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Applicability of functional genomics tools for water quality assessment

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Chapter 7: Summary, discussion and outlook

There is an increasing public concern about the possible threat of toxic compounds in the environment, as their presence has been linked to several disorders related to reproduction, metabolism and stress in animals and even in humans. Indeed, several compounds in the environment have been demonstrated to interfere with endocrine pathways, mainly the estrogenic pathway. However, information on interference of contaminants in water with other endocrine pathways is largely lacking. The research presented in this thesis describes the development, validation and application of a range of reporter gene assays that can detect activity on specific nuclear hormone receptor pathways and on pathways related to genotoxic stress. Results show that compounds are present in the surface water that interfere with one or several of these pathways, and first steps have been made in identifying some of the compounds responsible, including pharmaceutical drugs acting on glucocorticoid receptor. A summary of the findings is presented below.

Summary of the results

In the first chapter, the problem description is given and background information is provided. This chapter elaborates on the current challenges in water quality monitoring leading to the research described in this thesis. In order to place the selection of used and developed assays into perspective, the toxicological pathways (on a cellular level) and adverse outcome pathways (on a population level) frameworks are introduced. The principle of reporter gene assays and gene expression profiling is explained with a focus on nuclear receptors and DNA damage responses.

Chapter two describes the results of the first screening survey using a panel of reporter gene assays, which were performed on a diverse set of Dutch water samples including drinking water, surface water and several types of effluent. All water samples were screened for activity on four nuclear hormone receptors: the estrogen receptor, androgen receptor, progesterone receptor and glucocorticoid receptor. Previous research had shown that estrogenic compounds are present in the Dutch aquatic environment (Vethaak *et al.*, 2005), but information regarding the activity of compounds with other endocrine pathways was lacking. The results showed that compounds can be present in the aquatic environment that act on all four receptors. Especially the activity on the glucocorticoid receptor was prominent, as this receptor is involved in regulation of the immune system, stress responses and glucose metabolism and the contaminants found in water might therefore be linked to adverse effects on humans and wildlife.

In chapter three, compounds potentially responsible for the glucocorticoid responses described in chapter two were investigated. This initial investigation focused on pharmaceuticals only, because the glucocorticoid receptor is a target for frequently described medications. For practical reasons, this identification study focused on hospital effluent samples only, as these samples had the highest level of glucocorticoid activity in the initial screen described in chapter two. It was shown that a large part of the observed glucocorticoid activity in the effluent sample could be contributed to known pharmaceuticals that are specifically designed to act on this receptor.

Before assays might claim a role in water quality assessment and control, they need to be properly validated beyond the academic setting. An important aspect of such a validation is to show the ability to produce specific, robust and reproducible responses within and between different laboratories. This is described in chapter four for the AR CALUX, a bioassay that responds to androgenic compounds specifically and was used in the screening of androgenic and antiandrogenic activity in the water samples in chapter three. The first step in such a validation process is to introduce the assays at different laboratories for interlaboratory calibration and validation purposes. This is done by analyzing of a training set of pure compounds (positive and negative controls) that allow the assessment of the performance within and between individual laboratories. The results from chapter four show that the AR CALUX assay is a robust assay that has successfully been introduced at different laboratories. The results also show that the AR CALUX is selective, with no interference from other nuclear receptor pathways, which is important in determining specific pathway responses present in complex mixtures like water samples and other environmental matrices.

