Position paper how bioassay derived data can be applied for water quality assessment

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Summary

In vitro bioassays are techniques that promise great steps forward in drinking water security. The technique is based on engineered cells that show a reaction if the sample they are exposed to contains toxins. A wide range of in vitro bioassays has been applied for water quality assessment for:

- Hazard identification and,
- Testing of treatment efficiency of novel drinking water treatment methodologies.

These tests are mainly used as a diagnostic research and play an important role in the assessment of chemical mixtures. However, to date, regulations and water quality guidelines focus on individual chemicals. Nevertheless, in vitro bioassays for human health hazard have been accepted in other regulatory frameworks, as food safety and chemical regulation. Examples from these international regulatory policies can act as successful examples for the roadmap to implementation for the acceptance of *in vitro* bioassays in drinking water quality regulation.

A crucial step on the road to implementation of *in vitro* bioassays is their validation to demonstrate the reliability and reproducibility of a test method. However, demonstrating the validity of a method is a complicated process. All steps in the formal validation are time consuming and costly. Formal validation is performed by special institutes such as the centres for Validation of Alternatives Methods (VAM). Harmonisation of a test method (such as achieving ISO-standards and OECD guidelines) is another dominant prerequisite for regulatory acceptance and facilitates a widespread use.

Regulatory requirements in food safety and chemical regulation have shown that three factors play an important role in the acceptance of *in vitro* bioassays for human health evaluation. These factors are: the necessity, the usefulness and the performance. First, the necessity could be determined by the regulatory requirements, such as the prohibited use of *in vivo* assays (or animal tests) in risk assessment of cosmetics. Second, the usefulness implies the benefits of *in vitro* bioassay. Third, the performance is determined by validation requirements such as the reliability and the reproducibility. Ideally, the assay results should reflect the potency of the chemical and there should be a dose-response relation between the concentration of the chemical and the effect measured in the assay.

Overall, *in vitro* bioassays in water quality assessment have proven their usefulness. Testing with *in vitro* bioassays has advantages over analytical techniques, such as the relatively low costs, the possibility of high-throughput methods, the derivation of mixture effects, and the preference over *in vivo* tests due to ethical reasons. However, several factors that hinder the implementation of *in vitro* bioassays in regulatory frameworks are:

1. The costly and time-consuming formal validation procedures
2. The need for a battery of *in vitro* tests to replace one *in vivo* test
3. The proven relevance to apply *in vitro* bioassays for drinking water

Besides the validation procedures, a further step to improve the regulatory acceptance is the derivation of human health based guideline values. This threshold can act as a filter mechanism where detailed evaluation is only performed in samples exceeding that trigger value.

1 Introduction

Chemical analysis provides a quantitative assessment of single concentrations of target chemicals. However, results are limited by the sensitivity and resolution of the applied analytical method. In addition, at present it is not possible to measure the complete chemosphere and evaluate the toxicity for each compound. Lastly, the significance of single compounds in water for human health can remain obscure when no background information on toxicology is available. On the contrary, bioanalytical tools can provide information on all bioactive micropollutants with a specific mode of action in a sample, including known and unknown compounds. Therefore, *in vitro* bioassays can play an important role in the assessment of chemical mixtures.

Bioanalytical tools for water quality determination can be roughly divided into two categories namely *in vivo* bioassays and *in vitro* bioassays. The first category mainly covers tools which make use of living organisms such as rats, mice, daphnids, algae, chirononomids and so on. An extensive review on regulatory acceptance of these tools has been published by Power and Boumphrey (2004). One of the accepted use of *in vivo* test results is the application of the TEQ concept (see section food safety), occasionally applied in water quality legislation, for example in British Columbia (Nagpal *et al.*, 2013). In addition, the reader is referred to a recent review of Kienle *et al.* (2011, in prep) focusing on bioassays for waste water quality assessment. The present review focuses specifically on *in vitro* bioassays for the assessment of human health aspects. These *in vitro* bioassays mainly make use of bacterial, yeast and mammalian cells or tissues. Such assays often focus on a specific endpoint and can provide information on a given mode of toxic action (MOA), such as estrogenity and genotoxicity. The assessment of cytotoxicity gives information on general toxicity.

Due to the reasons stated above, bioassays should be applied complementary to chemical analysis. Of course, *in vitro* studies can only identify a selection of the adverse effects that may be inflicted by chemicals. The validation of *in vitro* assays is laborious. However, the DG Environment, one of the Directorates-General, and services that make up the European Commission, recognizes the need for non-target guidelines such as the establishment of bioassay trigger values. So far, *in vitro* bioassays for drinking water quality monitoring are mainly used as a diagnostic research tools for (i) hazard identification and (ii) testing of treatment efficiency of novel drinking water treatment methodologies. A wide range of *in vitro* bioassays has been applied for water quality assessment (see e.g. Escher and Leusch, 2012). The most prominent *in vitro* bioassays for human health assessment detect endpoints such as cytotoxicity, genotoxicity and endocrine disruption. For example, the combined risk of estrogenic chemicals in surface waters has been assessed with the BEQ/TEQ concept (Anderson, 2012, Kienle *et al.*, 2012), but at present there is no formal implementation in water quality regulation.

