Chapter 1

General introduction
Surface water and groundwater contain vast numbers of chemicals from either natural origin or introduced as pollutants from human activity\textsuperscript{1–6}. A large number of these chemicals present in the aquatic environment have the potential to be harmful to ecology or the human body when ingested\textsuperscript{7–12}. As both surface water and groundwater are used as source for drinking water, improper purification treatment can result in exposure of drinking water consumers to potentially toxic chemicals. In the Netherlands, approximately 60\% of drinking water is sourced from groundwater and 40\% from surface water\textsuperscript{13}. Due to its relative purity, groundwater is often only treated with aeration, filtration and softening whereas surface water, which is generally more prone to pollution with organic chemicals, requires more extensive treatment, consisting of combinations of e.g. sedimentation, coagulation/flocculation, sand filtration, dune or river bank infiltration and activated carbon filtration. In addition, at some plants, also membrane filtration or advanced oxidative processes using ozonation or UV/peroxide are applied\textsuperscript{14}. To ensure safe drinking water, the water source quality is routinely monitored by water laboratories. If excessive concentrations of contaminants are detected, water intake can be temporarily halted. To avoid shortages in the water supply, water sources need to be protected from contamination which remains one of the major challenges within the water sector\textsuperscript{15}. Managing the release of contaminants at the source is targeted as a way to reduce their presence in the aquatic environment and relieve the strain on water purification. Further quality control during the purification processes and of the final produced drinking water ensures proper removal of contaminants. Drinking water companies in Europe have to fulfill the quality standards and monitoring requirements mentioned in national legislation and in the European Drinking Water Directive. To protect the water bodies in Europe, having source for drinking water production as one of their functions, environmentally relevant contaminants have been included in the list of priority substances of the European Union (EU) Water Framework Directive (WFD\textsuperscript{16}). They are actively monitored by national governments and water boards.

Two important classes of contaminants monitored in the aquatic environment are endocrine disruptive chemicals (EDCs) and mutagens. EDCs are compounds that alter the function of the hormone system, for instance by mimicking endogenous hormones and binding to their respective receptors. Resulting receptor activation or inhibition disrupts signaling of endogenous hormones and can lead to disorders in (sexual)
development or formation of certain cancers\textsuperscript{17–19}. Exposure to mutagenic compounds can cause disruption of DNA replication resulting in genetic mutations that increase the risk of cancer development. (Synthetic) hormones and their metabolites, including androgens, estrogens, glucocorticoids, have been determined as a main contributor to steroid hormone receptor endocrine disruptive potency in wastewater and surface water\textsuperscript{20–26}. Non-hormonal EDCs have also been identified and include pesticides, flame retardants, personal care product additives, perfluorinated compounds and plasticizers\textsuperscript{27}. Mutagens, like polycyclic aromatic hydrocarbons (PAHs), have been found in the aquatic environment mostly in nonpolar matrices like suspended particulate matter (SPM)\textsuperscript{28}. In addition, mutagenic transformation products are also formed, and successfully removed, during the UV/peroxide oxidation and ozonation in drinking water purification\textsuperscript{29–31}.

While compounds actively monitored in accordance with the WFD are well characterized and potent toxic chemicals, novel contaminants remain to be discovered\textsuperscript{32,33}. Expanding the knowledge on environmentally relevant and bioactive contaminants is therefore an important step towards further improving the quality of the aquatic environment and to ensure safe drinking water in the future\textsuperscript{34}. Providing water laboratories with the technology to identify novel toxic contaminants during routine analysis of water samples would accelerate such efforts greatly.