Chapter five describes the development of a panel of novel assays aimed at detecting genotoxic compounds. The cell lines developed utilize human cells and detect activity on two (partly overlapping) pathways important for cells coping with genotoxic stress, the p53 pathway for direct DNA damage and Nrf2 pathway for oxidative stress. Additionally, as the width of the window

between activation of these pathways and cytotoxicity is rather small, the Cytotox CALUX has been developed that acts as a control for cell viability and luciferase expression. All these bioassays utilize the same U2-OS cell line, also used in the development of the nuclear receptor assays, together with the same luciferase reporter as readout. By applying these reporter gene assays within the same basic cell line in a panel setup, artifacts are kept to a minimum, which is important when analyzing complex mixtures like environmental samples. The panel of cell lines was validated using a set of 61 compounds, including genotoxic compounds with different modes of action, non-genotoxic compounds and compounds that are known to cause false positive results in *in vitro* genotoxicity assays. Results show that the cell line panel is able to detect genotoxic compounds with high sensitivity (95%) and specificity (85%) and can be performed in a high throughput format as part of a cell line panel that can screen for multiple modes of action in parallel.

Chapter six describes the comparison of analysis of water samples based on specific cell-based bioassays as described in previous chapters with a holistic approach using gene expression profiling. Water samples are analyzed for biological activity on a range of nuclear receptors using pathway specific bioassays, which included the assays described in chapter two, supplemented with assays responding to the peroxisome proliferator-activated receptor gamma (PPAR γ) and the aryl hydrocarbon receptor (AhR). The environmental samples were also tested for activity on nuclear hormone receptor pathways, by comparing the gene expression signatures elicited by the environmental samples to compounds-specific signatures derived from pure compounds. For this microarray based gene expression profiling, T47D cells were utilized as this cell line expresses a wide range of nuclear receptors endogenously. The results from this study showed that the gene expression responses using microarrays are reproducible, but were prone to false negative results, due to the cross-talk between pathways and associated suppressive effects. These effects are known to exist and are normal in cellular biology, but they can hamper utilizing those responses as the basis for the detection of compounds in complex mixtures like environmental samples. By selection the appropriate cell line-response combination, specific bioassays do not suffer from these effects and allow not only a more defined and sensitive detection of activity but can also quantify the response.

In the following of this chapter 7, the results obtained will be discussed and an outlook is presented regarding the possible use and applicability of functional genomics tools in future water quality control.

Presence and risk of endocrine activities in the environment

The results from the studies described in this thesis show that endocrine activities in the aquatic environment are widespread. Historically, most analysis of endocrine disruptors in the environment has focused on estrogens, which was linked to feminization observed in fish. However, activity on other hormone receptors such as the glucocorticoid and androgen receptor might be of equal importance. Only recently, activity on these other receptors has gained more attention in relation to endocrine disruption and related adverse effects (OECD, 2012; Christen *et al.*, 2010). Toxicological profiling of pure compounds has shown that many environmentally relevant compound can indeed act on these receptors, including non-steroidal compounds (Huang *et al.*, 2011; Martin *et al.*, 2010). Chemical analysis (Chang *et al.*, 2007; Liu *et al.*, 2011; Chang *et al.*, 2009) and biological effect based analysis (Van der Linden *et al.*, 2008; Stavreva *et al.*, 2012;

Escher and Leusch, 2012) have shown that compounds that are known to act on a wide range of nuclear hormone receptors are indeed present in the environment. Effect directed analysis (EDA), a combination of biological assays with sophisticated chemical analysis techniques, can elucidate whether the observed biological effects can be fully explained by identified compounds or whether there are other contributors to the observed activity.

As the awareness about other types of endocrine compounds than estrogens is relatively recent, little is known about the removal of (to some extent unknown) endocrine compounds in water treatment processes, including drinking water treatment. First results suggest that the removal of non-estrogenic endocrine compounds can differ from the removal of estrogenic compounds, depending on the treatment steps applied (Fan *et al.*, 2011; Arsand *et al.*, 2013; Yang *et al.*, 2012). Recent results on effect based bioassays suggest that the endocrine activity can also increase during treatment, possibly due to breakdown products originating from the wastewater treatment process (Kienle *et al.*, 2011). No endocrine effects have been detected in any of the drinking water samples tested. And as drinking water can be regarded as the most important route of exposure for humans, the human risk regarding exposure to compounds active on the endocrine system seems to be negligible if extensive purification processes are applied. However, most nuclear hormone receptors are relatively conserved throughout the animal kingdom (Gunnarsson *et al.*, 2008) as are other stress responses including the response to genotoxic compounds (Wahl and Carr, 2001; Sancar *et al.*, 2004). Therefore, effects on aquatic species might be expected as they are (partly) living in the untreated surface water and are chronically exposed to the compounds present. Indeed, estrogens can have adverse effects on fish reproduction at low, ng per liter concentrations by acting on the fish estrogen receptors (Kidd *et al.*, 2007). Similarly, environmentally relevant concentrations of synthetic glucocorticoids or progestins have been shown to induce adverse effects on fish, by acting on fish receptors that are similar to the originally targeted human receptors (Kugathas and Sumpter, 2011; Zucchi *et al.*, 2012).

