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Effect-based evaluation of chemical pollutants and mixture effects in the aquatic environment – implications for water reuse

Kim Frieberg

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Kim Frieberg

Faculty of Veterinary Medicine and Animal Science
Department of Animal Biosciences
Uppsala

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Swedish University of Agricultural Sciences, Department of HBIO, Uppsala, Sweden

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Abstract

It is known that pollution of organic micropollutants of the aquatic environment occurs through insufficient wastewater treatment. Environmental water samples therefore contain a complex mixture of known and unknown compounds and their transformation by-products. Effect-based analytical methods using cultured cells can be used to evaluate the impact of the complex mixture of contaminants that occur in environmental water samples. This thesis aimed to investigate the occurrence of pollutants in different water samples, using effect-based methods as tool for evaluating levels of pollutants through various water treatment methods, sample preparation methods and for a complex mixture assessment.

First, effect-based methods were used to evaluate a full-scale system of intentional reuse of treated wastewater for drinking water production. Where the pollutant burden in the water could be followed through various wastewater treatment steps, out into the recipient water body and finally through drinking water treatment. The highlight of this study was that no negative impact on drinking water quality could be detected from reusing treated wastewater in this system. Next, four different solid-phase extraction methods previously applied in effect-based water quality assessment was evaluated. By assessing three different sample types, treated wastewater, procedural blank and a spiked sample it was concluded that the choice of preparation method was more important for analysing the presence of estrogenic compounds as compared to androgenic compounds in water samples. Lastly, pollutant mixture complexity was investigated by fractioning treated wastewater using high-performance liquid chromatography and comparing bioactivity in the whole, highly complex sample, with sample fractions of reduced complexity. We found that the detected oxidative stress response and aryl hydrocarbon receptor activity was fully dependent on mixture effects, highlighting the implications of the complex mixture of pollutants that occur in the aquatic environment.

Keywords: Effect-based analysis, Reporter gene assays, Pollution of the aquatic environment, Wastewater effluent, Wastewater reuse, Solid-phase extraction, complex mixture assessment

Effect-based evaluation of chemical pollutants and mixture effects in the aquatic environment – implications for water reuse

Sammanfattning

Det är välkänt att förorening av den akvatiska miljön sker genom ofullständig rening av avloppsvatten. Sjöar och vattendrag innehåller därmed en komplex blandning av kända och okända ämnen och dess transformationsprodukter. Effekt-baserade analysmetoder baserade på odlade celler kan användas för att utröna effekter av denna typ av förorening av vattenmiljön. Denna avhandling ämnade undersöka närvaron av organiska microföroreningar i olika vattenprover genom att använda effektbaserade metoder för att utröna reningseffektivitet i olika typer av vattenrenings processer. Effektbaserade metoder användes även för att undersöka och jämföra olika extraktionsmetoder av-, och blandningseffekter i, renat avloppsvatten.

Först undersöktes återbruk av renat avloppsvatten i ett fullskaligt test av kopplad avloppsrening och dricksvattenproduktion. Där följdes effekten av förorenat vatten genom flera avloppsreningssteg, ut i miljön i recipienten och in genom dricksvattenproduktion. Det största fyndet i den studien var att kopplingen av renat avloppsvatten in i dricksvattenproduktion kunde ske utan att några negativa effekter på vattenkvalitén kunde påvisas i det färdiga dricksvattnet. Vidare undersöktes fyra olika fastfasextraktionsmetoder som tidigare används i effekt-baserade studier gällande vattenkvalitet. Genom att analysera tre prover, renat avloppsvatten, en process blank och ett spikat vattenprov kunde vi komma fram till att valet av extraktionsmetod tycktes vara mer avgörande vid analys av östrogena ämnen än androgena ämnen i vattenmiljön.

Till sist undersöktes blandningseffekter i renat avloppsvatten. Genom fraktionering av vattenprovet via högpresterande vätskekromatografi, till 54 nya prover, med reducerad komplexitet, kunde effekter i hela provet jämföras med effekter i de mindre komplexa provfraktionerna. Studien visade att effekter som oxidativ stress och arylkolvätereceptor-aktivering var helt och hållet beroende av blandningseffekter, vilket påvisar problematiken kring närvaron av blandningen av förorenade ämnen såsom de förekommer i vattenmiljön.

Nyckelord: Effekt-baserad analys, Reportergenanalys, Förorening av vattenmiljön, Renat avloppsvatten, Återanvändning av renat avloppsvatten, Fastfasextraktion, Blandningseffekter

Preface

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

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

Dedication

To anyone else out there who had difficulty grasping the concept of multiplication with negative numbers in high school, and thought that science was not for you. This thesis is proof that it does not take a specific kind of mind to be able to be interested in, and enjoy, science.

Contents

List of publications

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

I. **Frieberg K**., Gago-Ferrero P., Bijlsma L., Ahrens L., Wiberg K., Hernández., Oskarsson A., Lundqvist J. (2023). Effect-based evaluation of water quality in a system of indirect reuse of wastewater for drinking water production. Water Research, 242 (120147).

