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Towards improved drinking water safety

The use of *in vitro* bioassays to optimize treatment processes and assess chemical hazards

Maria Yu

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Maria Yu
Faculty of Veterinary Medicine and Animal Science
Department of Biomedicine and Veterinary Public Health Sciences
Uppsala

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Cover: *To reflect upon clean and wholesome drinking water*
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Swedish University of Agricultural Sciences, Department of Biomedical Science and Veterinary Public Health, Uppsala, Sweden

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Abstract

Drinking water safety has been primarily dependent on the monitoring of only a limited number of individual chemicals via chemical analysis, which is insufficient to characterize the myriad of hazardous chemicals present in water. Effect-based methods (EBMs) using *in vitro* bioassays are useful in that they evaluate biological effects from all the known and unknown chemicals in the water and draw on toxicological principles. This thesis aimed to explore the versatility of EBMs for different applications related to drinking water safety. Firstly, we demonstrated how EBMs can be used to compare pilot-scale vs. full-scale treatment processes at a large-scale municipal drinking water treatment plant (DWTP). More importantly, a potentially serious health-relevant effect, genotoxicity, was detected in the raw water as well as finished drinking water from this DWTP. Next, we evaluated the process of artificial infiltration as a treatment method at another municipal drinking water production system. This was in consideration of the fact that water sourced from this type of purification method is prone to contamination with chemical hazards. In addition to these two field studies, we assessed the impact of sample acidification and extract storage time on the outcomes of certain *in vitro* bioassays, which has been largely under-emphasized when designing sampling strategies for studies using EBMs. For this we collected samples from a wastewater treatment plant that had future water reuse plans for irrigation and potentially as a drinking water source. Our findings revealed that sample acidification did have impacts depending on the selected bioassay(s) and treatment process. Bioactivities in the sample extracts also did not remain unchanged following storage for approximately one year. In the final study, we used EBMs to evaluate two common mycotoxins and their derivatives in the presence of an exogenous metabolic activation system. We chose to study mycotoxins given that their occurrence in surface and drinking waters has been receiving increasing attention. Our findings highlighted that the inclusion of exogenous metabolic activation is useful for detecting the biological effects of mycotoxin metabolites in *in vitro* bioassays.

Keywords: Drinking water, effect-based methods, exogenous metabolic components, *in vitro* bioassays, mycotoxins, sampling strategies, water quality assessments, water treatment processes

Preface

”Water is not a commercial product like any other, but rather a heritage which must be protected, defended and treated as such.”

-From EU Water Framework Directive (2000/60/EG)

Contents

List of publications

This thesis is based on the work contained in the following papers, referred to by Roman numerals in the thesis text:

I. **Yu M**., Lavonen E., Oskarsson A., Lundqvist J. (2021). Removal of oxidative stress and genotoxic activities during drinking water production by ozonation and granular activated carbon filtration. Environ Sci Eur, 33(1):124.

II. **Yu M**., Mapuskar S., Lavonen E., Oskarsson A., McCleaf, P., Lundqvist J. (2022). Artificial infiltration in drinking water production: addressing chemical hazards using effect-based methods. Water Research, 221:118776.

III. **Yu M**., Lavonen E., Oskarsson A., Lundqvist J.. (2023). Impact of sample acidification and extract storage on hormone receptor-mediated and oxidative stress activities in wastewater. Journal of Water & Health, (manuscript submitted)

IV. **Yu M**., Oskarsson A., Lundqvist J.. Effects on estrogenic, androgenic and genotoxic activities of zearalenone and deoxynivalenol in the presence of exogenous metabolic activation. (manuscript)

Supporting information for Papers **I** and **II** are not included in this thesis, but can be found online with the respective papers.

The contribution of Maria Yu (**MY**) to the papers included in this thesis was as follows:

I. **MY** was involved in the coordination of the study project. **MY** conducted the sampling, experiments, and data handling. **MY** wrote the manuscript with input from all authors.

II. **MY** was involved in the planning of the study project. **MY** coordinated and conducted the sampling, experiments, and data handling. **MY** wrote the manuscript with input from all authors.

III. **MY** was involved in the planning of the study project. **MY** coordinated and conducted the sampling, experiments, and data handling. **MY** wrote the manuscript with input from all authors.

IV. **MY** was involved in the planning of the study project. **MY** coordinated and conducted the experiments and data handling. **MY** wrote the manuscript with input from all authors.

Unpublished study

The following study was conducted during MY's Ph.D. program. The work conducted in the study served as a prerequisite for a major aspect of one of her thesis studies (**Paper IV**). As such, it is mentioned here. However, preparation of the manuscript was not complete by the time of the thesis and therefore, is not part of the thesis evaluation.

I. **Selin E., Yu M**., Mattsson A., Oskarsson A., Lundqvist J. *Preliminary title*: Estrogen and androgen receptor transactivation assays –impact of metabolic enzymes

List of tables

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Abbreviations

3-aDON	3-acetyl deoxynivalenol
15-aDON	15-acetyl deoxynivalenol
α-ZEL	alpha-zearalenol
AhR	Aryl hydrocarbon receptor
AR	Androgen receptor
ATP	Adenosine triphosphate
BAC	Biologically activated carbon
BEQ	Biological equivalent concentration
CEC	Concentration effect curve
CHO	Chinese hamster ovary
CI	Confidence interval
CONTAM	Panel on Contaminants in the Food Chain
DBP	Disinfection by-product
DF	Disc filtration
DMSO	Dimethyl sulfoxide
DON	Deoxynivalenol
DWTP	Drinking water treatment plant
EBCT	Empty bed contact time
EBMs	Effect-based methods

EC	Effect concentration
EFSA	European Food Safety Authority
EMA	Ethidium monoazide
ER	Estrogen receptor
EtOH	Ethanol
EU	European Union
GAC	Granular activated carbon
GST	Glutathione S-transferases
HCI	Hydrochloric acid
HLB	Hydrophilic/Lipophilic Balanced
ISO	International Organization for Standardization
LOD	Limit of detection
LL	Lower limit (of confidence interval)
MAR	Managed aquifer recharge
MAS	Metabolic activation systems
MBR	Membrane bioreactor
MN	Micronuclei
MTS	3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium
NADPH	Nicotinamide adenine dinucleotide phosphate
NAT	N-acetyl transferases
Nrf2	Nuclear factor erythroid 2-related factor 2
O_3	Ozonation
OECD	Organization for Economic Co-operation and Development
OMP	Organic micropollutant
PAPS	3-phosphoadenosine-5-phosphosulphate
PHI	Phase I (cofactors)

PHII	Phase II (cofactors)
QA/QC	Quality assurance/quality control
RBMP	River basin management plan
REF	Relative enrichment factor
RGA	Reporter gene assay
SD	Standard deviation
SOP	Standard operating procedure
SPE	Solid phase extraction
SULT	Sulphotransferases
TK	Thymidine kinase
UDPGA	Uridine diphosphate glucuronic acid
UGT	UDP-dependent glucuronosyl transferases
UL	Upper limit (of confidence interval)
UV	Ultraviolet
WWTP	Wastewater treatment plant
ZEN	Zearalenone

1. Introduction

Presented in this chapter are brief overviews of key foundational concepts that formed the basis of the thesis work. Each subsection provides relevant background information for Papers **I** to **IV** and follows the order of the papers presented in this thesis.

1.1 Safeguarding drinking water from a chemical perspective

The production and usage of manufactured chemicals in society are becoming a pressing issue due to the ubiquitous release of potentially hazardous chemicals into the environment, particularly in large quantities (UNEP, 2019). Further, information on the potential adverse effects on human and environmental health remains limited to an outdated set of regulated chemicals established decades ago. Many of the newer chemicals have been labelled as "emerging contaminants" because they have been traditionally unmonitored or unregulated, but of increasing concern to public health in recent years (Rosenfeld & Feng, 2011). This is because most of these compounds are not yet fully understood from a toxicological perspective (Richard et al., 2009) and many are not or cannot yet be monitored in water treatment systems. Examples of emerging contaminants, or organic micropollutants (OMPs), include pharmaceuticals, pesticides, industrial chemicals, steroids and hormones, surfactants, perfluorinated compounds, and personal care products. The European Commission (EC) recently recognized that the current EU legislation does not have sufficient provisions specific to micropollutants, and that further work is needed to address these contaminants of emerging concerns (EIB, 2023). Further, the EC set out to revise their drinking water directive (DWD) to modernize their

20-year-old DWD (98/83/EC) such that higher standards be set for drinking water. Under the recently recast directive (2020/2184), water intended for human consumption is to be wholesome, clean, and free from any substances (and any micro-organisms and parasites) which, in numbers or concentrations, constitute a potential danger to human health (Drinking Water Directive, 2020).

To that, water sources, especially surface water, can become polluted with complex chemical mixtures of natural and synthetic organic compounds released from a variety of anthropogenic activities such as agriculture, urban storm water discharge, wastewater effluents, and air pollution. These same water sources are often used in drinking water production which raises concerns regarding the safety of drinking water. OMPs, for instance, can adversely impact drinking water quality. They are most typically polar to semi-polar organic compounds that are present in environmental waters at low concentrations (less than ng/L to μg/L range). Here in Sweden, OMPs have been detected in several of its largest lakes which are also used as drinking water reservoirs (Malnes et al., 2022; Rehrl et al., 2020). In addition, when water contaminants pass through drinking water treatment systems, the formation of known and unknown metabolites, degradation products, and disinfection by-products can also be problematic.

Drinking water is a unique food item in that it is essential for life. In an average diet, water is predominantly obtained through the consumption of drinking water and beverages (80% of daily intake). The average recommended daily consumption of drinking water is 2-2.5 L/day for adults (EFSA NDA, 2010). As such, when drinking water is consumed for extended periods of a lifespan (years to decades), even very low levels of contaminants in the drinking water can become a health risk due to cumulative intake and life-long exposure. More attention and priority should therefore be given to the compromised quality of drinking water sources due to chemical pollutants as they pose a potential threat to public health. It is important to detect and reduce adverse effects associated with the complex mixtures of chemicals present in water, rather than rely solely on the determination of concentrations of only a few individual chemicals.

1.2 Effect-based methods using *in vitro* bioassays in water quality monitoring

Routine water quality monitoring at DWTPs typically involves chemical analyses of individual chemicals. To this, the existing regulations and water quality guidelines focus on certain target chemicals. However, this approach does not account for the complex mixture effects of the multitude of chemicals present in the water. As a complement to this single-chemical approach, effect-based methods (EBMs), where the total effects of relevant toxicity parameters in a water sample are measured, have emerged as a promising tool in water quality monitoring (Brack et al., 2019; Dévier et al., 2011). As shown in Figure 1, the number of studies applying EBMs in water quality assessments of drinking water has been increasing over the past several decades. These EBMs provide the benefit of detecting mixture effects of all bioactive chemicals in a sample, including both known and unknown chemicals (Escher et al., 2021). To this, *in vitro* reporter gene assays (RGAs) have been developed to address certain cellular-level modes of action such as nuclear receptor mediated-modulation associated with endocrine effects.

It is in fact known from previous studies using both chemical fingerprinting and EBMs that detected chemicals in drinking water and wastewater samples could only explain a very small percentage of the observed bioactivity, for some endpoints as low as 0.1% (Escher et al., 2013; Gómez et al., 2021; Tang et al., 2013, 2014). Thus, the main biological effects originated from unknown chemicals not analysed or detected by standard chemical analytical methods or mixture effects. Further to the application of EBMs in water quality monitoring, they have been used to test the removal efficiencies of treatment processes utilized at DWTPs (and wastewater treatment plants) (Conley et al., 2017; Jugan et al., 2009; Lundqvist et al., 2019; Lv et al., 2016; Macova et al., 2011; Neale et al., 2020; Oskarsson et al., 2021; Rosenmai et al., 2018; Xiao et al., 2016) and reviewed in (Enault et al., 2023).

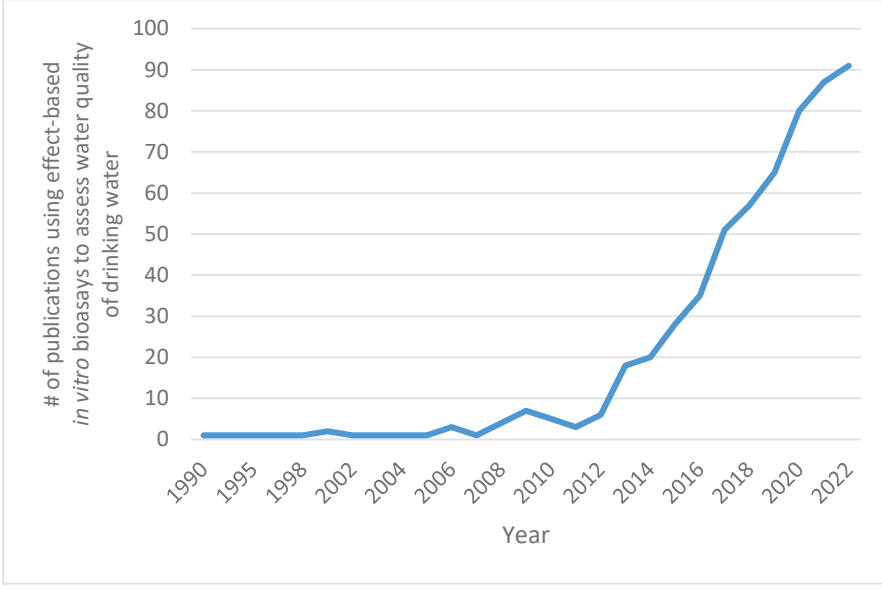


Figure 1 Time-trend of number of *in vitro* based publications in water quality testing of drinking water. Number of studies that have applied EBMs with *in vitro* bioassays in water quality assessments of drinking water since 1990. Search results from Scopus with the keywords "drinking water" AND "effect-based" OR "*in vitro* analysis" (22 August 2023).

In vitro RGAs using recombinant cell lines transfected with certain reporter gene plasmids are commonly used to study cellular signaling and transcriptional activity. In these genetically engineered cells, an exogenous coding reporter gene for a biological outcome using an observable parameter like bioluminescence is joined to a promoter region or responsive element in an expression vector to provide the means for promoter activity in the RGA. A commonly used reporter gene is the luciferase gene from the firefly *Photinus pyralis*. In the RGA, when an initiating event, such as a chemical stressor, triggers a particular cellular response, the activated luciferase reporter gene is transcribed to messenger RNA, which is then translated as an enzyme that can be measured by enzymatic assays. In the presence of the proper luminogenic substrate, the luciferase will catalyze a reaction that produces a bioluminescent signal. This signal is quantified with a luminometer and the intensity of the signal is correlated to the effect of the stressor on the expression of the target gene in a dose-dependent way. Further

details regarding the reporter cell lines used in these RGAs are provided in Chapter 3 (Section 3.6).

Chemical stressors can activate different cellular toxicity pathways which can be measured with different types of bioassays. Some of the more common bioassays applied to water samples are described briefly herein. There are bioassays indicative of xenobiotic metabolism receptors such as the aryl hydrocarbon receptor (AhR). The AhR is a ligand-activated transcription factor that was originally characterized as a receptor for 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) (Wang et al., 2016). The transcription factor plays a crucial role in the detoxification of several types of xenobiotics, such as polychlorinated dibenzo-p-dioxins, polychlorinated biphenyls, and polycyclic aromatic hydrocarbons. There are bioassays indicative of hormone receptor-mediated effects from endocrine-disrupting chemicals. These include synthetic hormones, industrial chemicals, and pesticides. Such chemicals can interfere with hormonal systems by interacting (e.g., agonism or antagonism) with hormone receptors, such as the estrogen receptor (ER) and androgen receptor (AR). There are bioassays that can also detect other modes of action such as the adaptive stress response. Adaptive stress response pathways are activated to restore cells back to homeostasis following damage from stressors, including organic chemicals. The oxidative stress response, for instance, is activated by chemicals that generate reactive oxygen species and electrophilic species. An important antioxidant defence mechanism involved is the Nrf2-Keap1 signalling pathway (Kobayashi et al., 2009). In the presence of reactive chemicals, the Nrf2 transcription factor dissociates from the negative regulator Keap1 and then translocates to the nucleus and activates the antioxidant response element (Zhang, 2006). Schematics of the basic principles of an RGA for two different activation pathways (receptor-mediated and oxidative stress) are provided in Figure 2.

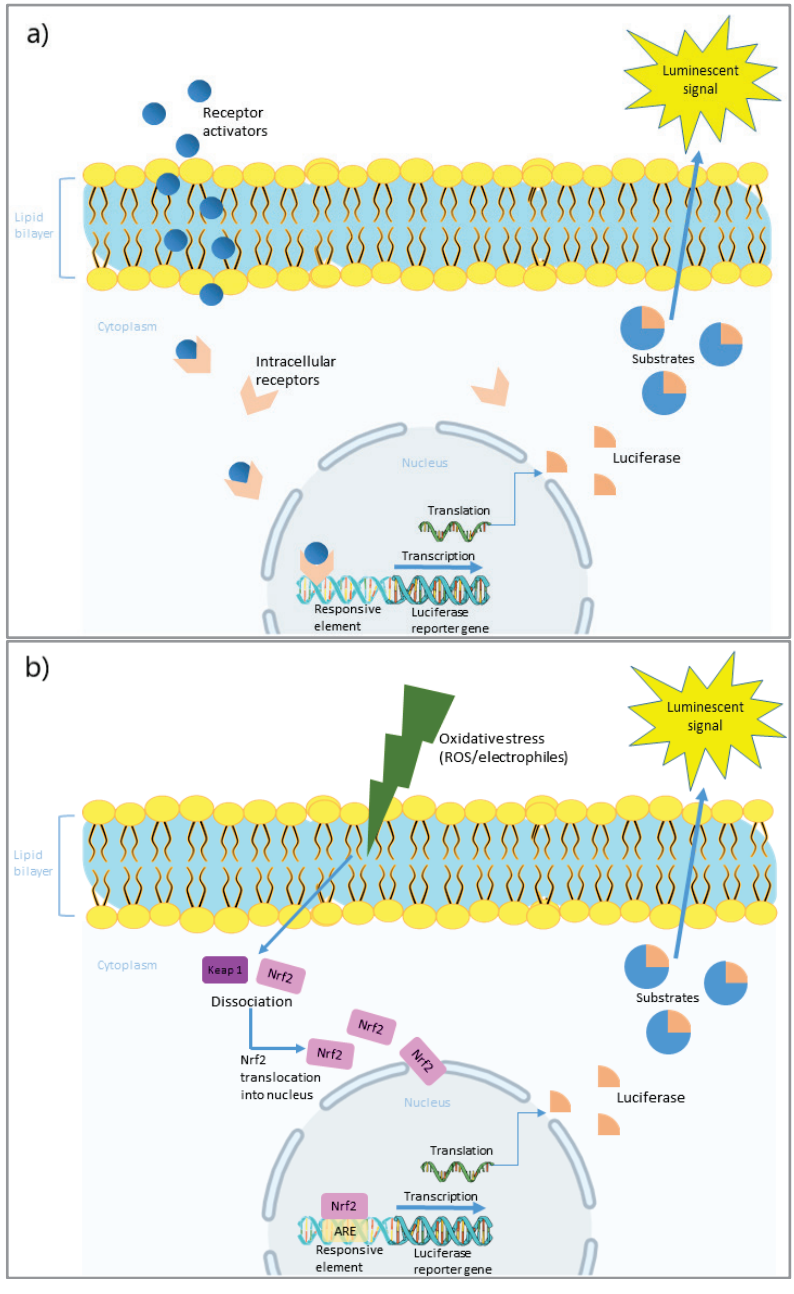


Figure 2 Schematic designs of the luciferase reporter gene assay for two types of activation pathways: Receptor-mediated (a) and oxidative stress response (b). Illustration created with BioRender.com.

The RGAs highlighted in this section assessing different modes of action, such as induction of xenobiotic metabolism, modulation of hormones, and adaptive stress responses have been identified as being the most responsive pathways relevant to drinking water quality (Dingemans et al., 2019; Escher et al., 2014; GWRC, 2020b). In water quality testing, given the presence of complex mixture of chemicals in water, one bioassay is insufficient to detect the effects of all known and unknown chemicals present. It is therefore recommended to select a practical test panel of at least three or four bioassays representative of different effects commonly detected in water extracts (GWRC, 2020b). Further, which bioassays to include can be determined based on the water type (e.g., drinking water, wastewater, surface water, etc.). Other bioassays for health-relevant endpoints such as genotoxicity and cytotoxicity which are not based on the reporter gene function are also available for water quality testing. These bioassays are described briefly later in Chapter 3 (Section 3.6).

1.3 Drinking water treatment methods as effective chemical barriers?

The presence of complex chemical mixtures in drinking water sources raises the important question of whether existing conventional water treatment methods are capable of removing the mix of hazardous chemicals present in our drinking water sources.

Conventional drinking water treatment methods generally involve physico-chemical processes that primarily work to eliminate pathogens as well as reduce turbidity, remove nutrients, metals, organic matter, color, and control taste and odor issues in the finished drinking water. However, it has been reported that many of these conventional treatment methods show incomplete removal of many OMPs, as many studies have reported the occurrence of OMPs in both raw water and finished water (Benotti et al., 2009; Huerta-Fontela et al., 2011; Kleywegt et al., 2011; Stackelberg et al., 2007; Tröger et al., 2018).

Ozonation (O_3) and activated carbon treatment methods have been suggested to be more effective treatment methods in managing the removal

of OMPs and disinfection by-product (DBP) precursors (Kim et al., 2005; Ullberg et al., 2021) than other conventional methods such as coagulation, sedimentation, and sand filtration. The main strengths of O_3 in water treatment include its disinfection capability as well as oxidation of harmful agents and substances. Activated carbon filters, such as granular activated carbon (GAC), can also remove OMPs (Boehler et al., 2012; Oskarsson et al., 2021; Tröger et al., 2018; Westerhoff et al., 2005) and DBP precursors as well as reduce DBP formation (Cuthbertson et al., 2019). Further, the combination of O_3 followed by GAC has been demonstrated to be very effective in removing trace organic chemicals (Borrull et al., 2021; Bourbigot et al., 1986; Reungoat et al., 2010; Sánchez-Polo et al., 2006) as GAC are common polishing techniques after O_3 to biodegrade O_3 by-products.

Since existing conventional treatment methods were not specially designed to remove residual concentrations of OMPs, and because these methods have been shown to be largely incapable of removing hazardous and biologically resistant pollutants, alternative treatment processes, such as the combination of O_3 and GAC, are needed to be further explored.

1.4 Artificial infiltration in drinking water production

With growing concerns over the deteriorating quality of surface water supplies around the world, groundwater is becoming a more important drinking water source (Förare, 2009). Pressures on the global groundwater resources are anticipated to increase as a result of population growths and increasing water demands due to climate change. In 27 of the European Union's (EU's) member states, for instance, 65% of drinking water supplies come from groundwater sources (European Environment Agency, 2022). When groundwater supplies become low, as may occur due to water scarcity conditions or from abstraction pressures, aquifers can be artificially recharged with surface waters. Managed aquifer recharge (MAR) is a common process wherein a groundwater aquifer is artificially recharged with surface water (Balke & Zhu, 2008; US NRC, 1994). This recharge can be done via various methods such as artificial infiltration basins, irrigation pits, redirection of the surface water across land surfaces, or via injection wells

into the subsurface. In Europe alone, more than 200 different MAR schemes, specifically riverbank filtration, are used in the production of drinking water (Sprenger et al., 2017). In Sweden, approximately 25% of the public drinking water is sourced from surface waters via artificial infiltration (Svenskt Vatten, n.d.).