Specific bioassays as environmental screening tools

To safeguard the quality of the aquatic environment, it is important to monitor (compounds with) potentially adverse effects. As chemical analysis alone cannot fully identify biologically relevant compounds in the water, additional methods are needed. Effect based assays are suitable candidates as they can be performed in a high throughput manner and can cost-effectively supply mechanistically relevant information. The toxicity pathway and the adverse outcome pathway framework (Ankley *et al.*, 2010) may serve as an excellent basis for the selection of bioassays that focus on the pathways relevant for assessing the risk of compounds present in the (aquatic) environment. Knowledge about these pathways can provide the necessary information to guide the development of predictive *in vitro* assays, as well as help these tests gain diagnostic credibility due to their links with the initiating event and the adverse outcome.

Many assays already exist that cover at least part of the known modes of action spectrum. Additional pathways can be included in the future, based on the advancement of knowledge about critical targets in cellular pathways and their link to adverse effects. Assays that focus on nuclear receptors are available, although some members that are clear pharmaceutical targets are still lacking, e.g. the mineral corticoid receptor and vitamin D receptor. Gaps regarding other pathways of interest are other important molecular targets of frequently applied pharmaceuticals like the

adrenoreceptors α and β (Christen *et al.*, 2010) and assays that monitor pathways related to adaptive stress and immune system responses. In addition to very specific mode of action pathways, xenobiotic receptor pathways could be included in a screening panel, e.g. the pregnane X receptor (PXR) and the constitutive androstane receptor (CAR), as they play important role in xenometabolism (Di Masi *et al.*, 2009). Many environmentally (and dietary) relevant compounds have been identified that can activate these receptors (Martin *et al.*, 2010; Hernandez *et al.*, 2009). However, the value of these receptors in screening water samples may be limited due to their responsiveness towards a wide range of compounds. In relatively clean matrices like drinking water, however, activity on these receptors may be of additive value as general indications of compounds present.

Of all the assays that can be found in literature, only a limited number has been validated beyond the academic setting (Hartung, 2009; Hartung, 2010). Even fewer assays have been applied in the detection of activity in the aquatic environment with (Escher and Leusch, 2012; GWRC, 2012). This formal assessment of reliability and relevance is what differentiates a model (e.g. a specific cell line or bioassay) from a test that can be applied routinely. Tests are subjected to quality assurance (QA) and will have to be fully validated and standardized to contribute to water quality monitoring and for governmental acceptance of the assays involved. And just like in chemical monitoring, ring trials will have to be conducted to ensure a reliable interlaboratory quality control system, for individual laboratories. Only a small set of mode of action based *in vitro* assays has already been fully validated and applied for water quality assessment. However, most of these assays focus on activity on nuclear hormone receptors. Assays for adaptive stress responses, focusing on oxidative stress, different aspects of genotoxicity and pathways related to the immune response are only scarcely applied in environmental monitoring (Escher and Leusch, 2012; Escher *et al.*, 2005; Escher *et al.*, 2012). Additionally, many assays focus on the activation of nuclear receptors and not on the disruption of metabolic processes. Disrupting of the conversion of hormones to inactive compounds or to compounds active on other hormone receptors can also potentially result in endocrine effects. Assays that focus on these important steps may also be included, e.g. by including cell lines that express (all the) key enzymes for steroidogenesis like the H295R cell line (Zhang *et al.*, 2005) in combination with specific endpoint assays.