II. **Frieberg K**., Oskarsson A., Lundqvist. An effect-based evaluation of recovery and blank activities on solid-phase extraction processes used for water quality assessment. (manuscript submitted).

III. **Frieberg K**., Lindblad S., Lundqvist J. Evaluation of mixture toxicity in treated wastewater using effect-based methods and sample fractionation assessment. (manuscript submitted)

Supporting information for papers I, II and III are included in the appendixes.

1. Introduction

1.1 Chemicals in the aquatic environment

Chemicals exist in the aquatic environment. Through various anthropogenic activities, or naturally occurring phenomenon's, chemical compounds escape their original purpose and migrates with surface water or domestic and industrial wastewater towards the aquatic environment. The load of chemical pollutants, or organic micropollutants as of this thesis focus, are known to originate from all areas of society. Man-made pharmaceuticals, hormones, herbicides and pesticides are especially problematic in the aquatic environment, as they were designed to have a biological effect. Other big groupings of micropollutants known to cause harm in the aquatic environment are industrial chemicals, flame-retardants, plasticisers, combustion by-products, residues form cosmetics and personal care products and surfactants, to mention a few.

The total load of pollutants in the aquatic environment is often described as an iceberg (Figure 1). With a small visible tip at the surface and the major part of the iceberg hidden below the surface. The tip represents known pollutants, with known structures and known biological effects, which are readily measured in water samples. The area below the surface represents unknown compounds with unknown structures, and compounds with unknown individual- and mixture effects and compounds that have biological effects below their chemical detection limits. Assessing the toxicological impact of the total pollutant burden of a water sample cannot be done with chemical analysis alone, due to the fact that chemical analysis only quantifies chemical components but tells little about their effects. However, complementing chemical analysis with effect-based analysis, or

effect-based methods, can reveal information of the submerged unknown part of the iceberg as well as disclose information about mixture effects from the total pollutant burden in a water sample.

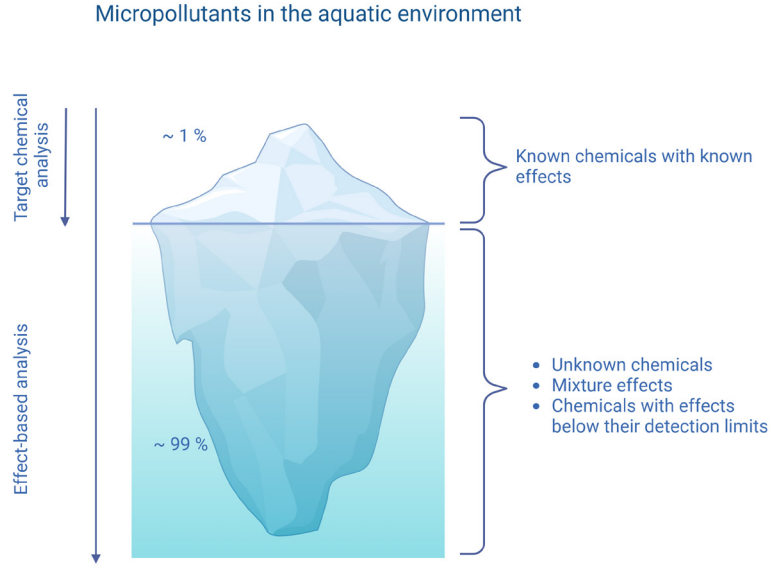

Figure 1: Displaying an iceberg as a representation of collective knowledge of the presence and impact of pollutants in the aquatic environment. With what is known, depicted as the tip of the iceberg and what is unknown depicted below the surface. Targeted chemical analysis can only detect chemical structures at the tip of the iceberg and tells little of the toxicological impact of their presence in the aquatic environment, alone or together in the complex mixture they exist in. Effect-based analysis aims to reflect the total impact of bioactive chemicals from the whole iceberg whether they are known or unknown. The figure was created using biorender.com

1.2 Effect-based methods – an overview

Effect-based methods are built around specific biological endpoints, or modes of action, and serves to reveal more detailed information than merely if a compound is toxic or not. One type of effect-based methods are *in vitro* bioassays, centred around genetically modified cells that have been manipulated to increased sensitivity for specific exposures, and to produce a signal to visualise this exposure. Assessing a water sample using several bioassays or endpoints in battery can reveal the toxicological impact of

known and unknown components of a water sample, and thus aiming to cover the whole span of the iceberg (Figure 1). When combining specific bioassays and chemical analysis to deduce chemical drivers behind observed effects, some endpoints have larger proportions of the iceberg on the surface than others [1]. Estrogenic effects is an example where it is typically known pollutants that can be linked to specific effects [2]. Oxidative stress is an example of and endpoint where most of the effects cannot be explained by known pollutants, and the unknown part of the iceberg can comprise up to 99 % of the detected effects [3] (Figure 1). Further reading on this in section 1.4.

