublethal toxic effects in zebrafish embryos (*Danio rerio*) after 96 h exposure to paraoxon-methyl. The inhibition of acetylcholinesterase (left abscissa; grey columns in % activity of the negative control = bright column) already starts at substance concentrations significantly below the EC_{10} value (right abscissa; line graph). Source: Kais et al. (2015).

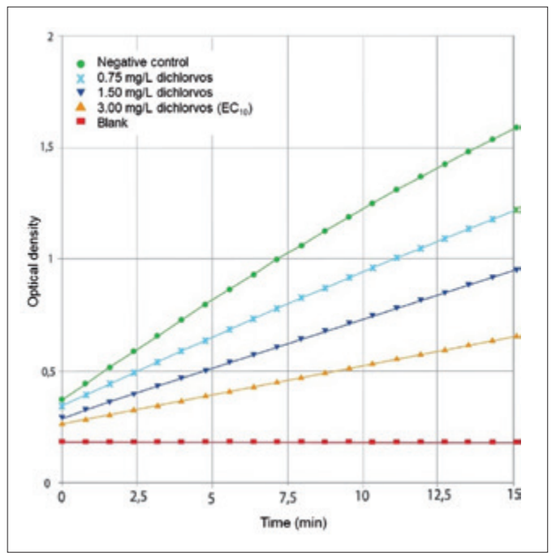
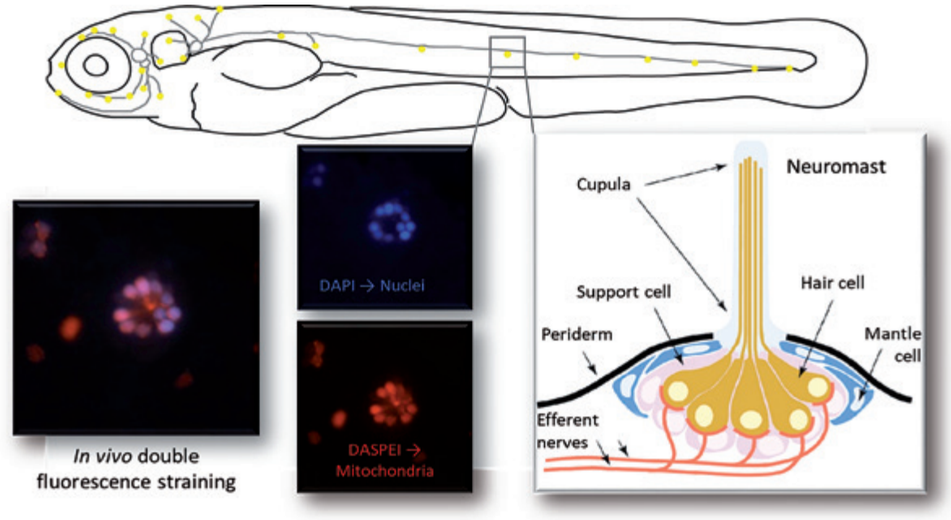


Figure 12: Neurotoxic effect of different concentrations of the phosphate ester pesticide dichlorvos on acetylcholinesterase in embryos of the zebrafish (*Danio rerio*). Non-exposed embryos serve as negative control; blank are determined without embryos. The activity of acetylcholinesterase can be tracked as a linear reaction over extended periods of time (Stengel et al., 2017).



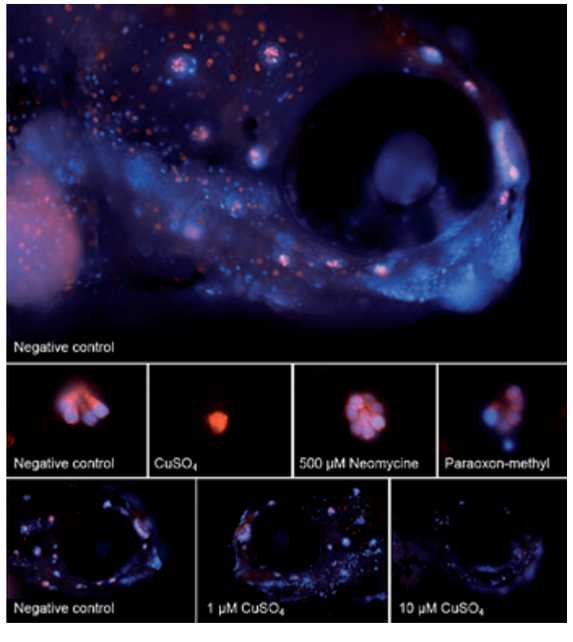


Figure 13: Based on a double-staining with DASPEI for mitochondria and DAPI for nuclei, neuromasts in the lateral organ of the zebrafish (*Danio rerio*) can be stained selectively *in vivo* (Stengel et al., 2017).

Figure 14: Fluorescence-optical representation of the neuromasts at the head of a 96 h old embryo of the zebrafish (*Danio rerio*) in control (large image, NC) and after exposure to neomycine, paraoxon-methyl and various concentrations of copper sulfate (Braunbeck et al., 2015).