The development of *in vitro* bioassays is promising for future applications. Nevertheless, there are currently no formal guideline values in water quality regulation for measurements resulting from *in vitro* bioassays (Tang *et al.*, 2013). To date, regulations and water quality guidelines focus on individual chemicals and only a few on mixture effects, for example in the assessment of Fresh and Marine Water Quality (ANZECC/ARMCANZ, 2000) and in the risk assessment of chemicals regarding combination effects from exposure to multiple chemicals (EU Council, 2009) and the cumulative effects of pesticides (USEPA, 2002). Therefore additional knowledge is needed for the correlation between bioanalytical measures and chemical analysis. Guidelines for *in vitro* bioassays –i.e. trigger values- can be useful to act as a first tier; the exceedance of a bioassay trigger value could serve as a trigger for a further chemical or effect-based assessment.

2 Purpose of the present overview

Amongst others, *in vivo* bioassays can be used as standard tools for characterising effluent quality and are well accepted by water quality regulators. The review of Power and Boumphrey (2004) provides a good overview taking into consideration the regulatory applications in various jurisdictions, with focus on the use of bioassays for effluent management. The aim of the present review is to provide a similar overview with a focus on the use of *in vitro* bioassays for human health effects.

This present study focusses on the roadmap to implementation of *in vitro* bioassays for human health in international regulatory frameworks dealing with drinking water quality. Regulatory acceptance is defined by the formal adoption of a (validated) test method by a regulatory agency/authority. To provide insights into this roadmap, a general overview of current *in vitro* bioassays in regulatory policies is provided. The scope of this document is an overview on the (international) regulatory acceptance of mammalian or bacterial cell-based bioassays for human health assessment. The focus is on drinking water quality assessment with an outlook to examples from other purposes such as food safety and chemical regulation. Such cases from other fields can be used as successful examples for future acceptance and implementation of *in vitro* bioassays in the framework of drinking water quality guidelines. The present overview can be used as a basis in future discussion with policy makers. The following section on validation provides information on the recommended validation procedures to obtain a quality assurance of a specific test.

3 Validation

A crucial step on the road to implementation of *in vitro* bioassays is their validation. In general, regulators and policymakers will accept *in vitro* test methods only after scientific validation. The validation assures that a test is reliable and reproducible. According to the Organisation for Economic Co-operation and Development (OECD), formal validation "contributes strongly to the international acceptance of any proposed test method" (Spielmann, 2000). As a result, the OECD has indicated that *in vitro* toxicity studies can be accepted for regulatory purposes only after a successful experimental validation study. However, demonstrating the validity of a method is a complicated process. All steps in the formal validation study have their challenges in terms of time, costs, and motivation (Spielmann, 2000).

Not all tests that are accepted in regulatory frameworks are officially validated. For example, some animal test methods and several *in vitro* bioassays (such as an dermal absorption test and the Ames test) which have been in use for a relatively long time, can be accepted based on in-house validation data. Conversely, validation does not automatically lead to regulatory acceptance. This is often a consequence of insufficient collaboration with the regulatory authorities (Shiffelers *et al.,* 2012). Therefore, an early involvement of regulatory authorities when validating a method is often considered as a critical success factor (Bottini *et al.,* 2008).

3.1 Formal validation

Official validation of *in vitro* assays is performed by the institutes concerning the Validation of Alternative methods (VAM): the EURL ECVAM (European Union Reference Laboratory for Alternatives to Animal Testing), the ESAC (ECVAM Scientific Advisory Committee), ICCVAM (the Interagency Coordinating Committee on the Validation of Alternative Methods) or the JaCVAM (Japanese Center for the Validation of Alternative Methods). The ECVAM and the other institutes are specialised in the validation of methods which reduce, refine or replace the use of animals for safety testing and efficacy/potency testing of chemicals, biologicals and vaccines. Upon request from institutes, industries and other stakeholders test are validated. More information on the validation method is given on their website (EURL ECVAM, 2013). Table 1 presents several validated *in vitro* bioassays.

Table 1: Overview of examples of validated *in vitro* bioassays, clustered by endpoints.

No.	Endpoint	*In vitro* assay(s)	Validated by
1	Developmental toxicity	Embryonic stem cell test (EST)	ECVAM
		Micromass (MM) test	ECVAM
2	Carcinogenicity	Bhas 42 cells based CTA	JaCVAM
		Syrian hamster embryo cell transformation (SHE) assay	ECVAM
		BALB/c 3T3 cell transformation assay	ECVAM
		C3H/10T1/2 cell transformation assay	Various authors

