

Use of effect-directed assays in assessing the quality of drinking water and its sources

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Abstract Current monitoring programs for drinking water or its sources only measure a limited amount of often well-known chemicals. To detect all harmful compounds present, and also the total effect of all compounds, *in vitro* effect-directed assays are a valuable addition. Relevant effects to analyze in drinking water and its sources are genotoxicity, endocrine disruption, developmental and teratogenic effects and possibly neurotoxicity and immunotoxicity, as these are effects that can occur at relatively low exposures already. A short overview is given of the various *in vitro* assays available for the mentioned effects. Application of these assays to various water types in the Netherlands has shown the presence of possibly harmful chemicals which were not detected by the current monitoring programs. In order to identify when a response of these tests is unacceptable, effect-directed water quality limits are necessary. A first idea on how to develop these, is by basing them on the ADI of a reference compound, assuming a worst case scenario for absorption, distribution, metabolism and elimination. Further work on this topic is considered necessary.

INTRODUCTION

In the sources for drinking water, thousands of industrial chemicals can be present (Schwarzenbach *et al.*, 2006). In the EU, 30,000 to 70,000 chemicals are in daily use (EINECS database), varying from industrial chemicals (such as solvents, petrochemicals), consumer chemicals (such as pharmaceuticals, personal-care products) to biocides. Due to global trends such as a growing and older population, increasing prosperity, urbanization and more extreme events (increasing transport rates between environmental compartments) (Diamond and Hodge, 2007), there is an increasing chemical pressure on drinking water sources. For example, only about 10% of European river water samples could be classified as "very clean" (Loos *et al.*, 2009). Pharmaceuticals, perturbation compounds, personal care products, detergents and endocrine disruptors could be found throughout Europe up to high ng/L median concentrations (Loos *et al.*,

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2009), many entering the water cycle via wastewater (Reemstma *et al.*, 2006). This occurrence of chemicals in sources of drinking water is, depending on the treatment processes, sometimes reflected in (much lower) occurrence of these chemicals in finished drinking water (Benotti *et al.*, 2009).

Only a very limited part of the chemicals which are found in drinking water sources are currently regulated via (drinking) water quality regulations. Additionally, current monitoring programs only measure a limited amount of often well-known chemicals. Especially the presence of so-called “emerging chemicals” such as pharmaceuticals, personal-care products, drugs-of-abuse, endocrine disruptors and nanochemicals may lead to consumers concern (e.g. Turgeon *et al.*, 2004). Emerging chemicals can be defined as newly detected chemicals, which are not covered by existing water quality legislation, and for which relatively little information is available on their environmental behaviour and (eco)toxicological properties.

Despite rapidly evolving chemical analytical techniques (Richardson, 2008), it is impossible to analyze and identify all different chemicals present in the aqueous environment. In addition, for many identified chemicals no statutory health-based (drinking) water quality guideline values have been established and no or scarce toxicological information is available to estimate human health risks (e.g. van Genderen *et al.*, 2000). Finally, our understanding of the combined mixture effects in these complex mixtures of many different chemicals occurring in low concentrations is still limited (McCarty and Borgert, 2006). The use of *in vitro* effect-directed assays can be an additional tool to interpret health risks of complex mixtures in drinking water and its sources. *In vitro* effect-directed bioassays do not determine the presence of (a group of) compounds directly, but determine their collective effect in a biological system such as cultured cells. Chemicals that cannot be revealed by analytical techniques but do attribute to the toxicological effects are included in the effect assays, and thus, the assays give a clue of the toxicity of the total mixture of chemicals present in the sample.

This paper discusses which effect assays are useful for assessing the quality of drinking water and its sources, and gives some examples on their application.

SELECTION OF RELEVANT *IN VITRO* EFFECT-DIRECTED ASSAYS

For quality assessment of the drinking water production chain, relevant effect-directed assays are related to carcinogenicity and genotoxicity, to hormonal disruption, or to developmental and teratogenic effects. These end-points are relevant human health effects which can occur after chronic exposure to

relatively low concentrations. Toxicity assays that focus on acute effects are considered as less relevant; human health effects after acute exposure are not expected given the drinking water quality in developed countries.