Table 3: Quantification of damage to the neuromasts in the lateral organ of the zebrafish (*Danio rerio*) after combined staining with DAPI and DASPEI

Degree of damage (score) / Quality of staining	
0	Hair cells present, strong fluorescence
1	Reduced hair cell count and/or reduced fluorescence
2	Few hair cells present, fluorescence strongly reduced
3	No more fluorescence detectable

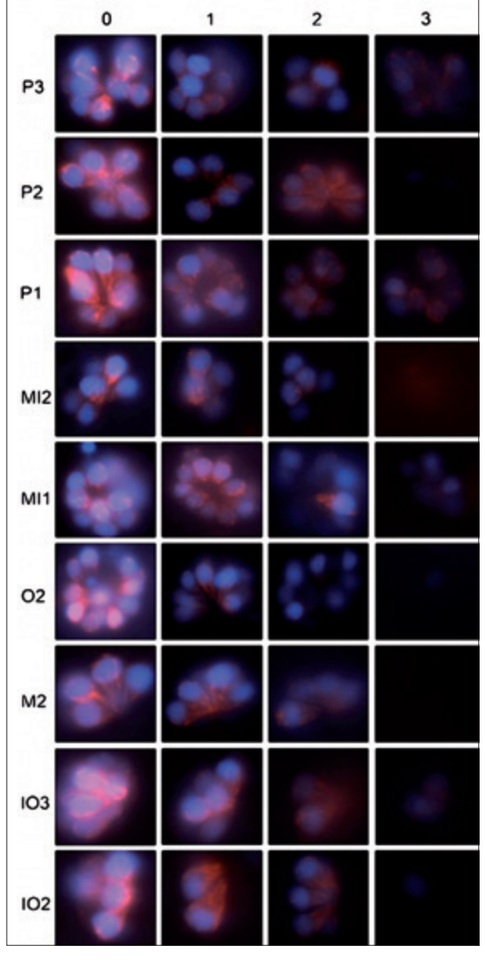


Figure 15: Four-stage scoring for substance-related damage to neuromasts in 96 h old embryos of the zebrafish (*Danio rerio*; Stengel et al., 2017).

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4. Module endocrine effects

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4 Module endocrine effects

Henner Hollert, Thomas-Benjamin Seiler, Jochen Kuckelkorn, Sibylle Maletz, Regine Redelstein, Petra Waldmann, Birgit Kneib-Kissinger, Walter Schadenboeck

4.1 Definition of the mode of action and scientific background

An endocrine active substance (endocrine disruptor or endocrine-disrupting chemical abbreviated EDC) is an "exogenous substance or mixture that alters function(s) of the endocrine system and consequently causes an adverse health effect in an intact organism, or its progeny, or (sub)populations" according to the WHO (2002).

Homeostasis, reproduction and the development and behavior of an individual are among the essential functions of the endocrine system. These and other functions are regulated in the target tissue through hormones and hormonal receptors. Via interactions of the EDCs with the synthesis, secretion, transport and binding behavior or through activation or elimination of the body´s natural hormones the concentration of hormones in the body can increase or decrease and therefore (negatively) influence these functions (Burkhardt-Holm, 2010).

Endocrine active substances can be found in a variety of everyday items as well as chemical formulas (Duis et al., 2014). Through these they can enter the water cycle through diverse exposure pathways (e.g. rainwater drainage, sewer systems and sewage treatment plants, leaching & groundwater runoff, erosion, deposition, plastic materials in contact with drinking water). In nearly all ecosystems that underlie anthropogenic influences, anthropogenic substances have been detected. A part of these substances have been proven to show endocrine activity (Gavrilescu et al., 2015). Consequently, every human could be exposed to these substances in small doses. Furthermore, there is evidence that endocrine active substances cause adverse effects in relatively low concentrations (Vandenberg et al., 2012). In ecotoxicology 5 ng/L ethinylestradiol could be shown to have effects on a fish on a population scale (Kidd et al., 2007). Moreover, some known endocrine disruptors are also known to be highly accumulative (e.g. PCB, DDT, benzo[a]pyrene, dioxins) and can therefore reach higher concentrations in tissues over the course of time.

Occurence	Name	Application
Endogenous hormones and metabolites	Estrone (E1) 17β-Estradiol (E2) Estriol (E3) Testosterone (T)	
Artificial hormones	17α-Ethinylestradiol (EE2)	Birth control pill
Phytoestrogens	Isoflavones Lignans	Food supplements Sedatives (alternative medicine)
Industrial chemicals	PCBs Flame retardants (e.g. PBDEs) Plasticizers (e.g. phthalates, TBBPA, BPA)	Hydraulic fluids in paint, insulating agents, plastics Electronic devices Upholstered furniture and carpets Foils, cables and rubber products
Pesticides and metabolites	DDT Lindan	Plant production products and biocides
Byproducts of industrial processes	Benzo[a]pyrene Dioxine	Combustion products
Heavy metals	Cadmium Lead	Corrosion protection (semiconductor manufacture), accumulators Automotive industry / chemical industry

Since the 1990s, endocrine active substances in aquatic bodies have come into the focus of both the public interest and the scientific community due to their potentially adverse effects for animals and humans. Endocrine active substances are not only toxic for the reproductive system, but can also be genotoxic, neurotoxic as well as immunotoxic (see Figure 16, Choi et al., 2004). The importance of endocrine disruptors in regulatory practices is constantly increasing and put into an international perspective by Hecker & Hollert (2011).

In 2013, the United Nations Environment Program (UNEP) and WHO released "State of the Science of Endocrine Disrupting Chemicals – 2012"a 289-page update of the International Program on Chemical Safety (IPCS) document from 2002 (WHO – World Health Organization, 2002; WHO – World Health Organization and UNEP – United Nations Environment Programme, 2013). The report summarizes the current global status of scientific knowledge of the effects of EDCs on ecosystems and humans.