		Survival assay	Various authors
		HaCaT cell transformation assay	Various authors
		MSU-1 cell transformation assay	Various authors
			Various authors
3	Hepatotoxicity	Hepatocytes	Various authors
		Liver slice system	Various authors
		Hepatic cultures	Various authors
			Various authors
4	Nephrotoxicity	CAKI-1 cells	Various authors
		MDCK cells	Various authors
		Renal ephithelial cell lines (LLC-PK, LLC-RK, OK, JTZ-12, MDCK, MDBK)	Various authors
		Human embronic kidney cells	Various authors
		LLC-PK1-FBPase	Various authors
		OK-GNG+	Various authors
		Rat SKPT O 193 cl2	Various authors
		HK 2 cells	Various authors
		NHK cells	Various authors
5	Reproductive system toxicity	Embryonic stem cell test (EST)	ECVAM
		LUMI-cell ER assay (BG1Luc ER TA)	ICCVAM
		MELN assay	ECVAM
		hER-HeLa-9903 assay	OECD
		H295R Steroidogenesis Assay	OECD
		MCF-7 cell proliferation test method	ICCVAM
6a	Skin toxicity		
	Skin corrosion	EST-1000 human reconstructed epidermis	ESAC/OECD

		Corrositex® noncellular membrane	ICCVAM/OECD
		EpiSkin® human skin model	ESAC/OECD
		EpiDermTM human skin model	ESAC/OECD
		SkinEthicTM human skin model	ESAC/OECD
		Vitrolife-Skin human reconstructed epidermis	JaaVAM/OECD
	Skin irritation	EpiSkin® skin irritation test (with MTT reduction)	ESAC/OECD
		EpiDermTM skin irritation test (with MTT reduction)	ESAC
		EpiDermTM SIT model (EPI-200)	ESAC/OECD
6b	Skin sensitization	Direct peptide reactivity assay	ECVAM
		Myeloid U937 skin sensitization test	ECVAM
		Human cell line activitation test (h-CLAT)	ECVAM
6c	Skin irritancy	*In vitro* Skin Irritation	OECD
6d	Eye irritation	SkinEthic HCE	ECVAM
		EpiOcular	ECVAM

An *in vitro* bioassay must be scientifically robust to become validated. The validation procedure consists of the technical validation of the test, the development of the Standard Operating Protocol (SOP) for routine application and the quality assurance and quality control (QA/QC). For more details the reader is referred to Escher and Leusch (2012), Chapter 10. In short, the validation is based on (i) accuracy (result close to true value), (ii) precision (closeness of results of repeated individual measurements), (iii) robustness (sensitivity to operational variations), (iv) selectivity (measure for matrix interference), (v) sensitivity (response to varying amounts of target compounds in concentrations at the limit of quantification (LOQ), (vi) specificity (reaction to a wide variety of chemicals) and (vii) sample stability (to test the influence of sample preparation).

Following the validation and the definition of a SOP for routine application, QA/QC procedures need to ensure the consistent test performance over time. These procedures are necessary to establish an additional degree of confidence in the accuracy, reliability and consistency of the data (confirm ISO standard 17025).

3.2 Harmonization

Another important step towards regulatory acceptance is the harmonization of bioassay test methods by establishing standards (International Organization for Standardization, ISO) and guidelines (Organisation for Economic Co-operation and Development, OECD). Harmonization is one of the dominant prerequisites for regulatory acceptance and facilitates a widespread use. At the same time, harmonization is a very lengthy and difficult process, because all member states of the responsible organisation must reach consensus.

ISO standards ensure that products and services are safe, reliable and of good quality. The standardization of an *in vitro* bioassay provides a level of reliability needed for implementation in regulatory frameworks. The number of current ISO standards concerning the use of *in vitro* bioassays tests is relatively scarce. At present, four tests are implemented in ISO standards (see Table 2). For application to water and waste water protocols exists for a mammalian cell-based *in vitro* test for genotoxicity on V79 cells (micronucleus assay) (ISO en NEN 21427-2 version 2009), for bacterial-based assays such as the UMU assay to assess genotoxic effects (ISO 13829:2000) and for the Ames test to assess mutagenic effects (ISO 11350:2012). Currently BioDetection Systems (BDS) and the Swiss Centre for Applied Ecotoxicology of Eawag and EPFL are involved in the design of ISO-standards for the determination of *in vitro* reporter gene assays for the detection of estrogenic activity in (waste)water based on yeast cells and on human cell lines (*Van der Linden and Kienle, pers. comm.*). The bioassays the Yeast Estrogen Screen (YES) with *Saccharomyces cerevisiae* (ISO/AWI 19040-1) and the YES with *Arxula adeninivorans* (ISO/AWI 19040-2) are to be standardised. Additionally a new work item proposal for several reporter gene assays based on human cell lines (ER CALUX, MELN en ERalpha CALUX) has been submitted recently (www.nen.nl). ISO-standards for *in vitro* test have been established in other areas of application as well, such as for medical devices and food safety. For example an ISO-standard to detect genotoxicity, carcinogenicity and reproductive toxicity of compounds in medical devices is described by SO/DIS 10993-3:2011.

Table 2: NEN (Dutch standards) and ISO-standards for the use of *in vitro* bioassays.