However, artificially recharged groundwater can become polluted with many of the same pollutants that enter surface waters including toxic metals, pesticides, industrial chemicals, microorganisms, natural toxins, and a variety of OMPs via point and non-point (diffuse) source emissions (Albergamo et al., 2019; Böhlke, 2002; Díaz-Cruz & Barceló, 2008; Maeng et al., 2011; Sasakova et al., 2018). According to the 2016 River basin management plan (RBMP) under the Water Framework Directive, 24% of the total groundwater body area in the 27 EU member states was reported to be of poor chemical status (European Environment Agency, 2022). In Sweden, a contamination scenario in the artificially infiltrated source water of a Swedish drinking water treatment plant (DWTP) was recently reported (Oskarsson et al., 2021). The results of that study highlighted that further effect-based research into the artificial infiltration process and the associated risks due to chemical contamination is needed.

1.5 Improving sampling strategies in *in vitro*-based testing

While EBMs have been increasingly included in water quality monitoring, particularly in the past decade, a critical step towards acceptance of *in vitro* bioassays for regulatory testing purposes is their validation to demonstrate the reliability and reproducibility of these test methods. Some transactivation bioassays have been validated by the Organization for Economic Co-operation and Development (OECD), such as for the detection of estrogenic agonist-activity of chemicals (OECD TG 455) and detection of androgenic agonist and antagonist activity of chemicals (OCED TG 458). However, these guidelines do not include guidance on sampling strategies. To this, there is a necessity to harmonize not only the bioassay test methods themselves but also the sampling strategies involved in EBMs. This can be

achieved by establishing Standard Operating Procedures (SOPs) and quality assurance and quality control (QA/QC) measures.

To ensure that bioassay results are meaningful and accurate assessments of the whole sample, it is imperative to develop an appropriate sampling strategy with suitable sample preparation and processing techniques to maintain the sample integrity. Sample acidification is one such technique which, among other purposes, can limit the microbial activity in water which can otherwise potentially biodegrade or biotransform the OMPs present in the sample. Such acidification techniques can be easily performed in the field immediately following sample collection to a target pH. While sample acidification techniques have been reported in certain studies utilizing *in vitro* bioassays, as summarized recently by Robitaille et al., 2022 in Table 5 of their study (Robitaille et al., 2022), there remains a lack of standardized protocols for sample preparation techniques when using EBMs to assess water quality. Further, there is a paucity of research investigating if acidification itself impacts the outcome of bioassays. There are only a handful of publications that have addressed sample pH adjustment, all with varying conclusions (Abbas et al., 2019; Escher et al., 2005; Šauer et al., 2018).

1.6 Mycotoxins – an understudied hazard to human health from the perspective of drinking water

Natural toxins produced by fungi, such as mycotoxins, are considered a concern to human and animal health due to the contamination of food crops or feed. They have consequently received considerable attention in this regard. Certain common mycotoxins produced by the *Fusarium* species such as deoxynivalenol (DON) and zearalenone (ZEN) along with their derivatives, for instance, are among the most abundant mycotoxins contaminating food and feed worldwide. Less focus, however, has been given to the fact that these mycotoxins can also release and transfer to water bodies, including drinking water sources. To this, both of these mycotoxins have been detected in surface waters (Bucheli et al., 2008; Gromadzka et al., 2009; Kolpin et al., 2014; Schenzel et al., 2012).

The toxicity of DON and ZEN along with their derivatives *in vitro* at the level of nuclear receptor signalling can occur and causes agonistic and/or antagonistic endocrine effects (Demaegdt et al., 2016). ZEN and some of its metabolites, such as α-zearalenol (α-ZEL), are well-established endocrine disrupters (Metzler et al., 2010) and have been shown to have strong estrogenic potencies *in vitro* (Demaegdt et al., 2016). Whereas the potential of DON and its derivatives to act as endocrine disruptors has been comparatively less studied but continues to be investigated.

Other important health-relevant effects of these two mycotoxins and their derivatives, such as genotoxicity, have also been reported as highlighted by the EFSA Panel on Contaminants in the Food Chain (CONTAM) (EFSA CONTAM, 2017; Panel, 2017). ZEN was found to be clastogenic and aneugenic in a variety of cell culture systems, but negative in bacterial mutation studies, as referenced in (EFSA CONTAM, 2017). The EFSA CONTAM Panel considered DON to be genotoxic *in vitro*; however, its acetyl derivative, 3-acetyl deoxynivalenol (3-aDON), was inactive in the bacterial mutation test (EFSA CONTAM, 2017). No *in vitro* genotoxicity data was available for 15-acetyl deoxynivalenol (15-aDON), another derivative of DON (EFSA CONTAM, 2017).

To fully evaluate a compound's toxic potential, it is important to gather information also on the toxic activities of its metabolites. However, to date, there remains a lack of research incorporating exogenous metabolic components into *in vitro* testing, particularly for endocrine disruption (Jacobs et al., 2013; van Vugt-Lussenburg et al., 2018). Many genetically engineered cell lines used in RGAs often have a limited capacity for xenobiotic metabolism/biotransformation due to a lack of the necessary enzymes. This is important to consider as the biotransformation of some xenobiotics can result in the formation of highly reactive metabolites (i.e. bioactivation). In the case of endocrine disruption, Phase I (e.g., oxidation) or Phase II (e.g., conjugation) biotransformation reactions can convert pro-estrogens to active estrogens or inactivate parent chemicals that are active estrogens. Otherwise, false positive data (due to lack of detoxification) or false negative data (due to lack of bioactivation) may result from the *in vitro* tests without the incorporation of metabolic components.

In the case of ZEN and its metabolites, testing their endocrine effects at the molecular level with RGAs in the presence of exogenous metabolic components would be highly relevant given that these compounds can, for instance, interact with the estrogen receptor (Cozzini & Dellafiora, 2012; Mostrom, 2011) which then bind to estrogen-responsive elements and activates gene transcription. The effects of DON and its derivatives on hormonal receptors have been comparatively less studied and therefore warrant interest.

2. Objectives

The overarching aim of this thesis was to use EBMs, based on a panel of *in vitro* RGAs, in various applications. This included evaluations of different water treatment schemes designed to remove bioactive compounds as well as to study a particular class of compounds of emerging relevance to water safety, that being mycotoxins. The thesis work was comprised of four studies. In the first study (**Paper I**), we applied a panel of *in vitro* bioassays to assess the treatment efficiency of two pilot-scale treatments: O_3 and GAC filtration at a Swedish DWTP (Norrvatten Görvälnverket). The pilot-scale systems were studied alongside a full-scale treatment process consisting of BAC filtration, UV disinfection, monochloramine dosing. Both systems were fed the same raw water treated with coagulation/flocculation/ sedimentation and sand filtration. In the second study (**Paper II**), we assessed the effectiveness of artificial infiltration as a water treatment method involved in drinking water production (Uppsala Vatten) again using a panel of *in vitro* bioassays. In the third study (**Paper III**), the impact of sample acidification on certain bioactivities (e.g., hormone receptor-mediated effects and oxidative stress response) was assessed using *in vitro* bioassays. And finally in the fourth study (**Paper IV**), using *in vitro* bioassays retrofitted with exogenous metabolic activation systems (MAS), two common mycotoxins (DON and ZEN) and their derivatives were tested for hormone receptor-mediated effects along with micronuclei (MN) formations. A graphical summary of the studies is provided in Figure 3.

In brief, the following research questions were considered in each study:

- How do the removal efficiencies of the pilot-scale O_3 and GAC treatment steps at Görvälnverket DWTP compare to the existing full-scale treatment process? (**Paper I**)

- How effective is artificial infiltration as a pre-treatment of source water in drinking water production? (**Paper II**)
- How does sample acidification impact the bioactivities in water sample extracts? And do the bioactivities of the sample extracts change over time? (**Paper III**)
- How does the incorporation of exogenous MAS impact the bioactivities of DON, ZEN, and their derivatives? (**Paper IV**)

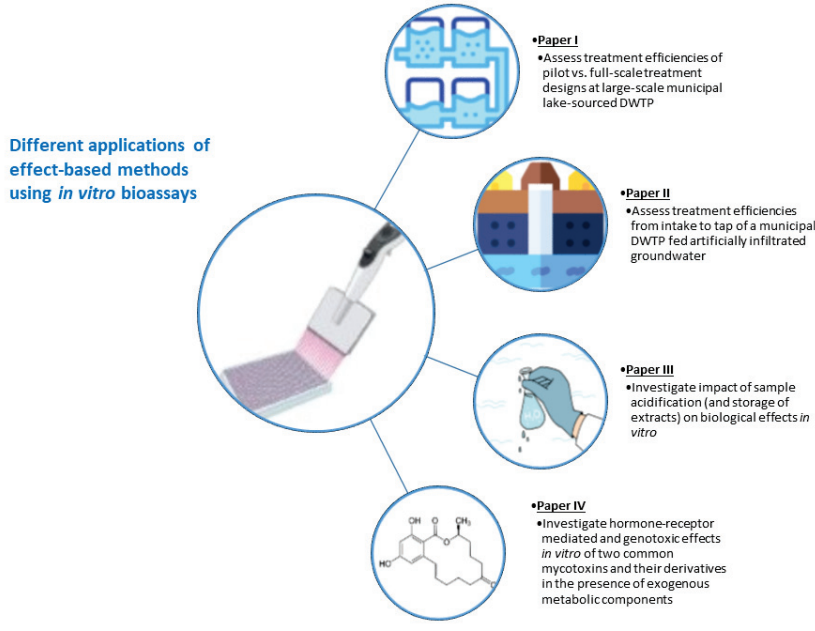


Figure 3 Graphical overview of studies undertaken for this thesis work.

3. Materials and methods

The following sections briefly describe the experimental designs, inclusive of key materials and methods, used in the studies (**Papers I to IV**). More detailed descriptions of each design can be found in the respective papers provided in the appendices of this thesis.

3.1 Site Descriptions (Papers I-III)

3.1.1 Norrvatten DWTP (Paper I)

Norrvatten is the fourth largest drinking water producer in Sweden and produces around 50 million m^3 of drinking water annually at Görvälnverket. This DWTP operates in the Stockholm region (Järfälla municipality) and services almost 700,000 consumers in several municipalities. The DWTP draws untreated raw water from Lake Mälaren, the third-largest freshwater lake in Sweden. The lake also receives effluent from several wastewater treatment plants located upstream of the DWTP. Görvälnverket was built in 1929 and the facility has undergone several expansions over the decades. To meet the increasing demand for drinking water from the growing municipalities, Norrvatten is planning for an expansion of treatment and production capacity of the DWTP.

Raw water from Lake Mälaren entering Görvälnverket undergoes several conventional treatment processes consisting of micro sieving followed by coagulation treatment using aluminum sulfate, flocculation and sedimentation/flotation, rapid sand filtration, BAC filtration, UV disinfection, and lastly dosing with monochloramine (NH_2Cl) for secondary disinfection and lime for alkalinization and pH adjustment (Fig. 1). The BAC

filters have a running time of approximately 10-15 years and a short empty bed contact time (EBCT) of approximately 4-6 minutes.

In May 2018, a pilot-scale water treatment system consisting of O_3 pre-treatment and GAC columns was installed at Görvälnverket to evaluate the efficacy of these two methods at removing e.g., OMP and DOC. This pilot-scale system receives incoming water treated with coagulation, sedimentation, and sand filtration from the full-scale treatment system at a flow rate of 610-720 L/hr. Two pilot-scale processes were investigated: (1) pilot-scale A wherein the incoming water undergoes O_3 then GAC filtration; and (2) pilot-scale B wherein the incoming water directly undergoes GAC filtration without pre-ozonation. For pilot-scale A, feed water was pH-adjusted to 6.5 prior to ozonation (target residual of 1 mg O_3/L after 4.2-4.9 min reaction) while the GAC column in the pilot-scale B process received water at ambient coagulation pH (6.5-6.8). The GAC column in pilot-scale B is used to: a) directly assess differences in GAC efficacy with and without pre-ozonation; and b) to investigate the gradual saturation of a GAC filter with longer EBCT and compare it to the full-scale short EBCT BAC treatment. The EBCTs for pilot-scale A and B were 20 minutes. The full- and pilot-scale treatment processes are illustrated below in Figure 4.

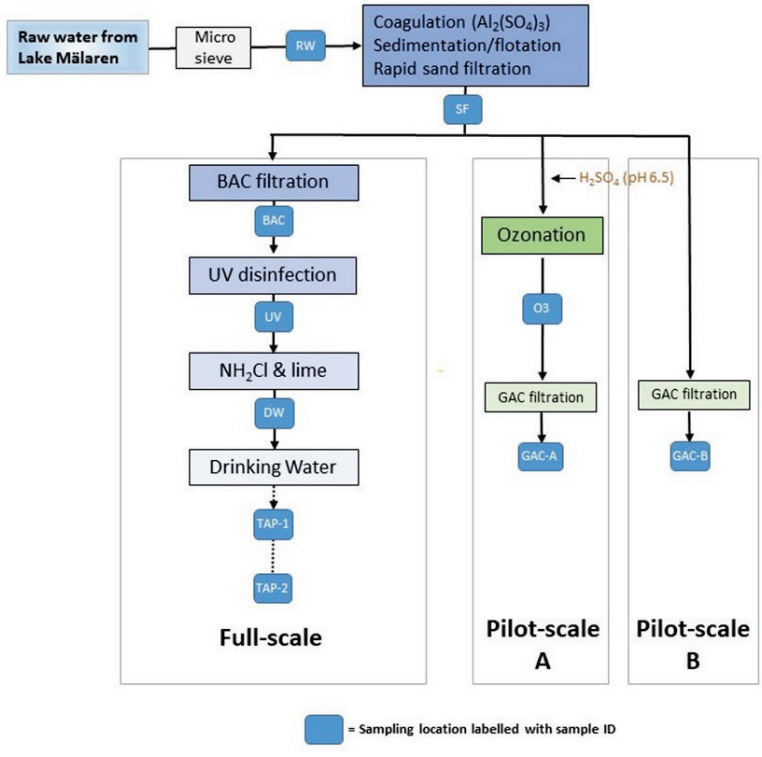
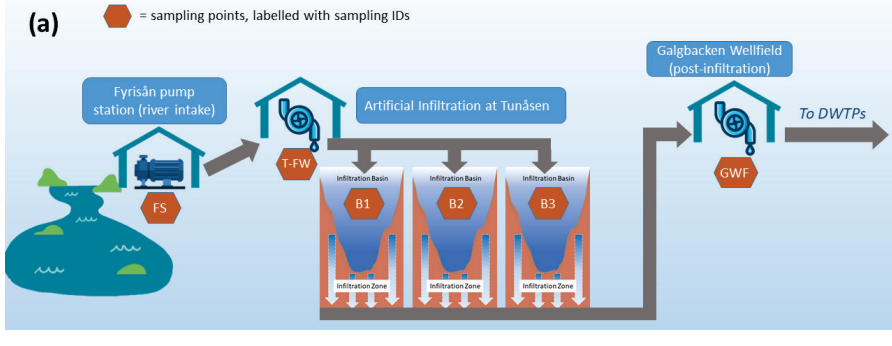


Figure 4 Simplified diagram of full- and pilot-scale treatment processes at Norrvatten DWTP. Treatment steps for the full-scale and the two pilot-scale treatment systems investigated at Görvälnverket. The sampling points for all sampling events are indicated with symbols. Figure reproduced from Yu et al., 2021 (Appx I – **Paper I**).

3.1.2 Uppsala Vatten DWTP (Paper II)

Each year, Uppsala Vatten och Avfall AB (Uppsala Vatten) supplies approximately 17 million m^3 of drinking water for consumers in the city of Uppsala. The drinking water supply is sourced primarily from groundwater extracted from the Uppsala esker. However, to compensate for water abstraction, a managed aquifer recharge system has been utilized since 1966 to infiltrate surface water from the Fyris River (and additionally from Lake Tämnaren during the summer months). At the source water intake, the raw river water first undergoes rapid sand filtration and then is pumped uphill to multiple infiltration basins situated north of the Greater Uppsala area in a

nature area (referred to as Tunåsen). The water from the basins percolates into the subsurface and mixes with the naturally formed groundwater as it flows through the aquifer. It takes approximately six to eight months for the infiltrated water to travel to four wellfields that supply two DWTPs (Gränby and Bäcklösa). The groundwater abstracted at the respective four wellfields varies in the proportion of infiltrated water from 15-20%, 40-45%, 45-50%, to 80-90%. At both DWTPs the incoming infiltrated water undergoes similar treatments including: aeration, hardness removal (pellet reactors), sand filtration, and then disinfection via chlorination (with sodium hypochlorite). However, ten GAC filters are also installed at the Bäcklösa DWTP between the sand filtration and chlorination treatment steps. The finished drinking water is stored in underground reservoirs at the DWTPs before entering the distribution network which consists of two municipal water towers and 650 km of pipelines that serve residential, commercial, and industrial water users. An average of 48,300 m^3 of finished drinking water per day was distributed from these two DWTPs in 2021 to serve approximately 190,000 consumers in the city of Uppsala. An illustration of the drinking water production process is provided in Figure 5 and a more detailed explanation is provided in the specific paper (Appx II – **Paper II**). A photo of one of the artificial basins is provided in Figure 6.



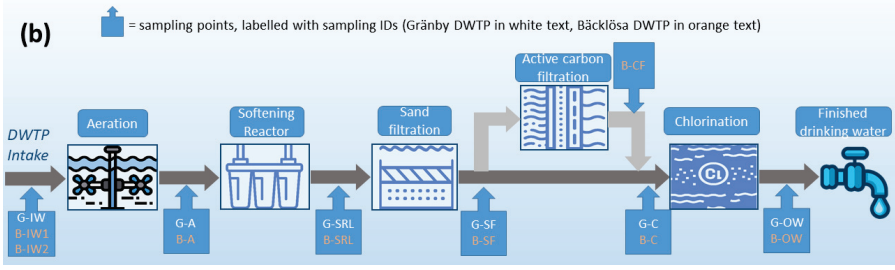


Figure 5 Simplified diagram of Uppsala Vatten's drinking water production process. The artificial infiltration pre-treatment process from the raw water source to one of the wellfields is shown in (a) prior to downstream water purification in the two DWTPs (b). Note that activated carbon filtration treatment is utilized at Bäcklösa (sampling IDs denoted with "B"), but not at Gränby (sampling IDs denoted with "G"). Figure reproduced from Yu et al., 2022 (Appx II – **Paper II**).

Figure 6 Site photo of an artificial infiltration basin of Uppsala Vatten. View of one of the three artificial infiltration basins sampled. Uppsala Vatten DWTP (Paper II). Photo credit: Maria Yu

3.1.3 Kivik WWTP (Paper III)

In southern Sweden, the Simrishamn municipality operates the Kivik WWTP which services the town of Kivik with approximately 890 inhabitants. The facility was reconstructed in 2019/20 after the municipality received financial support from the Swedish Environmental Protection Agency to

implement advanced treatment for the removal of OMPs such as pharmaceutical residues from the treated wastewater. The WWTP was originally constructed over 50 years ago. The treatment process at this WWTP consists of pre-treatment with cleaning grates, grease separation and sand capture, followed by chemical precipitation and disc filtration. A photo of the pre-treatment reservoir prior to the disc filtration is provided in Figure 7. This is then followed by a membrane bioreactor (MBR) with two parallel ultrafilters and a mixing reservoir, followed by two parallel GAC filters. Following treatment, the treated water is pumped to the adjacent Hanö Bay on the east coast of Skåne, South Sweden. The plant is designed for a flow of 180 m^3/h. An important consideration in the reconstruction of the plant was to be able to reuse the treated water for irrigation, swimming pools and re-infiltration without unnecessary discharges to the sea. The feasibility of using the treated water even as a drinking water source has also been evaluated recently (Takman et al., 2023).

Figure 7 Site photo of the pre-treatment reservoir sampled at the Kivik WWTP. View of the pre-treated wastewater influent to the disc filtration treatment step at the Kivik WWTP (Appx III - Paper III). Photo credit: Maria Yu

3.2 Sampling strategies (Papers I to III)

The sampling designs developed for each of the three papers are summarized briefly in Table 1. As highlighted by Escher et al. (2021), the design of the sampling strategies should be tailored to the purpose and objectives of the water quality assessment as well as sample context (Escher et al., 2021). For the Norrvatten (**Paper I**) and Uppsala Vatten (**Paper II**) studies, the purpose was to assess the removal efficiencies of each of the treatment steps. As such, grab samples were collected before and after each treatment step. For the Kivik study, while the main purpose was to assess the impact of sample acidification, this would inherently involve some observation regarding the overall treatment process efficiency. As such, grab samples were collected from the inlet (following pre-screening) and outlet of the WWTP.

The volume of sample to be collected will vary depending on the expected level of chemical contamination. Smaller volumes are recommended for wastewater influent and effluent, whereas larger volumes are needed for drinking water and surface waters (Escher et al., 2021). As such, the sample volumes selected for the three studies in this thesis followed these recommendations. The higher volumes collected in the Norrvatten study vs. the Uppsala Vatten study are explained further below regarding the sample extractions.

In the case of the Norrvatten study, while the original intent was to conduct one sampling event, the detection of certain bioactivities (e.g., genotoxicity) in some of the samples prompted an additional follow-up sampling event. Given that genotoxicity was then also detected in the finished water in the second sampling event, a third follow-up sampling event was conducted and expanded to include samples from the DWTP's distribution network.

In all three studies, samples were transported immediately back to the laboratory where they were stored at -20 °C until sample extractions.

Table 1 General overview of sampling designs for Paper I to III

	Norrvatten DWTP (Paper I)	Uppsala Vatten DWTP (Paper II)	Kivik WWTP (Paper III)
Type of source water type at intake	Raw water from a lake	Raw water from a river	Wastewater influent
Type of sampling	Grab	Grab	Grab
Sample volume	5 L	2 L	1 L
# of sampling events	3	1	1

3.3 Sample acidification (Paper III)

An important research question that arose following the completion of the Norrvatten and Uppsala Vatten studies is the inclusion of sample acidification during collection. As mentioned in the International Organization for Standardization (ISO) 5667-3:2003 standard (Sampling-Part 3: Guidance on the preservation and handling of water samples), fresh waters, wastewaters, and groundwaters are particularly susceptible to changes as a result of physical, chemical, or biological reactions which may take place between the time of sampling and the commencement of analysis. As such, if proper precautions are not taken to preserve stability during sampling, transport, and storage, the outcomes of the subsequent analyses may not accurately reflect what existed at the time of sampling (e.g., in the case of chemical analysis, the concentrations determined may differ from those existing at the time of sampling). Acidifying a sample can reduce microbial activity, which could otherwise potentially cause biodegradation or biotransformation of the OMPs present in the sample (GWRC, 2020a). Acidification prior to solid phase extraction has been shown to also improve the extraction of weak acids (Escher et al., 2005). In general, very few water quality assessments employing *in vitro* bioassays have specifically assessed the impact of sample acidification on bioactivities. One other study that investigated this aspect reported that sample acidification and extraction methods can critically affect the outcome of bioassays when assessing the toxicity of water samples (Abbas et al., 2019).