The vast number of (potentially) endocrine active substances, the diversity of known and potential modes of action of some of these substances, as well as the effectiveness of some substance in relatively low concentrations confirm the relevance of this research field (Caliman & Gavrilescu, 2009; Leusch, 2008; Moltmann et al., 2007). The ecotoxicologically relevant trace substances include, in particular, those that already cause significant (endocrine) effects in relatively low concentrations such as 17α-ethinylestradiol (Kidd et al., 2007). They occur in the environment in concentrations as low as <10 ng/L to approximately 1 µg/L (Fent, 2013). In general, substances that are known to be highly persistent, bioaccumulative and /or have high biological activity are especially important. However, high polarity and low sorption are main properties which enable their entry into the drinking water system (Grummt, 2011).

Among the drinking water-relevant, endocrine active substances (trace substances) are substances such as pharmaceuticals, pesticides, detergents, flame retardants and industrial chemicals (Eldridge & Stevens, 2016; Tijani et al., 2016). They can reach ground and surface waters through industrial effluents, agriculture, run-off from surfaces or through sewage treatment plants, which only

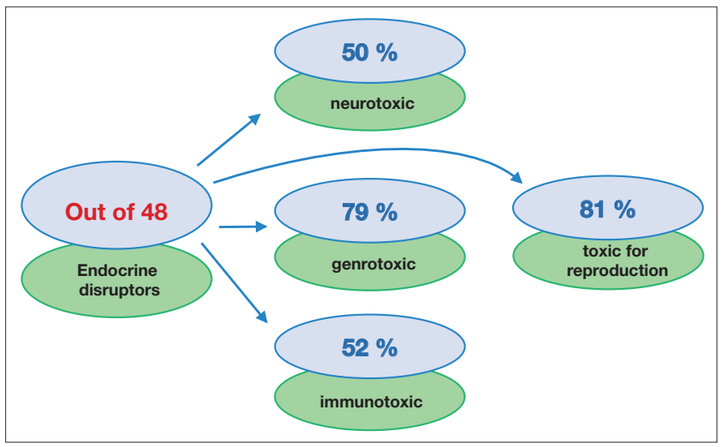

Figure 16: Percentage distribution of adverse effects of endocrine disruptors
(Choi et al., 2004)

can only eliminate them partially, if at all (Caliman et al., 2009; Chou et al., 2016; Kassotis et al. 2016).

Numerous studies have shown a direct influence of endocrine active substances on various aquatic organisms such as snails and fish (Jobling et al., 2004; Kidd et al., 2007; Oehlmann et al., 2007; Oehlmann et al., 2000; Pal et al., 2010; Schmitt et al., 2008; Schulte-Oehlmann et al., 2000; Sohoni et al., 2001; Tillmann et al., 2001). Systematic test series do not exist for drinking water, but this should be demanded for the long term. The indications from ecological tests series (early warning system) and the issue of synthetic material in contact with drinking water do not necessarily exclude endocrine risk potentials.

Furthermore, EDCs can also be considered as causes of human health impairments (Dallinga et al., 2002; Morales-Suarez-Varela et al., 2011; Safe, 2002; Toppari et al., 1996). It has been suggested that xenoestrogens influence the beginning of puberty of young girls (Aksglaede et al., 2009; Mouritsen et al., 2010), the fertility of women (Zama et al., 2016; Crain et al., 2008) and disorders of the menstrual cycle of women (Cooper et al., 2005; Farr et al., 2004). Furthermore, there are indications that EDCs decrease the sperm quality of men (Guo et al., 2000). The influence of EDCs on hormone-driven cancer types e.g. breast cancer is undisputed (Doherty, 2010). Another study found endocrine disrupting substances in carbonated water from plastic bottles (Wagner & Oehlmann, 2009). Zhang et al. (2017) found increased concentrations of fluoren-9-bisphenol (BHPF), a substitute substance of the highly controversial bisphenol A, in water from babies' and children's bottles made from different plastics. The substance produced a high anti-estrogenic effect in tests with mice; such as disruptions during pregnancy and an influence on the birthweight of the progeny. BHPF is, as of now, not approved in Europe for the usage in synthetic materials that come into contact with food products.

4.2 State of regulation

So far there are no compulsory, binding, legally mandated decision criteria for the definition and classification of endocrine disruptors (EDCs) in the EU. Consequently, there is still no extensive regulation of EDCs in the field of environmental and health protection. For instance, there are neither thresholds for endocrine activity in drinking water, ground water or surface waters nor the obligation to measure them. However, this is supposed to be addressed in the new EU Drinking Water Directive, which is currently in progress.

Recent activities of the European Commission to enhance regulations of EDCs have been massively influenced by the pesticide industry and lobby groups. Currently, the European Commission is attempting to establish a definition for the substance class of EDCs, which should serve as the basis for appropriate classification and regulation. The outcome of these efforts is however still unresolved.

Among these measures that have been taken so far is the restriction of known EDCs such as bisphenol A, nonylphenol or diethylhexylphthalate (DEHP). Compared to the great number of potential EDCs and their widespread use in many everyday products, these measures are far from sufficient.

The iincreased awareness of EDCs impact on humans has created an influence on the market. Especially for babies' and children's drinking bottles; the consumer is increasingly paying attention to labels such as "BPA- and phthalate free" and „plasticizer-free"

4.3 Test strategy for the assessment of endocrine activity

After the analysis of laboratory results the following hierarchic test strategy has been proposed (Figure 17).

With the respective test systems, concentration-effect-relations as well as the lowest observed effect concentration (LOEC) shall be determined, and then will be included in the HRIV-concept. Unless the attached working instructions state otherwise, the cytotoxic effects should not be higher than 20%.

If the chemical structure of the test substance is known, it should be assessed using QSAR-programs before performing the first steps. These can predict (potential) active metabolites and simplify the decision regarding the order of the tests related to the metabolic activation of the test substance (e.g. with rat liver homogenate S9).