ISO-standard	Title	Description
NEN-EN-ISO 21427-2:2009	*Water quality - Evaluation of genotoxicity by measurement of the induction of micronuclei - Part 2: Mixed population method using the cell line V79*	This part of ISO 21427 specifies a method for the determination of genotoxicity of water and waste water using a mammalian *in vitro* test which detects damage, induced by water-soluble substances, to the chromosomes or the mitotic apparatus of V79 cells from the Chinese hamster. The micronucleus test allows the identification of substances that cause cytogenetic damage which results in the formation of micronuclei containing lagging chromosome fragments and/or whole chromosomes. The assay is based on the increase in the frequency of micronucleated cells after incubation with and without metabolic activation.
ISO 13829:2000	*Water quality - Determination of the genotoxicity of water and waste water using the umu-test*	No extra information available online.
ISO 11350:2012	*Water quality - Determination of the genotoxicity of water and waste water -- Salmonella/microsome fluctuation test (Ames fluctuation test)*	No extra information available online.

NEN-EN-ISO 10993-3:2011	*Biological evaluation of medical devices - Part 3: Tests for genotoxicity, carcinogenicity and reproductive toxicity*	This part of ISO 10993 specifies strategies for hazard identification and tests on medical devices for the following biological aspects: genotoxicity, carcinogenicity and reproductive and developmental toxicity. This part of ISO 10993 is applicable when the need to evaluate a medical device for potential genotoxicity, carcinogenicity, or reproductive toxicity has been established.

The **OECD testing guidelines** are a collection of the most relevant internationally agreed test methods for chemicals used by government, industry and independent laboratories to determine the safety of chemicals and chemical preparations, including pesticides and industrial chemicals. OECD guidelines serve as a harmonization guide for all member states. The OECD guidelines for the testing of chemicals are divided in the sections: physicochemical properties, effects on biotic systems, degradation and accumulation, health effects, and other test guidelines. Within the section health effects, a number of *in vitro* tests used to detect and identify potential hazards of new and existing chemical substances, chemical preparations and chemical mixtures are described. An example for the assessment of estrogenic effects is test No. 455 (2012): *Performance-Based Test Guideline for Stably Transfected Transactivation In vitro Assays to Detect Estrogen Receptor Agonists*. For the assessment of mutagenicity a guideline for the Ames test is available (OECD 471). A more advanced version of this test, the Ames II procedure uses different—albeit functionally comparable—Salmonella tester strains for the detection of base pair mutations, which is not strictly conform with the guidelines used for regulatory approval (Kamber *et al.*, 2009). In Table 3 a condensed overview of OECD guidelines for *in vitro* bioassays is given.

Table 3: A selection of available *in vitro* methods to detect genotoxicity and endocrine disruption, with references to the respective regulations and OECD guidelines. Source: ECVAM, 2013.

Test Method	Council Regulation (EC) No 440/2008	OECD	Test Method
Bacterial reverse mutation test (Ames test)	B.13-14	471	Gene mutations
In vitro Mammalian chromosome aberration test	B.10	473	Structural aberrations
In vitro Mammalian cell gene mutation test	B.17	476	Gene mutations
In vitro Mammalian cell micronucleus test	B.49	487	Structural and numerical aberrations
Syrian Hamster Embryo (SHE) cell transformation assay	-	Draft	Cell transformation
ERá-HeLa-9903 cell line, derived from a human cervical tumor, and the BG1Luc ER TA assay using the BG1Luc-4E2 cell line, derived from a human ovarian adenocarcinoma	-	455	Estrogen Receptor Agonists (ER TAs)
In vitro screen (human H295R adreno-carcinoma cell line) for chemical effects on steroidogenesis, specifically the production	-	456	Steroidogenesis

of 17ß-estradiol (E2) and testosterone (T).			

The ICH (International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use) and the VICH (International Cooperation on Harmonisation of Technical Requirements for Registration of Veterinary Products) strive for harmonisation to ensure that safe, effective, and high quality (veterinary) medicines are developed and registered in the most resource-efficient manner. Guideline S2 (R1) for the assessment of the genotoxicity of pharmaceuticals under the ICH includes *in vitro* tests (EMEA, 2012)), see Table 5. Positive results lead to further *in vivo* testing to evaluate the potential risk for patients.

A similar assessment of the genotoxicity of veterinary drugs is established by the VICH Guideline GL23(R) (FDA, 2012).

Table 4: *In vitro* tests mentioned in the ICH and VICH harmonization guidelines.

Bioassay	Mode of action/endpoint	Regulatory for	Country	Reference
1. Test for gene mutation in bacteria. 2. Cytogenetic test for chromosomal damage (the *in vitro* metaphase chromosome aberration test or the *in vitro* micronucleus test), or an *in vitro* mouse lymphoma Tk gene mutation assay.	Genotoxicity	Pharmaceuticals	International	ICH Guideline S2 (R1), 2011
Similar to pharmaceuticals	Genotoxicity	Veterinary drugs	International	VICH Guideline GL23(R), 2012

4 Regulatory acceptance

In the following paragraphs, examples of regulatory acceptance of *in vitro* bioassays in the areas of drinking water quality assessment, food safety, and chemical safety are summarized.

4.1 Drinking water quality

The regulatory framework of drinking water quality concerns the quality of water intended for human consumption. In the regulations quality standards are set. Examples of regulations are the Drinking Water Directive in the European Union, The Safe Drinking Water Act (SDWA) in The United States of America. Drinking Water Protection Act in Canada and the Australian Drinking Water Guidelines (2011) in Australia. Currently, in the drinking water frameworks there is no acceptance of the use of *in vitro* bioassays for water quality assessment for human health.