At the time of sampling for the Kivik study (**Paper III**), grab samples were collected from each sampling point using a 12-L polyethylene bucket. From this bucket, two 1-L sterile PET bottles (VWR® collection) were filled. The use of the bucket was intentional to ensure that the two bottles were filled from the same grab sample. To one of these bottles, approximately 3 to 4 mL of 1M hydrochloric acid (HCI) was immediately added to a target pH range of 2 to 3. All unacidifed and acidified samples were transported immediately back to the laboratory where they were stored at -20 °C until sample extractions.

3.4 Sample extractions (Papers I to III)

For all three studies, the collected samples were concentrated via solid phase extraction (SPE). SPE is the most commonly used method to concentrate water samples prior to bioanalyses (Neale et al., 2018). Further, as EBMs mainly focus on complex mixtures of OMPs, SPE methods also serve to extract the OMPs from the matrix and inorganics present in a water sample (Escher et al., 2021). This is particularly important in the case of raw water and drinking water samples wherein OMPs are often present at very low concentrations. SPE cartridges or disks containing a sorbent to retain particular substances of interest (e.g., OMPs) are used in the extraction process. The sorbed compounds are then eluted with solvents and further evaporated to a target volume to become a concentrated extract, which is then run in the bioanalyses.

For the Norrvatten study (**Paper I**), samples were concentrated using HLB[1] extraction disks (Atlantic HLB-H Disks, diameter 47 mm; Horizon Technology, Salem, NH, USA) and with an automatic SPE system (SPE-DEX 4790, Horizon Technology, Salem, NH, USA). This instrument can accommodate large sample volumes up to 8-L and the SPE discs can handle the extraction of large sample volumes while maintaining fast flow rates. Between the time of sample collections for the Norrvatten and Uppsala

[1] The polymeric HLB (Hydrophilic/Lipophilic Balanced) media in this disk comes in low, medium and high capacity formulations. These disks can be used for drinking and wastewater applications. The HLB sorbent contain a copolymer mix that allows for extraction of a wide range of both hydrophilic and hydrophobic compounds.

Vatten studies, a new, more modern automated SPE system was purchased: the SPE-03, 8-Channel Automated SPE System (PromoChrom Technologies, Canada). This more compact SPE system utilizes SPE cartridges and can accommodate sample volumes of up to 4-L. The samples collected for the Uppsala Vatten and Kivik studies were concentrated using this instrument and with 6 mL SPE cartridges (6cc, 200 mg sorbent weight, Oasis Prime HLB cartridge, Waters Corporation). For comparison's sake, photos of each SPE system are provided in Figure 8.

For all three studies, the SPE processes used the same solvents (95% ethanol (EtOH) and Milli-Q® water) and involved the same extraction sequence: pre-treatment/conditioning, loading of sample volumes, extraction, followed by rinsing and evaporation.

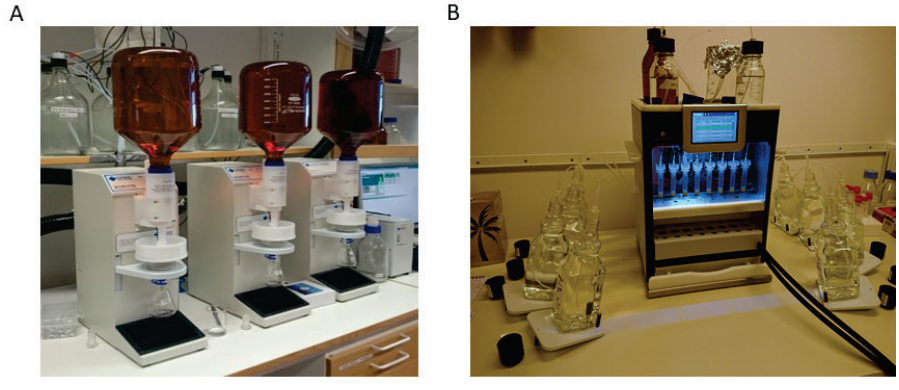


Figure 8 Photos of the two SPE instruments used in the thesis studies. (A) SPE-DEX 4790 (Horizon Technology, Salem, NH, USA) used for solid phase extraction of samples in Norvatten study (**Paper I**). (B) SPE-03, 8-Channel Automated SPE System (PromoChrom Technologies) used in Uppsala Vatten and Kivik studies (**Papers II** and **III**). Photo credit: Maria Yu. Note: as Photo A was resized, it is not to scale.

In all studies, each water sample was enriched by a factor of 5000. Additional information regarding the sample preparations is provided in the respective papers. All sample extracts were stored at -20°C and protected from light.

3.5 Mycotoxin test compounds (Paper IV)

The following mycotoxins were assessed:

- zearalenone (CAS 17924-92-4)
- α-zearalenol (CAS 36455-72-8)
- deoxynivalenol (CAS 296.32)
- 3-acetyl deoxynivalenol (CAS 50722-38-8)
- 15-acetyl deoxynivalenol (CAS 88337-96-6)

All mycotoxin test compounds were purchased in solid form and dissolved in dimethyl sulfoxide (DMSO, ≥99.9%, Sigma-Aldrich Germany) to prepare stock solutions. Serial dilutions of test concentrations were prepared in DMSO for all test compounds. All stock solutions of the mycotoxins and test concentrations were stored at - 20°C and protected from light.

3.6 *In vitro* bioassays

In the realms of EBMs and water quality assessments, *in vitro* bioassays have been developed for a host of different cellular-level endpoints. It is therefore of critical importance to select the most relevant endpoints to fit the purpose of the study and type of water sample such that the most relevant compounds from a toxicological point of view are investigated. This was highlighted in a 2014 large-scale study involving the application of 103 unique *in vitro* bioassays in different water types (e.g., wastewater, recycled water, and drinking water) to evaluate which pathways were most relevant for water quality testing (Escher et al., 2014). The study concluded that the most responsive and relevant endpoints were related to hormone-mediated modes of action (ER and AR), xenobiotic metabolism (AhR), and reactive modes of action (e.g., genotoxicity and oxidative stress).

The panel of bioassays, therefore, selected for each of the papers in this thesis are summarized in Table 2. In the Norrvatten study (**Paper I**), genotoxicity was included because some samples were bioactive in the Nrf2 assay. This is because activation of the Nrf2 pathway has been reported to have the potential to indirectly result in genotoxic effects (Escher et al., 2012; Van der Linden et al., 2014). In the Uppsala Vatten study (**Paper II**), no samples were active in the Nrf2 assay. For the Kivik study (**Paper III**), the bioassays were selected based on their relevance to compounds commonly

detected in wastewater extracts. The selection of the bioassays for the mycotoxins study (**Paper IV**) was based on relevant literature regarding some of the more common adverse effects associated with the compounds of interest.

Table 2 Summary of bioassays assessed in each paper (**I** to **IV**) of this thesis.

Effect of interest	Bioactivity Assayed	Norrvatten (Paper I)	Uppsala Vatten (Paper II)	Kivik WWTP (Paper III)	Mycotoxins (Paper IV)
Oxidative stress response	Nrf2 activity	✔	✔	✔	
Genotoxicity	Micronuclei formation	✔			✔
Xenobiotic metabolism	Aryl hydrocarbon receptor activation	✔	✔		
Modulation of hormone systems	Estrogen receptor agonism	✔	✔	✔	✔
	Estrogen receptor antagonism				✔
	Androgen receptor agonism	✔	✔	✔	✔
	Androgen receptor antagonism	✔	✔	✔	✔

3.6.1 Luciferase reporter gene assays (RGAs) and recombinant cell lines tested

As introduced in Chapter 1 (Section 1.2), *in vitro* luciferase RGAs were used to assess the hormone-mediated effects (ER and AR), xenobiotic metabolism (AhR), and oxidative stress response (Nrf2). For each assay, the associated recombinant mammalian cell line stably transfected with the assay-specific response element is described briefly herein:

The Nrf2 assay was conducted on the human mammary MCF7-derived reporter cell line which contains eight copies of the rat Glutathione-S-transferase (GST) antioxidant response element (referred to as MCF7AREc32). The cells were a kind gift from Prof Ronald Wolf (University of Dundee, Nethergate, Scotland).

The AhR assay was conducted on the mouse hepatoma Hepa1c1c7 cell line transfected with a reporter plasmid containing seven copies of dioxin-responsive elements (referred to as DR-EcoScreen). The cells were obtained from Hiro Biotech via the Japanese Collection of Research Bioresources (JCRB) Cell Bank (JCRB1630), National Institutes of Biomedical Innovation, Health and Nutrition (Ibaraki city, Osaka, Japan).

The ER assay was conducted on the human breast carcinoma cell line MCF-7, stably transfected with an estrogen receptor-sensitive luciferase plasmid (referred to as VM7Luc4E2). The cells were kindly donated by the late Professor Michael Denison (University of California, USA).

The AR assay was conducted on the Chinese hamster ovary cell line (CHO) stably transfected with an androgen receptor responsive luciferase plasmid and an expression vector for the human androgen receptor and glucocorticoid receptor knockout (referred to as AR-EcoScreen GR-KO M1). The cells were obtained from the JCRB (JCRB1761).

3.6.2 MTS and ATP-based assays for cytotoxicity

In all studies, cytotoxicity of the sample extracts (for **Papers I** to **III**) and test compounds (**Paper IV**) was initially assessed in all cell lines with cell viability assays. The main purpose of the cytotoxicity testing was to ensure that the bioanalyses be performed under non-cytotoxic conditions such that that a specific response was not masked by cytotoxicity.

For Paper **I** to **III**, cytotoxicity of the sample extracts in the MCF7AREc32, DR-EcoScreen, and AR-EcoScreen GR-KO M1 cell lines was tested with the MTS-based colorimetric assay (Cell Titer 96® Aqueous One Solution Cell Proliferation Assay, Promega, USA). The assay is based on the reduction of the tetrazolium compound, MTS, by viable cells to

generate a coloured formazan product that is soluble in cell culture media. The formazan dye produced by viable cells is then quantified by measuring the absorbance at 490 nm. The quantity of formazan product as measured by the amount of 490nm absorbance is directly proportional to the number of living cells. For the VM7Luc4E2 cell line, cytotoxicity was measured using the ATP-based assay (CellTiter-Glo® Luminescent Cell Viability Assay, Promega, USA). The CellTiter-Glo Luminescent Cell Viability Assay is a homogeneous method of determining the number of viable cells in culture based on quantitation of the ATP present. The cell is the source of ATP, so the luminescent signal produced is proportional to the number of viable cells.

For **Paper IV**, cytotoxicity of the mycotoxin test compounds in the VM7Luc4E2 and AR-EcoScreen GR KO M1 cell lines was tested with the ATP-based assay. Initial trials with just the test compounds (i.e., without exogenous metabolic components) were conducted to determine suitable non-cytotoxic concentration ranges. As well, to verify that the exogenous metabolic components themselves did not induce cytotoxicity, cell viabilities in the presence of the tested MAS concentrations were also assessed.

3.6.3 Micronucleus assay for genotoxicity

The genotoxic potentials of the sample extracts (for **Paper I**) and mycotoxin test compounds (**Paper IV**) were assessed in the human lymphoblast TK6 cells using the micronuclei (MN) assay and analysed via flow cytometry. The MN assay is an *in vitro* method that detects genetic damage caused by chemicals through the presence of micronuclei (MN) in the cytoplasm of interphase cells. MN are formed due to breakage of chromatin or chromosomes, chromosome abnormalities, or from an entire chromosome that may have lagged behind in anaphase (Guy, 2014).

In **Paper IV**, the genotoxic potential of the test compounds in the presence of exogenous metabolic components was also evaluated and conducted in general accordance with the OECD guideline No. 487 (OECD, 2016). The protocol followed the treatment schedule described in the OECD guideline for the evaluation of experimental conditions with and without metabolic activation.

Cell analysis and enumeration of MN were conducted using the Litron Laboratories *In Vitro* MicroFlow Kit, which is a flow cytometric method for scoring MN in cultured mammalian cells. The principle of this kit involves a 2-color sequential staining technique with ethidium monoazide (EMA) and then SYTOX Green to differentiate MN from chromatin fragments derived from apoptotic or necrotic cells. The MN scoring was performed using a FACSVerse (BD Biosciences, Franklin Lakes, NJ, USA). For data acquisition, approximately 20,000 events were collected. A template of data plots and gating regions was created following instructions provided by the manufacturer of the staining kit (Litron Laboratories, USA), and included gates to eliminate doublets and the chromatin of dead/dying cells. Cell cycle characteristics were monitored using a histogram. The final scoring of micronuclei was expressed as the percentage of micronuclei among the nucleated events meeting the gating criteria.

In addition to MN scoring, the health of treated cells was inferred from the percentage of particles stained with EMA. A cytotoxicity limit based on a less than 4-fold %EMA-positive event increase over the solvent control was applied to eliminate overly cytotoxic concentrations, as recommended by the manufacturer of the kit.

3.7 Exogenous metabolic components (Paper IV)

Technical details regarding preparation of the metabolic components are provided in **Paper IV**. Presented herein is background information on each of the metabolic components themselves.

As mentioned previously in the introduction, the metabolic capabilities of many genetically engineered cell lines used in *in vitro* bioassays are either absent or of low competence because they do not express a full complement of metabolizing enzymes. While this limitation has been raised (Jacobs et al., 2013), incorporation of metabolism into *in vitro* tests particularly for endocrine active substances has been identified as a matter of urgency (OECD, 2014). In general, xenobiotic metabolism can be categorized into two phases (Phase I and II). In Phase I metabolism, the parent substance undergoes oxidation, reduction, or hydrolysis into more polar metabolites.

The cytochrome P450 (P450) isoforms are key enzymes for phase I reactions. In Phase II metabolism, conjugation of the metabolites with polar molecules such as glucose, sulphate, amino acids, glutathione or glucuronic acid occurs to generate metabolites that are more soluble and thus easily eliminated. Key Phase II enzymes include N-acetyl transferases (NAT), UDP-dependent glucuronosyl transferase (UGT), sulphotransferases (SULT), and glutathione S-transferases (GST) (Jacobs et al., 2008).

3.7.1 Incorporating exogenous metabolic components into bioassays

Subcellular fractions obtained from successive centrifugations of liver tissue homogenates can be added to *in vitro* test methods as a metabolically competent source. The hepatic S9 fraction, which is obtained after the first centrifugation step at about 9000 g, contains microsomal and cytosol fractions (Coecke et al., 2006) and is widely used in *in vitro* models, particularly for genotoxicity testing. S9 fractions derived from rat livers, for instance, are commonly incorporated as an exogenous metabolising system in routine *in vitro* genotoxicity screening of chemical compounds. In the mycotoxins study, commercially available S9 fractions derived from a pool of male Sprague Dawley rat livers induced with Phenobarbital/β-Naphtoflavone were used (Xenometrix, Art. No.: PRS-PB01). The rat hepatic S9 fraction has been demonstrated to be compatible with hormone receptor-mediated transcriptional activation bioassays (Charles et al., 2000; Jacobs et al., 2013; Mollergues et al., 2017).

Phase I and II cofactors

Since many of the enzymes in the S9 fractions are considerably diluted, specific cofactors for Phase I and Phase II enzymes are also required to promote metabolism. As P450 enzymes are known to catalyze a variety of Phase I oxidation (and some reduction) reactions, an NADPH regenerating system (Promega, Cat# V9510) was incorporated as the Phase I cofactors. The system consists of a solution of NADP+ and glucose-6-phosphate and a solution of glucose-6-phosphate dehydrogenase, which when combined before use, generates NADPH which then serves as the source of electrons for the P450 oxidative reactions (Meunier et al., 2004). For the key Phase II reactions: reduced L-glutathione was incorporated for the glutathione conjugation which is facilitated by glutathione transferase enzymes; uridine

diphosphate glucuronic acid (UDPGA) was incorporated as a substrate for UDP-glucuronosyl transferases involved in glucuronide conjugation; and 3-phosphoadenosine-5-phosphosulphate (PAPS) was incorporated as a coenzyme for sulfotransferases involved in sulphate conjugation.

Further details regarding the respective concentrations of the metabolic components are provided in **Paper IV**. Optimization and validation of the protocol that was developed to incorporate the exogenous metabolic components into the ER and AR bioassays was previously completed in an unpublished study which is not presented in this thesis work.

3.8 Data handling

The data handling approach was generally the same among all four studies for the collected data. Some key concepts are presented herein as background information. Complete details on the data handling and analyses are otherwise found in the respective Materials & Method sections of all papers.

The GraphPad Prism software (versions 8.3.0 & 9.3.1) was used for all statistical modelling, analysis, and plotting. Data storage was maintained with Microsoft Excel 2016. Statistical methodologies utilized are elaborated upon in each respective paper.

3.8.1 Relative enrichment factor of samples (Papers I to III)

For the bioassays tested in **Papers I to III**, the concentration metric for the sample extracts was expressed in units of relative enrichment factor (REF). This is because the samples contain undefined complex mixtures of chemicals. The enrichment and dilution of the samples constitute the REF and was calculated using the following equations (a) to (c), as described by Escher et al. (2014) (Escher et al., 2014):

$$REF = enrichment\ factor_{SPE} * dilution\ factor_{bioassay} \quad \text{(a)}$$

The dilution and enrichment factors were calculated by the following equations:

$$enrichment\ factor_{SPE} = \frac{volume\ water}{volume\ extract} \quad \text{(b)}$$

$$dilution\ factor_{bioassay} = \frac{volume\ of\ extract\ added\ to\ bioassay}{total\ volume\ of\ bioassay} \quad \text{(c)}$$

All sample extracts were dissolved in ethanol (EtOH, 99.7%) such that the final concentration of EtOH in the culture medium was 1%.

3.8.2 Derivation of effect concentrations (Papers I to III)

For Papers **I** to **III**, data analysis included the derivation of effect concentrations: the effect concentrations causing a 10% effect (EC_{10}) for the ER and AR agonism assays and the effect concentration causing a 30% inhibitory effect (IC_{30}) for the AR antagonism assays. The effect concentrations were expressed as REF. To obtain the effect concentrations, concentration-effect curves (CECs) were generated from the data sets of sample extracts tested in 2-fold dilution series. To generate CECs for the ER and AR agonism assays, the mean activities of the vehicle controls were first subtracted from all sample activities; all adjusted values were then normalized to the mean activities of the vehicle controls (set to 0%) and then to the maximum mean activities of the highest concentration of the respective reference compound (assay maximum, set to 100%). The normalized data set was then fitted to a four-parameter sigmoidal non-linear regression model and the EC_{10} value interpolated from the CEC. For the AR antagonism assay, the mean activities of the unspiked vehicle control were first subtracted from all sample activities; all adjusted values were then normalized to the mean activities of the unspiked vehicle control and then to the mean activities of the spiked vehicle control. The normalized data set was then fitted to a four-parameter sigmoidal non-linear regression model and the IC_{30} value interpolated from the CEC. For the Nrf2 assay where no maximum effect can be reached, all sample activities were normalized to the mean activity of the vehicle control and then fitted to a linear regression model. The concentration causing a 1.5-fold induction ($EC_{IR1.5}$) was interpolated from the CEC.

3.8.3 Bioanalytical equivalent concentrations (BEQ) (Papers I to III)

For Papers **I** to **III**, the effect concentration values were further translated into bioanalytical equivalent concentrations (BEQ) by dividing the EC_{10}/IC_{30} values of the samples (EC_{10}/IC_{30}, sample) from that of the respective reference compound (EC_{10}/IC_{30}, ref) of the particular assay. See equation (d).

$$BEQ = \frac{(EC_x \; or \; IC_x)_{ref}}{(EC_x \; or \; IC_x)_{sample}} \qquad \text{(d)}$$

Determining the BEQ value is a way of translating the measured effect of the sample extract to the concentration of a known reference compound. This corrects for differences in sensitivity between experiments and also allows for comparisons to other studies that report with the same metric.

3.8.4 Test concentrations of mycotoxins (Paper IV)

For the mycotoxins study (**Paper IV**), because single compounds were studied, the prepared test concentrations could be expressed in standard units of concentration (e.g., molarity or mass per volume). All test concentrations were dissolved in DMSO such that the final concentration of DMSO in the culture medium was 1%.

4. Results and discussion

This chapter highlights some of the key findings of each paper and discusses these findings in a broader scientific context. The complete results and discussions of each study are presented in their respective papers (**I to IV**).

4.1 EBMs as useful bioanalytical tools in assessing treatment methods in drinking water production (Papers I & II)

In this thesis work, EBMs involving *in vitro* bioassays were applied to different drinking water production scenarios to assess and compare treatment methods.

4.1.1 Norrvatten (Paper I)

Based on the panel of bioassays assessed in the Norrvatten study (**Paper I**), it was demonstrated that the current full-scale treatment technologies at this DWTP were unable to consistently remove compounds inducing the observed bioactivities across several sampling events. In comparison, the pilot-scale treatment processes showed better treatment efficiencies. A heatmap illustrating the overall results is provided in Figure 9.

While the use of effect-based *in vitro* bioassays to assess the treatment efficiencies of DWTPs has been done before, that repeat sampling events were conducted is a highlight of this study, and not often done in other studies. It is from the repeated sampling events, that certain undesirable biological effects were detected, such as genotoxicity, which is further discussed in the next section. That seasonal fluctuations in biological effects were detected not only in the raw water source but also following respective

treatments within the facility serves as useful monitoring data, much like the routine monitoring of chemical and microbiological parameters in water quality. The fluctuations captured from routine monitoring data can also assist the utility operator in tracking potential sources of the effects as well as adjust the operating conditions of the treatment technologies.

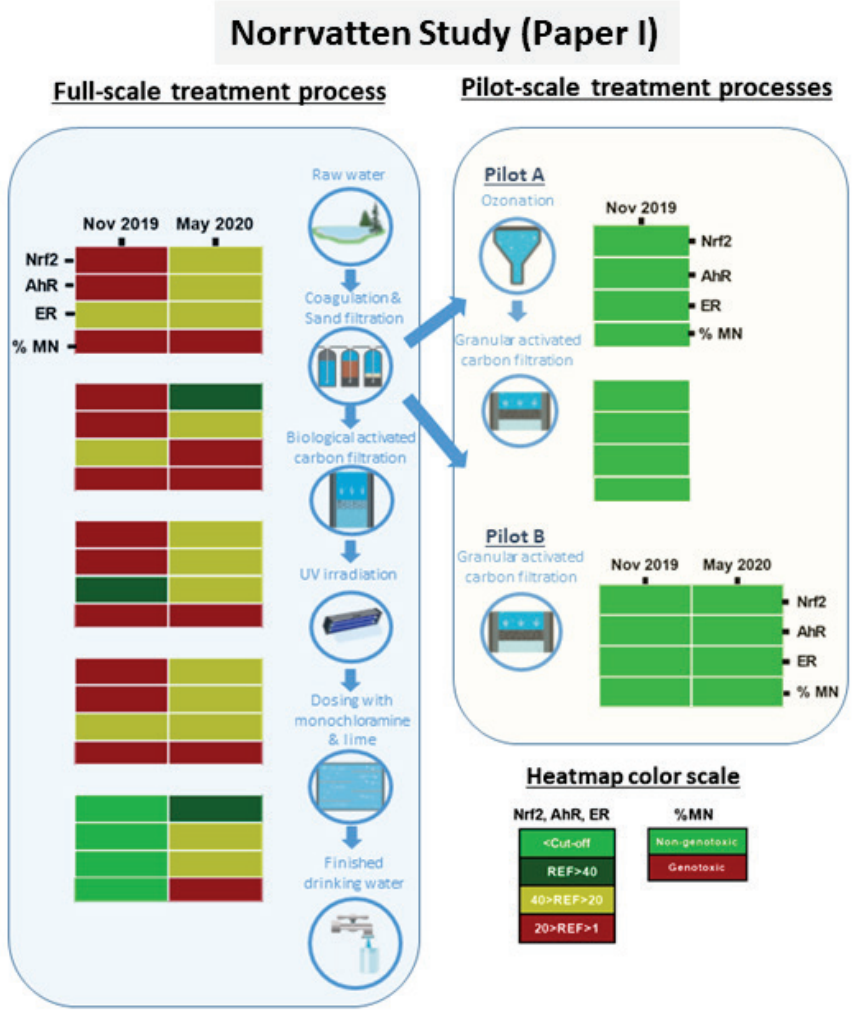


Figure 9 Heat map summarizing the results from the Norrvatten study. Shown are the effect concentrations (expressed as REF) detected in the panel of bioassays assessed for each treatment step of the full-scale and pilot-scale treatment processes in the Norrvatten study (**Paper I**). The chart was created based on the $EC_{IR1.5}$ values (Nrf2

assay), EC_{10} values (AhR & ER assays), and % Micronuclei Formations (MN assay) determined from samples collected in November 2019 and in May 2020.