The investigated substance is assessed for endocrine activity in the first screening-step. During this first run, receptor-mediated tests are suggested, that allow a high throughput and fast execution but can nevertheless sensitively determine a potential effect of the substance. If the single substances cannot be metabolized in the test system, an additional metabolic activation of the substance using an S9-mix (metabolic complementation with S9) is recommended, to also

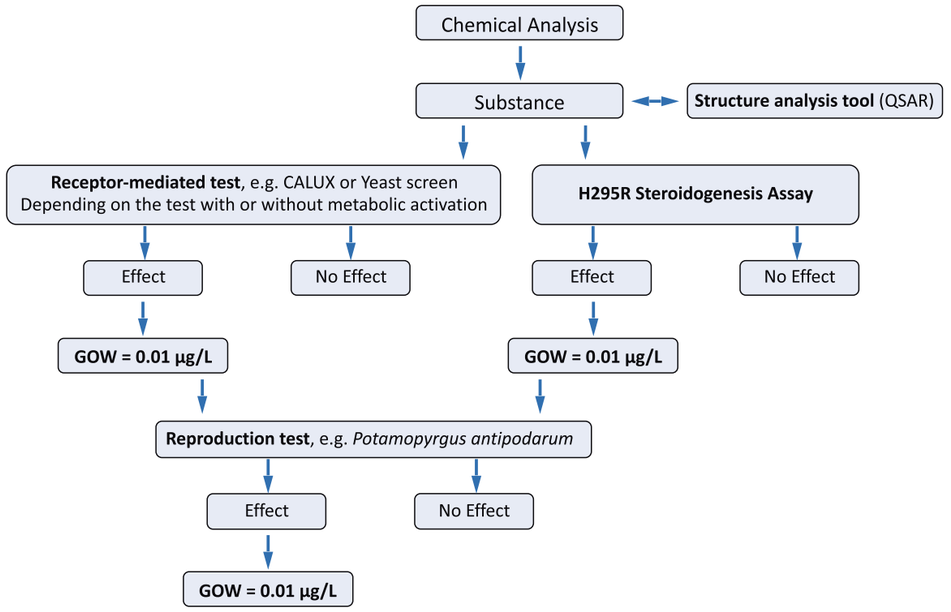

Figure 17: Schematic overview of the test strategy for endocrine effects. The concentration is given an equivalent of the respective hormone.

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account for endocrinal active metabolites that could form in mammals. This way additional information of human-toxicological relevance can be obtained. For the further evaluation of a substance, a detectable endocrine effect should be used to perform a chemical-analytical determination of possible metabolites (Brinkmann et al., 2014). In case of a positive result, the substance should be evaluated according to the HRIV-concept.

It is recommended to also run a non-receptor-mediated test in parallel. Substances that are endocrinal active without receptor binding can still be detected e.g. through the influence on the steroidogenesis using this test. These tests also offer the possibility to elucidate further mechanism-specific modes of action. For the endocrine effect, the substance will be evaluated using the HRIV-concept.

An additional possibility for evaluation is the reproduction test using the New Zealand mud snail *Potamopyrgus antipodarum* (Figure 18). In contrast to the previously explained *In vitro*-test systems, a specimen-based test is conducted. Endocrine effects directly influence the number of progenies of the mother in this test, which allows a limited inference of potential effects on the population of the species. This test is conducted for 28 days and is suitable for long-term assessments of a substance appearing in drinking water.

In the following segment, the test system for the assessment of (potential) endocrine active substances as suggested in the module Endocrine Effects within the test strategy are explained briefly.

Detailed test protocols, guidelines and SOPs are provided in 4.4.

4.4 Test systems

ERα- and AR-CALUX®-Assay

The ERα- and AR-Calux® Assays (Estrogen-/Androgen-Responsive Chemical-Activated LUciferase gene eXpression) determine receptor-mediated endocrine activity using the human osteosarcoma cell line U2OS. The cell line has been transfected with the reporter plasmids pEREtata-Luc or pAREtata-Luc as well as the expression plasmids pSG5-neo-hERα or pSG5-neo-hAR. Due to the binding of the agonist-receptor-complexes with the respective responsive element the enzyme luciferase is transcribed (Legler et al. 1999, Sonneveld et al. 2005). The luciferase production increases as more complexes with estrogens/androgens or estrogen/androgen-like substances or substance mixtures are provided by the drinking water. The substances can also be metabolically activated through the addition of "S9-Mix "(rat liver homogenate) (Kuckelkorn et al. 2018). After the addition of the substrate luciferine the estrogen/androgen-like activity can be determined through a luminescence measurement in comparison with a reference exposure with either 17ß-estradiol or dihydrotestosterone. An estimate of the endocrine activity can be made by calculating the estradiol-/testosterone-equivalence-factor

$$(EEF/TEF = \frac{EC50\ (E2/T)}{EC50\ (Probe)})$$

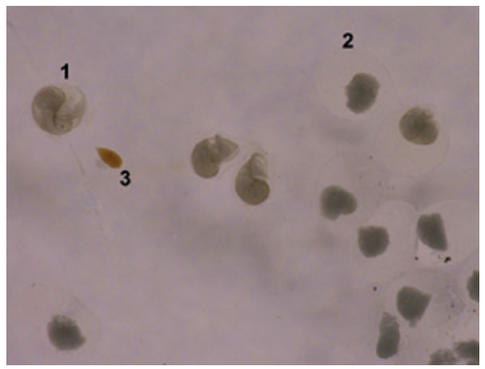
of the dose-response-curves. Anti-estrogenic and anti-androgenic-like effects can be determined and quantified with the use of basis concentrations of 17β-estradiol or dihydrotestosterone and the usage of standards of the respective receptor-antagonists tamoxifen or flutamide during the entire test. The ERα-/AR-Calux® was recommended by the Global Water Research Council based on an inter-laboratory comparison of complex environmental samples for the assessment of ground waters and waste waters (GWRC 2008). Additionally, the use of human cell based *In vitro*-reporter gene-test systems have been proposed in a current template of ISO-norms (ISO 19040 Water quality – Determination of the estrogenic potential of water and waste water; Section 3).