Hormonal (or endocrine) disruption occurs when chemicals disturb the delicate balance of the levels of the various hormones. Hormones are very potent chemical messengers that travel from one organ to another, specific target organ, to regulate a variety of physiological processes in the human body. For example, the estrogenic hormones regulate the various female reproductive processes, as the menstrual cycle and breast development. The androgenic hormones regulate the typical male processes, such as body hair growth and sperm production. Progesteragens play a pivotal role during pregnancy and thyroid hormones are important for fetal development and energy metabolism. Glucocorticoid hormones play a role in inflammation and in the availability of glucose (hence the name glucocorticoids) for the energy metabolism. When a chemical compound structurally resembles one of these hormones sufficiently to bind to the specific receptor for this hormone, the action of this hormone is unnecessarily increased. Alternatively, a chemical can block the action of a hormone by occupying its receptor without leading to action. Both processes can lead to a disruption of the finely regulated functions of the hormones. Presently, several effect-directed bioassays are available to measure estrogen, androgen, progesteron, glucocorticoid and thyroid activity of contaminants in human and yeast cells (e.g. Jin, 1997; Someveld, 2005; Marchesini, 2006).

Genotoxic chemicals are compounds that damage DNA. They can for example bind to the DNA, preventing it from being copied or read. When certain genes on the DNA can not be read, certain essential cellular components cannot be produced anymore, which may be lethal to the cell. The cell therefore has several repair systems, to cut out the piece of DNA with the chemical bound to it and repair the DNA. Some of these repair systems can make errors, leading to changes (mutations) in the DNA. These can be very small changes, such as the change of a nucleotide (i.e. gene mutations), or changes on a larger scale, such as chromosomal breaks (i.e. chromosomal aberrations). Such changes in the DNA can cumulatively lead to a distorted function of a cell, thereby becoming a cancer cell. Thus, genotoxic chemicals can ultimately cause cancer. Mutations in germ cells can lead to changes in the offspring, which can cause congenital diseases. For detection of genotoxicity, various tests are available. Genotoxicity can be tested by measuring the formation of DNA damage itself (gene mutations or chromosomal aberrations) or by measuring the induction of the various DNA repair enzymes. As DNA damage can occur through different mechanisms, a battery of tests is necessary in both cases. However, for the repair enzymes, no full battery covering all repair types for the different types of damage is available.

Therefore, a battery consisting of a gene mutation test and a chromosomal aberration test is recommended (Heringa, 2005; Pfuhrer *et al.*, 2007).

Finally, developmental and teratogenic effects are relevant effects of drinking water contaminants. Teratogenic effects are structural birth defects caused during pregnancy by chemicals passing from the mother to the foetus. A well known example of such an effect are the limb malformations caused by the sedative Softenon in the 1960s. Developmental effects can occur during the whole lifestage from conception to adult, and can range from subtle effects on cognitive function (for example the loss of IQ by lead exposure) to gross effects such as growth retardation. There are several *in vivo* assays to cover adverse developmental (and teratogenic) effects using *Daphnia*, fish or tadpoles as model systems (e.g. Abe *et al.*, 2000; Berry *et al.*, 2007; Gutleb *et al.*, 2007). These are not used in screening of drinking water quality at the moment, but seem promising and relevant tools also as model systems for humans (Berry *et al.*, 2007).

Other toxicological end-points that might be relevant to study in future work are assays related to immunotoxicity and neurotoxicity.

COMPARING ROBUSTNESS AND SENSITIVITY OF VARIOUS *IN VITRO* EFFECT ASSAYS

Effect assays for endocrine disruption

Recently, Leusch (2008) evaluated the performance of five *in vitro* effect-directed assays to assess estrogenic activity in the same water samples; the yeast estrogen screen (YES), ER-CALUX, MELN, T47D-KBluc and E-screen. These assays were selected from an initial broader set of 24 bioassays. Both ER-CALUX and E-screen were shown to be robust and able to sensitively detect estrogenic activity in water, at concentrations of 0.1 to 320 ng/L EEQ (equivalents of the most potent estrogenic chemical). The YES and MELN assay gave respectively less sensitive and less quantitative results and results also depended on the matrices. Data were too limited to draw firm conclusions for the KBluc assay.

Similar comparative studies have not yet been performed for other effect assays related to hormone disruption. However, based on 41 literature references the sensitivity, robustness, time to result and operational specifications were evaluated for 20 available effect assays for hormonal disruption in the context of the TECHNEAU project (Table 1; Mons, 2008). The assays are based on mammalian or yeast cells, and are reporter gene assays or cell proliferation assays. As especially for recently developed assays available literature is still scarce, this evaluation should be viewed as indicative. For more detailed information we refer to Annex I of Mons, 2008.