4.1.2 Uppsala Vatten (Paper II)

While the treatment processes assessed in the Norrvatten study (**Paper I**) would be considered fairly conventional, the next study (Uppsala Vatten, **Paper II**) was undertaken to assess the artificial infiltration treatment process in drinking water production, which to-date has been comparatively under-studied using effect-based *in vitro* bioassays (Jia et al., 2015; Oskarsson et al., 2021). Further, contamination of the artificial infiltration zone with hazardous chemicals has been previously reported elsewhere in Sweden (Oskarsson et al., 2021). In the Uppsala Vatten study, bioactivities (AhR and ER) that were detected in the infiltration basins were no longer detectable downstream following the artificial infiltration process (Figure 10). Thus, using effect-based *in vitro* bioassays, the treatment efficiency of the artificial infiltration process was shown to be effective for these parameters.

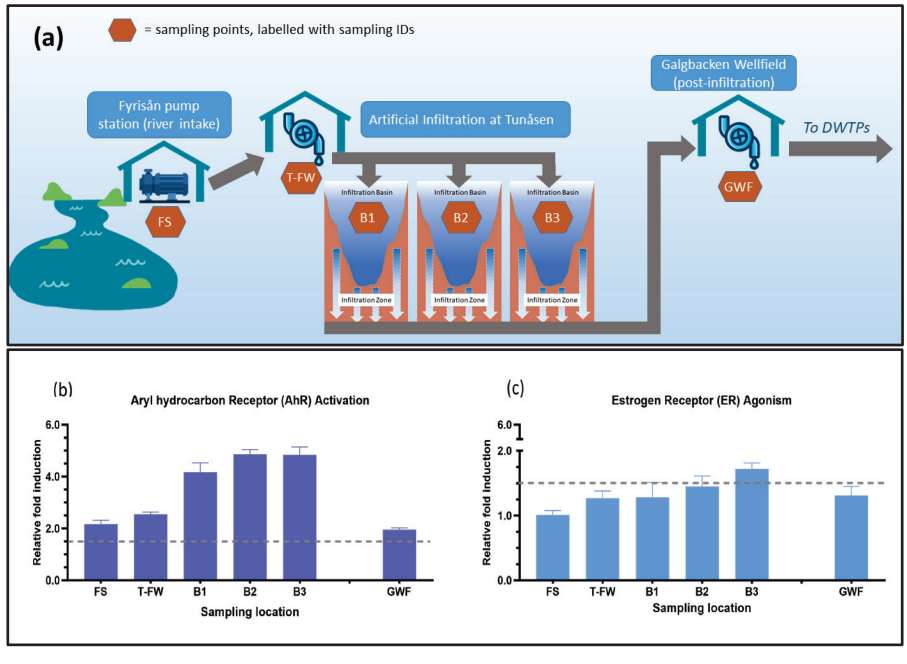


Figure 10 Diagram showing the sample points between the river intake and wellfield and results from the Uppsala Vatten study. Panel (a) is a schematic showing the treatment

steps only between the river intake and wellfield for the Uppsala Vatten study (**Paper II**). The AhR and ER activities detected at REF 50 at each sampling location in the schematic are shown in panels (b) and (c), respectively.

It is worthwhile to mention that the findings of the two studies (**Papers I** and **II**) highlight an important aspect of study designs when using EBMs. More specifically, different biological effects were detected between the two studies - e.g., predominantly AhR and Nrf2 activities (and genotoxicity) in the Norrvatten study vs. AhR and ER activities in the Uppsala Vatten study. This emphasizes the importance of assessing a panel of several bioassays representing a broad range of cellular-level response pathways which can provide an integrated snapshot of what effects are detected in a water sample which might be otherwise limited if using only single bioassays. This also illustrates the presence of complex chemical mixtures in different water sources.

4.2 Detection of genotoxicity in drinking water (Paper I)

An important finding of the Norrvatten study (**Paper I**) was the detection of genotoxicity in the raw water as well as finished drinking water from the full-scale system in some of the sampling events (Figure 11). This is potentially a serious health effect because DNA damage at the cellular level can lead to cancer and other diseases. Importantly, such effects may also occur at very low doses (no threshold dose) for which there is no safe exposure level if it is a direct-acting effect. Moreover, all of the regulated chemical parameters monitored for at the DWTP were at acceptable levels. As our study has shown, the use of EBMs specific for genotoxicity is fundamental for the protection of human health, considering that genotoxic substances can occur in drinking water (Ceretti et al., 2016; Feretti et al., 2020). In other studies that combined targeted chemical analyses with effect-based *in vitro* bioassays, the detected chemicals were often found to either not contribute or only marginally contribute to the observed biological effects (Escher et al., 2013; Neale et al., 2017; Oskarsson et al., 2021; Tang et al., 2014). In these studies, the detected chemicals typically explained only a small fraction (i.e., less than 1%) of the observed biological effects.

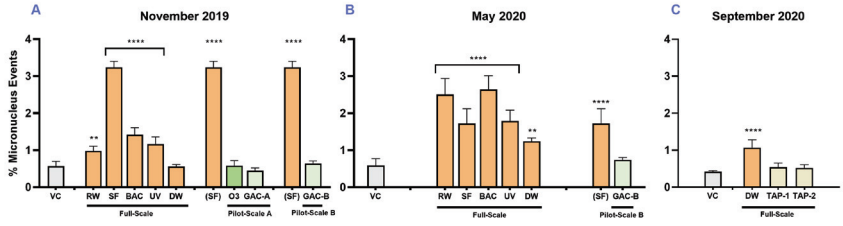


Figure 11 Results of the MN assay from the Norrvatten study. Shown are the micronuclei formations in TK6 cells exposed to the full-scale vs. the pilot-scale treated sample extracts at REF 50 collected in November 2019 (A), in May 2020 (B), and in September 2020 (C). Treatment groups (n = 4) were compared to the vehicle control (n = 8). Data bars for each sampling point are presented as mean ± standard deviation. * represents significant differences from the vehicle control (*=$p \leq 0.05$, **=$p \leq 0.01$, ***=$p \leq 0.001$, ****= $p \leq 0.0001$). NOTE: For comparison's sake between the full- and pilot-scale processes, the micronuclei formations for the SF sampling point are shown for all 3 treatment systems. Figure reproduced from Yu et al., 2021 (Appx I – **Paper I**).

4.3 To acidify or not acidify during sampling (Paper III)

While studies such as the first two of this thesis work have demonstrated the useful applications of effect-based *in vitro* bioassays in water quality assessments (Brack et al., 2019; Neale & Escher, 2019); less focus, as previously mentioned, has been given to certain aspects related to this type of testing such as proper sample handling. Sample acidification is an important aspect of sample handling to maintain the stability of the sample contents until time of analysis. Yet there remains a paucity of research into this aspect as it relates to effect-based *in vitro* testing. The findings from the Kivik study (**Paper III**) were compelling in that the impact of acidification on bioactivities in wastewater samples varied before vs. after disc filtration treatment. These differences could be attributed to a pH-dependent increase in the partitioning of bioactive compounds to solids in the acidified sample collected before the disc filtration treatment samples (which were subsequently removed in the SPE process before bioanalysis); and inhibition of microbial degradation of bioactive compounds in the acidified sample collected following the disc filtration treatment. In addition, all sample extracts were re-tested in the same bioassays after approximately one year of cold (-20°C) storage to address the secondary objective of assessing the stability of bioactivities in the sample extracts over time. Decreased ER and AR activities were observed in the sample extracts. A summary of the results

before and after disc filtration treatment is provided in Table 3. A complete summary of all results from all sample points is provided in the relevant manuscript. (Appx III). Taken together, the findings of the study revealed that sample acidification and storage time need to be optimized depending on the sample and the bioassay to accurately assess water quality as certain treatment processes may impact the resulting chemical compositions of the samples. As there remains a lack of standardized protocols for sample preparation techniques when using EBMs to assess water quality, the findings of the current study are highly relevant.

Table 3 Summary of BEQs for two of the sample points from the Kivik study. Presented are the BEQ values for the unacidified (without HCl) and acidified (with HCl) samples before and after disc filtration from the 2022 and 2023 analyses. Presented below each BEQ value are the respective 95% confidence intervals (LL, UL) in parentheses.

	Influent to disc filtration				Effluent of disc filtration			
	2022		2023		2022		2023	
Assay	Without HCl	With HCl	Without HCl	With HCl	Without HCl	With HCl	Without HCl	With HCl
ER activity (ng E2eq/L)	2.14 (1.25,5.53)	2.62 (1.87,12.1)	0.019 (0.01,0.03)	0.013 (0.01,0.02)	0.71 (0.003,1.41)	0.81 (0.28,1.90)	<LOD	0.044 (0.03,0.06)
AR agonist activity (ng DHTeq/L)	4.85 (2.75,6.95)	<LOD	0.06 (0.03,0.09)	<LOD	4.43 (3.20,5.66)	16.7 (5.15,28.3)	0.03 (0.01,0.04)	0.23 (0.10,0.60)
Nrf2 activity (µg tBHQeq/L)	16.2 (11.9,20.5)	<LOD	12.1 (10.4,13.8)	<LOD	10.4 (9.04,11.7)	15.9 (14.1,17.6)	9.30 (3.52,15.1)	25.6 (23.5,27.8)

LOD: Limit of detection

4.4 EBMs to evaluate the bioactivities of two common mycotoxins and their derivatives *in vitro* with exogenous metabolic components (Paper IV)

While effect-based *in vitro* bioassays are useful in water quality assessments, they can also be applied to the testing of substances from a toxicology perspective (European Commission, 2023). Whereas the previous three studies in this thesis work focused on biological effects from water

contaminants such as OMPs, the presence of mycotoxins and their derivatives/metabolites in water bodies have been identified as contaminants of increasing concern, particularly with respect to drinking water safety (Gromadzka et al., 2009; Hageskal et al., 2009; Székács, 2021). In **Paper IV**, bioactivities of deoxynivalenol (DON), and its derivatives 3-acetyl DON (3-aDON) and 15-acetyl DON (15-aDON) along with zearalenone (ZEN) and one of its primary metabolites, α-zearalenol (α-ZEL) were assessed in the presence of exogenous metabolic components *in vitro* with a focus on hormone-receptor mediated and genotoxic effects. To the best of our knowledge, such a study has not been previously conducted on these compounds for the hormone-receptor endpoints with the incorporation of exogenous metabolic components.

An important finding was that the *in vitro* ER bioassay used in the current study proved to be a highly sensitive method to detect very low concentrations (in the pM range) of the ZEN compounds in aqueous solutions. This is particularly useful towards, for instance, the screening of water samples for ZEN and α-ZEL as they are well-known potent estrogens and known to co-occur such as in cereals and production animals (EFSA CONTAM, 2017), and evaluated *in vitro* (Demaegdt et al., 2016).

Another (rather surprising) finding of the study was the decrease in the estrogenic activity of ZEN in the presence of the exogenous S9 and phase I cofactors (Figure 12). It is surprising because α-ZEL, as one of the expected primary phase I metabolites of ZEN, is known to be more estrogenic than ZEN. A number of possible explanations for this finding are presented in **Paper IV**. One such explanation is the production of other novel (mono-hydroxylated) metabolites which have lower estrogenic activities than α-ZEL or ZEN. In addition, species differences have been reported in terms of which metabolites are formed from the metabolism of ZEN (Malekinejad et al., 2006; Yang et al., 2017).

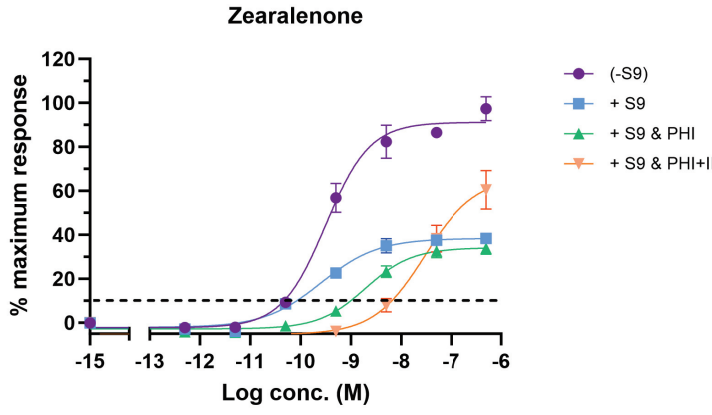


Figure 12 CEC of the ER agonistic effects of ZEN in the presence of exogenous MAS. The test compound was assayed in the absence of MAS (purple, circles), in the presence of S9 alone (light blue, squares), S9 with Phase I cofactors (green, triangles), and S9 with Phase I and Phase II cofactors (light orange, inverted triangles). Data presented as mean±SD.

In general, the information gathered in this study will help to better elucidate the impact of metabolism on bioactivities of these common mycotoxins *in vitro* at the level of receptor-binding related to endocrine effects. Such information will also be useful contributions to *in vitro/in vivo* modelling and the development of relevant adverse outcome pathways for these mycotoxins.

An evaluation of the genotoxic potential of ZEN *in vitro* in the presence of the exogenous metabolic components did not find any induction of genotoxicity (i.e., micronuclei formations) of ZEN following metabolism. This suggests that the metabolites of ZEN formed in the present study were not genotoxic. While ZEN has been determined to be clastogenic by the European Food Safety Authority (EFSA) Panel on Contaminants in the Food Chain (EFSA CONTAM, 2011), the potential of ZEN to cause micronuclei formations remains inconclusive (Rencüzoğulları & Aydin, 2019). DON was also evaluated in this thesis study and found to not be genotoxic at any of the non-cytotoxic test concentrations assessed. Further, no induction of genotoxicity was detected in the presence of the exogenous metabolic components. The EFSA Panel on Contaminants in the Food Chain considers

DON to be genotoxic *in vitro*, however this determination was informed by other types of genotoxic endpoints other than micronuclei formations. As such, the results of this study provide additional insights into the genotoxicity of these two compounds.

5. General conclusions and future perspectives

The studies comprising this thesis work set out to demonstrate the utility of EBMs using *in vitro* bioassays in several different applications. The study presented in **Paper I** was a classic example of how EBMs can be used to assess the treatment efficiencies of different water treatment processes in drinking water production. In this case, it was to compare pilot- vs. full-scale treatment schemes at a large-scale municipal DWTP to inform the decision-making process regarding future upgrades to the existing facility. A prominent highlight of that study was the presence of genotoxic compounds in the raw water as well as finished drinking water, which were detected from the inclusion of repeat sampling events. Moreover, the current monitoring programme at the DWTP was unable to detect the presence of genotoxic compounds in the water. This critical finding prompted the operator of the DWTP to take action and implement an intensive monthly monitoring campaign over one year to discern the source(s). In the next study (**Paper II**), EBMs using a panel of *in vitro* bioassays were applied again to assess the treatment efficiency of another municipal DWTP from source to tap, but one that involved artificially infiltrated river water as its water source. Such a study using EBMs was the first to be undertaken for the operator of this water utility. For **Paper III**, to identify if sample acidification (and long-term storage) is an important aspect to consider in the designs of EBM studies, the study was undertaken at a WWTP which has future plans to reuse the finished water for irrigation, swimming pools and re-infiltration, and even potentially as a drinking water source. The findings of **Paper III** revealed that the decision to incorporate sample acidification may need to be customized depending on the assays of interest and treatment technologies to be assessed. For **Paper IV**, as mycotoxins have been identified as

contaminants of concern in waterbodies and drinking water, the study involved testing certain common mycotoxins using effect-based *in vitro* bioassays for three specific endpoints. More importantly, the bioactivities of metabolic biotransformations of these compounds were assessed *in vitro* in the presence of exogenous metabolic components. The findings from this paper demonstrated how the inclusion of exogenous metabolic activation is useful in detecting biological effects of metabolites in *in vitro* bioassays for hormone-receptor mediated effects.

To close, the work completed in this thesis aimed to address current knowledge gaps in the research related to the respective studies in **Papers I to IV**. More studies using EBMs to assess DWTPs, such as **Paper I**, are needed towards the acceptance and implementation of *in vitro* bioassays into drinking water quality frameworks and regulatory water quality testing to minimize the risks of populations being exposed to unknown chemical pollutants. For **Paper II**, the artificial infiltration process in drinking water production remains under studied, particularly using EBMs. Given the global prevalence of this type of treatment in the management of water resources, and the increasing attention being given to water accessibility and water scarcity issues due to urbanization and climate change, more research into the effectiveness of this treatment method from a drinking water safety perspective is needed. **Paper III** addressed an under-emphasized aspect of sample handling and storage with respect to EBMs, and further showed that these aspects should not be overlooked. Moreover, such studies are needed towards the ultimate goal of developing standardized protocols and eventual acceptance of EBMs for regulatory testing purposes. **Paper IV** demonstrated that exogenous metabolic components can be integrated into *in vitro* bioassays when testing the effects of two common mycotoxins and their derivatives on important cellular pathways related to endocrine disruption and genotoxicity.

There are several future perspectives regarding the different applications of EBMs demonstrated in this thesis work. From **Papers I and II**, more can be explored in terms of: the inclusion of EBMs in water safety planning at DWTPs as well as the incorporation of EBMs into routine water quality monitoring and the development of real-time alerting systems for effect-based endpoints. From **Paper III,** as already mentioned, there remains a need

to standardize and validate protocols involving EBMs in order to be accepted as regulatory-endorsed tools in water quality assessments and management. The information and insights gleaned from **Paper IV** would serve useful in more informed risk assessments for these compounds of interest.

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Popular science summary

Chemicals are omnipresent in our daily lives with over tens of thousands of chemicals in commercial use on a global scale. These include pharmaceuticals, surfactants, personal care products, pesticides, biocides, and many others together with numerous transformation products. With that, issues of chemical pollution in the environment and implications to human and environmental health are increasing as chemicals and transformation products put pressure on ecosystems and drinking water resources. While regulatory frameworks on water quality do address the chemical status of water, the existing monitoring requirements based on only a limited number of chemicals fall critically short in characterizing the plethora of known (and unknown) chemicals which can adversely affect the quality of water. As such, chemical monitoring alone of only individual chemicals will become increasingly less informative in drinking water safety. To tackle the conundrum of mixtures, effect-based methods (EBMs) using *in vitro* bioassays have emerged in the past few decades as useful tools in water quality assessments, and have been gaining momentum based on the growing number of research studies demonstrating their applicability. Whereas chemical analysis is a quantitative approach for key target chemicals, EBMs assess cellular-level effects and can offer an integrative snapshot of cumulative effects on various modes of action from chemical mixtures.

From a toxicology perspective, *in vitro* bioassays have gained popularity in recent years. With the launch of the "Toxicity Testing in the 21st Century" (Tox21) program in 2007, the US National Research Council promoted the use of *in vitro* bioassays as alternatives to animal testing. This program envisions a fundamentally new direction to toxicity testing based on evaluations of perturbations to toxicity pathways and molecular interactions

between the chemical and biological target. This *in vitro* approach, together with *in silico* and *in chemico* methods, have been coined New Approach Methodologies (NAMs). Such NAMs have the advantage of being less expensive and time-consuming than animal testing and more ethical from an animal welfare aspect.

Toxicity testing with *in vitro* bioassays is already implemented in regulatory frameworks in other fields such as food safety and chemical regulation. In contrast, most drinking water frameworks currently do not involve the use of *in vitro* bioassays for water quality assessment for human health. However, that is not to say that the value of EBMs has not gone unnoticed. More attention is being put on revising the tools and paradigms used to assess the hazards of chemicals in the environment based on modern 21st century toxicology. The European Commission, for instance, states that "effect-based tools are especially suitable as part of investigative monitoring programs for which the regulatory requirement are less formally determined" in the context of the Water Framework Directive (2000/60/EC). More recently, the potential application of EBMs in water quality assessments has been recognized by the World Health Organization and in the Australian guidelines for water reuse. The recently recast Drinking Water Directive (2020/2184) was also a step in the right direction towards updating the legislative framework to address new challenges faced by the drinking water sector in the European Union.

The work presented in this thesis focused on 21st century toxicology to study water quality and explored the different applications of EBMs in research topics relevant to drinking water safety. The thesis started with addressing an important question regarding the effectiveness of water treatment technologies as chemical barriers. Drinking water treatment technologies have traditionally focused on removing potential pathogens in the water to make it safe for consumption; however, many of these technologies were not specifically designed to remove chemical pollutants. We demonstrated that EBMs are highly sensitive in detecting biological effects, which may not have been otherwise identified from the parameters being routinely monitored for in the drinking water system. We also evaluated an artificial infiltration treatment method in drinking water production. Given increasing issues related to water stress and contamination

of surface waters, a reliance on other sources of drinking water such as groundwater would be expected to grow. To that, contamination of groundwater should not be overlooked. In addition to conducting field studies with EBMs, this thesis work also considered how EBMs can be improved – specifically, sampling handling. This is necessary as there is a lack of standardised methods relevant to the application of EBMs to environmental samples. Lastly, much akin to a typical toxicology study, we applied EBMs to investigate *in vitro*-level effects of two common mycotoxins and their derivatives. These mycotoxins were studied in the context of water quality and drinking water safety based on increasing attention being given to these compounds as emerging contaminants of concern in water resources and drinking water. A novel element of this study was the incorporation of exogenous metabolic components into the bioassays. In doing so, we addressed a key research topic in the realm of EBMs that has remained relatively unexplored but requires further attention.

Taken together, the work of this thesis showed the diversity of EBM applications. Such work will hopefully serve as insightful contributions toward wider implementation of such methods in water management and eventual acceptance of EBMs in drinking water quality frameworks. In doing so, a better roadmap can be built that involves a multidisciplinary approach to improving drinking water safety. From a toxicology perspective, this work could promote the inclusion of EBMs in hazard identification and risk assessments as well. Finally, it is this author's hope that the next time you, as the reader, look at a glass of drinking water you will take but a brief moment to reflect on what is meant by clean and wholesome water.