In the EU, awareness of micropollution of the aquatic environment is growing. Through the water framework directive [4] and the environmental quality standards [5] there are now target levels for priority substances [6] and requirements of water quality monitoring in the work towards a non-toxic environment. However, the legislation is lacking in the sense that its fundaments is based on the tip of the iceberg (Figure 1) and often limited on single chemical presence and risk assessments, and much remains to be investigated, discovered and described about the impact of the unknown part of the iceberg.

1.3 Freshwater cycle

In the previous section, it was established that micropollutants occur in the aquatic environment. Wastewater treatment and the treated wastewater effluent has been identified as a point source of pollution of recipient waters [2, 7-14]. One reason for that is because the bulk of chemical used in domestic households migrate from their original purpose and are washed away through wastewater drains and enters municipal wastewater treatment. Wastewater treatment was first introduced to combat issues of eutrophication, needing to be a solution to remove excess nutrients in treated waters. Conventional wastewater treatment methods were therefore not designed to remove *more* things from the water other than phosphorous, nitrogen and excess carbon. A majority of micropollutants can therefore pass through conventional wastewater treatment into the environment, and possibly into source water for drinking water production, as many drinking

water facilities are situated downstream wastewater treatment plants [1, 15-17]. Further reading on this in section 1.3.3 and Figure 3.

1.3.1 Wastewater treatment as barrier for chemical pollution

The connection between inefficient wastewater treatment and micropollution is highlighted in recent EU legislation. Advanced treatment methods for micropollutant removal is a required implementation for wastewater treatment plants, of a certain size, through the 2024 version of the urban wastewater directive [18]. Wastewater treatment is usually described in terms of primary, secondary and tertiary treatment steps (Figure 2). With the new EU legislation, the term quaternary or advanced treatment is also added to the list. In broad terms, the treatment steps can be summarised as follows: Primary treatment refers to physical or mechanical treatment through screens, grit chamber and a first sedimentation of suspended particles (Figure 2). Primary treatments aims to remove large objects, debris as hair, sand, and stones to prepare the incoming water for further treatment. Secondary treatment refers to biological treatment using microorganisms to remove phosphorous, nitrogen and organic material. Secondary treatment usually involves activated sludge where bacterial degradation of nitrogen occurs as well as further sedimentation processes (Figure 2). Tertiary treatment refers to chemical treatment where chemicals or precipitating agents are added to further decrease nutrient content in the water. Quaternary or advanced treatment refers specifically to treatment techniques that aims to remove micropollutants.

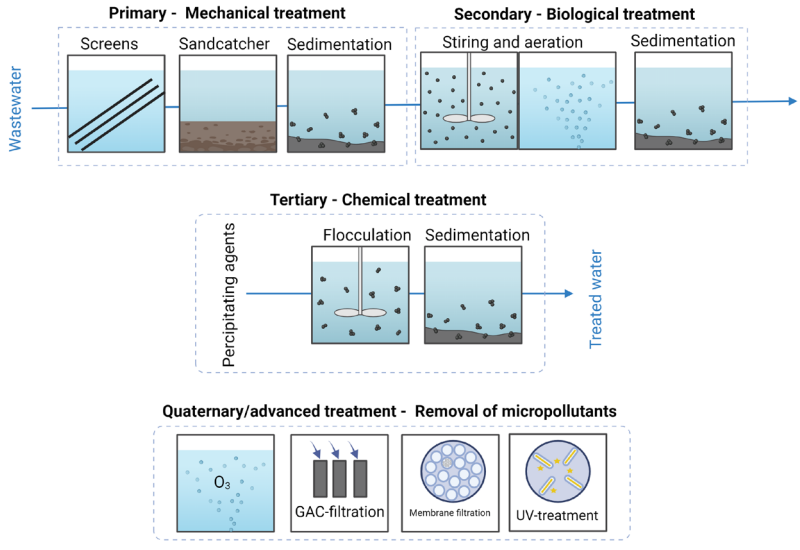

Figure 2: Simplified conventional wastewater treatment consisting of primary-, secondary and tertiary treatment methods, primarily removing nutrients from the treated water. To remove micropollutants from wastewater, advanced or quaternary treatment methods are required. The figure was created using biorender.com

In Sweden, 96 % of all suburban wastewater underwent secondary treatment in 2020 [19, 20]. However, there were about 1 million properties not connected to municipal water treatment facilities [19] and many more that are connected to local small treatment facilities. Facilities with advanced or quaternary treatment methods are mostly larger (incoming load of ≤ 150 000 PE, person equivalents) treatment plants, primarily situated in the larger cities of the country, constituting about 10 % of the total number treatment plants [19]. Examples of advanced or quaternary types of treatment methods are ozonation, UV-radiation treatment, membrane-filtration, and granulated activated carbon (GAC) filtration (Figure 2) [21-23]. It serves well to realize that the range of chemo-physical properties of incoming load of pollutants to a wastewater treatment facility is vast, and while one treatment technique would work well for one type of pollutant, it might have little effect on another.