In Australia, a communication and education "campaign" is providing a good basis for regulators (pers. comm. F. Leusch). *In vitro* bioassays are now specifically mentioned as useful tools in the monitoring section of the Australian Guidelines for Water Recycling: Phase 2 (Augmentation of Drinking Water Supplies): "*In vitro* tests have been used to measure chemical quality of Australian sewage (Leusch *et al.* 2005 and 2006, Muller *et al.* 2007), and a similar approach could be used to monitor the quality of source waters, and of partially and completely treated recycled water. Detection of biological activity should lead to further investigations into the cause of that activity. Biological tests can be used as a screening and prioritisation tool for subsequent chemical analysis".

Whilst there is currently no formal implementation of *in vitro* bioassays in drinking water quality regulation, examples in other fields can provide information on the roadmap to implementation. In the following sections examples of accepted *in vitro* bioassays in food safety and chemical regulation are given.

4.2 Food safety

In food and feed safety the General Food Law (EU) aims at ensuring a high level of protection of human life and human health, taking into account the protection of animal health and welfare, plant health and the environment. The Regulation establishes rules based on scientific and technical evaluations undertaken by the European Food Safety Authority (EFSA) and the American FDA (food and drug administration).

In drinking water quality regulation most guidelines are based on individual chemicals. In food safety standards also exist for groups of chemicals, such as dioxins (Table 6). The use of *in vitro* bioassays has successfully been implemented in the regulation for dioxin-like chemicals by applying the toxic equivalents (TEQ) concept (van den Berg *et al.*, 2000) for assessing food safety in the EU (Hoogenboom *et al.*, 2010). For each dioxin and dioxin-like compound a toxic equivalency factor (TEF) is defined. The toxic potential of the mixture is the sum of the respective substance concentrations times the equivalency factor for each compound. *In vitro* bioassays are used to determine the relative effect potencies. Bioanalytical equivalent concentrations (BEQ) can be calculated from the relative effect potencies and the measured concentrations or directly from bioassay results (e.g. the DR-CALUX). The BEQ concept expresses the effect by relating it to the effect elicited by a known reference chemical. This concept can only be applied to chemicals that act via a well-defined common mechanism such as receptor mediation. For the risk assessment of genotoxicity in food, a tiered approach is proposed by the EFSA (2011). *In vitro* bioassays (Ames test and micronucleus test) are recommended for an assessment of the genotoxic potency, followed by advanced toxicity studies and exposure evaluation.

For animal feed safety many *in vitro* test are implemented in regulatory purposes, for example for the combined effect measurements of dioxins, endocrine disruptors, antibiotics and antibodies. However, these tests are implemented to protect animal health. These test all act as a first tier followed by chemical analyses.

Table 5: Implemented method in food safety regulation using an *in vitro* bioassays for evaluation of human health risks.

Bioassay	Mode of action/endpoint	Regulatory for	Country	Reference
CALUX-DR	BEQ-concept for dioxins and dioxine-like chemicals	Food	European Union	Hoogenboom *et al.* 2010
Ames fluctuation assay is proposed	Genotoxicity	Food		EFSA, 2011

4.3 Chemical regulation

Another example for the acceptance of *in vitro* bioassay is the framework of safety assessment for chemicals with a focus on genotoxicity testing (Table 7). The European Union (EU) established many regulations aiming at the protection of human health. In general, the assessment of genotoxic hazard to humans follows a tiered approach, beginning with a basic battery of *in vitro* bioassays, in some cases (if the *in vitro* bioassay yields a positive result) followed by *in vivo* testing. Examples of the implementation of *in vitro* toxicity tests in European regulation are: industrial compounds in the REACH regulation (Registration, Evaluation, Authorisation and Restriction of Chemicals), the hazard classification of chemicals (carcinogenic, mutagenic or reproductive), and the regulation of cosmetic products, biocides and plant protection products. In addition, international regulations for pharmaceuticals and veterinary drugs also allow for screening with *in vitro* bioassays. For industrial chemicals and biocidal products a positive outcome in one or more of the *in vitro* genotoxicity tests requires confirmation by appropriate follow-up *in vivo* testing (EC no. 1907/2006; EC no. 528/2012). For pharmaceuticals, veterinary drugs and plant protection products the *in vitro* testing battery (irrespective of the outcome) must always be followed by *in vivo* testing (ICH, 2011; VICH, 2012; EC no. 1107/2009b and 283/284-2013). For cosmetics ingredients and products, *in vivo* testing is prohibited in the EU (EC no. 1223/2009) and only *in vitro* tests are used. More information on the different regulations is provided below.

Table 6: Implemented methods in chemical regulation using *in vitro* bioassays for evaluation of human health risks.