Populärvetenskaplig sammanfattning

Tiotusentals kemiska ämnen har tillverkats, används och riskerar spridas i miljön. Exempel på dessa inkluderar läkemedel, ytaktiva ämnen, hygienprodukter, bekämpningsmedel, biocider och många andra. Vidare kan dessa ämnen brytas ned till olika omvandlingsprodukter och metaboliter. Detta har lett till ett ökat fokus på frågor kring kemisk förorening i miljön och konsekvenserna för människors hälsa och miljötillståndet, när kemikalier och omvandlingsprodukter förorenar ekosystem och dricksvattenresurser. Dagens regelverk för vattenkvalitet och vattnets kemiska status fokuserar på haltbestämning av ett begränsat antal kemikalier. Detta angreppsätt är otillräckligt med tanke på det stora antalet, både kända och okända, kemikalier som kan förorena vattnet. Att enbart fokusera på ett fåtal välkända föroreningar innebär att man riskerar underskatta hälso- och miljöriskerna som kommer från okända ämnen, ämnen som inte kan detekteras med kemisk analys, omvandlingsprodukter samt blandningseffekter som kan uppstå då flera kemikalier samverkar. Effektbaserade metoder (EBM), här utförda som in vitro¬-bioanalyser i odlade däggdjursceller, har under de senaste decennierna växt fram som användbara verktyg för att upptäcka kemiska föroreningar i olika typer av vatten. Ett växande antal forskningsstudier visar på dessa metoders tillämpbarhet. Haltbestämning med kemisk analys är en kvantitativ metod som fokuserar på enskilda kemikalier. EBM mäter istället den totala toxiska effekten av alla kemikalier i ett prov som kan orsaka en viss typ av toxicitet. Därmed får man en helhetsbild av föroreningarna i provet, inkluderande såväl kända som okända ämnen och blandningseffekter.

I denna avhandling har fokus varit på att undersöka hur EBMs kan användas i olika tillämpningar för att bedöma vattenkvalitet och bidra till en

förbättrad dricksvattensäkerhet. I avhandlingen användes EBMs för att utvärdera effektiviteten i kemiska barriärer i dricksvattenproduktion. Vi kunde där visa att EBMs var användbara och kunde upptäcka kemiska föroreningar i vattnet som hade riskerat passera oupptäckta om enbart klassisk kemisk analys hade använts. Vidare användes EBMs för att undersöka hur effektivt kemiska föroreningar avskiljs vid artificiell infiltration i dricksvattenproduktion. Avhandlingen inkluderar även metodutveckling av EBM-metodiken, med fokus på provtagning och provhantering. Dessa är mycket viktiga frågor eftersom det fortfarande pågår ett arbete med standardisering av såväl analysmetoderna som provtagningsstrategier och provhantering. Avslutningsvis användes EBMs för att undersöka toxiciteten av två mykotoxiner och deras derivat. Dessa två mykotoxiner har rapporterats kunna förorena dricksvatten, varför toxicitetstestning av dessa substanser var viktigt ur ett dricksvattensäkerhetsperspektiv.

Sammanfattningsvis ger denna avhandling flera exempel på hur EBMs kan användas för att förbättra dricksvattensäkerheten och därmed förhoppningsvis bidra till att dessa metoder används mer rutinmässigt inom dricksvattenområdet.

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Appendix I

Paper I

Removal of oxidative stress and genotoxic activities during drinking water production by ozonation and granular activated carbon filtration

Appendix II

Paper II

Artificial infiltration in drinking water production: Addressing chemical hazards using effect-based methods

Appendix III

Paper III

Impact of sample acidification and extract storage on hormone receptor-mediated and oxidative stress activities in wastewater

Appendix IV

Paper III

Effects on estrogenic, androgenic and genotoxic activities of zearalenone and deoxynivalenol in the presence of exogenous metabolic activation

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RESEARCH

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Removal of oxidative stress and genotoxic activities during drinking water production by ozonation and granular activated carbon filtration

Maria Yu[1*], Elin Lavonen[2,3], Agneta Oskarsson[1] and Johan Lundqvist[1]

Abstract

Background: Bioanalytical tools have been shown to be useful in drinking water quality assessments. Here, we applied a panel of in vitro bioassays to assess the treatment efficiency of two pilot-scale treatments: ozonation and granular activated carbon (GAC) filtration at a drinking water treatment plant (DWTP). The pilot-scale systems were studied alongside a full-scale treatment process consisting of biological activated carbon (BAC) filtration, UV disinfection, and monochloramine dosing. Both systems were fed the same raw water treated with coagulation/flocculation/sedimentation and sand filtration. The endpoints studied were oxidative stress (Nrf2 activity), genotoxicity (micronuclei formations), aryl hydrocarbon receptor (AhR) activation, as well as estrogen receptor (ER) and androgen receptor (AR) activity.

Results: Nrf2, AhR, and ER activities and genotoxic effects were detected in the incoming raw water and variability was observed between the sampling events. Compared to most of the samples taken from the full-scale treatment system, lower Nrf2, AhR, and ER bioactivities as well as genotoxicity were observed in all samples from the pilot-scale systems across all sampling events. The most pronounced treatment effect was a 12-fold reduction in Nrf2 activity and a sixfold decrease in micronuclei formations following ozonation alone. GAC filtration alone resulted in sevenfold and fivefold reductions in Nrf2 activity and genotoxicity, respectively, in the same sampling event. Higher bioactivities were detected in most samples from the full-scale system suggesting a lack of treatment effect. No androgenic nor anti-androgenic activities were observed in any sample across all sampling events.

Conclusions: Using effect-based methods, we have shown the presence of bioactive chemicals in the raw water used for drinking water production, including oxidative stress, AhR and ER activities as well as genotoxicity. The currently used treatment technologies were unable to fully remove the observed bioactivities. Ozonation and GAC filtration showed a high treatment efficiency and were able to consistently remove the bioactivities observed in the incoming water. This is important knowledge for the optimization of existing drinking water treatment designs and the utilization of alternative treatment technologies.

Keywords: Drinking water, In vitro bioassays, Toxicity, Treatment efficacy, Repeat sampling, Pilot-scale

Introduction

Drinking water sources, particularly surface water, are increasingly subject to contamination risks from various anthropogenic activities including agricultural land use, urban stormwater discharge, wastewater treatment plant

*Correspondence: maria.yu@slu.se
[1] Department of Biomedical Sciences and Veterinary Public Health, Swedish University of Agricultural Sciences, Box 7028, 750 07 Uppsala, Sweden
Full list of author information is available at the end of the article

outlets and long-distance air pollution. As a result, there is growing concern over the presence of micropollutants in water sources and their potential negative effects on the environment and in drinking water production. Further, the presence of naturally occurring toxicants in raw water, as well as the formation of disinfection byproducts (DBPs) during drinking water production, pose potential threats to human health [1–3]. Such challenges facing the drinking water sector, thus, call for continued research to not only better understand and predict the removal rates of treatment technologies and support the optimization of water purification strategies, but also to inform more comprehensive water quality frameworks to ultimately safeguard the hygienic quality of drinking water.

Effective water treatment processes are essential to produce safe drinking water under varying source-water quality conditions. Drinking water purification methods commonly utilized at drinking water treatment plants (DWTPs) include different combinations of the following basic physical and chemical processes: coagulation, sedimentation, filtration, and disinfection [chlorination or ultraviolet (UV) irradiation]. These methods, however, do not completely eliminate micropollutants that may remain in the treated drinking water [4] and lead to human exposure to hazardous compounds or mixtures. As such, the choice and combination of treatment methods are important considerations in the design of DWTPs. Chlorination remains a common disinfection method at DWTPs around the world. In the US alone, disinfection using free chlorine continues to be the most widely used disinfectant, as reported by 70% of respondents in a recent survey summarizing common disinfection practices among drinking water utilities [5]. Cost has been reported as the primary factor as to why the utility operators have not considered switching to other disinfection methods such as ozone or UV [5]. Similarly, chlorination is widely used in other countries such as South Africa, Canada, and Australia [6]. However, a major downside of using chlorine is the potential formation of DBPs such as trihalomethanes (THMs) and haloacetic acids (HAAs). Alternative disinfectants such as chloramines, ozone, chlorine dioxide, and UV disinfection are, however, gaining popularity. Ozonation (O_3) and activated carbon treatments have been suggested to be more effective treatment methods in managing the removal of organic micropollutants (OMPs) and DBP precursors [7, 8] than other methods such as coagulation, sedimentation, and rapid or slow sand filtration. Certainly, water treatment methods continue to be modernized with the development of advanced treatment alternatives. While each treatment method has its advantages and disadvantages, it is undoubtedly beneficial to pilot-test treatment designs prior to implementation to ensure the designs are appropriately suited for implementation into full-scale capacities or when considering the optimization of existing treatment system processes.

The efficiencies of treatment processes at a DWTP are routinely tracked via drinking water quality monitoring. However, such monitoring is typically only conducted for the limited number of chemical parameters listed in the drinking water regulations, such as metals, pesticides, a few DBPs, and polycyclic aromatic hydrocarbons. Such parameters alone are insufficient in characterizing water quality given that numerous chemicals are likely to be present and potentially at low concentrations. As such, effect-based monitoring using bioanalytical tools (i.e., in vitro bioassays) has been suggested as a promising complement to existing water quality assessments [9–17] and can provide valuable information related to treatment efficiencies. Importantly, more studies using bioanalytical tools to assess DWTPs are needed to provide support towards the acceptance and implementation of in vitro bioassays in drinking water quality regulations.

In the current study, a panel of cell-based reporter gene assays assessing effects from multiple toxicity pathways relevant to human health were used to investigate the treatment efficiencies of a pilot-scale drinking water system. Specifically, the study compared the efficacy of pilot-scale GAC filtration as well as the combination of ozonation and GAC filtration vs. full-scale biological activated carbon (BAC) filtration, UV disinfection, and monochloramine dosing at removing/reducing bioactivities. Both treatment systems were fed the same raw water that had undergone primary treatment (coagulation, sedimentation/flotation, sand filtration) in the full-scale. We hypothesized that the ozonation and GAC filtration methods would be more effective at reducing bioactivities. The selected bioassays targeted reactive modes of action such as cytotoxicity and genotoxicity as well as adaptive stress responses and receptor-based effects including oxidative stress, aryl hydrocarbon receptor (AhR) activation, and hormone-mediated modes of action. These particular endpoints, which relate to adaptive stress response, xenobiotic metabolism, and modulation of hormone systems, have been identified as being the most responsive toxicity pathways in the case of drinking water [2, 18, 19]. This study aimed to: (1) provide knowledge on the efficacy of the pilot-scale ozonation and GAC filtration treatments; (2) assess temporal differences in water quality; and (3) report findings regarding bioactivities observed in the raw water and the full-scale treatment process. While the efficacy of the pilot-scale ozone (O_3) and GAC had been previously assessed in the context of OMP levels in drinking water production [8], the bioanalytical approach of the current study will

provide further useful information regarding the removal efficiencies of the two treatment technologies. This could be of particular relevance as a decision-making tool in the potential implementation of the alternate treatment technologies into full-scale capacities or when considering the optimization of the existing treatment system processes.

Materials and methods

Conventional full-scale and pilot-scale treatment systems

Görvälnverket is one of three DWTPs operating in the Stockholm region of Sweden. This facility draws untreated raw water from Lake Mälaren and services almost 700,000 consumers in several regional municipalities. Lake Mälaren is the third largest freshwater lake in Sweden and also receives effluent from several wastewater treatment plants located upstream of the DWTP [20]. Görvälnverket was built in 1929 and the facility has undergone several upgrades over the years. To meet the increasing demand for drinking water from the growing municipalities, additional upgrades are currently being considered for the DWTP.

Raw water entering Görvälnverket undergoes several conventional treatment processes consisting of: micro-sieving followed by coagulation treatment using aluminum sulfate, flocculation and sedimentation/flotation, rapid sand filtration, biologically activated carbon (BAC) filtration, UV disinfection, and lastly dosing with monochloramine (NH_2Cl) for secondary disinfection and lime for alkalinization and pH adjustment (Table 1, Fig. 1). The BAC filters have a running time of approximately 10–15 years and a short empty bed contact time (EBCT) of approximately 4–6 min.

A pilot-scale water treatment system consisting of ozonation pre-treatment and GAC columns (Table 1, Fig. 1) was installed at Görvälnverket in May 2018 to evaluate the efficacy of these two methods in removing, e.g., OMP and DOC removal. This pilot-scale system receives incoming water treated with coagulation, sedimentation, and sand filtration from the full-scale treatment system at a flow rate of 610–720 L/h. In this study, two pilot-scale processes were investigated: (1) pilot-scale A wherein the incoming primary-treated water undergoes ozonation then GAC filtration; and (2) pilot-scale B wherein the incoming primary-treated water undergoes GAC filtration without pre-ozonation. For pilot-scale A, feed water was pH-adjusted to 6.5 prior to ozonation (target residual of 1 mg O_3/L after 4.2–4.9 min reaction) while the pilot-scale B GAC column received water at ambient coagulation pH (6.5–6.8). The GAC column in pilot-scale B is used to: (a) directly assess differences in GAC efficacy with and without pre-ozonation; and (b) to investigate the gradual saturation of a GAC filter with longer EBCT and compare it to the full-scale short EBCT BAC treatment. The EBCTs for pilot-scale A and B were 20 min.

Table 1 Water sample identifications (IDs) and description of treatment and sampling locations at Görvälnverket DWTP

Sample ID	Sampling point	Treatment/location description
Full-scale treatment process		
RW	Incoming raw water	Incoming raw water to Görvälnverket from lake Mälaren (Görväln basin) at 4 m or 22 m depth, after micro sieve, ambient pH
SF	After coagulation and sand filtration	After coagulation/flocculation, sedimentation (or flotation) and sand filtration, pH ≈ 6.5–6.8
BAC	After BAC filter	After the oldest full-scale filter (approx. 13 years, EBCT 5–6 min), Norit® 830 W, pH ≈ 6.5–6.8
UV	After UV	After UV disinfection (400 J/m^2), pH ≈ 6.5–6.8
DW	After NH_2Cl—finished drinking water	After the addition of monochloramine (0.2–0.38 mg excess chlorine/L) and lime, pH ≈ 8–8.3
TAP-1	Tap water location #1	From a faucet at a location approximately 6 km in the distribution network from Görvälnverket
TAP-2	Tap water location #2	From a faucet at a location approximately 50 km in the distribution network from Görvälnverket
Pilot-scale treatment process A		
O_3	After ozone treatment	After approximately 20 min of ozonation of sand filtrate water from full-scale, pH ≈ 6.5
GAC-A	After GAC-A	Ozonated sand filtrate feed water, after treatment through a Norit® 1240 W GAC filter column, EBCT 20 min, pH ≈ 6.5
Pilot-scale treatment process B		
GAC-B	After GAC-B	Sand filtrate feed water, no ozonation, after treatment through a Norit® 1240 W GAC filter column, EBCT 20 min, pH ≈ 6.5–6.8

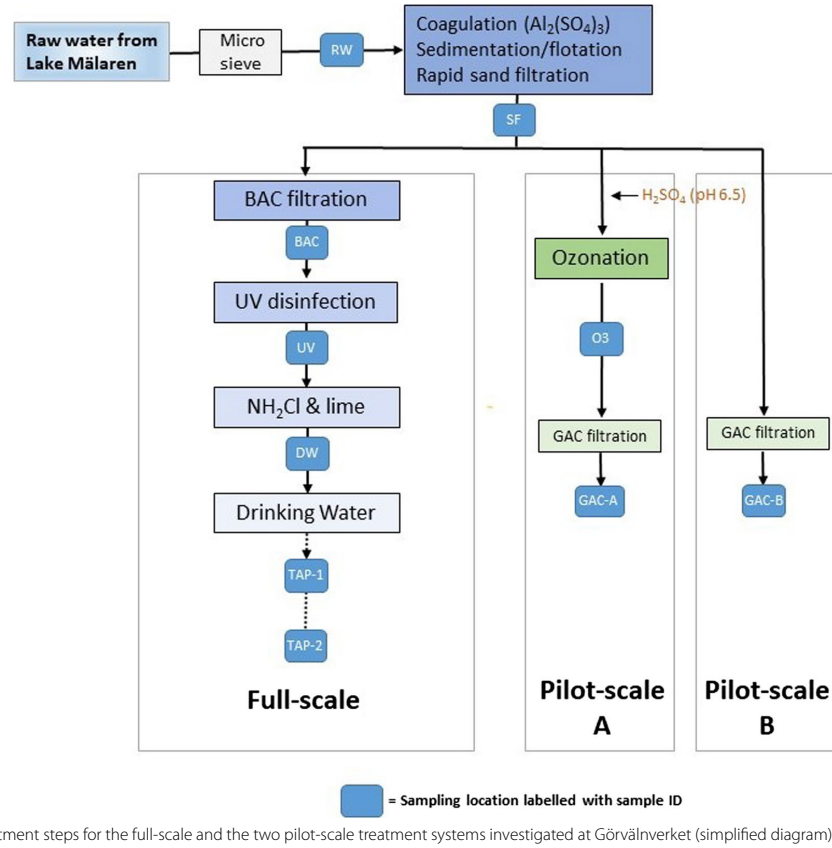

Fig. 1 Treatment steps for the full-scale and the two pilot-scale treatment systems investigated at Görvälnverket (simplified diagram). The sampling points for all sampling events are indicated with symbols

Sample collection

Water samples were initially collected at the Görvälnverket DWTP in November 2019. Grab samples (approximately 5 L) of water were collected from multiple points in both the full-scale and pilot-scale treatment systems (Table 1, Fig. 1). The water samples were collected in 12-L stainless steel (Sharpsville) containers and transported immediately back to the laboratory where they were stored at −20 °C until sample preparation. Procedural controls included ultrapure water (Milli-Q®) sourced from the laboratory.

Based on the results from the November 2019 samples, a follow-up sampling event was carried out in May 2020. The May 2020 sampling event targeted only the full-scale treatment system and one sample from the pilot-scale B system (the pilot-scale A system was no longer operational). An additional follow-up sampling event was conducted in September 2020 to determine if the bioactivities observed in the full-scale DWTP were persisting along the distribution network. As such, the September event focused only on the DWTP outlet as well as two consumer tap water sampling points along the distribution network, located approximately 6 km and 50 km from Görvälnverket. All repeat samples were collected from the same locations at Görvälnverket in all sampling events, then prepared and stored in the same manner.

Sample preparation

The extraction of the water samples (5 L) was conducted with an automatic solid-phase extraction system (SPEDEX 4790, Horizon Technology, Salem, NH, USA) using HLB extraction disks (Atlantic HLB-H Disks, diameter 47 mm; Horizon Technology, Salem, NH, USA).

Additional information regarding the sample preparations is provided in the Additional file 1: Section S1.

When incubated with the cells, the concentrated water samples were diluted 100-fold with cell medium to get a final plate concentration of 1% ethanol to obtain a relative enrichment factor (REF) of 50 in the bioassays: 5000 (enrichment factor at SPE) × 0.01 (dilution factor at bioassay). REF > 1 denotes an enriched water sample and REF < 1 denotes a diluted water sample. The enrichment and dilution of the samples constitute the REF, as described by Escher et al. [19].

Bioassays

A summary of the bioassays is provided in Table 2 and more detailed descriptions of the bioanalytical methods and positive controls are provided in the Additional file 1: Section S1. The concentrated water samples along with procedural, vehicle, and positive controls were tested for Nrf2, AhR, ER, and AR agonist and antagonist activities in reporter gene assays. Genotoxicity was assessed using the in vitro mammalian cell micronucleus test (MCN) and analyzed with a MicroFlow Kit (Litron Laboratories, USA). Cytotoxicity was initially tested in all cell lines and defined as a cell viability of 0.8 compared to the vehicle control, set at 1. For the MCN test, cytotoxicity was also assessed following the manufacturer's kit protocol (fourfold EMA-positive event increase over vehicle control). The main purpose of the cell viability testing was to ensure that the bioanalytical assessment of specific parameters was performed under non-cytotoxic conditions. Each sample was analyzed at the highest non-cytotoxic REF value.

As an initial screening, all water samples were analyzed for bioactivity at REF 50 in all bioassays. Each bioassay was conducted at least two times to prove biological reproducibility. This study presents the results from one of the representative experimental runs for each endpoint. In all assays except the MCN, samples showing bioactivity above the respective cut-off levels at REF 50 were then analyzed again in dilution series from REF 50 to REF 1.56 (dilution factor = 2). As it has been suggested that activation of the Nrf2 pathway can also indirectly result in genotoxic effects [21, 22], the MCN test was performed on samples that displayed Nrf2 activity above the cut-off. In all experimental runs, four technical replicates for each sample were tested. In each reporter gene assay, reference compounds (positive controls) were analyzed in parallel with the water samples. The positive controls were analyzed in 6–12 concentrations to obtain standard curves. The compounds used in each assay are listed in Table 2 and described further in the Additional file 1: Section S1.

Data evaluation

Bioactivities observed in the initial screening at REF 50 were expressed as fold change normalized to the vehicle controls, set to 1 in all assays except the MCN. In the MCN assay, the genotoxicity of the water samples was assessed by comparing the micronuclei formation rates (%) to that in the vehicle control. For Nrf2, where no maximum effect can be reached, the standard curve for the reference compound was based on a linear regression of activities normalized to the mean activity of the vehicle control. For AhR, AR, and ER, the standard curves for the reference compounds were obtained by fitting data to a four-parameter sigmoidal curve fit.

For the dilution series, concentration–effect curves (CECs) were obtained from the dilution series for those samples that showed bioactivity above the respective cut-off levels at REF 50. CECs were fit to a four-parameter

Table 2 Summary of the applied bioanalytical methods

Biological effect	Cellular endpoint	Cell line	Reference compound/positive control	LOD (fold change)	Cut-off (fold change)
Adaptive stress response	Oxidative stress response (Nrf2 activity)	MCF7AREc32	tBHQ (0.78–50 µM)	1.31–1.40	1.5
Reactivity	Genotoxicity (micronuclei formation)	TK6	Mitomycin C (100 nM)	–	*
Xenobiotic metabolism	Aryl hydrocarbon receptor activation	DR-EcoScreen	TCDD (0.5–1000 pM)	1.12–1.50	1.5
Modulation of hormone systems	Estrogen receptor agonism	VM7Luc4E2	17β-estradiol (0.358–367.1 pM)	1.56–1.70	2.0
	Androgen receptor agonism	AR-EcoScreen GR KO M1	DHT (0.001–1000 nM)	1.20–1.50	1.5
	Androgen receptor antagonism	AR-EcoScreen GR KO M1	OHF (0.01–10,000 nM)	0.76–0.88	0.70

*Genotoxicity was determined as statistical significance of micronuclei formations compared to that in the vehicle control ($p < 0.05$)

sigmoidal curve fit for the reference compounds. For all data sets, the mean activity of the vehicle control was first subtracted from all replicates. All adjusted values were then normalized first to the mean activity of the vehicle control, then to the percent of maximum activity of the reference compound. For AhR and ER, linear regression was performed on the normalized data with *y*-axis intercept fixed at zero and the estimated slope from the regression was used to determine the concentration causing 10% effect (EC10) expressed as REFs, as proposed by Escher et al. [23]. As there is no clear maximum response for Nrf2 activity, fold inductions were normalized to that of the vehicle control then fitted to a linear regression model. The concentration, expressed as REF, causing a 1.5-fold induction ($EC_{IR1.5}$) was estimated from the model. The EC values for all samples are provided in the Additional file 1: Table S2. All statistical analyses as well as graphical presentations were performed using GraphPad Prism (version 8.3.0).