**Toxicity testing**

Traditionally, target analysis is used to detect and quantify well-defined toxicologically relevant contaminants (e.g. pesticides, EDCs or mutagens). Compounds in sample extracts are chromatographically separated with liquid- (LC) or gas chromatography (GC) and masses of precursor ions and their fragments are recorded with a mass spectrometer (MS). When the retention time (RT) and mass spectrum of a specific compound correlate with that of a compound that is screened for, the compound can be confirmed and quantified. Target analysis, however, focusses on a limited set of known (priority) compounds and while specific and sensitive, it is unable to detect (novel) toxicologically relevant compounds that have not yet been defined before. By testing the toxicity of a sample in an endpoint-specific bioassay, the presence of toxic substances can be monitored based on the measured biological activity rather than by the presence of known compounds. Such bioassays make use of proteins or living cells from an organism in which the effect of a molecule (including small drug-like
molecules or proteins) on a specific molecular or cellular process induces a measurable (and quantifiable) response. Therefore, bioassay testing enables measurement of the total activity by all contaminants in a mixture that contribute to the specific effect and is less prone to underestimation of the toxic potency than chemical analysis of individual compounds. While currently not yet implemented in WFD monitoring activities\textsuperscript{16}, efforts are made to implement effect-based monitoring strategies using bioassays in the WFD in addition to chemical-analytical monitoring strategies\textsuperscript{35–38}.

**Effect-directed analysis**

Although effect-based monitoring provides knowledge about the total toxicity of all compounds present in the mixture, it does not indicate which compounds are (mainly) responsible for the observed bioassay response. Identification of emerging bioactive compounds, however, is necessary to extend existing target analysis databases and improve detection of toxicologically relevant compounds during routine monitoring or to guide efforts to reduce their emission into the aquatic environment altogether. To achieve both detection and identification of emerging compounds, bioassay testing and chemical-analytical methods are combined in an effect-directed analysis (EDA) approach\textsuperscript{39}. In EDA, fractions collected following chromatographic separation of a sample are individually tested in a bioassay to determine active fractions. Active fractions are then selected and further fractionated if necessary. Finally, they are analyzed by LC or GC coupled to high-resolution MS (HRMS) to identify and, ideally, quantify the compound(s) responsible for the observed bioassay response.

Exact masses in bioactive fractions that are not explained by target analysis are selected for identification and subjected to non-targeted analysis\textsuperscript{40,41}. Without prior information on the compound responsible for the observed bioactivity, mass specific features in the mass chromatogram are analyzed in order to resolve its identity. Molecular formulas are determined based on the exact mass, its corresponding isotopic distribution and suspected elemental composition. Candidate structures with the correlating molecular formula are retrieved from compound databases and their mass spectra are compared to spectra from mass libraries to tentatively identify the structure. Tentatively identified candidates are then compared against compound standards tested on GC or LC/HRMS and in the investigated bioassay to confirm their identity and their endpoint-specific bioactive potency, respectively\textsuperscript{42}.
While commonly applied in environmental research, EDA also found use in food analysis, cosmetics industry, drug development and other fields where it is commonly referred to as bioassay-guided fractionation\(^4\). Natural extracts, metabolic mixtures or combinatorial libraries are subjected to EDA for the identification of novel flavors, fragrances, pesticides, antimicrobial agents or for identification of novel compounds for use as lead in drug development. New developments in EDA for the purpose of environmental monitoring, therefore, may be applied in these fields as well.

Classical EDA strategies, however, are too time consuming to identify emerging compounds during routine (drinking) water quality control. Collection of a limited number of large volume fractions (e.g. 3 mL), each with a long collection time (e.g. 3 minutes), results in a large number of compounds being collected in a single fraction\(^3\). Consequently, the mass of the active compound must be distilled from a large number of measured masses. Increasing the number of fractions and thereby the resolution, reduces the number of masses per fraction to be identified and improves the focus of identification. This requires bioassay setups that (1) are suitable to test large numbers of fractions, and (2) produce a measurable response in reduced test volumes.

Bioassays

The capacity of a sample to interact with steroid hormone receptors is commonly tested in mammalian cell-based luciferase reporter gene bioassays, whereas it’s mutagenic activity is commonly tested in the bacterial Ames test.