Bioassay	Mode of action/endpoint	Regulatory for	Country	Reference
1. *In vitro* gene mutation study in bacteria. 2. *In vitro* carcinogenity study in mammalian cells or *in vitro* micronucleus study 3. *In vitro* gene mutation study in mammalian cells	Genotoxicity	Industrial compounds under CLP regulation and REACH	European Union	The CLP Regulation and the REACH Regulation (EC) No 1907/2006

1. bacterial reverse mutation test 2. micronucleus test 3. mammalian chromosome aberration test	Genotoxicity clastogenicity and aneugenicity	Cosmetics	European Union	Regulation (EC) No 1223/2009
1. *in vitro* test for gene mutations in bacteria 2. *in vitro* cytogenicity test in mammalian cells 3. *in vitro* gene mutation test in mammalian cells	Genotoxicity	Biocides	European Union	Regulation (EU) No 528/2012
1. bacterial assay for gene mutation (Ames test), 2. combined test for structural and numerical chromosome aberrations in mammalian cells, 3. test for gene mutation in mammalian cells	Genotoxicity	Plant protection product	European Union	Regulation (EC) No 1107/2009

The REACH Regulation (EC) No 1907/2006 requires all companies manufacturing or placing a substance on the EU market in quantities greater than one tonne per year to register that substance with the European Chemicals Agency (ECHA). The assessment of genotoxicity is required for all compounds under REACH. For compounds with production levels between 1-10 tonnes, studies with an *in vitro* test (Ames test) are applied. Compounds with production levels over 10 tonnes are tested with two extra *in vitro* studies with mammalian cells assessing cytotoxicity and genotoxicity (RIVM, 2007). A positive result in any of the *in vitro* genotoxicity studies, will lead to further *in vivo* testing. Compounds with a production level >1000 ton are, in addition, subjected to a long term *in vivo* study for carcinogenic potency.

The REACH Regulation is closely interlinked with the CLP regulation (Classification, Labelling and Packaging), which ensures that the hazards presented by chemicals are clearly communicated to workers and consumers. The hazard classification of chemicals is legally established in the European Union (EC 1272/2008 Annex I) and harmonized with the United Nations. Compounds are classified as carcinogenic, mutagenic and reproductive (CMR). *In vitro* bioassays are part of the testing package (see EC Regulation 1272/2008).

The Regulation (EC) No 1223/2009 establishes rules for cosmetic products made available on the market for a high level of protection of human health. At present, three *in vitro* bioassays are recommended for the basic level testing of cosmetic substances: 1) a bacterial reverse mutation test for gene mutation, 2) the micronucleus test or 3) a mammalian chromosome aberration test for clastogenicity (mutagenesis which can lead to carcinogenesis) and aneugenicity (loss of chromosomes during cell duplication). *In vivo* testing is no longer allowed and yet no validated methods are available that allow the follow-up of positive results from standard *in vitro* assays (see EU Regulation 1223/2009).

The Regulation (EU) No 528/2012 establishes rules concerning biocides. For mutagenicity, after an assessment of available *in vivo* genotoxicity data, three tests are required: 1) an *in vitro* test for gene mutations in bacteria, 2) an *in vitro* cytogenicity test in mammalian cells and 3) an *in vitro* gene mutation test in mammalian cells. A positive result in any of the *in vitro* genotoxicity studies leads to the consideration of an appropriate *in vivo* genotoxicity study (see Regulation 528/2012 and ECHA, 2013).

Regulation (EC) No 1107/2009 establishes rules any plant protection product made available on the market has to fulfil. Three *in vitro* mutagenicity tests shall be performed: 1. bacterial assay for gene mutation (Ames test), 2. combined test for structural and numerical chromosome aberrations in mammalian cells, 3. test for gene mutation in mammalian cells. If all the results of the *in vitro* studies are negative, at least one

in vivo study has to confirm it. For substances with positive test results obtained in any *in vitro* test, additional testing (including *in vivo* tests) shall be considered taking into account all relevant information, and using the same endpoint as in the *in vitro* test (see Regulation (EU) 528/2012).

The ICH initiative includes the drug regulatory authorities of the European Union (European Agency for the Evaluation of Medicinal Products, EMEA), Japan (Ministry of Health, Labour and Welfare, JMHLW), and the USA (Food and Drug Administration, US FDA), The guidelines are at present implemented by regulatory authorities of the ICH countries. The VICH guidance has been adopted, for the most part, by the regulatory bodies of the USA, EU, Japan, Australia, New Zealand and Canada.

5 Discussion

5.1 Requirements for regulatory acceptance

Developing and validating *in vitro* bioassays for regulatory testing purposes can only make sense if, at the end of the process, regulatory authorities accept its use as relevant for consumer safety. In general, three factors play an important role in the acceptance of *in vitro* bioassays for human health evaluation:

- The necessity
- The usefulness
- The performance/reliability

The **necessity** for the implementation of *in vitro* bioassays in the regulatory framework could be the regulatory requirements that act as a trigger for testing purposes. With regard to drinking water quality, however, so far guidelines mainly specify permitted concentrations of individual chemicals or groups of very closely related chemicals and no guidelines have been established for the use of *in vitro* bioassay results. When *in vivo* assays are not allowed (such as for testing cosmetics) or not feasible (such as for screening drinking water quality), *in vitro* assays will be the techniques of choice to reveal a potential human health hazard.