No matter the pathway of interest under investigation, complex interaction between different pathways can seriously hamper the reliability of results when analyzing complex environmental mixtures. Important examples of reporter gene assays that focus on a specific type of activity but are less selective due to the cell line-reporter construct combination. For example, a MDA-MB-453 based reporter gene assay for the detection of androgenic activity expresses both the androgen receptor and the glucocorticoid receptor (Wilson *et al.*, 2002). As all these receptors activate the same responsive element, the bioassay using a cell line expression both types of receptors responds equally to androgens and glucocorticoids. Similarly, a T47D cell line based androgenic assay also expresses the PR receptor (Blankvoort *et al.*, 2001), resulting in a response to both androgens and progesterin. Discrimination between the two responses can be determined to some extent, but requires additional testing in the presence of a selective antagonist. By avoiding or eliminating potentially interfering aspects of a bioassay, the number of artefacts can be limited, keeping expensive follow up studies regarding the chemical identity of the causative compounds to a minimum.

Microarrays as environmental screening tools

An important novel approach to screen for adverse effects in the environment is by considering the whole genome expression profile in response to exposure to a (mixture of) contaminants (Van Aggelen *et al.*, 2010). This can be done by measuring gene expression levels of all known genes using microarray, by quantifying mRNA levels. Although the mRNA is not the final product of a gene, its levels do provide insight into the expression of genes in response to the exposure. Numerous examples can be found in literature that show that gene expression profiles can be used to determine important pathways activated in response to compound exposure (Hamadeh *et al.*, 2002; Garcia-Reyero *et al.*, 2008) and as a result several authors have highlighted that genomics data can be used to identify chemical causation of effects induced by complex mixtures. The use of gene expression data has several advantages over specific effect-based bioassays, most importantly the potential to include all pathways in the assessment, rather than only on a *a priori* selection.

Gene expression responses in complex cellular systems reflect the biological interactions based on one or more initiating events, assessed by pathway specific assays and as such they may serve as a link between bioassay responses and *in vivo* predictions. However, screening tools in water quality assessment are aimed at identifying potential risks and interactions depend on the type of cell line species under investigation. Therefore, pathway interaction can result in a misclassification of activities present in samples. Specific effect based assays can be optimized for the pathway of interest, keeping interaction to a minimum while providing a quantification rather than a qualification of the level of effect. Results also suggest that specific bioassays are more sensitive with regard to identifying initiating event (chapter 6 of this thesis), possibly in part due to the minimized interaction. Because of this, *in vitro* expression profiling is unable to replace mode-of-action based bioassays, tuned to a range of specific nuclear receptor based effects in water quality assessment and environmental monitoring.

Water quality criteria for effects based responses

Most current legislative guidelines for safety and quality of e.g. water are based on concentrations of single compounds. And even though several types of activity have been shown to be present in the environment that could be related to adverse effects, bioassays still mainly remain research tools and no bioassay based guidelines are in place for water quality monitoring. Therefore, there is a need to shift from a chemical by chemical approach to a more combined approach where bioassays and chemical analysis can supplement each other, each proving essential information regarding the presence of unwanted compounds in the water (see figure 1). Water industries and regulators, sometimes driven by public concern, are generally positive towards the idea of effect based analysis. However, they are somehow - besides the concern about additional costs - not daring to place enough trust in these new tools to steer away from the already established but clearly non-perfect *in vivo* test systems and individual chemical limit values once in place.