For Nrf2 activity, a fold induction of 1.5 compared to the normalized vehicle control was used as the cut-off level for bioactivity, as recommended by Escher et al. [21]. For AhR, AR, and ER, cut-off levels for bioactivity were based on the limit of detection (LOD) for that assay, which was defined as 1 plus 3 times the standard deviation (SD) of the normalized vehicle control [21]. A cut-off level for a positive response was then set for each assay as a value exceeding the LOD value. In instances when the LOD was below 1.5, the cut-off level was set to 1.5, and if the LOD was between 1.5 and 2, the cut-off level was set at 2. For AR antagonist activity, the LOD was calculated as 1 minus 3 times the SD of the normalized vehicle control, and a cut-off level of 0.7 was set. For genotoxicity, statistical significance from the vehicle control was assessed in place of a cut-off level, following the guidance in the OECD's TG487 acceptability [24]. A summary of the bioassays and concentration ranges of the reference compounds is provided in Table 2.

For the Nrf2, AhR, AR, and ER assays, $EC_{IR1.5}$ and EC_{10} values were used to convert the bioactivities measured in the samples into biological equivalent concentrations (BEQs) of the respective positive controls using the following Eq. (1) adapted from Jia et al. [25]:

$$BEQ_{bio} = \frac{(EC_{10} \text{ or } EC_{IR1.5})_{positive\ control}}{(EC_{10} \text{ or } EC_{IR1.5})_{sample}}. \quad (1)$$

To account for differences in sensitivity in the experimental runs between November, May, and September, the BEQ values were calculated to enable comparisons of the data sets between the three sampling events. Based on the BEQ values, the removal efficacies could then be compared to each other.

For the MCN assay, the results in each sampling event were compared to the vehicle control using a one-way ANOVA comparison followed by Dunnett post hoc test. Genotoxicity was defined as a statistically significant increase in the number of micronuclei (% micronuclei events) compared to the vehicle control. *P*-values less than 0.05 were considered statistically significant. To determine if the results could be compared between the three sampling events, a multiple comparison test of the positive control (Mitomycin C) data was first conducted. No significant differences between the mean % micronuclei events across all three sampling events were detected (Additional file 1: Fig. S7). The statistical analysis and graphical presentations were performed using GraphPad Prism (version 8.3.0).

Results and discussion

Cell viability

All samples were initially tested for cytotoxicity at REF 50 in all assays. In the Nrf2, AhR, ER, and AR assays, none of the water samples exerted cytotoxicity, defined as cell viability below 0.8 compared to vehicle control (Additional file 1: Fig. S1). This demonstrated that the bioassays were conducted under conditions where the cell viability was not compromised. For the MCN assay, no cytotoxicity as defined by the manufacturer's kit protocol (fourfold EMA-positive event increase over vehicle control) was observed in any of the samples at the highest REF value of 50 (Additional file 1: Fig. S8).

Initial screening of pilot- and full-scale samples

In the samples from November 2019, we observed Nrf2, AhR, and ER activities above cut-off levels at REF 50 and statistically significant genotoxic activities in the incoming raw water (Fig. 2A, D, G; Fig. 3A). Further, the Nrf2, AhR and genotoxic activities increased after the initial coagulation and sand filtration step. In general, the activities were not decreased to below cut-off levels by the coagulation and SF, BAC or UV treatments. The finished drinking water after dosing with monochloramine, on the other hand, was inactive in all four assays. In the pilot-scale systems, both ozonation and GAC filtration considerably reduced the observed bioactivities to below the respective cut-off levels across all assays.

In the follow-up sampling in May 2020, the Nrf2 activities at REF 50 in the incoming raw water were lower than in November. Again, none of the treatment steps in the full-scale system reduced the bioactivities, except the reduction of genotoxic activity after monochloramine dosing. The pilot-scale GAC-B system, on the other hand, reduced all the activities to or below the cut-offs (Fig. 2B, E, H; Fig. 3B). The highest reduction following the GAC treatment was a 2.3-fold decrease in AhR activity. This

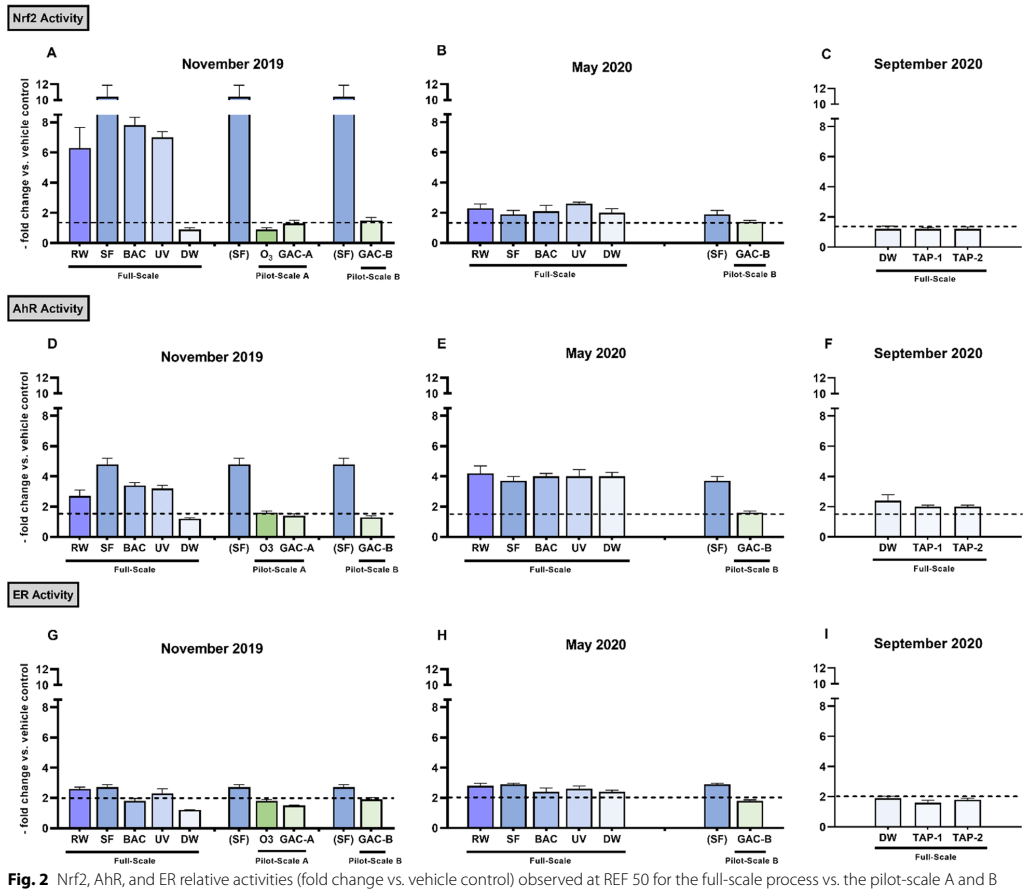

Fig. 2 Nrf2, AhR, and ER relative activities (fold change vs. vehicle control) observed at REF 50 for the full-scale process vs. the pilot-scale A and B treatment processes in November 2019 (**A**, **D**, **G**), in May 2020 (**B**, **E**, **H**), and in September 2020 (**C**, **F**, **I**). Treatment groups ($n = 4$) were normalized to the vehicle control ($n = 8$) set to 1. The dotted lines represent the respective cut-off levels. Data presented as mean ± standard deviation. For comparison's sake, the bioactivities for SF have been repeated for all treatment processes in panels **A**, **B**, **D**, **E**, **G**, and **H**

indicates that, even after being in operation for nearly 2 years, adsorption properties in this filter still remain, as the filter performed better when compared to the full-scale BAC. Alternatively, there may be beneficial effects from the longer EBCT in the pilot filter.

In September 2020, an additional sampling event was carried out to specifically study the outgoing drinking water and two tap locations in the distribution network. AhR activity at REF 50 above cut-off (Fig. 2F) and genotoxicity (Fig. 3C) were observed. However, genotoxicity was not detected in the two samples from the distribution network indicating that removal or transformation of micropollutants may have occurred during distribution.

As an additional step in the data interpretation, to compare differences in bioactivities between each of the treatment steps, results were statistically evaluated using a one-way ANOVA comparison followed by Dunnett post hoc test, performed in GraphPad Prism (version 8.3.0). A table summarizing the statistical analysis is provided in the Additional file 1: Table S1. Neither androgenic nor anti-androgenic activities were observed in any sample across all sampling events. The results are presented in the Additional file 1: Fig. S9.

In summary, the removal efficiencies of the treatment steps in the full-scale system varied between the sampling events and most treatments showed little or no

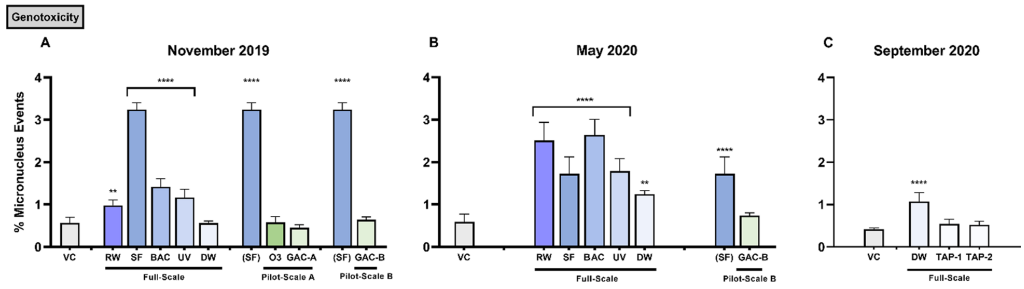

Fig. 3 Micronuclei formation rates at REF 50 from the full-scale process vs. the pilot-scale A and B treatment processes in November 2019 (**A**), in May 2020 (**B**), and in September 2020 (**C**). Treatment groups ($n=4$) were compared to the vehicle control ($n=8$). Data presented as mean ± standard deviation. * represents significant differences from the vehicle control (*$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$, ****$p \leq 0.0001$). For comparison's sake, the micronuclei formation rate for SF has been repeated for all treatment processes in panels **A** and **B**

effect on the bioactivities detected in the incoming water. Both ozone and GAC in the pilot-scale systems, on the other hand, effectively reduced observed Nrf2, AhR, and ER activities to or below the cut-off levels and to no statistically significant inductions compared to the vehicle control for genotoxic activities. In general, ozonation treatment is likely to continuously provide a decrease in observed activities. GAC filtration, particularly without pre-ozonation, will likely require regular regeneration to maintain its removal capabilities. However, our results indicate that for an EBCT of 20 min, regeneration may not be needed until after more than 2 years of running time. As the ozonation appeared to remove all activities to levels below the cut-off in the pilot-scale A system, it would suggest that regeneration of the subsequent GAC in the combined treatment would not be needed. However, a biofilter step would still be needed downstream of ozonation to obtain biostability.

Bioactive samples, defined as above the respective cut-off levels at REF 50, were also analyzed in dilution-series (Additional file 1: Figs. S3 to S5) in order to determine effect concentrations (ECs) and bioequivalent concentrations (BEQs). These results are discussed in further detail below.

Seasonal differences in bioactivities between sampling events

We observed seasonal differences in the bioactivities in the raw water samples between the November and May sampling events. For Nrf2, the activity was noticeably higher (8.4 times) in November than May based on the BEQ values (Table 3); while the AhR activity was higher in May than November, albeit by only 1.8 times. Further, the genotoxicity was higher in the raw water in May than in November (Fig. 3). These inconsistent bioactivities across the sampling events can be attributed to the fact that temporal variation in the quality and micropollutant profile of source water is expected to occur. Such factors have been discussed in other DWTP studies which also reported seasonal/temporal variations for similar endpoints such as Nrf2 activity [26, 27], AhR and androgenic activities [28], and genotoxicity [29–32]. For instance, Hebert et al. [26] measured Nrf2 activities in water samples collected from the outlets of three DWTPs in France across several sampling events (November 2015 and March, May, September 2016). The DWTPS used a combination of clarification, sand filtration, ozonation, GAC filtration, and UV. They reported higher Nrf2 activities in May and September compared to November and March for all three DWTPs, likely due to higher levels of some DBPs in September as a result of warmer temperatures. In our study, the highest Nrf2 activity was detected in November. Based on monitoring data provided by Norrvatten, the temperature of the incoming raw water was slightly higher in November 2019 than in May 2020. Hebert et al. [26] also speculated that the increased effect seen in September may be due to the formation of undetected non-volatile or semi-volatile DBPs or due to other existing micropollutants in the source water. Further, treatment processes could increase effects such as genotoxicity in the water and genotoxic dissolved organic matter might be released or formed during purification processes [32]. Other possible influences may be related to interactions with organic matter [33] and the biostability of the water [34], the presence of natural toxins [35] in the raw water, or the formation of transformation products during a treatment process [36]. In our study, it could thus be hypothesized that the compositions of bioactive compounds present in the samples were different between the sampling events, due to temporal variations

Table 3 Summary of bioequivalent concentrations (BEQs) based on the ECIR1.5 for Nrf2 and EC10 values for the AhR, and ER assays

Assay	RW		SF		BAC		UV		DW			GAC-B	
	Nov-19	May-20	Nov-19	May-20	Nov-19	May-20	Nov-19	May-20	Nov-19	May-20	Sep-20	Nov-19	May-20
Nrf2 activity tBHQEQ (μM)	0.42	0.05	0.86	0.04	0.69	0.05	0.65	0.06	(IA)	0.05	(IA)	(IA)	(IA)
AhR activity TCDDEQ (pM)	0.06	0.11	0.08	0.09	0.07	0.12	0.06	0.10	(IA)	0.08	0.16	(IA)	(IA)
ER activity E2EQ (pM)	0.07	0.07	0.07	0.09	0.03	0.06	0.05	0.07	(IA)	0.06	(IA)	(IA)	(IA)

"(IA)"—inactive. If the bioactivity at the highest REF value in the dilution series was below the cut-off limit for bioactivity, the EC and BEQ values were not calculated

in the incoming source water, and that these different causative compounds are not equally responsive to the different treatment technologies used.

Discussion on pilot-scale treatment technologies

This study highlights the efficacy of ozonation and GAC filtration in removing bioactive and genotoxic compounds compared to the full-scale treatments. For instance, the tBHQEQ was decreased from 0.86 µM (November) and 0.04 µM (May) in the primary-treated (SF) water entering the pilot-scale GAC-B system to below the cut-off for Nrf2 activity following GAC filtration alone in both sampling events. Similarly, the E2EQ in the SF-treated water was reduced to below the cut-off for estrogenic activity from 0.07 pM in November and 0.09 pM in May. For AhR activity, the TCDDEQ in the SF-treated water decreased from 0.08 pM (November) and 0.09 pM (May) to below the cut-off following GAC filtration alone. Similarly, Nrf2, AhR, ER bioactivities and micronuclei formations were all lower at REF 50 in the O_3 and GAC-A treated samples collected in November compared to the incoming water following the initial coagulation and rapid sand filtration step. It is worthwhile to mention that the performance of the pilot-scale systems at removing OMPs was previously tested in a 1-year pilot-scale study between May 2018 and July 2019, alongside the full-scale system [8]. The lowest levels of OMPs were observed in GAC effluents from ozonated feed water demonstrating the efficacy of combining ozone with GAC for managing OMP levels [8]. Similar to the observation made in this study regarding the efficiency of the pilot-scale ozonation treatment, Jia et al. [25] reported that ozone technology was able to significantly remove Nrf2 activity (AREc32) with BEQ reduction values between 60 and 80%. While BEQ values for the pilot-scale ozonation were not determined in this study, the fact that Nrf2 activities at REF50 decreased to below cut-off following ozonation suggests high BEQ reduction. In another study, Shi et al. [37] reported higher overall treatment efficiencies in removing genotoxic, mutagenic, dioxin-like and estrogenic pollutants at DWTPs that used primary treatment methods coupled with ozone-activated carbon similar to Görvalnverket vs. those DWTPs that did not include ozone-activated carbon.

Other studies based on chemical profiling of the water samples have reported the efficacy of ozonation and GAC filtration in removing micropollutants [7] as well as NOM and precursors of DBPs products [7, 38–42]. As well, the efficacy of GAC filtration (from a DBP perspective) has been reported elsewhere [43] and at other Swedish DWTPs [4, 15]. Also, ozonation alone has been shown to be an effective treatment for antibiotics [31] and estrogenic chemicals [44–46]. The estrogenic activities of 17α-ethinylestradiol and bisphenol A, for instance, dramatically decreased following ozonation treatment in a MCF-7 cell proliferation assay [46]. In the ERα CALUX bioassay, the combination of coagulation, sedimentation, sand filtration and chlorination with ozone-activated carbon adsorption was shown to efficiently remove estrogenic potentials from source water better than coagulation, sedimentation, sand filtration and chlorination alone [37]. GAC filtration alone has also been shown to remove more organic compounds, including pharmaceuticals and steroids, than disinfection with sodium hypochlorite or clarification [47].

However, the age of the respective treatment technologies should also be considered when comparing removal performances. In the case of the full-scale BAC vs. the pilot-scale GAC, for instance, it would be worthwhile to compare their effectiveness based on the age range and treated bed volumes of the filter units given that the effectiveness of a filter will likely decrease with time as the filter ages [48]. In an earlier study, Cuthbertson et al. [43] reported that calculated cytotoxicity and genotoxicity were considerably lower following GAC treatment at younger service lives. Further, an increase in GAC run time resulted in an increase of DBPs under simulated distribution system conditions which corresponded to increased calculated cytotoxicity and genotoxicity. The toxicity in their study was determined by the TIC-Tox method. The age and regeneration frequency of a GAC is essential for its function as a chemical barrier. The GAC in the full-scale system has been operating for over 10 years and is currently functioning as a BAC. In comparison, the GAC filters of the pilot-scale systems were installed in May 2018. As such, the dynamics of a GAC filter in its function as either an adsorption filter (GAC) or a biological filter (BAC) can represent two very different removal processes and capabilities. Nevertheless, both ozonation and GAC filtration or GAC filtration alone show great promise as barriers against bioactive compounds in drinking water production [8] and in the overall reduction of DBP formations [43]. Similar observations have been demonstrated elsewhere [15, 49].

Discussion on full-scale treatment technologies

In November, Nrf2, AhR, and genotoxic activities measured in the incoming raw water increased following the initial coagulation and rapid sand filtration steps (Fig. 2; Table 3). The tBHQEQ and TCDDEQ were approximately 2 and 1.3 times higher, respectively, following the conventional coagulation treatment. In the ER assay, the estrogenic activity remained comparable after this initial treatment step. Taken together, the results suggest there was little or no removal effect following this primary

treatment step of the full-scale system. Other studies have reported that chemical precipitation processes using coagulants like aluminum sulfate, which is used at Görvälnverket, result in minimal removal of most endocrine-disrupting compounds (EDCs) [45, 50].

Overall, most of the remaining treatments in the full-scale system did not reduce the bioactivities to below cut-off levels across both sampling events, except for BAC in the ER assay and monochloramine dosing in all assays in November. The lack of treatment effects is also reflected in the relatively low BEQ reduction values between these treatment steps, observed for multiple toxicity endpoints. For Nrf2 activity, the highest BEQ reduction value achieved was 20% (SF in May), while the highest BEQ reduction value for AhR activity was 18.2% (SF in May). Rosenmai et al. [16] had previously conducted a bioanalytical study at Görvälnverket and reported findings similar to our observations in May wherein the coagulation treatment, GAC filtration, UV disinfection, and monochloramine dosing of the full-scale system did not decrease Nrf2, AhR, or ER activities. Also consistent with our results of little treatment effect, Lundqvist et al. [17] reported almost similar activities in the inlet (53 ng/L TCDDEQ) and outlet samples (45–52 ng/L TCDDEQ) collected from a DWTP that employed treatments similar to Görvälnverket (e.g., coagulation, sedimentation, rapid then slow sand filtration, UV, NH_2Cl dosing) in the AhR reporter gene assay. As well, Macova et al. [51] reported no change in activities in the AhR CAFLUX assay between the inlet and outlet samples from a DWTP that used coagulation, flocculation, sedimentation, filtration, and chlorination.

On the other hand, Escher et al. [21] reported higher Nrf2 activities in AREc32 cells in water samples from the outlet (4.16×10^4 ng/L tBHQEQ) compared to the inlet (1.83×10^4 ng/L tBHQEQ) of a DWTP that utilized treatments similar to at Görvälnverket (e.g., coagulation, flocculation, sedimentation, filtration, chlorination). The authors attributed the increase to the formation of DBPs as a result of chlorination.

As well, in a recent bioanalytical study that investigated multiple DWTPs, Oskarsson et al. [15] reported better treatment effects at two Swedish DWTPs that employed treatment trains similar to that at Görvälnverket (e.g., rapid sand filtration, GAC, UV irradiation, chlorination). At those two DWTPs, AhR activities were reduced from 31 and 34 pM TCDDEQ at the inlets to below cut-off at the outlets.

In available literature investigating genotoxicity of finished drinking water samples, conflicting results have been reported. For instance, some studies investigating the genotoxicity in drinking water samples observed decreases in micronuclei inductions following various conventional water treatment methods similar to those employed at Görvälnverket [37, 52]. Several other studies using different established cell lines reported no significant differences in the frequency of micronucleus events between control samples and samples representing conventional treatment methods (e.g., pre-disinfection with chlorine dioxide, coagulation, sand- and GAC-filtration, post-chlorination), therefore suggesting no treatment effects [29, 53–55].

In November, the full-scale BAC filtration and monochloramine secondary disinfection appeared to reduce estrogenic activities, albeit low estrogen activities were detected overall; however, in May there appeared to be little treatment effect. Neale et al. [27] studied two DWTPs that employ similar treatment sequences: (pre-ozonation), clarification, sand filtration, ozonation, GAC, and then UV treatment followed by chlorination. In their study, estrogenic activity was detected in all source water samples as well; however, the treatment processes reduced the activity to below the limit of detection. As discussed above, ozonation has been shown to reduce estrogenic chemicals.

With respect to the September sampling event focused on the finished drinking water and the distribution network, passage through the distribution network appeared to reduce genotoxic activity. Removal of bioactivities in water samples collected from Görvälnverket's distribution network had previously been reported [16]. It has been suggested that decreasing bioactivities along a distribution network could be due to the binding of bioactive compounds to the pipe surface or the biofilm, or the degradation/inactivation of the compounds by microorganisms present in the biofilm [56]. As such, the removal of genotoxic activity in the distribution network in the present study may be explained by interactions between genotoxic compounds and the biofilm.

Regarding the lack of AR activity observed in the current study, similar results were reported by Rosenmai et al. [16] from Görvälnverket and in other countries wherein the activation or inhibition of AR was not commonly detected [27, 28, 57–61]. Further, AR antagonistic activity was detected in drinking water from other DWTPs in Sweden [15]. Still, AR agonistic activity has also been reported in treated water [62].

Conclusions

In this study, we observed oxidative stress, genotoxicity, AhR, and ER agonist activities in raw water used for drinking water production over the course of repeated sampling events. In general, most of the full-scale treatment methods were unable to fully remove the compounds causing these activities. On the other hand, pilot-scale treatment with either ozonation or GAC

filtration was more efficient in removing the compounds causing oxidative stress, genotoxicity, AhR and ER agonist induction. Both treatment methods appeared to perform better than the conventional full-scale treatment methods utilized at this subject DWTP.

The variability in treatment effects in the full-scale system observed in this study, which has also been reported in other studies on similar treatment technologies, suggests that the treatment process as well as the characteristics of the source water are important factors when assessing the toxic potential of treatment-processed drinking water. Another important consideration is the seasonal variation in the quality of the raw water in that the physico-chemical profile of the incoming water (e.g., temperature, organic matter, micropollutant profile) would differ between sampling events which could be linked to different reactivities during the treatment processes.