The **usefulness** implies the benefits of *in vitro* bioassays. The latter have the advantage that they can act as high-throughput tests at relatively low costs compared to chemical analyses. Second, they allow the assessment of mixture effects (such as concentration additivity or synergism) that cannot be derived from chemical analyses. Bioassays offer a third advantage above chemical analytical tools because of their effect-related results. Serious human health consequences (e.g. genotoxicity) of exposure to chemicals can be revealed by *in vitro* bioassays, whereas they cannot be inferred from chemical analysis without toxicological data. In situations where *in vivo* assays are used for hazard assessment, the reduction of animal tests is an ethical aspect which contributes to the usefulness of *in vitro* bioassays. Besides, *in vitro* assays are in some instances more predictive for human health hazard than assays involving experimental animals, and require a smaller investment in time and money.

The **performance** of a bioassay needs to be appropriate. The steps of validation and harmonisation of *in vitro* bioassays are explained in Chapter 2. Besides, ideally there should be a dose-response relation between the concentration of the chemical and the effect measured in the assay. The assay results should reflect the potency of the chemical, and the link between the test result and the adverse human health effects has to be established scientifically.

5.2 Reasons for successful implementations

Examples for a successful implementation of *in vitro* bioassays can be found in regulations on food safety and in the chemical regulations of industrial compounds (REACH), cosmetics, biocides and plant protection products.

The TEQ/BEQ method applied in food and even water quality is successful, if the performance of the test is good. Dioxin-like compounds each have a similar mechanism of action (on the Arylhydrocarbon (Ah) receptor). A certain level of dioxins determines the risk of adverse human health effects. Therefore trigger values can be derived, above which human health effects can be expected. Endocrine disrupting compounds have a similar specific mode of action (estrogenic, androgenic, progestagenic and glucocorticoid). This approach can be applied for the assessment of groups of compounds with a similar/specific mode of action. *In vitro* tests for genotoxicity are successfully implemented in regulations because of their necessity and usefulness. In the regulation of cosmetics, *in vitro* bioassays are strongly

needed as a risk assessment tool, because *in vivo* testing is prohibited due to ethical reasons. As genotoxicity is an important endpoint with high relevance to human health, *in vitro* bioassays for its assessment are considered as very useful. In the pharmaceutical industry, genotoxic effects of candidate compounds result in the termination of the development of the product. Furthermore, the usefulness of the genotoxic tests is influenced by the time and costs that are consumed by animal testing. Besides, *in vivo* genotoxicity tests are no absolute predictors for human health concerns and the measurement of 'key events' by proper *in vitro* test can give a good estimate of the potential hazard. With regard to performance, *in vitro* genotoxicity bioassays have a long history of application. For example, the Ames test has been used in water quality studies at KWR since 1980 (Sjerps *et al.*, 2013). Therefore data from this test on many compounds are available and its performance is well known. A clear link between the test result and the adverse human health effect exists (Claxton *et al.*, 2010), which triggers acceptance of the test. Standards for the Ames test have been derived by ISO since 2005 and by OECD since 1997 (Table 2). However, the Ames test has not been formally validated by ECVAM or similar organisations,

One of the reasons why many *in vitro* bioassays are not implemented in the regulatory framework, is the process of the official validation of the test method. To officially validate a test a lot of data are needed in a long and cumbersome process. Another obstacle for some applications is the need for a battery of *in vitro* assays to replace a single *in vivo* test, because of the variety of modes of action and biological effects that chemicals may have. In addition, not for all *in vitro* bioassays the direct relevance for the *in vivo* situation is proven, which hinders interpretation of the test results. Further development and validation of *in vitro* tests is certainly needed to proof their reliability for hazard assessment (see Adler *et al.*, 2011). For these reasons, it is more convenient for the industry to comply to the current regulations, which often prescribe *in vivo* testing methods. Besides, *in vitro* bioassay testing on a voluntary basis costs time and money and is may not be profitable for the industrial companies.

5.3 Further steps

Provided that *in vitro* bioassays relevant for an assessment of drinking water quality are validated and/or scientifically sound, the derivation of human health-based guideline values for bioassay results is the most important point for regulatory acceptance in the field of water quality monitoring. This raises the question on how to determine the threshold level. *In vitro* bioassays provide a good prioritisation mechanism, where a detailed evaluation is performed only in samples exceeding a given threshold.

Several approaches to derive a trigger value are proposed by Escher and Leusch (2012):

1. No observed effect of the undiluted water sample – only further investigation if response is above level of quantification (applicable for non-specific and reactive toxicity)
2. Definition of effect-based trigger values – using the TEQ-concept, chemicals with known guideline values are tested in the bioassay to derive the relevant trigger value (applicable for specific toxicity)
3. Redefinition of effect-based guideline values – link the response observed in the bioassay to an adverse human health outcome

The first approach is a conservative method where any effect besides 'no effect' leads to further evaluation. An exposure measurement is not included. The second approach is only appropriate if the TEQ-concept can be applied. The third approach deviates from the expected route, the derivation of a testing method from a guideline, to a direct link of the test result to a guideline value.