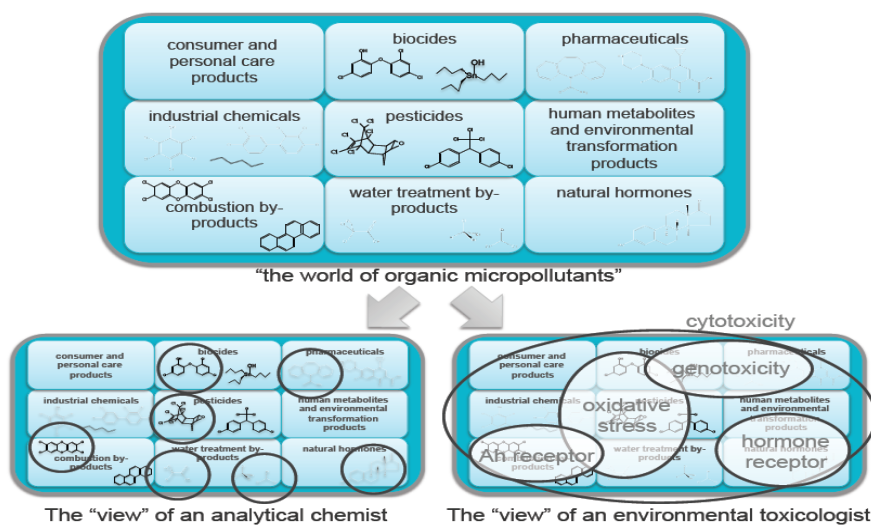


Figure 1. The realm of environmental chemicals and the targets for chemical analysis (left) and bioanalytical tools (right). The circles indicate subsets of chemicals or mode of action groups that are covered by various types of bioassays (Taken from Escher and Leusch, 2012).

In order to make the transition possible, several steps are identified to be important (Velleneuve and Garcia-Reyero, 2013; Hartung and Daston, 2009). First of all there is the need to link responses measured at the cellular level to adverse outcomes on the animal or even population level. Secondly, biologically based, quantitative extrapolation tools are needed that allow to model data on initiating events (cell or tissue level) to individuals across species. While this is relatively straight for pure compounds, it is a daunting task for complex mixtures like environmental samples. It is therefore important to relate integrated bioassay responses to the culprit chemical present in the environment (Villeneuve and Garcia-Reyero, 2013). Clear case studies are needed to change toxicological practice to suit the novel needs of risk assessment. However, it is not realistic nor productive to wait for the perfect assay panel to be available that will be able to fully predict in vivo toxicity, but mode of action based assays can already add valuable information to the currently established methods today (Dietrich, 2010; Escher and Leusch, 2012). A key element missing in the current bioassay screening approach is the absence of limit values. An important aspect of bioanalytical tools is that they can quantify the total level of effects present in complex mixtures in a sample extract, but a quantitative limit value of some kind is needed to give the assay response meaning. Once such values are derived, bioassays can be ideal screening or prioritization tools that can be used in a tiered approach for initial screening. Only samples that exceed a given threshold would require follow up, e.g. chemical analysis to identify causative compounds or alternative assays for confirmation (see figure 2). So the most important question that remains is: at what level should we set thresholds?