Reporter gene bioassays consist of a monolayer of cells grown in medium at the bottom of multi-well plates. To detect (synthetic) steroid hormones and hormone mimicking compounds, different reporter gene assays exist that report a sample’s activity on nuclear hormone receptors (NHR) (e.g. androgen (AR), estrogen (ER), dioxin (DR) or glucocorticoid receptors (GR)). Once exposed, activated NHRs bind the DNA and initiate transcription and translation of a reporter gene encoding for an easily quantifiable protein, e.g. luciferase. Luciferase is an enzyme which catalyzes the reaction of luciferin into light (\(\text{D-luciferin} + \text{ATP} + \text{O}_2 \rightarrow \text{oxyluciferin} + \text{PPi} + \text{AMP} + \text{CO}_2 + \text{light}\)). The intensity of light (luminescence) produced is quantified as measure for receptor activation. Reporter gene assays are increasingly applied in environmental monitoring and have been used in EDA\(^4\). Because these luciferase reporter gene bioassays are commonly performed in 96-well plates, they are not yet compatible with
high-resolution fractionation. The sensitivity of these bioassays, however, make them good candidates for miniaturization to high-density well plates (384 and 1536 wells).

The Ames test is the standard for toxicological testing for mutagenicity\textsuperscript{46}. The assay is performed with Salmonella bacteria made histidine auxotrophic by a point mutation in a gene of the histidine synthesis pathway. Exposure to a mutagen can revert the mutation and restore histidine autotrophy. Growth of reverted bacteria on histidine-free medium after 48 hours is used as marker for mutagenicity. The first type of Ames test was performed on solid agar medium plates on which the response was quantified by counting the number of bacterial colonies formed. In order to improve the throughput of this labor intensive technique, the Ames fluctuation test (Ames II) was developed which is performed in liquid medium in multi-well plates\textsuperscript{47}. Reversion is visualized by the colorimetric change of a pH-sensitive indicator in the medium as the medium is acidified by bacterial growth. The reversion measured in a single well is a yes-no response which provides no information on the strength of the response. To allow quantification of the response, bacteria from each exposure are divided over 48 wells and mutagenic potency is determined as the number of wells (out of 48) that show a colorimetric change. While the original Ames test remains the ‘golden standard’ in environmental testing for mutagenicity, the fluctuation test has been validated in interlaboratory studies and is gradually being adopted as the new standard\textsuperscript{29,48–50}. The fluctuation test has also been used in EDA\textsuperscript{51}, however, the incubation time and number of wells required do not allow rapid screening of high-resolution fractionated samples. Various studies have focused on improving the throughput of the Ames (fluctuation) test by genetically modifying the bacteria to express (bacterial) luciferase reporters\textsuperscript{52–54}. The use of bioluminescence in the Ames test would allow sensitive detection of revertants at an early stage in a dose-response manner. This would prove a solution to the limitations of the Ames fluctuation test for use in high-resolution EDA. However, no combination of rapid testing and miniaturization has yet been developed.

**Scope and outline of the thesis**

The aim of this thesis was to increase the throughput of current EDA strategies, allowing to work towards routine EDA application for identification of bioactive emerging contaminants by
1) developing miniaturized bioassays for (rapid) testing of large numbers of samples or fractions,

2) integrating miniaturized bioassays into a high-throughput EDA (HT-EDA) approach in which the bioassay response to high-resolution fractionated samples can be directly linked to HRMS data recorded in parallel, and

3) demonstrating the complete high-resolution EDA work-flow through its application on relevant aquatic samples.