1.3.2 Transformation products and treatment by-products

Micropollutants can transform in many different ways in the aquatic environment and through wastewater treatment. Abiotic factors like sun exposure can lead to photodegradation or spontaneous oxidation can occur in surface waters, biotic factors as biodegradation can transform, partially or fully degrade (mineralise) a mother compound. The transformation of a compound most often leads to a less persistent, less bio-accumulative and less toxic end-product but could potentially become a more persistent, bio-accumulative and toxic end-product [24]. Biological degradation that occurs in wastewater treatment through bacterial activated sludge treatment is extensive, but does most often not lead to full degradation of micropollutants (as previously discussed this is why removal micropollutants needs more advanced treatment techniques). Pollutants can also be removed from the wastewater by adsorption to sludge particles without transforming at all, but could then potentially become a hazard elsewhere, if the sludge is used for fertilizing soil. Therefore, to remove and not just transfer micropollutants, more powerful degradation process as compared to biodegradation, needs to be implemented. These types of methods are often referred to as advanced oxidation processes and includes ozonation, UV-radiation and different chlorination treatments among others[25]. Advanced oxidation processes has the capacity to split and degrade pollutants, but as with any other transformative process the end products could potentially be a more toxic transformation product or treatment by-product [23, 24]. Because of the potential formation of by-products, oxidation processes are often, but not always, paired with pollutant collecting processed like GAC, membrane filtration or further degradation of activated sludge processes to remove unwanted treatment by-products [26], especially when these reactive methods are applied in drinking water treatment facilities.

One specific type of treatment by-products are disinfection by-products (DBPs) that can form when combating microbial presence with reactive processed like chlorination treatment. Over 700 disinfection by-products has been identified, and two major subgroups of DBPs are trihalomethanes and haloacetic acids, which are both considered carcinogen compounds [27]. DBPs can form when the disinfectant, chlorine, or other, react with dissolved organic matter in the treated water [28, 29]. The formation of DBPs is dependent on properties of the treated water, such as dissolved organic

matter, ammonia content, as well as contact time with the disinfectant, temperature and the dose of chlorine [30-32]. Previous effect-based studies has shown DBP's to activate the oxidative stress response via the Nrf2 pathway [30, 33-35]. Chlorination treatment has historically most commonly been applied in drinking water treatment contexts, but as reuse of treated wastewater is gaining attention so is chlorination treatment of wastewater [36, 37].

1.3.3 Reuse of treated wastewater

In the face of climate change and water scarcity, reusing treated wastewater for different purposes would be an act towards improved sustainability. Due to the geographical localisation of wastewater treatment plants and drinking water treatment plants in the same watershed, *de facto* reuse of wastewater is actually implemented in many places in Sweden (Figure 3). One hundred and eighty drinking water production plants in Sweden use surface water as sole source for drinking water production. Typically, the largest drinking water producers for the largest cities in Sweden are using surface water affected by treated wastewater as a source for drinking water production. Apart from de facto reuse, as described above, the reuse of treated wastewater can be intentional, and can then be divided into two categories. Indirect reuse would mean that effluent wastewater is discharged into recipient surface waters before purposely reused. Direct reuse would mean that the treated wastewater is directly redistributed, via pipelining, without contact with environmental water sources prior reuse. The latter alternative can be a solution to avoid extensive evaporation in regions of high temperatures and severe drought conditions. The growing global freshwater shortage has increased interest in this matter and pushed for reuse innovation. In high-income countries severely affected by drought as Italy, Spain, Greece, Malta, and USA, Australia and Israel, non-potable reuse infrastructure is already in place [38].

In Europe, specific water reuse legislation as the Water Reuse Regulation [39] and Urban Wastewater Treatment Directive [18] supports a framework for wastewater reuse. Italy and Spain has the highest wastewater reuse in Europe. In Spain 2022, 343 hm^3 treated wastewater was purposely reclaimed and was redistributed for agricultural purposes (62%), irrigation of urban green areas (18 %), industrial processes (17 %), street cleaning (2%) and

recharging of aquifers (~1%) [38]. Direct reuse for drinking water production, potable water reuse, is legislated against in Europe and Australia, because of risks of pathogenic microbial presence. In the USA however, the states of Texas, Colorado and California have recent regulations in place for direct potable reuse of treated wastewater (EPA 2024). Another example of a pioneering country for direct potable reuse in Africa, is Namibia.

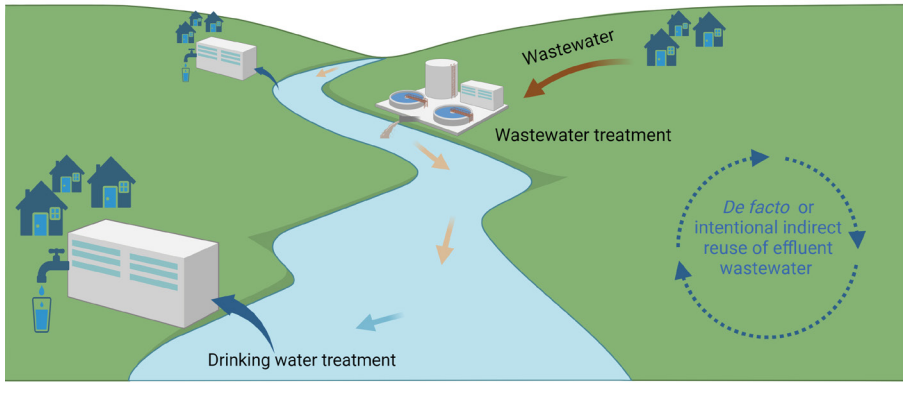

Figure 3. Displaying an interconnection in the water cycle between treated wastewater and drinking water production. In many places (in Sweden), *de facto* reuse of treated wastewater effluent is a reality where drinking water producers draws source water from surface water downstream wastewater treatment. Another situation could exist in areas suffering water shortages where treated wastewater effluent is *intentionally* redirected to replenish waterbodies that are used as source water for drinking water production.