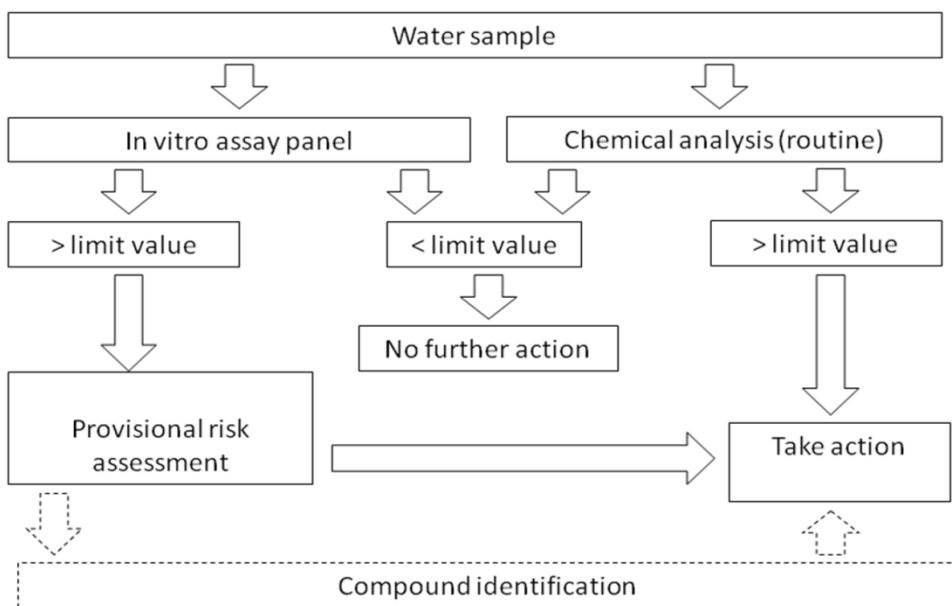


Figure 2. Testing strategy for the monitoring of water quality using bioassays and chemical analysis. Water quality is monitored using bioassays, in parallel with routine chemical analysis. In case of thresholds are exceeded, a provisional risk assessment can be made based on the mode of action. When needed, sophisticated chemical analysis can be utilized for the identification of the compounds responsible and risk can be assessed on a compound basis.

The application of bioanalytical tools and approaches is already in place in the field of dioxin testing, where the total amount of dioxin-like activity assessed by a bioassay (DR CALUX) can limit the need for chemical confirmation analysis of dioxin-like compounds. Only samples in which the total amount of dioxin-like activity exceeds the limit value set for dioxins only (the most strict limit value), require chemical confirmation. Similarly, based on knowledge regarding the (possible presence) of potent compounds, a "trigger value" might be derived for other pathways of interest. These can be derived for different pathways and can be based on known compounds for which toxicological information is available, e.g. Acceptable Daily Intake (ADI) for humans or NOEL for animal studies. These values can be combined with their pharmacokinetic factors and potency in the specific bioassays. The value derived is the concentration that can still be regarded safe, even if all activity could be attributed to the most potent compounds, thus encompassing a worst case scenario. All values below this threshold level can then be waived, as adverse effects are not anticipated. All values above this level require a more detailed examination, as adverse effects cannot be excluded. This follow-up can then rely on more traditional toxicity testing. Such an approach has been proposed recently (Brand et al., submitted), and might serve as an encouragement to develop and explore other effect-directed bioassays and adherent trigger values.

Concluding remarks

By integrating different monitoring strategies, including chemical analysis and a suite of specific mode of action based bioassays, a more accurate and predictive picture can be obtained regarding the presence of biologically active compounds in the environment. To suite such an intelligent testing strategy, these assays have to respond specifically to a specific mode of action, to gain a clear picture of the types of activity present in a complex mixture and to avoid unnecessary follow-up. By using this data in computational, mechanistically based models that include pathways present in many different species, human and ecotoxicological risks of environmental chemicals can be predicted more accurately. Major hurdles exist, including identifying and analyzing all the major pathways while still being costs-effective, keeping the analysis costs to a minimum. The starting point of such an endeavour may be pathways for which low trigger values are derived, e.g. genotoxicity and hormone receptors. Additionally, pathways should be included for which levels higher than the trigger value can be expected, based on predicted environmental concentrations by combining compound activity and use (Von der Ohe *et al.*, 2011). The initial set of tools may not, or may even never be, perfect (Hartung, 2009; Dietrich, 2010), but at least they will provide a more science-based assessment of the risks, including estimates of uncertainty. Future development will continuously increase our understanding between exposure and effects. It is now time to demonstrate and provide regulators with clear examples identifying key initiating events in the aquatic environment and let genomic based tools claim their important role in safeguarding the quality of the water. The work described in this thesis is aimed at providing some of these important initial steps.