Effect assays for genotoxicity

Similarly, high-throughput effect assays for genotoxicity were evaluated in the context of the TECHNEAU project (Mons, 2008). Here, 13 different bioassays were evaluated based upon 49 individual literature studies (Table 2). Existing assays are gene mutation assays, chromosomal aberration tests or DNA repair assays. Often the enzyme mix S9 is added to simulate metabolic processes that may lead to the formation of genotoxic metabolic products. As Table 2 shows, information is far from complete.

Table 2. Indicative evaluation of different high-throughput effect assays for genotoxicity (Mons, 2008).

	Sensitivity		Robustness		Time to result	Operational specifications	
	Sources	Drinking water	Operational robustness	Selectivity		Ease-of-use	Maintenance requirements
<i>Gene mutation assays</i>							
Ames test	2	2	4	4	3	4	3
Vibrio harveyi test	2	2			3		
<i>Chromosomal aberration tests</i>							
Comet assay	3	3	4		3		
Micronucleus assay					2-3		
Alkaline elution assay				2	2-3		
Polymerase inhibition assay					3-4		
<i>DNA repair assays</i>							
UMU test	4	4	3-4	3	3	3	
SOS Chromotest					4		
Vitotox [®]					4		
Mutatox [®]	3	3	4	3	4		
Greenscreen [®]					3		
Greenscreen HC					3		
MCF-7-p53R2					3		

1 = very poor, 2 = poor, 3 = average, 4 = good, 5 = very good; when insufficient information was available, the box was left empty.

As stated before, a battery consisting of a gene mutation test and a chromosomal aberration test is recommended (Heringa, 2005; Fuhler *et al.*, 2007). Such a combination has been shown to have good sensitivity (i.e. a high degree of correct positives) for rodent carcinogenicity, but seems not to be able to escape a poor specificity (i.e. a high degree of false positives) (Kirkland *et al.*, 2005). To assess genotoxicity in surface water, a combination of the Comet assay with human lymphocytes next to the Ames or umu-test has been proven useful (Grunmt and Erprobung, 2001; Minnear and Plewa, 2003). If one of the tests is positive in such a scheme, a third assay should be performed, preferably in mammalian cells. If a genotoxic sample remains genotoxic after conventional water treatment, additional research, including unravelling of responsible chemicals and risk assessment, will be necessary.

APPLICATION IN DRINKING WATER AND ITS SOURCES AND UNRAVELLING RESPONSIBLE COMPOUNDS

Often sample preparation and concentration are needed before aqueous samples can be tested in effect assays, because of the detection limits of the assays and because water samples need to be cleaned of excess dirt and microorganisms to allow the cells of the test to function properly. During this sample preparation, the original chemical mixture should preferably not be modified due to e.g. volatilization or sorption. Detection limits of the *in vitro* effect-directed bioassays, including sample concentration, should be sufficiently low to detect water concentrations that are relevant for human health risks.

The identity of the compounds responsible for the toxicological effect remains unknown, unless the effect directed bioassays are combined with analytical chemical techniques in a toxicity identification evaluation (Houtman *et al.*, 2006). Especially accurate mass screening and identification using LC-LTQ FT Orbitrap MS has proven useful in elucidating unknown compounds responsible for observed effects in *in vitro* effect assays (Hogendoorn *et al.*, 2009). This asks for sample preparation techniques that are suitable for both chemical analysis and evaluation in effect assays. Using solid phase extraction (SPE) with modern, broad-spectrum extracting columns, this can be accomplished.

Van der Linden *et al.* (2008) applied the various CALUX assays for hormonal disruption to various effluents, surface waters and drinking waters in the Netherlands. Estrogen, androgen, progesterone and glucocorticoid activities were shown in effluents and surface waters, but not in drinking water. They showed that androgen and progesterone activities were very low, but especially glucocorticoid activities were high compared to the other hormonal effects.

Schriks *et al.* (in preparation) identified and quantified the compounds responsible for the observed effects in these samples, which were mostly immunosuppressive pharmaceuticals used against diseases such as asthma and eczema. These compounds were not included in any regular monitoring programme thus far.

Heringa *et al.* (in preparation) have applied the Ames II, Comet and micronucleus assays to a sewage water treatment plant effluent, several surface waters and several contaminated ground waters in the Netherlands. They found genotoxic activity in the effluent, one surface water and one ground water in the Ames II and/or Comet assays, but no responses were measured with the micronucleus assay. The responsible chemicals have not been elucidated, but the occurrence of detectable levels of genotoxic compounds in these water samples was not known so far. It remains to be investigated whether the responsible genotoxic compounds pass the treatment steps in drinking water preparation.