1.4 Effect-based methods for using bioassays for water quality monitoring

Most commonly, water quality assessments are preformed using chemical analysis. Wastewater treatment facilities and monitoring of surface waters are focused on target chemicals due to existing legislation (see section 1.2). This approach only includes a small part of the unknown complex mixture of pollutants that might be present. As a complement to chemical analysis, including effect-based methods, can aid in gaining knowledge of the biological, or toxicological impact, of the total pollutant burden in a specific

water sample. Effect-based methods can detect the total toxicity of a water sample whether caused by known or unknown compounds, if a compound is active for the specific endpoint, it will contribute to the total read-out of an effect-based assessment. Effect-based methods entails an array of different methods, but for the purpose of this thesis, the focus lies with cell-based reporter gene assays.

Cell-based reporter gene assays have been developed to address effects at cellular-level in coherence with the 3R's [40] principle towards reducing animal testing. The principle behind a toxicological investigation at a molecular level, rather than higher up in a scale of biological complexity, is: even though a molecule binding to a receptor cannot be interpolated as to giving effects on an organism level, a whole organism effect might have started with just that molecular initiating event of a compound binding to a receptor (Figure 3). This rationale is also the basis of the Tox 21 programme, toxicology in the 21st century, were governmental bodies in the U.S.A., National Institute of Health, Environmental Protection Agency, and the Food and Drug Administration joint efforts to advocate for advancing molecular toxicology testing as alternative to traditional *in vivo* toxicology testing [41].

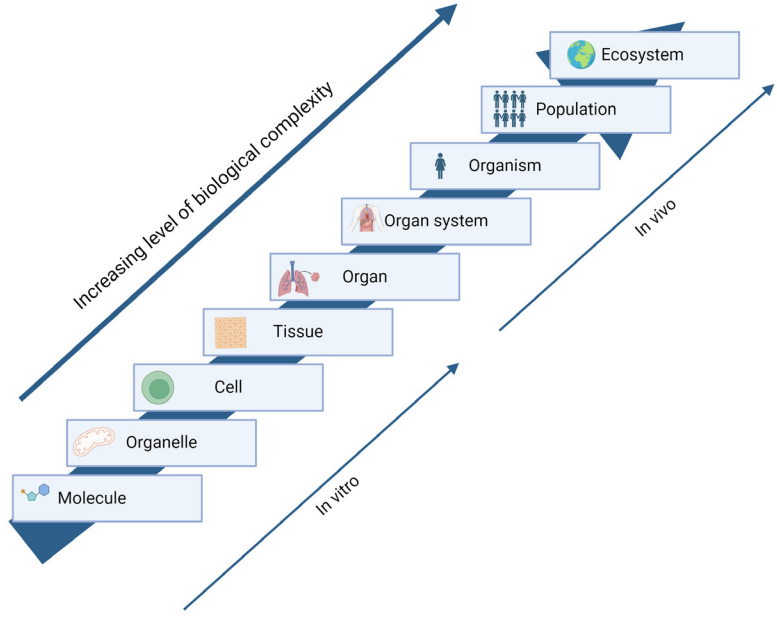

Figure 4: Displaying hierarchy of biological complexity, placing *in vitro* cell-based reporter gene assays at the lower end of the range. The detection of a compound initiating a genetic response in a cell can be evaluated using *in vitro* methods. The effects of an initiating event can result in adverse outcomes higher up in the hierarchy, as altered tissue or organ function, which in extension could lead to whole organism effects as impaired development or disease. Although molecular responses does not directly imply effects higher up in the biological hierarchy, they are a prerequisite for whole organism responses. Assessing for whole organism effects needs to be performed using appropriate methods for detection for *in vivo*. However, working in the lower end of the hierarchy is in line with the Toxicology in the 21st century programme, Tox21, of advancing molecular toxicology testing and increasing understanding of chemically induced bioactivity in alternative methods to traditional *in vivo* testing. It is also in line with the principle of the three R's of reducing and replacing animal testing. Adopted & modified from Escher *et al*. 2021. Figure was created using biorender.com

1.5 In vitro bioassays for water quality assessment

1.5.1 Cell-based reporter gene assays

For this thesis work, recombinant mammalian cell lines transfected with reporter genes have been used to study molecular initiating events – a molecule binding to a receptor or triggering a specific response in the cell. These genetically engineered cells have been altered to express a specific gene in an amplified way, and has compared to a natural or native cell, an enforced response machinery with a final luminescent product to quantify the triggering event (Figure 5). In detail, the luciferase gene from fireflies, is incorporated in to the cellular genome to be activated upon binding to a specific receptor in the cell. The reporter gene is then transcribed to mRNA which is then translated into an enzyme. When an experiment is terminated, substrate for the luciferase enzyme is added to the cells and a measurable luminescent signal is created (Figure 5). For each specific assay, the luminescent signal is directly proportional to the amount of initiating compounds.