Using a panel of cell-based bioassays, this study highlighted that ozonation and GAC filtration methods are effective at reducing bioactivities. The study also demonstrated the usefulness of conducting a pilot-scale assessment combined with bioanalytical methods as a valuable approach to test water treatment techniques before full-scale implementation. As such, the conclusions made in this study regarding both the pilot-scale and full-scale drinking water treatment methods provide important insights into the optimization of existing drinking water treatment designs and support the need for further research into the removal of micropollutants in drinking water. The findings of this study thus suggest that micropollutant removal appears to be dependent on the treatment type, season, and quality of the source water.

Further work with this study could include: chemical analyses and an effect-based analysis (EDA) of the water samples to identify the causative compounds of the observed biological effects; composite sampling over a longer time period in a day; and cost–benefit analysis of the pilot-scale systems. The results of this study will hopefully contribute to the growing body of research supporting more efficient implementation of bioanalytical tools into operational practices at DWTPs, water safety planning, and incorporation into more comprehensive regulatory frameworks for water quality monitoring. Lastly, while the focus of this study was on drinking water treatments, it would be remiss to not also mention environmental implications. Given that source waters of DWTPs are often impacted by human activities, it can be hypothesized that the effects observed in this study are likely attributed to environmental pollutants. Implications such as this draw evidence-based attention towards the need to improve the management and protection of our water resources.

Abbreviations

AhR: Aryl hydrocarbon receptor; AR: Androgen receptor; BAC: Biologically activated carbon; BEQ: Biological equivalent concentration; CEC: Concentration effect curve; DBP: Disinfection by-product; DWTP: Drinking water treatment plant; EBCT: Empty bed contact time; EC: Effect concentration; ER: Estrogen receptor; GAC: Granular activated carbon; HAAs: Haloacetic acids; LOD: Limit of detection; MCN: Micronucleus; NOM: Natural organic matter; O_3: Ozonation; OMP: Organic micropollutant; REF: Relative enrichment factor; SD: Standard deviation; SPE: Solid-phase extraction; THMs: Trihalo-methanes; UV: Ultraviolet.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12302-021-00567-y.

Additional file 1: Section S1. Detailed information on sample preparation. **Section S2.** Detailed information on bioassays and analysis. **Table S1.** ANOVA and post hoc Dunnett comparisons of mean bioactivities measured at REF50 in each treatment step in the Nrf2, AhR, and ER assays. **Table S2.** ECIR1.5 (Nrf2) and EC10 (AhR, ER) values of the water samples expressed as REF. **Figure S1.** Cell viabilities for MCF7AREc32, DR EcoScreen, and AR EcoScreen GR-KO were analyzed by MTS activity and by ATPase activity for VM7Luc4E2. **Figure S2.** Standard curves for the positive controls in the Nrf2 (tBHQ), AhR (TCDD), and ER (E2) assays from the initial screening assays tested at REF50 for each sampling event. **Figure S3.** Concentration–effect curves for Nrf2-active samples in November 2019, May 2020, and September 2020. **Figure S4.** Concentration–effect curves for AhR-active samples in November 2019, May 2020, and September 2020. **Figure S5.** Concentration–effect curves for ER-active samples in November 2019, May 2020, and September 2020. **Figure S6.** Standard curves for the positive controls in the Nrf2 (tBHQ), AhR (TCDD), and ER agonist (E2) assays in the dilutions series for each sampling event. **Figure S7.** Comparison of micronuclei formations from the positive control at REF 50 across all three sampling events. **Figure S8.** Cytotoxicity for select samples ($n=4$) from the full- and pilot-scale treatment systems analyzed at REF 50 in November 2019, May 2020, and September compared to the vehicle control ($n=8$). **Figure S9.** AR agonist and antagonist activities (fold change vs. vehicle control) observed at REF 50 for the full-scale process vs. the pilot-scale A and B treatment processes in November 2019), in May 2020, and in September 2020. **Figure S10.** Standard curves for the positive controls in the AR agonist (DHT) and antagonist (OHF) assays from the initial screenings at REF50 for each sampling event.

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Authors' contributions

JL and MY planned the study project. JL coordinated the collaborations. MY conducted the sampling, experiments, and data handling. JL, AO, and EL supervised the findings of the study. MY wrote the manuscript with input from all authors. JL, AO, and EL provided critical feedback and helped shape the manuscript. All authors read and approved the final manuscript.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Declarations

Ethics approval and consent to participate
Not applicable.

Consent for publication
Not applicable.

Competing interests
The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: J.L. and A.O. are the founders and owners of BioCell Analytica Uppsala AB, a company providing effect-based testing services to the water sector.

Author details
[1]Department of Biomedical Sciences and Veterinary Public Health, Swedish University of Agricultural Sciences, Box 7028, 750 07 Uppsala, Sweden. [2]Veolia Water Technologies, Vretenvägen 9, 17154 Solna, Sweden. [3]Norrvatten, Quality and Development, 16902 Solna, Sweden.

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Artificial infiltration in drinking water production: Addressing chemical hazards using effect-based methods

Maria Yu [a,*], Shreya Mapuskar [a], Elin Lavonen [b], Agneta Oskarsson [a,b], Philip McCleaf [c], Johan Lundqvist [a,b]

[a] Department of Biomedical Sciences and Veterinary Public Health, Swedish University of Agricultural Sciences, Box 7028, Uppsala 750 07, Sweden
[b] BioCell Analytica, Ulls väg 29C, Uppsala 756 51, Sweden
[c] Uppsala Vatten och Avfall AB, Box 1444, Uppsala 751 44, Sweden

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ABSTRACT

Artificial infiltration is an established managed aquifer recharge method that is commonly incorporated into drinking water processes. However, groundwater sourced from this type of purification method is prone to contamination with chemical hazards. Such an instance was previously shown at a Swedish DWTP where the river water was contaminated by hazardous chemicals during artificial infiltration. Further, there remains a paucity of research studying the quality of drinking water following this type of treatment from an effect-based bioanalytical perspective. In the current study, an effect-based assessment for chemical hazards was conducted for a Swedish drinking water system comprised of two DWTPs fed artificially-infiltrated river water. In this system, artificial infiltration of the river water takes approximately six to eight months. A sampling event was conducted in the autumn season and the samples were enriched by solid phase extraction. A panel of cell-based reporter gene assays representing several toxicity pathways was selected: oxidative stress response (Nrf2 activity), aryl hydrocarbon receptor (AhR) activation, and hormone receptor-mediated effects (estrogen receptor [ER], androgen receptor [AR]). AhR and ER bioactivities were detected in samples collected from the river intake and in the open-air infiltration basins prior to artificial infiltration. However, the AhR activity decreased and ER activity was effectively removed following artificial infiltration. In the Nrf2 and AR assays, no bioactivities above cut-off levels were detected in any samples collected along the entire treatment process of the drinking water production from source to tap. Using a suite of bioassays, the current study highlighted the effectiveness of artificial infiltration in reducing bioactive compounds in this raw river water. Although artificial infiltration is a common purification method in drinking water production, the limited number of effect-based studies evaluating the effectiveness of this method emphasizes the need for further research to better understand the risks and benefits of this water treatment process.

1. Introduction

Globally, groundwater is commonly used as a freshwater supply for drinking purposes. To sustainably manage this resource, groundwater can be replenished through a process of managed aquifer recharge (MAR) (Balke and Zhu, 2008) wherein the aquifer is artificially recharged with surface water (US National Research Council, 1994). This can be done by various methods such as via infiltration basins, irrigation pits, redirection of the surface water across land surfaces, or via injection wells into the subsurface. In Europe alone, more than 200 different MAR schemes, specifically riverbank filtration, are used in the production of drinking water (Sprenger et al., 2017). In Sweden, for instance, approximately 25% of the public drinking water is sourced from surface waters via artificial infiltration, a method of MAR (Svenskt Vatten, 2021).

However, artificially recharged groundwater can become contaminated by many of the same pollutants that enter surface waters including toxic metals, pesticides, industrial chemicals, microorganisms, natural toxins, and a variety of micropollutants (MPs) via diffuse (non-point) sources (Albergamo et al., 2019; Böhlke, 2002; Díaz-Cruz and Barceló, 2008; Maeng et al., 2011; Sasakova et al., 2018). In a previous study using *in vitro* bioassays, we detected a contamination scenario in the

* Corresponding author.
E-mail address: maria.yu@slu.se (M. Yu).

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artificially infiltrated source water of a Swedish drinking water treatment plant (DWTP) (Oskarsson et al., 2021). In that study, high oxidative-stress activity and anti-androgenic effects were detected in the outlet of the DWTP but not in the raw river water samples collected. Further, a chemical analysis of the samples revealed that the detected organic MPs did not contribute to the observed effects. The results of that study highlighted that further effect-based research into the artificial infiltration process and the associated risks due to chemical contamination is clearly needed.

Effect-based methods using *in vitro* bioassays provide useful information on the total effect and moreover, the toxic potential of a sample for a specific toxicity pathway, integrating both known and unknown chemicals as well as mixture effects (Brack et al., 2019; Escher et al., 2020). The application of such methods in the assessment of drinking water production is not new and has been used in hazard identification as well as in assessing the efficacies of drinking water treatment technologies. However, very few studies have investigated the artificial infiltration process in drinking water production using such a bioanalytical approach.

The current study thus aimed to perform an effect-based assessment of chemical hazards of another Swedish drinking water system comprised of two DWTPs fed artificially-infiltrated groundwater. A panel of *in vitro* reporter gene assays was used representing several common toxicity pathways relevant to human health, such as oxidative stress (Nrf2 activation), aryl hydrocarbon receptor (AhR) activation, and hormone-mediated effects. The selection of bioassays followed what is generally recommended to be comprehensive of effects commonly detected in water samples (Escher et al., 2021). This study also monitored the bioactivities across all subsequent treatment steps in two conventional DWTPs fed the infiltrated groundwater as well as in their respective distribution networks.

2. Materials and methods

2.1. Drinking water production in Uppsala, Sweden

In Uppsala, the drinking water supply is sourced primarily from groundwater extracted from the Uppsala esker. To compensate for water abstraction, a managed aquifer recharge system has been utilized since 1966 to infiltrate surface water from the Fyris River (and additionally from Lake Tämnaren during the summer months). At the source water intake, the raw river first undergoes rapid sand filtration and then is pumped uphill to multiple infiltration basins situated north of the Greater Uppsala area in a nature area (referred to as Tunåsen). The water from the basins percolates into the subsurface and mixes with the naturally formed groundwater as it flows through the aquifer. It takes approximately six to eight months for the infiltrated water to travel to four wellfields that supply two DWTPs (Gränby and Bäcklösa). The groundwater abstracted at the respective four wellfields vary in the proportion of infiltrated water from 15–20%, 40–45%, 45–50%, to 80–90%. At both DWTPs the incoming infiltrated water undergoes similar treatments including: aeration, hardness removal (pellet reactors), sand filtration, and then disinfection via chlorination (with sodium hypochlorite). However, ten granular activated carbon (GAC) filters are also installed at the Bäcklösa DWTP between the sand filtration and chlorination treatment steps. The finished drinking water is stored in underground reservoirs at the DWTPs before entering the distribution network which consists of two municipal water towers and 440 km of pipelines that serve residential, commercial, and industrial water users. An average of 48,300 m^3 of finished drinking water per day was distributed from these two DWTPs in 2021 to serve approximately 190,000 consumers in the city of Uppsala. A more detailed explanation of the drinking water production process is provided in the Supplementary Information (S1).

2.2. Sample collection and preparation

Water samples were collected in late September and early October 2020. Grab samples (2 L) were collected from 22 sampling sites representing the full treatment cycle of the drinking water production process from source to tap (Table 1, Fig. 1). The water samples were collected in two 1-L sterile PET bottles (VWR® collection) and transported immediately to the laboratory where they were stored at -20 °C until sample preparation within 45 days. This specific type of bottle has previously been demonstrated not to contaminate water samples with any activity in the assays assessed in this study (Lundqvist et al., 2021). Procedural controls of ultrapure water (Milli-Q®) sourced from the laboratory were also included.

The samples collected from the three basins sampled at Tunåsen were first filtered using 0.45 μm PES filters under vacuum due to their turbid nature and the presence of visible debris (e.g., dead vegetation). All water samples (2 L) were extracted via solid-phase extraction (SPE) using a SPE-03 8-Channel Automated SPE System (PromoChrom Technologies) and 6-mL HLB cartridges (6cc Oasis Prime HLB cartridge, sorbent weight 200 mg, Waters Corporation). The sample extraction process consisted of: preconditioning with ethanol, loading of water volume, extraction with ethanol, followed by rinsing and evaporation. All samples were re-suspended with ethanol to obtain a final extract volume of 0.4 mL. Each water sample was thus enriched by a factor of 5000. Additional information regarding the sample preparations is provided in the supplementary information (S2.1).

2.3. Bioassays

The concentrated water samples along with procedural (Milli-Q®) controls, vehicle negative (1% ethanol) controls, positive controls, and reference compounds were tested in luciferase reporter gene assays. The assays were selected based on their relevance to effects commonly detected in drinking water extracts and representation of different cellular toxicity pathways relevant to human health. The following endpoints were thus assessed: oxidative stress response (Nrf2 activation), aryl hydrocarbon receptor (AhR) activation, estrogen receptor (ER) activation, androgen receptor (AR) activation and inhibition. Cytotoxicity was initially assessed in all cell lines with cell viability assays (MTS for all assays except ER activity, where the ATP assay was used). The main purpose of the cell viability testing was to ensure that the bioanalytical assessment of specific parameters was performed

Table 1
Description of sampling locations and sample IDs.

Sample ID	Treatment/Location Description
FS	Fyris River pump station before infiltration
T-FW	Tunåsen pre-infiltration water
B1	Tunåsen basin 1
B2	Tunåsen basin 2
B3	Tunåsen basin 3
GWF	Galgbacken wellfield
G-IW	Gränby - incoming water from Galgbacken wellfield
G-A	Gränby - after aeration
G-SRL	Gränby - after softening reactor line 1
G-SF	Gränby - after sand filters 1 to 3 (of 6)
G-C	Gränby - before chlorination (composite sample of all 6 sand filters)
G-OW	Gränby - outgoing water
G-TAP	Gränby - tap water location approximately 2.6 km from DWTP
B-IW1	Bäcklösa - incoming water from Sunnersta wellfield
B-IW2	Bäcklösa - incoming water from Stadsträdgården wellfield
B-A	Bäcklösa - after aeration
B-SRL	Bäcklösa - after softening reactor line 1
B-SF	Bäcklösa - after sandfilter 1-3
B-CF	Bäcklösa - after active carbon filters
B-C	Bäcklösa - before chlorination
B-OW	Bäcklösa - outgoing water
B-TAP	Bäcklösa - tap water location approximately 2.4 km from DWTP

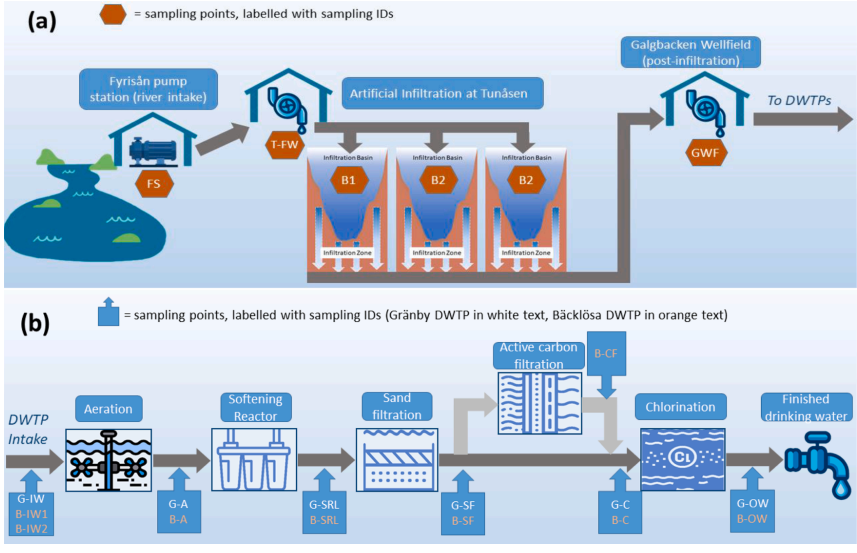

Fig. 1. Simplified diagram of Uppsala Vatten's artificial infiltration pre-treatment process from the raw water source to one of the wellfields (a) prior to downstream water purification in the two DWTPs (b). Note that activated carbon filtration treatment is utilized at Bäcklösa (sampling IDs denoted with "B"), but not at Gränby (sampling IDs denoted with "G"). Refer to Table 1 for sampling location descriptions.

under non-cytotoxic conditions. Cell viability of < 0.80 of the vehicle control was defined as cytotoxic.

The concentrations of the tested samples were expressed in units of relative enrichment factor (REF). When incubated with the cells, the 5000-fold enriched samples and controls were diluted 100-fold with cell medium to attain a final well concentration of 1% ethanol and a REF of 50 (as well as 200 for some samples) in all bioassays. The enrichment and dilution of the samples together constitute the REF (Escher et al., 2014). A REF of 1 is interpreted as the unconcentrated native sample while a REF of 50, for instance, indicates that the sample was enriched 50 times in the bioassay.

Detailed descriptions of the bioanalytical methods are provided in the supplementary information (Sections S2.2 to 2.5). For all bioassays, the concentrated water samples and controls were analyzed in quadruplicate. All bioassays were repeated at least once to prove biological reproducibility. In brief, all activity experiments were conducted in white-walled 384-well cell culture plates with transparent bottoms (Corning Incorporated). Cells were seeded in the plates and incubated for 24 h. The cells were then exposed to the concentrated water samples for another 24 h. On the third day, bioactivity (i.e., luminescence) was measured on a TECAN Spark® Multimode Microplate Reader using the Luciferase® Reporter Assay System (Promega), according to the manufacturer's instructions. Vehicle controls and a dilution series of reference compounds were included on every experimental plate for each assay. For the ER assay, a weak positive control (p,p'-methoxychlor) was also included. A summary of the bioassays and concentration ranges of the reference compounds are provided in Table 2.

Table 2
Summary of the applied bioassays.

Target Cellular Endpoint	Cell Line	Reference Compound & Conc. range
Oxidative stress response (Nrf2 activity)	MCF7AREc32	tBHQ (0.78-50 μM)
Aryl hydrocarbon receptor activation	DR-EcoScreen	TCDD (0.5-1000 pM)
Estrogen receptor agonism	VM7Luc4E2	17ß-estradiol (E2) (0.36- 370 pM)
Androgen receptor agonism	AR-EcoScreen GR KO M1	DHT (0.001-1000 nM)
Androgen receptor antagonism	AR-EcoScreen GR KO M1	OHF (0.01-10000 nM)

2.4. Data evaluation

All concentrated water samples were initially analyzed for bioactivity at a concentration of relative enrichment factor (REF) 50 in all bioassays. Bioactivities in each sample were expressed as the mean fold change normalized to the mean fold change in the vehicle controls, set to 1. For Nrf2 activity, where no maximum effect can be reached, the standard curve for the reference compound was based on a linear regression of activities normalized to the mean activity of the vehicle control. For AhR, AR, and ER, the standard curves for the reference compounds were obtained by fitting data (x-axes were log-transformed) to a four-parameter sigmoidal curve.

Cut-off levels for a positive response in bioactivity were determined as follows: for Nrf2, a fold ratio of 1.5 compared to the normalized vehicle control was used as the cut-off level for bioactivity, as recommended by Escher et al. (2012). For AhR, AR, and ER, cut-off levels for bioactivity were based on the limit of detection (LOD) for that assay, which was defined as 1 plus 3 times the standard deviation (SD) of the normalized vehicle control. A cut-off level for a positive response was then set for each assay as a value exceeding the LOD value. In instances when the LOD was below 1.5, the cut-off level was set to 1.5, and if the LOD was between 1.5 and 2, the cut-off level was set at 2. For AR antagonist activity, the LOD was calculated as 1 min 3 times the SD of the normalized vehicle control, and a cut-off level of 0.7 was set. For some samples, differences in bioactivities at REF50 were statistically evaluated using a one-way ANOVA followed by a Šidák's multiple comparisons test, performed in GraphPad Prism (v. 9.3.1). Statistical

significance was defined as $p < 0.05$.

Samples collected from the inlets, outlets, and distribution networks of Gränby and Bäcklösa were tested further in dilutions series up to REF 200 to calculate bioanalytical equivalent (BEQ) concentrations. In one of the dilution series, a Mann-Whitney test was performed to circumvent a lack of sample volume. Further details of this instance are provided in Section 3.2.2. Mean activities were normalized first to the vehicle control then to the assay maximum, defined as the highest concentration of the reference compound of the respective assay. The normalized data were then fit to four-parameter sigmoidal curves to generate concentration-effect curves (CECs) and analyzed via non-linear regression. The concentrations causing a 10% effect (EC10), expressed as REF, were then interpolated from the curves. The EC values were further translated into BEQ concentrations in units of ng/L or μg/L, using the EC_{10} values of the sample ($EC_{10,\ sample}$) and the reference compounds ($EC_{10,\ ref}$) of the particular assay using Equation (a). A more detailed explanation of the selection of the bioassays and samples for the dilutions series is provided in Section 3.2.2. All statistical analyses as well as graphical presentations were performed using GraphPad Prism (v. 9.3.1).

$$BEQ_{bio} = \frac{(EC_{10}\ or\ EC_{IR1.5})_{ref}}{(EC_{10}\ or\ EC_{IR1.5})_{sample}} \quad \text{(a)}$$

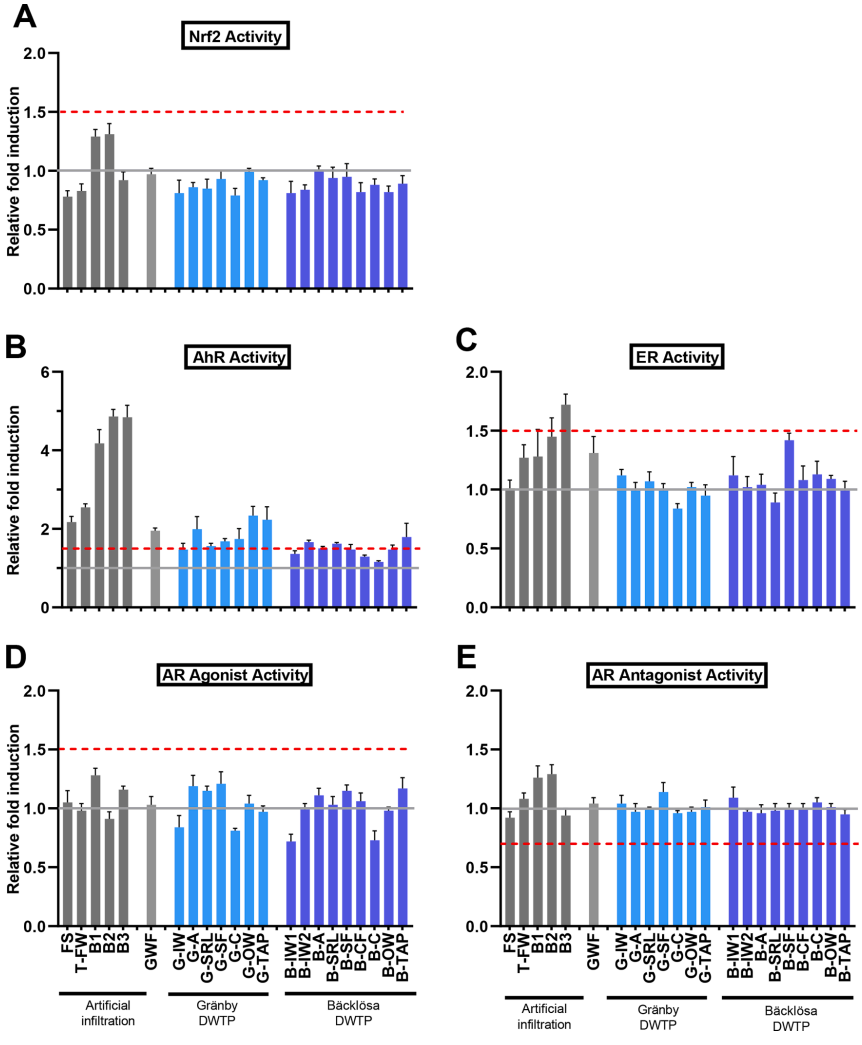

Fig. 2. Relative fold inductions (vs. vehicle control) observed at REF 50 for Nrf2 (A), AhR (B), ER (C), and AR agonist (D) and antagonist (E) activities. Treatment groups (n = 4) were normalized to vehicle controls (n = 8) set to 1 (grey line). The dotted red lines represent the respective cut-off levels. Data presented as mean ± SD. Refer to Table 1 for sampling location descriptions.