RYES/RYAS

The yeast strains used (RYES/RYAS, D.P. McDonnell, 1991) have been genetically modified so that the human estrogen-/androgen-receptor is formed in the presence of copper ions. Furthermore, the fusion product of a specific response gene (estrogen responsive element (ERE) or androgen responsive element (ARE)) and a reporter gene (LacZ, codes for β-galactosidase) have been inserted using a plasmid. The estrogen/androgen active substance binds to the specific receptor and activates it. This active receptor acts as a transcription factor and binds the respective fusion products (ERE/ARE), so that LacZ is transcribed and β-galactosidase is formed. The enzyme cleaves a colourless substrate into galactose and a dye, which is then photometrically detected. The test is currently going through the ISO/DIN-standardization process. The receptor plasmid of the RYES (YePtrpER) carries the gene for the human estrogen receptor α and a selection marker (tryptophan auxotrophy). The reporter plasmid (YrpE2ura) of this strain carries two copies of a gene (vitellogenin) that can be activated by the estrogen receptor, which is also coupled with the reporter gene (lacZ gene) and the selection marker (uracil auxotrophy). The reporter plasmid of the RYAS strain (Yep24leu2) carries the gene for the human androgen receptor and a selection marker (leucine auxotrophy). The reporter plasmid (YrpG2his) carries two copies of an androgen response gene, which is coupled to the lacZ gene and a selection marker (histidine auxotrophy).

H295R Steroidgenesis Assay

The H295R steroidogenesis assays is an *in vitro*-test system for the identification of non-receptor-bound effects of endocrine disruptors (Hecker et al. 2011). The used cell line H295R is a human adrenal cortex carcinoma cell, which produces all relevant enzymes for the synthesis of steroids such as estradiol, testosterone and progesterone as well as glucocorticoids (Gazdar et al. 1990, Staels et al. 1993). Multiple substances can inhibit or induce the expression and activity of enzymes. A disruption of the steroid synthesis can be measured through the concentration of produced steroids or via competitive enzyme-linked immunosorbent assays (ELISA) or can be measured analytically using LC/MS-MS. Changes of gene regulation can furthermore be observed using quantitative real-time PCR (qRT-PCR). The exposed cells are also checked for cell vitality to estimate the influence of possible cytotoxicity on the results. The H295R steroidogenesis assay is performed according to OECD Guideline 456 (OECD 2011).



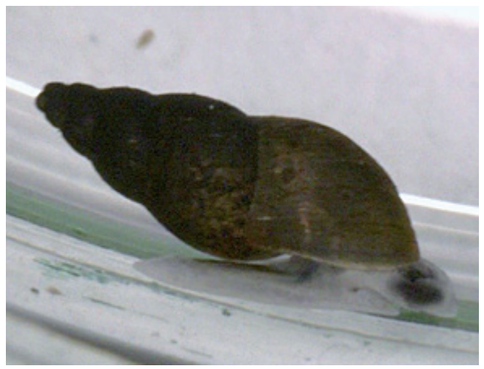


Figure 18: Developmental stages (left): 1 Embryo with shell, 2 Embryo without shell, 3 Snail embryos; (Adult parent) (right)

Reproduction test with *Potamopyrgus antipodarum*

The reproduction test with the New Zealand mud snail *Potamopyrgus antipodarum* detects endocrine substances in water and sediments within a time frame of 28 days as an *In vivo*-test (Duft et al. 2007, Schmitt et al. 2013) according to the OECD-Guideline for Testing of Chemicals 242 (Schmitt et al 2013, OECD 2016). Due to the parthenogenetic reproduction of the female snails (Ovoviviparity) it is possible to determine the number of embryos and reproductive output per adult snail. The substances and mixtures of substances in drinking water samples can be classified according to the dose-dependent endocrine activity either as an inhibition or induction of reproduction (Duft et al. 2003). Data from this test system allows a direct estimation of the effects on the whole population. The test with P. antipodarum has been internationally validated since 2012 and has been approved as OECD Guideline 242 since 2016.

4.5 Test protocols of the test procedures and evaluation of results

For the standardized implementation of the bioassays, already established and evaluated test protocols of the OECD and university research institutes were used.

- OECD Guideline for the Testing of Chemicals 456 - H295R Steroidogenesis Assay (28.07.2011)
- OECD Guideline for the Testing of Chemicals 242: *Potamopyrgus antipodarum* Reproduction Test (29.07.2016)
- Department Aquatic Ecotoxicology, Goethe University Frankfurt a. M. Schmitt et al. Draft Guideline for Testing of Chemicals – Reproduction test with the mudsnail *Potamopyrgus antipodarum* (01.2013)
- LFG Ökosystemanalyse, RWTH Aachen University. Maletz et al. und Kuckelkorn et al. Standard Operating Procedure (SOP) – ERα und AR Calux (12.02.2016 und 03.11.2016)

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Frequently asked questions (FAQ) about HRIV 5

5 Frequently asked questions (FAQ) about HRIV

1. For which substances are HRIVs available?

In principle, HRIVs are available for all substances with concentrations > 0.1 µg/L in drinking water which are not yet adequately assessed in terms of human toxicology – so limit or guideline values for these substances are not yet established. For substances with chemical structures that lead to major concerns regarding genotoxicity, appropriate initial evaluation will also be made at concentrations < 0.1 µg/L.