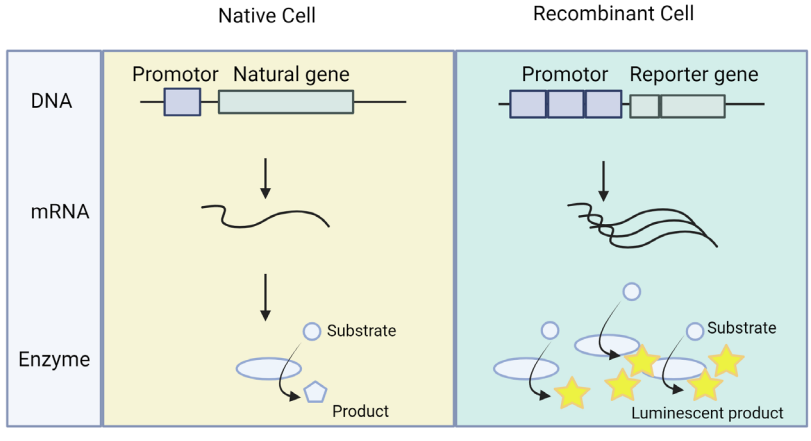

Figure 5: Fundamentals of reporter gene assays, displaying the broad differences between a native- and a recombinant cell. The recombinant cell has an amplified genetic response machinery with a final luminescent signal that can be readily measured using spectrometry. The luminescent signal is directly proportional to the activation of the reporter gene, i.e. the more activation the stronger luminescent signal. Adopted & modified from Escher *et al*. 2021. Figure was created using biorender.com

1.5.2 A battery of bioassays

Due to the complex nature of pollutants known to be present in the aquatic environment, using single bioassays or endpoints, is not enough to draw conclusions about the chemical state or pollution of a body of water. Rather, it is common to use a battery of bioassays, covering a range of endpoints, for a holistic overview of the toxicological impact of the pollutant burden. The battery design now commonly applied for water quality assessments has years in the making, rooting from a place of what methods were available, and developed through what kind of effects was commonly found in the aquatic environment. A large collaboration study including 20 laboratories applied 103 different bioassays to a shared set of 10 different type of water samples, including treated wastewater. From this study, the authors found that among the most responsive health-relevant endpoints were aryl hydrocarbon receptor (AhR) activation, hormone-mediated receptor activation as estrogen receptor (ER) activation and androgen receptor (AR) agonistic activity as well as activation of the oxidative stress response pathway [42]. The same endpoints are highlighted through the Global Water Research Coalition that has published guidelines on recommendations of which endpoints to include for specific types of water analysis. Where assessment of Estrogen receptor (ER) activity, Aryl hydrocarbon receptor (AhR) activity and oxidative stress assessment in recommended for analysing wastewater [43].

Sex hormone receptor mediated effects; Androgen receptor (AR) and Estrogen receptor (ER) activity are often included because it is known from field studies, that compounds disrupting these pathways can have negative effects on reproduction in wild fish populations [44, 45]. Aryl hydrocarbon receptor (AhR) mediated effects are also often included because of adverse effects in wild animal population connected to pollutants acting on this receptor. The decline in the Baltic seal population and eggshell thinning in wild birds are two classical toxicology examples of AhR mediated effects [46]. Oxidative stress response (Nrf2 activity) and Inflammatory response (Nfκβ activity) are both broader endpoints indicative of general stress in the cells, often activated by reactive compounds [33, 47].

1.5.3 Cell viability testing

Cell viability testing is used to detect disruption of basal cellular mechanisms and integrity of the cells. Measures of cellular metabolism assays, MTS and ATPase assays Section 3.4., are assessed in parallel to reporter gene assessments to ensure meaningful results. Because assessing reporter gene activity in a water sample that induces cytotoxicity, could read out as false-negative results.

1.5.4 Water sample preparation & procedural blanks

Because micropollutants are often present in the aquatic environment at low individual concentration, it is custom for both chemical and effect-based analysis of water quality, to concentrate water samples prior analysis to enable detection. Increasing the concentration of a water sample also enables analysis in a concentration-response manner where a range of concentrations of a water sample can be analysed to investigate concentration-dependent effects. Typically, water sample preparation is performed by first filtering the water to rid the sample of large particles that could interfere with downstream processes and the bioassay itself. Secondly, the micropollutants are extracted the from the water matrix by passing the sample over a sorbent material that collects the pollutants and releases the water, thereby increasing the concentration of the micropollutants. This sample preparation process is known as solid-phase extraction, and further details on this method can be found in section 3.2. In parallel to preparing a water sample the extraction procedure is paired with a blank sample of pure water as a procedural control. The procedural blank is included to ensure that the extraction process did not contaminate the sample with bioactive compounds from the preparation process, as the bioassay cannot differentiate process contaminants from pollutants present in the sample.