3. Results and discussion

3.1. Cytotoxicity

All samples were initially tested for cytotoxicity at REF 50 in all assays. Thereafter, samples that were to be assessed further in dilutions series were tested for cytotoxicity up to REF 200. In all assays, none of the water samples exerted cytotoxicity (Supplementary Information, Fig. S1) which demonstrated that the bioassays were conducted under conditions where the cell viability was not compromised.

3.2. Initial screening of bioactivities

Initially, all samples were analyzed at REF 50 in bioassays for oxidative stress (Nrf2 activity), AhR, and ER agonistic activities, as well as AR agonistic and antagonistic activities (Fig. 2). In general, the majority of the samples were inactive for most of the studied endpoints at REF 50. None of the samples exerted oxidative stress (Fig. 2A) or AR agonist (Fig. 2D) or antagonistic activity (Fig. 2E) above cut-off levels and only one sample showed estrogenicity at REF 50 (Fig. 2C). For AhR, however, several raw water samples taken in the basins prior to infiltration exerted relatively high activities at REF 50 (Fig. 2B). AhR activity was lower in the post-infiltration wellfield sample and significantly different than the activities detected in the basin samples ($p<0.0001$, Šídák's multiple comparisons test, $\alpha= 0.05$). The AhR activity remained at or just above the detection limit in most post-infiltration samples collected downstream in the DWTPs and distribution networks.

3.2.1. Treatment effects of artificial infiltration

In the soil subsurface, chemical contaminants can undergo biodegradation and attenuation over time via various biotic and abiotic processes during migration. They may also be removed from the aqueous environment by adherence to soils. Many studies have reported on the fate and degradation of various chemical contaminants in wastewater effluents treated by natural attenuation in the soil subsurface (Cordy et al., 2004; Drewes et al., 2003; Hoon et al., 2007). Further to this, artificial recharge through infiltration basins has been reported to improve recharged water quality by eliminating various pesticides, pharmaceuticals, and pathogens (Dragon et al., 2018; Maeng et al., 2011; Nagy-Kovács et al., 2018; Tröger et al., 2020; Valhondo et al., 2020; Verstraeten et al., 2003). Moreover, biological toxicity assays have been used to evaluate the safety of reclaimed wastewater and recycled water quality (Leusch and Snyder, 2015; Xu et al., 2020). However, there appears to be a paucity of bioanalytical studies investigating the effectiveness of artificial infiltration processes in drinking water production. Nevertheless, the results of the current study are discussed below in the context of a limited number of studies relevant to the treatment efficiency of artificial infiltration processes in water purification.

In the Nrf2 and AR assays, no bioactivities above cut-off levels (or below in the case of AR antagonist activity) were detected at REF 50 at any of the sampling points between the river source, the Galgbacken wellfield, and the infiltrated water intakes at the two DWTPs. This is in contrast to the findings of Oskarsson et al. (2021)'s study wherein high Nrf2 and anti-androgenic activities were detected in samples collected from abstraction wells and the outlet of the DWTP over different seasons (Oskarsson et al., 2021). That particular DWTP draws artificially infiltrated water from a large river source which receives treated wastewater, storm water discharges, and effluents from industries. The artificial infiltration in that study had been in place since the 1950s. The Nrf2 and anti-androgenic activities in the raw water source to be infiltrated did not show any detectable Nrf2 or anti-androgenic activities, so contamination of the water occurred during the infiltration process. Targeted chemical analysis of the infiltrated water samples detected 17 of 163 analysed MPs (Tröger et al., 2020). A mixture of all the analysed MPs (each at a concentration of 1 μg/L, which was far higher than the concentrations of the 17 detected chemicals) did not induce Nrf2 or anti-AR activities. Thus, it was concluded that the detected MPs were not responsible for the bioactivities observed in the infiltrated water samples. Possible explanations of the observed effects included: the release of contaminants into the infiltrated water retained in the infiltration soil in the past, and/or the release of natural bioactive compounds (toxins) formed by microorganisms present in the infiltration environment. Still, the effectiveness of artificial infiltration in reducing reactive oxygen species (ROS) and anti-androgenic activity has been demonstrated elsewhere in another bioanalytical study that assessed infiltrated wastewater effluent (Jia et al., 2015). The authors reported that infiltration attenuated mutagenic and oxidative stress effects with BEQ reductions up to >97% and >93%, respectively. On another note, the presence of plants as a filtering layer in natural water purification systems has been demonstrated to biodegrade some pollutants. In such an example wherein a drinking water source was purified through a large-scale constructed wetland, decreases in ROS levels as well as in cytotoxicity and anti-androgen activity following purification was reported (Xu et al., 2019). Other studies using bioassays also reported a lack of AR activation or inhibition in finished drinking water samples (Jones et al., 2020; Leusch et al., 2018; Neale et al., 2020; Valcárcel et al., 2018). In contrast to AR activity, Nrf2 activity is often detected in river waters in other parts of the world (Neale et al., 2017; Wang et al., 2013). Further, the oxidative stress response is quite commonly detected in a variety of water types and therefore a highly relevant parameter in water quality assessments (Escher et al., 2014). Overall, the lack of AR and Nrf2 activities detected in any of the samples in the current study, particularly in the source river water, suggests the low presence of bioactive compounds during this sampling event for these two endpoints.

In contrast to the non-detectable bioactivities in the other bioassays tested, water samples collected before the artificial infiltration were above the cut-off level for AhR activity. In particular, bioactivities were higher in the samples collected from the three open-air infiltration basins compared to the source river water even though there is no treatment in between these sampling points. This may be attributed to the fact that the water in the open-air infiltration basins may undergo physical, chemical, or biochemical changes while exposed to sunlight, temperature fluctuations, and other ambient conditions which may affect water quality before entering the groundwater. Natural sunlight irradiation, for instance, plays an important role in transforming MPs and dissolved organic matter in other water environments such as open storage of reclaimed water and in natural surface waters due to natural processes (e.g., photolysis) (Bahnmüller et al., 2014; Tixier et al., 2002; Wang et al., 2021). At the same time, phototoxic products may also be formed during the photolysis of organic contaminants such as dioxin-like bromocarbazoles and chlorocarbazoles (Mumbo et al., 2017) and pharmaceutical mixtures (Wang and Lin, 2014). In short, while identification of the compounds inducing the AhR activity was not in the scope of the current study, the higher AhR bioactivities detected in the basin samples compared to the preceding sampling points provide interesting insight into the presence of AhR-inducing chemical hazards in the basins.

In contrast to the elevated AhR bioactivities detected in the infiltration basin samples, the bioactivity in the sample collected from the subsequent Galgbacken wellfield (GWF) was much lower, with a 2.5-fold decrease compared to the highest bioactivities measured in the basin samples, albeit still above the cut-off level. The lower bioactivities measured in the wellfield sample following infiltration may be due to several explanations. First, the infiltration basins contain approximately one meter of sand directly in contact with the underlying natural esker formation. Water from the basins undergoes infiltration at a rate of approximately 3.5-4.5 m^3/m^2 per day. A biological growth or "schmutzdecke" typically occurs on the sand surface and is a key factor in the treatment process. Physical, chemical, or biochemical changes in the water matrix can occur within the schmultzdecke and the

unsaturated and saturated natural esker material underlying the infiltration basins. The schmultzdecke functions as a biologically active filter and may account for the AhR activity due to the adsorption of contaminants during this process. Next, natural attenuation due to dilution and mixing with the natural groundwater as well as adsorption occurs within the esker material during the transit time from infiltration to extraction which is approximately 6–8 months. Oskarsson et al. (2021) also reported removal of AhR activity detected in raw river water following the artificial infiltration treatment (Oskarsson et al., 2021). Still, in another study of a DWTP-fed riverbank filtrate, AhR-mediated effects at an EC_{10} value of approximately REF 8 (Albergamo et al., 2020) were detected in the filtrate. The raw anaerobic riverbank filtrate in that study had an average infiltration time of 30 years. Taking into account the results from these other studies of the infiltration process, the transit or residence time of the infiltrated water seems to be a notable factor. On the whole, compared to the positive AhR responses in the less treated basin samples, the clear lower response following artificial infiltration is a compelling observation of the current study.

In the ER assay, bioactivity above the cut-off level was detected in only one sample (Basin 3), albeit marginally. Furthermore, no estrogenic activity above the cutoff level was detected in the sample collected from the wellfield (following infiltration) nor in the two DWTP's inlet samples. Riverbank filtration piloted for water supply systems has been demonstrated to remove thyroid-disrupting chemicals as well in the recombinant thyroid hormone receptor (TR) gene yeast assay (Valcárcel et al., 2018). While there appears to be a lack of toxicological effect-based studies investigating the degradation of estrogenic compounds in artificially infiltrated drinking water sources, other studies using chemical analyses have investigated the occurrence and elimination of endocrine-disrupting compounds in groundwater recharge systems in Germany. For instance, a study that investigated the removal of steroids during two different groundwater recharge systems (riverbank infiltration and artificial groundwater replenishment) observed significant decreases in the selected estrogenic compounds following these two processes (Zuehlke et al., 2004). Similarly, a study that monitored the concentrations of 10 natural and synthetic estrogens and progestogens in water samples collected from two artificial recharge plants located in Sweden and Denmark detected only one compound (estrone-3-sulfate) following the recharge processes (Kuster et al., 2010). As such, while no to low estrogenic effects were detected in the current study, the presence of hormones in MAR systems has been observed in other non-bioanalytical studies.

In brief, the lower AhR bioactivities and lack of ER bioactivities in the wellfield and DWTP intake samples compared to the preceding raw water samples (river and basins) where activities were detected above the respective cut-off levels would suggest that artificial infiltration is an effective natural purification method in this study. This is in contrast to the findings of our previous study at another Swedish DWTP utilizing artificial infiltration wherein the artificial infiltration process appeared to be a source of contamination (Oskarsson et al., 2021). As mentioned previously, the contrasting findings may be attributed to several factors regarding the removal efficiency of the artificial infiltration process. One explanation may be due to differences in the residence (or travel) time of the raw water in the subsurface. The infiltrated water in the current study takes approximately six to eight months to reach the wellfields supplying the two DWTPs. The infiltrated water in the previous study takes seven to thirty days to percolate through the subsurface from the infiltration basins. A longer travel time could, therefore, result in greater removal or dilution of bioactivity compounds to undetectable concentrations. An alternative explanation could be due to the accumulation of contaminants in the subsurface in the case of the DWTP in our previous study (Oskarsson et al., 2021). As revealed by Oskarsson et al. (2021) and elsewhere, the artificial infiltration of aquifers may lead to the eventual mobilization of toxic, naturally occurring contaminants into the water, thereby compromising the water quality (Fakhreddine et al., 2021; Oskarsson et al., 2021). Further, certain classes of hydrophilic organics that enter riverbank filtration systems can persist and migrate over prolonger time scales (e.g., decades) (Albergamo et al., 2019).

The fact that bioactivities above cut-off levels following artificial infiltration were detected in the AhR assay, but not in any of the other bioassays in this study may suggest that certain compounds present in infiltrated water cannot be as effectively removed during subsurface attenuation as others due to their resistance to biodegradation and the hydrophilic nature of the compounds, even at low concentrations in the groundwater. This has, for instance, been demonstrated for certain pharmaceuticals (e.g., carbamazepine and primidone), personal care products (PPCPs), and endocrine-disrupting compounds (Benotti et al., 2012; Debroux et al., 2012; Heberer et al., 2004; Hrkal et al., 2018). Compound-specific characteristics such as hydrophilicity and recalcitrance may limit the amount of compound that will adsorb to soils or that can be biodegraded by the soil microbial community (Maeng et al., 2011). Also, mobility during subsurface flow/riverbank filtration depends on the polarity of the MPs (Mishra et al., 2021). Next, the fate of organic compounds and degree of attenuation during artificial recharge is influenced by multiple factors such as the retardation factor, the distance and time spent in travel, depth to water table, sediment porosity and permeability, groundwater flow, and the hydrogeologic characteristics of the aquifer (Mishra et al., 2021; Petrovic et al., 2009). Such characteristics of the aquifer include its lithology, hydraulic and textural properties of the soil, temperature, and the microbial environment. Among these factors, redox conditions of the aquifer play a significant role in that certain pollutants are preferably removed under some particular redox conditions (Barbieri et al., 2011; Valhondo et al., 2015).

Finally, there is the temporal aspect of the sampling strategy in the current study. Given that all samples were collected on the same day in this study, it is likely that the composition of the water samples collected along each step of the total treatment process from the river water source would differ from each other. Further to this, only one sampling event was conducted for this study. Seasonal differences in the quality and chemical profile of the raw water were therefore not assessed. As described by Jokela et al. (2017), fluctuations in commonly monitored water quality parameters alone related to the organic matter content of river waters are typical (Jokela et al., 2017).

Overall, artificial filtration as a natural water purification method has been shown to have its benefits as well as limitations, mainly in that it does not result in the complete removal of all bioactive MPs, and that it may be a cause of contamination of drinking water. This treatment method, therefore, can serve as an effective pre-treatment of raw water but should include some water quality monitoring, with additional purification required thereafter, in drinking water production.

3.2.2. *Water purification at Gränby and Bäcklösa DWTPs*

Following artificial infiltration, the surface water will have mixed with the groundwater (refer to Section 2.1) and then fed into the Gränby and Bäcklösa DWTPs for further purification. A secondary objective of the current study was to monitor the bioactivities across all treatment steps in the two DWTPs fed the infiltrated groundwater. In general, consistent with the lack of Nrf2, AR, and ER bioactivities detected in almost all samples collected between the river and wellfield locations, samples from both DWTPs were below cut-off levels at REF 50 in all assays tested, except in the AhR assay. Low AhR bioactivities either at or slightly above the cut-off level were detected at REF 50 in several samples collected from both DWTPs. It is important to mention that these AhR activities were either lower or similar to what was observed in the raw water in the basins prior to artificial infiltration. However, that AhR activities above cut-off were detected in some samples collected at the DWTPs suggests the limited removal effect of AhR-inducing compounds during the treatment processes utilized at the DWTPs.

As mentioned in Section 2.4, samples collected from the inlets, outlets, and distribution networks of both DWTPs were further analyzed in dilution series to obtain CECs. EC values and BEQs were then

determined to compare to other effect-based studies on DWTPs. This was done for the AhR and ER bioassays only based on the initial screening results, as these two assays showed more frequent bioactivities above cut-off levels at REF 50 compared to the Nrf2 and AR bioassays. However, given the low levels of bioactivities detected in some of the samples at REF 50, it was necessary to increase the sensitivity in the assays by increasing the highest tested concentration to REF 200. The CECs and calculated results are presented in Fig. 3 and Table 3, respectively. For the Gränby DWTP, dilution series were possible to study for the inlet sample but not for the outlet sample due to a lack of sample volume. However, a Mann-Whitney test of the REF 50 results for the outlet sample and the subsequent tap water sample yielded no significant differences in the AhR assay (p=0.686) or the ER assay (p=0.343). Consequently, dilution series of the tap water sample were completed instead. At the Bäcklösa DWTP, it should be pointed out that this plant receives water from two wellfields.

As mentioned previously, low AhR was detected overall in all samples collected from both DWTPs in the current study. Other effect-based studies on river water-sourced DWTPs utilizing similar conventional treatment methods have reported higher activities. Escher et al. (2014), for instance, reported an EC_{10} value of REF 8.6 in the 24 h AhR-CAFLUX assay in finished drinking water samples collected from a river water-sourced DWTP that utilized coagulation and filtration followed by chlorination and finishing with chloramination (Escher et al., 2014). A previous study at that same Australian DWTP reported AhR activity at 0.17 ng TCDD/L in the finished drinking water (Macova et al., 2011). In the ER bioassay, low estrogenic activities were detected in the current study in the finished drinking water samples collected from Gränby and Bäcklösa. Another effect-based study investigating river water-sourced DWTPs reported reduced estrogenic activity to below the limit of detection (EEQ_{bio} $<3.00 \times 10^{-2}$ ng E2/L) in finished water samples following conventional treatments (Neale et al., 2020). Similarly, ten DWTPs sourced from surface stream water, alluvial groundwater, and deeper groundwater in an area of high agricultural use in the USA reported a low prevalence in the detection of ER activity in the finished drinking water (Jones et al., 2020). Still, much higher activities have been reported at other DWTPs elsewhere, such as 0.035–1.51 ng EEQ/L in the E-screen assay in tap water samples collected from ten DWTPs located throughout Taiwan (Gou et al., 2016) and an EC_{10} value greater than REF 30 in the E-SCREEN assay in finished drinking water samples collected from a river water-sourced DWTP in Australia (Escher et al., 2014). Furthermore, the observed estrogenicity in the finished drinking water samples collected from both DWTPs in the current study were at concentrations far below the suggested threshold of concern of 1 ng estradiol/L recommended by the World Health Organization (World Health Organisation Europe, 2017) and included in the 2022 watch list (Council of the European Union, 2020) in the EU drinking water directive (Drinking Water Parameter Cooperation Project. Support to the Revision of Annex I Council Directive 98/83/EC on the Quality of Water Intended for Human Consumption (Drinking Water Directive), 2017).

Table 3
Summary of effect concentrations (EC10), in units of REF, and BEQs expressed as TCDD-eq and E2-eq obtained from the concentration-effect curves for select samples. BEQ values are presented both as molar concentrations (pM) and as pg/L (in parentheses).

Sampling point	AhR activity		Estrogen receptor activation	
	EC10 (REF)	TCDD-EQ (pM)	EC10 (REF)	E2-EQ (pM)
Gränby inlet (G-IW)	$9.81 \times 10^{+01}$	4.33×10^{-02} (13.9 pg/L)	$3.45 \times 10^{+01}$	4.10×10^{-02} (11.2 pg/L)
Gränby outlet (G-OW)	*(insufficient sample vol.)*		*(insufficient sample vol.)*	
Tap water location in distribution network (G-TAP)	$8.00 \times 10^{+01}$	5.31×10^{-02} (17.1 pg/L)	$8.53 \times 10^{+01}$	1.66×10^{-02} (4.52 pg/L)
Bäcklösa inlet 1 (B-IW1)	$1.16 \times 10^{+02}$	3.65×10^{-02} (11.8 pg/L)	$4.93 \times 10^{+01}$	2.86×10^{-02} (7.79 pg/L)
Bäcklösa inlet 2 (B-IW2)	$9.88 \times 10^{+01}$	4.30×10^{-02} (13.8 pg/L)	$7.06 \times 10^{+01}$	2.00×10^{-02} (5.45 pg/L)
Bäcklösa outlet (B-OW)	$1.20 \times 10^{+02}$	3.53×10^{-02} (11.4 pg/L)	$6.05 \times 10^{+01}$	2.33×10^{-02} (6.35 pg/L)
Tap water location in distribution network (B-TAP)	$1.11 \times 10^{+02}$	3.84×10^{-02} (12.4 pg/L)	$4.92 \times 10^{+01}$	2.87×10^{-02} (7.82 pg/L)

4. Conclusions

MAR techniques, such as artificial infiltration, are commonly utilized around the world to improve water quality and increase groundwater storage (Stefan and Ansems, 2018). However, groundwater aquifers are susceptible to contamination by many of the same MPs found in surface waters. Such an instance was previously shown at a Swedish DWTP where the river water source was contaminated by hazardous chemicals during artificial infiltration (Oskarsson et al., 2021). The current study

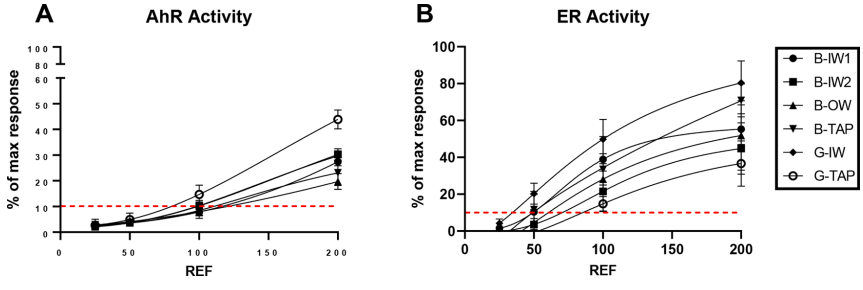

Fig. 3. Concentration-effect curves of AhR activity (A) and ER activity (B) for inlet, outlet, and tap water samples for Gränby and Bäcklösa DWTPs. The symbols denoting each sample are provided in the legend. Treatment groups (n = 4) were normalized to the vehicle control (n = 8), then to the maximum experimental response of the reference compound (TCDD for AhR, E2 for ER), set to 100. Data was fitted to four-parameter sigmoidal regression models. The dotted line indicates 10% activity of assay max. Data presented as mean±SD.

involved an effect-based evaluation of another Swedish DWTP that utilizes artificial infiltration in its drinking water production. In this case, the artificial infiltration process seemed effective in reducing AhR and ER bioactivities. What is important to highlight is that there are still a very limited number of relevant effect-based studies evaluating the effectiveness of artificial infiltration in removing chemical hazards. Given that artificial infiltration is commonly utilized around the world in drinking water production, further research, particularly using effect-based methods, is urgently needed to gain further understanding of the risks and benefits of this water treatment process.

Future work with the current study could include additional sampling to observe any temporal differences along the artificial infiltration process. It would also be worthwhile to investigate operational factors related to the infiltration process such as loading rates, basin material, and pre-treatment which may optimize the reduction of bioactivities.

Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: J. L. and A.O. are the founders and owners of BioCell Analytica Uppsala AB, a company providing effect-based testing services to the water sector. E.L is employed by BioCell Analytica Uppsala AB.

Data availability

Data will be made available on request.

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Supplementary materials

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This thesis aimed to explore the versatility of EBMs using *in vitro* bioassays for different applications related to drinking water safety. We applied EBMs to assess various water treatments as well as certain mycotoxins. We also investigated an import aspect of sampling involved in EBMs. Such work serve as insightful contributions towards wider implementation of such methods in water management and eventual acceptance of EBMs in drinking water quality frameworks.

Maria Yu received her doctoral education at the Department of Biomedical Sciences and Veterinary Public Health, Swedish University of Agricultural Sciences. Her BSc. and MET degrees were obtained from the University of Alberta and Simon Fraser University, respectively.

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