Background information:

A drinking water guideline value can be calculated based on ADI (Acceptable Daily Intake) values, i.e. the dose which can be daily ingested via food, water and air over a lifetime without health risks. Similar to an ADI value the drinking water guideline value indicates the maximum concentration of a particular substance in drinking water that can be ingested over a lifetime without health risk. According to WHO guidelines a daily intake of 2 litres of water is used for calculation. Contrary to the WHO recommendations a body mass of 70 kg (instead of 60 kg) and an allocation of the ADI for drinking water of 10 % vs. 20 % is used in EU.

Limit values are politically defined maximal concentrations of natural ingredients, residues concerning active substances and environmental contaminants in food, consumer products and environmental media that are embodied in laws and regulations. They have proven useful for regulatory requirements to manage chemicals and many other potentially noxious agents in all fields of human environment. Toxicologists, physicians, ecologists, environmental technicians and engineers provide options regarding need, type and level of limit values as scientifically (toxicologically, medically, environmentally) or technically (practical requirements, avoidance issues) derived concentrations (Dieter 2011). Limit values (like guideline values) are maximum values that human beings can be exposed to over a lifetime without risk to human health and therefore are still acceptable.

2. Who defines HRIVs?

The approach to derive HRIVs is published (Dieter 2014) and can be used by toxicologists. The UBA derives an HRIV for a defined substance, if the determined concentration exceeds the precautionary value for substances in drinking water of 0.1 µg/L and a benchmark for that assessment is needed, i.e. a water supplier, the appropriate health department, the regional government authority or another authority have requested the evaluation of the detected concentration (and therefore, if necessary, an HRIV).

The advantage of an HRIV derivation by the UBA is a consistent and nationwide application of the procedure and thus for the evaluation itself. HRIVs are not defined for substances in raw water (particularly surface waters), neither as a precautionary measure, if they are of no concern in drinking water.

3. How is the HRIV derived?

HRIV are derived according to the available data using following levels:

a. Are data regarding genotoxicity available? If not or existing data indicate genotoxicity, $HRIV_1$ = 0.1 µg/L is applied.

b. Special cases are a few genotoxic substances (e.g. nitrosamines) which are particularly relevant for human beings (e.g. due to a known metabolism that is relevant in humans). An $HRIV_0$ of 0.01 µg/L is valid for these substances, e.g. for N-nitrosodimethylamine (NDMA).

c. If genotoxicity can be excluded, then $HRIV_2$ = 0.3 µg/L is applied.

d. Are data regarding neuro- or immunotoxicity available? If neuro- and immunotoxicity can be excluded, then $HRIV_3$ = 1.0 µg/L is valid, otherwise $HRIV_2$ = 0.3 µg/L is used (see c.).

e. Are data regarding subchronical toxicity available? If subchronic toxicity can be excluded, then $HRIV_4$ = 3.0 µg/L is applied, otherwise $HRIV_3$ = 1.0 µg/L is valid (see d.).

f. Are data regarding chronic toxicity available? If chronic toxicity can be excluded for relevant levels, then > 3.0 µg/L or higher (depending on data availability) is applied; otherwise $HRIV_4$ = 3.0 µg/L (see e.) is valid.

Are no toxicological data for an endpoint available, an evaluation can be made by the corresponding test strategy of the guidelines. The determination of the corresponding level values ($HRIV_{0\text{-}4}$) is based on an analysis of 140 limit and guideline values of 6 large public authorities regarding 50 different substances with various modes of action since 1993 (Dieter 2014). For each of the endpoint-specific effects mentioned in the levels, the corresponding lowest value was used. This ensures that derived limit or guideline values depending on necessary subsequent animal testing data, most likely are higher than the respective HRIV. Thus, improved toxicological data of a relevant substance rather gives the all-clear, meaning the derived value is higher than the previous HRIV. This special precautionary nature of the HRIV versus the level of protection of a limit or guideline value can avoid a feelling of uncertainty among the population due to a reduction of a previous value.

4. What is meant by metabolism relevant for humans?

The human body can protect itself from harmful substances by various mechanisms. The passage through the intestinal wall into the bloodstream can be reduced or delayed and active substances can be metabolized in the liver via biotransformation and subsequently be excreted *via* the bile. A few drinking water relevant contaminants however have a chemical structure allowing these substances to pass these defence systems mostly unmodified. Thus, genotoxic effects may be triggered. Therefore, these substances exhibit a metabolism that is relevant in humans grading those to the special classification $HRIV_0$ (0.01 µg/L).

5. Where can I find already established HRIV?

A current list of HRIV can be found on the website of the UBA:
https://www.umweltbundesamt.de/themen/wasser/trinkwasser/trinkwasserqualitaet/toxikologie-des-trinkwassers

There you can also find the list of HRIVs regarding non-relevant metabolites (nrM; according to the Plant Protection Products Regulation) of active substances in plant protection products.

6. What is being done by UBA, if no HRIV is found in the tables for a given substance?

If there is no HRIV, it will be determined by the UBA on request.

7. How long will a given HRIV be valid?

A given HRIV will be valid until its reassessment on the basis of updated toxicological data. HRIVs may be updated or in best-case replaced by (limit or) guideline values. At this time the previous HRIV is no longer valid.