2. Objectives

The thesis is comprised of three studies. In the first study (Paper I), water quality was evaluated in a full-scale trial of indirect wastewater reuse for drinking water production, in the city of Barcelona. Chlorination treatment was applied to effluent wastewater prior discharge into the recipient river. The downstream presence of disinfection by-products was assessed using the Nrf2 assay for oxidative stress response assessment. Additional to the investigation of disinfection by products, the full-scale system from incoming wastewater to finished drinking water, was part of a water quality assessment, excluding and including disinfection of effluent wastewater, using a battery of seven bioassays.

In the second study (Paper II), an evaluation of sample preparation methods for *in vitro* bioassays was performed. Four previously published solid-phase extraction methods for water quality assessment were evaluated for effect-recovery and blank effects in three bioassays. A blank sample, a spiked sample with known bioassay inducers and treated wastewater was assessed.

In the third paper (Paper III), complex mixture toxicity was evaluated by fractioning extracted wastewater effluent using high performance liquid chromatography. The 54 sample fractions were assessed, along with the whole sample, in four bioassays commonly used for water quality assessment.

In summary, the following research questions were considered in the three papers:

- Does disinfection treatment, by chlorination, of effluent wastewater affect the oxidative stress response in downstream water? If so, does the effects carry through into finished drinking water? (Paper I)
- How does the different treatment steps affect water quality from incoming wastewater to finished drinking water in a full-scale trial of indirect wastewater reuse for drinking water production? (Paper I)
- How can the choice of sample extraction method impact the outcome of the bioassay? (Paper II)

- Is the bioactivity caused by a complex mixture (effluent wastewater), dependent on additive effects that can be explained by bioactivity in sample fractions? (Paper III)
- How much of the bioactivity in the whole sample can be explained by the sum bioactivity in the sample fractions? (Paper III)

3. Material & Methods

3.1 Water Sampling

How a water sample is collected is of course crucial for the interpretation of a bioassay. Water samples can be collected in different ways, for instance to capture time trends of the load of micropollutants using passive sampling utilizing absorption of micropollutants to different kinds of devices, or composite sampling by catching small fractions of a flow over time. Water samples can also represent a snapshot in time by simply grabbing a volume of a flow at a specific time point. Through wastewater treatment, or drinking water treatment, grab samples can be collected according to water flow through the treatment plant, so that the same bulk of water is sampled before and after specific treatment steps.

In Paper I and II grab water sampling was applied. In Paper I grab water sampling was applied to follow a bulk of water through a great length of water flow. From incoming untreated wastewater through wastewater treatment, out in the environment and in through drinking water treatment. Sampling was timely scheduled to sample the same bulk of water at the different locations to enable evaluation of the different treatment steps. Although time related composite samples would have been stimulating to have as a basis for this study, there were simply not enough resources available to sample in a different fashion. In Paper II, grab water sampling was applied because the main question was not related to any time trends or quality of wastewater treatment related to a possible variation of influx of micropollutants to the treatment plant at hand, but rather to have a complex water sample as a basis for assessment of different extraction techniques. In Paper III, 24 h composite samples of effluent water was collected, to have a rich complex mixture as a basis for mixture assessment.

3.2 Water Sample extraction

Solid-phase extraction (SPE) is one of the most common methods to use when preparing water samples for bioanalysis [48]. Other alternatives include large volume solid-phase extraction (LVSPE), Liquid-liquid extraction (LLE) or whole effluent toxicity (WET) testing. LVSPE is

typically used to sample waters of lower level of pollution, like surface water [48, 49] and is a bit over dimensioned for, but can also be used for highly polluted waters like wastewater [50]. LLE requires large volumes of solvent, is time consuming to perform as compared to SPE, and would be heavy-duty to perform for large number of samples in high-throughput assessments. WET testing is an option when assessing pollutant effects together with matrix effects, as this method does not select for organic pollutants but also includes metals and inorganics, and is perhaps closer to assessing the impact of the entire iceberg (Figure 1) as compared to the all of the above mentioned extraction methods. Historically WET testing has mostly been applied for *in vivo* aquatic organism assessments of lethality, as well as developmental and reproductive disruption. However, in the work towards reducing animal testing WET testing has been adopted for application in an i*n vitro* context [51, 52].

Solid-phase extraction is based on the principle that a mobile phase (water sample) is passed over a stationary phase (solid packing material). Compounds that are dissolved or suspended in the liquid will separate from the liquid and adhere onto the stationary phase, until released by an eluent solution of choice. Regardless of the extraction method of choice, the extraction process is limited to a specific range of pollutants, where for SPE, there will be hydrophilic compounds that does not transfer to the solid phase, and there will be hydrophobic compounds that does not leave the stationary phase when eluting the sample.