An example of the second approach is given by Brand *et al.* (2013). In this study trigger values are derived for the highly sensitive *in vitro* CALUX bioassays (estrogen (ERα), androgen (AR), progesterone (PR), and glucocorticoid (GR) receptor) for agonistic hormonal activities in (drinking) water. Trigger values range between 3.8 ng and 333 ng equivalents/L to reference compounds. These values can help to judge

measured agonistic hormonal activities in drinking water samples using the CALUX bioassays and help to decide whether a further examination of specific endocrine activity followed by a subsequent safety evaluation may be warranted.

As an alternative, the relevance of the response of an *in vitro* bioassay can be determined by relating it to the summed TTC-value (Van Wezel *et al.*, 2012). The TTC is the threshold of toxicological concern, which is the level of human exposure that is considered to be of negligible risk, despite the absence of toxicity data. The TTC may act as a conservative target value for genotoxic (0.01 µg/L), steroid endocrine (0.01 µg/L) and other chemicals (0.1 µg/L) in drinking water (Mons *et al.*, 2013). Likewise, summed TTC values have been established for genotoxic chemicals (0.01 µg/L), steroid hormones (0.1 µg/L) and all other organic compounds (1.0 µg/L). An *in vitro* bioassay can demonstrate the exceedance of the TTC, provided that of the bioassay is able to demonstrate the presence of reference compounds at the level of the TTC-value. When a water sample elicits a response in such a bioassay, adverse health effects cannot be excluded, whereas absence of a response in the bioassay would mean that health risks can be assumed to be negligible.

6 Conclusion

The factors that play an important role in the acceptance of *in vitro* bioassays for human health evaluation are: the necessity, the usefulness and the performance. The necessity for the implementation of *in vitro* bioassays in the regulatory framework is defined by the regulatory standards. With regard to drinking water quality, however, so far guidelines mainly specify permitted concentrations of individual chemicals or groups of very closely related chemicals and no guidelines have been established for the use of *in vitro* bioassay results. Secondly, the usefulness implies all the benefits of the *in vitro* bioassay, such as the relatively low costs, the possibility of high-throughput methods, the derivation of mixture effects, and the preference over *in vivo* tests due to ethical reasons. Several examples from regulatory frameworks have shown that important factors are: regulatory needs (as for the risk assessment of cosmetics), the derivation of trigger values (as for the BEQ method for dioxins), the link between the test result and the adverse human health result (as for the Ames test). However, formal validation procedures and the need for a battery of *in vitro* tests to replace one *in vivo* test hinder the implementation of *in vitro* bioassays in regulatory frameworks. Besides validation, a further step to improve the regulatory acceptance is the derivation of human health based guideline values. *In vitro* bioassays can act as a tool in prioritisation, where only the samples with a response above the threshold level have to be examined further. The derivation of a threshold level (trigger values, bioassay guideline values) is thus an important step towards acceptance.

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8 Glossary of terms

Ames test	*In vitro* bioassay to assess the mutagenic potential of chemical compounds
CLP	The CLP Regulation for "Classification, Labelling and Packaging" is a European Union regulation which aligns the European Union system of classification, labelling and packaging chemical substances and mixtures to the Globally Harmonised System (GHS)
DG Environment	The Directorate-General for the Environment is one of the more than 40 Directorates-General and services that make up the European Commission.
EFSA	The European Food Safety Authority (EFSA) is an independent European agency for risk assessment regarding food and feed safety in the European Union (EU)
ESAC	ECVAM Scientific Advisory Committee
EURL ECVAM	European Union Reference Laboratory for Alternatives to Animal Testing, European Centre for the Validation of Alternative Methods
FDA	The Food and Drug Administration (FDA or USFDA) is an agency of the United States Department of Health and Human Services and is responsible for protecting and promoting public health through the regulation and supervision of food safety.
ICH	The International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH) is unique in bringing together the regulatory authorities and pharmaceutical industry of Europe, Japan and the US to discuss scientific and technical aspects of drug registration.
ICCVAM Methods	The Interagency Coordinating Committee on the Validation of Alternative
ISO-standard	ISO International Standards ensure that products and services are safe, reliable and of good quality.
JaCVAM	Japanese Centre for the Validation of Alternative Methods
MOA	A mode of action (MoA) describes a functional or anatomical change, at the cellular level, resulting from the exposure of a living organism to a substance
NEN-standard	The Netherlands Standardization Institute (NEN) is a private, non-profit organization which develops of international and European standards,
QA/QC	Quality assurance and quality control
REACH	Registration, Evaluation, Authorisation and Restriction of Chemicals (REACH) is a European Union Regulation (2006) which addresses the production and use of chemical substances (i.e. everything made of atoms), and their potential impacts on both human health and the environment.
VAM	Validation of Alternative Methods
VICH	International Cooperation on Harmonisation of Technical Requirements for Registration of Veterinary Medicinal Products

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OECD Test Guideline 456 H295R Steroidogenesis Assay.

OECD Test Guideline 471 Bacterial reverse mutation test (Ames test).

OECD Test Guideline 473 *In vitro* Mammalian chromosome aberration test.

OECD Test Guideline 476 *In vitro* Mammalian cell gene mutation test.

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