8. What is being done, if the HRIV is exceeded?

An HRIV is clearly a value of prevention and sufficiently ensures, that a life-long exposure to a substance *via* 2 litres of drinking water per day will be without health risks for human beings (see 3.). Even a (short-term) excess by a factor of 10 should be without direct adverse health effects as long as the precautionary measure value mentioned below is complied with. In principle, an excess by a factor of 10 is possible, whereby the precautionary measure value is strongly event-related. In practice that means, it is decided on a case-by-case basis which measures are taken to ensure that the HRIV excess is being minimized. According to the Drinking Water Ordinance and next to the compliance of (guideline, HRIV, particularly) limit values, precautionary and minimization measures should always be introduced to achieve a lower value than the HRIV, for example. In addition to the reduction of concentrations, additional monitoring and verifications, the filling of toxicological data gaps is intended to ensure a better evaluation. The type of measures should always be decided on a case-by-case basis. Ultimately the determination of the precautionary measure value lies at the responsible health department. However, these departments can allow higher or lower excess of HRIVs independent from the UBA recommendation. According to the UBA point of view, values > 10 µg/L (precautionary measure value) are generally not tolerable over a long period of time.

9. Which transition periods until compliance with the HRIV can be tolerated by the health authorities?

In terms of health, the provision of § 6 (1) TrinkwV applies to substances with an existing HRIV. According to an explanatory memorandum to the TrinkwV, a regulation should be done, if "it is sufficiently probable, that a substance is able to have harmful effects on human health according to the state of scientific knowledge. A remote possibility or general concern of health hazard is not sufficient." It cannot be presumed, however, that concentrations exceeding the HRIV of a substance are unreserved acceptable for human health over a lifetime. Even if the HRIV is set in precautionary range, it is based on more than a remote possibility or general concern of health hazard. On the one hand, from a legal point of view, measures according to §§ 6 (4) and § 10 TrinkwV are only limited to parameters of annex 2 TrinkwV. On the other hand, according to § 9 (6) TrinkwV corrective measures, which can be ordered by the health department in the case of an excess of the chemical limit value, also apply to chemical substances with no defined limit values in the TrinkwV, that could be of relevance for human health in drinking water. On basis of scientific justification, the concept of HRIV developed by the UBA fills the legal gap between assessment and appearance of a substance without a limit value for drinking water and for which no guideline value can be derived due to insufficient toxicological data. As a result of this classification, the UBA recommends in case of an HRIV excess to proceed analogically to methods regarding excess of limit values (see, inter alia, guidelines for measures of implementation according to § 9 and § 10 TrinkwV 2001). Consequently, this does not preclude, that the competent authority first takes the "three-by-three-years-regulation" according to § 10 TrinkwV into consideration for measures, as a practical option for action, after detected excess of an HRIV. The expected duration of an excess is determined by the potential duration of the specific measures. The minimum requirement to measures is (analogously to excess of a chemical limit value), that the precautionary measure value is not exceeded during the whole duration of measures analogously to the UBA maximum value of measures (see guidelines for measures of implementation according to § 9 and § 10 TrinkwV 2001).

10. What is being done, if the HRIV is below the analytical detection limit of a substance?

In this case, as well as almost for all other value categories, the HRIV is considered to be met, when the substance is not detected. However, this should not exempt from the responsibility of lowering the detection limit to reliably monitor if the HRIV is met.

11. Is it possible to assess mixtures of substances by HRIVs?

Currently, there is no method available to evaluate mixtures of substances using the concept of HRIV. Therefore, HRIVs can only be derived for each single substance of the mixture. However, the HRIV concept is sufficiently designed for precaution, even if several substances of the same category are present as long as the corresponding HRIVs of all the single substances are met. Thus, there is no need to apply e.g. the addition rule[1] for substances with an effect threshold as long as no HRIV is being exceeded. "Health precaution" for substances without an effect threshold, i.e. with a genotoxic potency, means the restriction of each single substance of the mixture as the summation rule specifies. This means that the concentration-weighted sum of all "genotoxic potencies" correspond to a maximum risk index value of RI = 1 (Dieter 2014).

Literature:

Dieter HH (2011) Grenzwerte, Leitwerte, Orientierungswerte, Maßnahmewerte - Aktuelle Definitionen und Höchstwerte Bundesgesundheitsblatt 52:1202-1206

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1 For toxicological assessment of several substances with the same or similar effect end points / mode of actions in a substance mixture the risk index (RI) or the addition rule is applied: RI = c1/GK1 + c2/GK2 + … + cn/GK1 <=1 with c1, c2, … cn as measures substance concentration and GK1, GK2, … GKn as limit concentration of the substances within the exposition scenario.

Thus, the risk index represents the sum of quotients from the single measured exposition concentrations to the corresponding toxicological reference value, e.g. a toxicological founded and derived limit concentration for a certain exposition media (e.g. drinking water).

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List of abbreviations 7

ADI	Acceptable Daily Intake
BMBF	Federal Ministry of Education and Research
BMGS	Federal Ministry of Health and Social Security
BPA	bisphenol A
CDD^+-ELISA	Cell Death Detection (CDD^+)- Enzyme-linked Immunosorbent Assay (ELISA)
CLP	classification, labelling and packing
CYP	cytochrome P450
DCFH-DA	dichloro-dihydro-fluorescein diacetate
DDT	dichlorodiphenyltrichloroethane
DIN	German Institute for Standardization e. V.
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid (biomolecule and carrier of hereditary information)
DSMZ	German Collection of Microorganisms and Cell Cultures
EC-Wert	effective concentration or dose
EDC	endocrine disruptor or endocrine-disrupting chemical
EMA	ethidium monoazide
EU	European Union
EURL-ECVAM	European Union Reference Laboratory for Alternatives to Animal Testing
GOW	German abbreviation for HRIV („gesundheitlicher Orientierungswert“)
GSH	glutathione, reduced form
GST	glutathione-S-transferase
GSTT	glutathione-S-transferase Theta
H295R	human adrenal cortex carcinoma cell
HTS	High-Throughput-Screening
ICH	International Conference on Harmonisation of Technical Requirements
ISO	International Organization for Standardization
LC-MS/MS	liquid chromatography mass spectrometry / mass spectrometry
LOEC	Lowest Observed Effect Concentration
MMP	mitochondrial membrane potential
M_r	relative molecular mass
NAT	N-acetyltransferase