The aforementioned examples show that a combination of using sensitive *in vitro* effect assays and subsequent accurate mass screening to identify and quantify responsible chemicals is a powerful combination to improve the assessment of the drinking water quality.

INTERPRETATION OF EFFECT ASSAYS IN TERM OF HUMAN HEALTH RISKS

Currently, the significance of test results in the effect-directed bioassays in terms of human health risks is still in debate, as *in vitro* cell cultures are clearly very different from the whole of the human body. Current human-health based drinking water quality limits are generally based on Acceptable Daily Intake values (ADIs), the quantity of a compound (expressed per kg body weight per day) that can be ingested without adverse effects. This ADI is mostly based upon *in vivo* mammalian toxicological data, taking into account the exposure to one compound only. In *in vivo* assays adsorption, distribution, metabolism and elimination (ADME) of the compound will influence its toxicity. These factors will not always be well mimicked in *in vitro* assays. In order to come to effect-based quality limits for complex mixtures, it is proposed to base these on the Acceptable Daily Intake value (ADI) of a highly potent reference compound, and to translate this value into effect-based limits using worst case assumptions for ADME (Van der Oost, 2008). This method has the disadvantage of probably leading to overprotective limits in most cases, but seems the only way to effectively apply effect-directed assays in water quality assessment. Further work is necessary to develop such water quality limits expressed in terms of acceptable effects in *in vitro* assays.

CONCLUSIONS

- *In vitro* effect assays are useful in the assessment of (drinking) water quality as they detect the presence of harmful chemicals that analytical chemical methods might not pick up, and they show the cumulative effect of all chemicals present in the water.
- Relevant effects to analyze in drinking water and its sources are genotoxicity, endocrine disruption, developmental and teratogenic effects and possibly neurotoxicity and immunotoxicity, as these are effects that can occur at relatively low exposures already.
- There are various assays available for the mentioned effects, differing in sensitivity, specificity and robustness.
- Application of these assays to various water types in the Netherlands has shown the presence of possibly harmful chemicals which were not detected by the current monitoring programme.
- There is a first idea on how to develop water quality limits expressed in terms of acceptable effects in *in vitro* effect assays, by basing them on the ADI of a reference compound. Further work on this topic is necessary.

REFERENCES

- Abe, T., Saito, H., Niiikura, Y., Shigeoka, T. and Nakano, Y. (2000). Embryonic development assay with *Daphnia magna*: Application to toxicity of chlorophenols. *Water Science and Technology*, **42**, 297-304.
- Benotti, M.J., Trenholm, R.A., Vanderford, B.J., Holady, J.C., Stanford, B.D. and Snyder, S.A. (2009). Pharmaceuticals and endocrine disrupting compounds in U.S. drinking water. *Environmental Science and Technology*, **43**(3), 597-603.
- Berry, J.P., Gantat, M., Gibbs, P.D.L. and Schmale, M.C. (2007). The zebrafish (Danio rerio) embryo as a model system for identification and characterization of developmental toxins from marine and freshwater microalgae. *Comparative Biochemistry and Physiology C*, **145**, 61-72.
- Diamond, M.L. and Hodge, E. (2007). Urban contaminant dynamics: From source to effect. *Environmental Science and Technology*, **41**(11), 3796-3800.
- Grunmt, T. and Erprobung (2001). Vergleich, Weiterentwicklung und Beurteilung von Genotoxizitätstests für Oberflächengewässer. *Wasser Abwasser*, **142**(5), 346-355.
- Gutleb, A.C., Schriks, M., Mossink, L., Van den Berg, J.H.T. and Mürk, A.T. (2007). A synchronized amphibian metamorphosis assay as an improved tool to detect thyroid hormone disturbance by endocrine disruptors and apolar sediment extracts. *Chemosphere*, **70**, 93-100.
- Heringa, M. (2005). *Approach for Assessment of Carcinogenic Activity in Water - Part I*. BTO report 2005.022. KWR Watercycle Research Institute, Nieuwegein, The Netherlands.