To allow rapid high-throughput detection and identification of emerging mutagenic and endocrine disrupting compounds, currently available bioassay setups were to be made compatible to high-resolution fractionated samples. For the rapid and high-throughput detection of mutagens we developed a luminescent and miniaturized luminescent Ames fluctuation test variant (Chapter 2). A constitutively expressed luxCDABE bacterial luciferase gene cassette that produces its own substrate was introduced to allow rapid detection of revertant bacteria by their luminescence. The luminescent response was characterized in two Salmonella strains that report frame-shift (TA98) and base pair substitution (TA100) mutations in the presence and in the absence of S9 metabolic activation. A miniaturized test format was developed for high-throughput testing of large numbers of samples on a single plate and for testing of high-resolution fractionated samples. Suspended particulate matter samples, water samples and a set of 14 mutagens were tested to validate their response in the newly developed strains and in the downscaled test format against the response in the regular fluctuation test.

In order to selectively detect androgens, a glucocorticoid-insensitive glucocorticoid receptor (GR) knock-out (KO) mutant of the AR-EcoScreen bioassay was developed using CRISPR genome editing (Chapter 3). The AR-EcoScreen is a Chinese Hamster Ovary (CHO) cell-based reporter gene bioassay for the detection of (anti-)androgenic compounds, expressing the human androgen receptor AR and firefly luciferase under transcriptional control of the human AR responsive element (ARE). The bioassay has cross-reactivity with glucocorticoids as activation of the GR, endogenously expressed at low levels by the CHO cells, can stimulate reporter gene expression and translation. The GR KO mutant was characterized and its sensitivity and selectivity was compared to that of the unmodified AR-EcoScreen bioassay by testing (anti-)androgenic and glucocorticoid compounds and mixtures. Applicability was
demonstrated through analysis of passive sampler extracts. Lower responses in the GR KO mutant compared to the AR-EcoScreen indicated that the presence of glucocorticoids led to an overestimation of the androgenic activity and an underestimation of the anti-androgenic activity in the unmodified assay.

Cell based luciferase reporter gene assays for detection of androgen, estrogen and dioxin-like compounds were miniaturized and applied in EDA of passive sampler extracts (Chapter 4). The AR-EcoScreen, AR-EcoScreen GR KO mutant, ER-Luc and DR-Luc bioassays were miniaturized to 384-well plate format, characterized and compared to the standard 96-well format with agonistic and antagonistic reference compounds. The ER-Luc bioassay (VM7Luc4E2 formerly known as BG1Luc4E2) is a MCF7 human breast carcinoma cell-based luciferase reporter gene assay for the detection of estrogens. The DR-Luc is a H4IIIE rat hepatoma cell-based luciferase reporter gene assay expressive the aryl hydrocarbon receptor (AhR) for detection of dioxin-like compounds including PAHs. Passive sampler extracts were fractionated using LC-MS and collected in 64 or 192 fractions in well plates while mass chromatograms were recorded in parallel. In addition, an exposure method was developed to allow high-throughput exposure of multiple assays from a single fraction collection plate. The miniaturized assays were used to analyze 64 fractions (in triplicate exposures) from passive sampler extracts for agonistic and antagonistic activity. Analysis of high-resolution fractionated passive sampler extracts was demonstrated by testing 192 fractions (in single exposures). Finally, an identification strategy was developed and applied in the non-targeted analysis of masses recorded in bioactive fractions. Selected candidate structures were confirmed for their presence in the sample and their biological activity on the UPLC-ToF-MS and bioassays, respectively.

The combined application of high-resolution fractionation in combination with miniaturized AR, ER and GR reporter gene assays and the miniaturized luminescent Ames test in an HT-EDA platform for analysis of water samples was demonstrated in Chapter 5. Wastewater treatment plant influent and effluent and surface water sample extracts were prepared and screened in miniaturized assays for bioactivity. Extracts were chromatographically separated using UPLC-QToF MS while masses were recorded in parallel. Fractions were collected on a contact free high-resolution fraction collector developed in parallel to the work described in this thesis. Miniaturized assays were applied to high-resolution fractionated extracts (228 fractions). The
identification strategy was applied with minor modifications and mutagens and endocrine disruptors were (tentatively) identified and confirmed.

References


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Chapter 1 General introduction


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