NaWaM	sustainable water management, German: Nachhaltiges Wassermanagement
NHA	Normal Human Astrocytes (primary human astrocytes)
NOEL	No Observed Effect Level
NSE	neurite specific enolase
Ntera 2	human cell line that is able to differentiate
OECD	Organisation for Economic Co-operation and Development
OSIRIS	Optimised Strategies for Risk Assessment of Industrial Chemicals through Integration of Non-Test and Test Information
PCB	polychlorinated biphenyls
PI	propidium iodide
QSAR	Quantitative Structure-Activity Relationships
REACH	Registration, Evaluation, Authorisation and Restriction of Chemicals
RiSKWa	“Risk Management of new pollutants and pathogens in the water cycle”
ROS	Reactive Oxygen Species
RTCA™	Real-Time Cell Analyzer
S9-Mix	liver microsomal fraction of rats
SH-SY5Y	human neuroblastoma cell line
SULT	sulfotransferase
TA 98 / TA 100 / TA 102 / TA 1535 …	test strains of bacteria for the Ames test (*Salmonella typhimurium*)
TMRE	tetramethyl rhodamine ethyl ester
Tox-Box	acronym for the BMBF joint research project “Hazard-based risk management for anthropogenic trace substances to secure the drinking water supply”
TrinkwV	Drinking Water Ordinance
U2OS	human osteosarcoma cell line of thigh
U.S. EPA	U.S. Environmental Protection Agency
UBA	German Environment Agency
UFZ	Centre for Environmental Research Leipzig - Halle Ltd. / Helmholtz Centre for Environmental Research Ltd.
VICH	Veterinary International Conference on Harmonisation of Technical Requirements
VW_a	general precautionary value
WHO	World Health Organization

Availability of the protocols of the mentioned detection methods 8

Detection methods with available national or international standardized protocols or standards (OECD, ISO, DIN) are mentioned in the text and can be obtained from the corresponding source. The standard operating procedures (SOP) of not yet standardized test methods are available via the following project partners:

Detection methods to evaluate genotoxicity

- RheinEnergie AG
  Wasserlabor (WL)
  Dr. Meike Kramer
  (m.kramer@rheinenergie.com)
  Parkgürtel 24
  50823 Köln

Detection methods to evaluate neurotoxicity

- ***In vitro*-neurotoxicity**

  Umweltbundesamt
  Toxikologie des Trinkwassers und des Badebeckenwassers (FG II 3.6)
  Dr. Alexander Eckhardt
  (alexander.eckhardt@uba.de)
  Ralf Junek
  (ralf.junek@uba.de)
  Heinrich-Heine-Straße 12
  08645 Bad Elster
  Germany

- ***In vivo*-neurotoxicity**

  Universität Heidelberg
  Center for Organismal Studies
  Aquatische Ökologie und Toxikologie
  apl. Prof. Dr. Thomas Braunbeck
  (braunbeck@uni-hd.de)
  Im Neuenheimer Feld 504
  69120 Heidelberg
  Germany

Detection methods to evaluate endocrine effects

- RWTH Aachen
  Institut für Umweltforschung (Biologie V)
  Prof. Henner Hollert
  (hollert@bio.uni-frankfurt.de)
  Worringerweg 1
  52074 Aachen

  New Contact: Goethe University Frankfurt
  Biologicum
  Campus Riedberg
  Max-von-Laue-Str. 13
  60438 Frankfurt am Main
  Germany

- Umweltbundesamt
  Toxikologie des Trinkwassers und des Badebeckenwassers (FG II 3.6)
  Jochen Kuckelkorn
  (jochen.kuckelkorn@uba.de)
  Ralf Junek
  (ralf.junek@uba.de)
  Heinrich-Heine-Straße 12
  08645 Bad Elster
  Germany

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Editor:
Umweltbundesamt
Wörlitzer Platz 1
06844 Dessau-Roßlau

Contact person for the BMBF funding project “Risk Management of new pollutants and pathogens in the water cycle” RiSKWa:

BMBF:
Dr. Christian Alecke
Bundeministerium für Bildung und Forschung (BMBF)
Referat 724 - Ressourcen und Nachhaltigkeit
Heinemannstrasse 2
53175 Bonn
Tel.: +49 (0)228 9957-3890
Fax: +49 (0)228 9957-83890
E-Mail: christian.alecke@bmbf.bund.de

The Karlsruhe Project Management Agency:
Dr. Verena Höckele
Projektträger Karlsruhe, Karlsruher Institut für Technologie
Hermann-von-Helmholtz-Platz 1
76344 Eggenstein-Leopoldshafen
Tel.: +49 (0)721 608-24932
Fax: +49 (0)721 608-924932
E-Mail: verena.hoeckele@kit.edu

Contact person for the BMBF joint research project “Tox-Box – Hazard based risk management of anthropogenic trace substances to protect drinking water”:

Umweltbundesamt
Fachgebiet II 3.6 Toxikologie des Trink- und Badebeckenwassers
Heinrich-Heine-Strasse 12
08645 Bad Elster
Tel.: +49 (0)37437 76-354
Fax: +49 (0)37437 76-219
E-Mail: II3.6@uba.de

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