- Hogenboom, A.C., Van Leerdam, J.A. and De Voogt, P. (2009). Accurate mass screening and identification of emerging contaminants in environmental samples by liquid chromatography-hybrid linear ion trap Orbitrap mass spectrometry. *Journal of Chromatography A*, **1216**, 510–519.
- Houtman, C.J., Booij, P., Jover, E., Pascual del Rio, D., Swart, K., Van Velzen, M., Vreuls, J.J., Legler, J., Brouwer, A. and Lamoree, M.H. (2006). Estrogenic and dioxin-like compounds in sediment from Zierikzee harbour identified with CALUX assay-directed fractionation combined with one and two dimensional gas chromatography analyses. *Chemosphere*, **65**, 2244–2255.
- Jin, L., Tran, D.Q., Ide, C.F., McLachlan, J.A. and Arnold, S.F. (1997). Several synthetic chemicals inhibit progesterone receptor-mediated transactivation in yeast. *Biochim. Biophys. Res. Commun.*, **233**(1), 139–146.
- Kirkland, D., Aardema, M., Henderson, L. and Müller, L. (2005). Evaluation of the ability of a battery of three in vitro genotoxicity tests to discriminate rodent carcinogens and non-carcinogens. I. Sensitivity, specificity and relative predictivity. *Mutation Research*, **584**, 1–256.
- Leusch, F.D.L. (2008). *Tools to Detect Estrogenic Activity in Environmental Waters*. GWRC, London, UK.
- Loos, R., Gawlik, B.M., Locoro, G., Rimaviciute, E., Contini, S. and Bidoglio, G. (2009). EU-wide survey of polar organic persistent pollutants in European river waters. *Environmental Pollution*, **157**, 561–568.
- Marchesini, G.R., Meulenberg, E., Haasnoot, W., Mizuguchi, M. and Irth, H. (2006). Biosensor recognition of thyroid-disrupting chemicals using transport proteins. *Analytical Chemistry*, **78**, 1107–1114.
- McCarty, L.S. and Borgert, C.J. (2006). Review of the toxicity of chemical mixtures: Theory, policy, and regulatory practice. *Regulatory Toxicology and Pharmacology*, **45**, 119–143.
- Minear, R.A. and Plewa, M.J. (2003). *Comparative Genotoxicity Assessment of DBPs in Drinking Water*, Awwa Research Foundation and American Water Works Association, report number 90939.
- Mons, M. (2008). *Monitoring and Control of Drinking Water Quality – Inventory and Evaluation of Monitoring Technologies for Key-parameters*. TECHNEAU report D.3.1.3.
- Pfuhler, S., Albertini, S., Fautz, R., Herbold, B., Madle, S., Utesch, D. and Poth, A. (2007). Genetic toxicity assessment: employing the best science for human safety evaluation part IV: Recommendation of a working group of the Gesellschaft fuer Umwelt-Mutationsforschung (GUM) for a simple and straightforward approach to genotoxicity testing. *Toxicological Sciences*, **97**(2), 237–240.
- Reemtsma, T., Weiss, S., Mueller, J., Petrovic, M., Gonzalez, S., Barcelo, D., Ventura, F. and Knepper, T.P. (2006). Polar pollutants entry into the water cycle by municipal wastewater: A European perspective. *Environmental Science and Technology*, **40**, 5451–5458.
- Richardson, S.D. (2008). Environmental mass spectrometry: Emerging contaminants and current issues. *Analytical Chemistry*, **80**, 4373–4402.
- Schwarzenbach, R.P., Escher, B.I., Fenner, K., Hofstetter, T.B., Johnson, C.A., Von Gunten, U. and Wehrli, B. (2006). The challenge of micropollutants in aquatic systems. *Science*, **313**, 1072–1077.

- Sonneveld, E., Jansen, H.T., Ritco, J.A., Brouwer, A. and van der Burg, B. (2005). Development of androgen- and estrogen-responsive bioassays, members of a panel of human cell line-based highly selective steroid-responsive bioassays. *Toxicological Sciences*, **83**(1), 136-148.
- Turgeon, S., Rodriguez, M.J., Theriault, M. and Levallois, P. (2004). Perception of drinking water in the Quebec region (Canada): The influence of water quality and consumer location in the drinking water system. *Journal of Environmental Management*, **70**, 363-373.
- Van der Linden, S.C., Heringa, M.B., Man, H.Y., Sonneveld, E., Puijker, L.M., Brouwer, A. and Van der Burg, B. (2008). Detection of multiple hormonal activities in wastewater effluents and surface water, using a panel of steroid receptor CALUX bioassays. *Environmental Science and Technology*, **42**, 5814-5820.
- Van der Oost, R. (2008). *Vision Upon Mixture Toxicity in Drinking Water*. BTO report 2008.009. KWR Watercycle Research Institute, Nieuwegein, the Netherlands (in Dutch).
- van Genderen, J., Mons, M.N. and van Leeerdam, J.A. (2000) *Inventory and Toxicological Evaluation of Organic Micropollutants, revision 1999*. Report of the Association of River Waterworks - RIWA, Amsterdam.