Foremost, solid-phase extraction of water samples serves to extract micropollutants from the water matrix. Other factors present in any water sample, such as salts and metals as well as differing osmotic conditions and levels of pH, would interfere negatively with the bioassay. Secondly, the process also increases the concentration of the micropollutants, enabling detection of very low environmental levels of micropollutants (further reading in 4.4.1). Lastly, as previously mentioned, an important principle to carry onwards in this thesis, is that the choice of extraction method could affect the span of extracted micropollutants as different SPE methods, with differing sorbent material and differing conditioning- and elution solvents, could have different capacities to extract the unknown content of a water sample [53]. This fact is the whole investigational purpose of paper II.

3.2.1 Paper I

In Paper I, a custom Multi-layer SPE method was used. The column was packed with of Strata-X, Strata X-AW and StrataX-CW, Phenomex, and Isolut ENV+, Biotage, sorbent material. Sample preparation was part of a collaboration project and samples were extracted in Spain, and reconstituted upon arrival in Uppsala. Reconstituted sample extracts was in 1:4 methanol:ethanol (v/v).

3.2.2 Paper II

In paper II, a selection of SPE methods were investigated to examine if bioactivity would be affected by the choice of extraction method. Four SPE methods, Bond Elut (Agilent) (BE), a custom multi-layer column (ML), the same as in paper I, (Strata X, Strata X-CW, Strata X-AW, Phenomex, and Isolut ENV+, Biotage), Oasis PriME HLB (Waters) (HLB) and LiChrolut RP18 (Merk Millipore) (LIC), were chosen as subjects of investigation, based on the fact that they had all previously been used in published effect-based assessment of water quality.

The sorbent material in HLB is according to manufacturer a balanced hydrophilic and lipophilic co-polymer that retains both polar and non-polar compounds. The sorbent in BE, is according to manufacturer, stacked with cation exchange (SCX) and anion exchange (SAX) sorbents and is effective for the extraction of acidic, basic, and neutral analytes from biological samples (a modified styrene-divinylbenzene polymer that retains polar and non-polar compounds). LIC is described as suitable for non-polar extraction of aromatic compounds and compounds with alkyl chains in water solution. The sorbent material is a gel made of high porosity synthetic silica particles. The ML column was made from four different sorbents: Strata X, Strata X-CW and Strata X-AW from Phenomenex and Isolut ENV+ from Biotage, a combination of reversed-phase extraction, cat- and anion exchange, and non-polar exchange. By combining sorbent materials with differing properties in a multilayer column, the goal is to broaden the range of extracted pollutants. Full details on extraction processes can be found in Paper II. All the samples and SPE methods were processed on a SPE-03 8-channel automated system (PromoChrom Technologies). All eluents were dried on a SpeedVaC® System (AES 2010, Savant) and reconstituted in ethanol.

3.2.3 Paper III

In Paper III, the extraction process was performed on a SPE-DEX semi-automated extraction system (4790 SPE-DEX®; Horizon Technology) using HLB disk (47 mm, Atlantic HLB-M, Horizon Technology). In contrast to SPE cartridges that was used in the Papers I & II. This method was chosen as sample preparation was part of a collaboration project for LC-MS analysis and the extraction process was part of an established protocol for LC-MS analysis of water quality (further reading in section 3.3).

3.3 Fractionation of samples

For Paper III, mixture toxicity of effluent wastewater was assessed. Separating the different components of a complex environmental mixture into different fractions and running it against the whole sample in a bioassay, can reveal mixture-effects. In Paper III, extracted (Section 3.2.2) effluent wastewater was separated using high performance liquid chromatography (HPLC) FractioMate™ - set up and separated into 54 sample fractions on 96-well plates. HPLC is driven by high-pressure pumps that carries the sample in a liquid, or mobile phase, over a stationary phase, a column filled with adsorbent material. Solutes in the sample then interacts with the stationary phase, which creates a gradient in migration rates through the column, separating the compounds and portioning them accordingly into 54 fractions. More details on fractionation can be found in the Paper III.

3.4 In vitro bioassays for water quality assessment

3.4.1 Reporter gene assays

The panel of bioassays applied for each Paper (I-III) is summarised in Table 1. In paper I, a range of mammalian bioassays were chosen to assess water quality through the full-scale evaluation of wastewater reuse, to get an as a holistic overview of water quality as possible. In Paper II, AR agonist activity, ER agonist activity and Nrf2 activity were included to investigate effect recovery and blank effects that we had previously experienced for those endpoints (in Paper I). In Paper III, AR agonist activity, ER agonist activity, AhR activity and Nrf2 activity were chosen to assess mixture toxicity. AR and ER activity were included because of the severity of the

presence of these kind of effects and additionally, because ER agonist activity is probably the most commonly assessed and detected endpoint. AhR and Nrf2 activity were chosen for mixture assessment, because they are both activated by such a broad range of endpoints and would be especially interesting to assess for mixture toxicity.

Table 1: Applied bioassays in Papers I-III

Paper	I	II	III
Androgen receptor (AR^+) agonism	X	X	X
Androgen receptor (AR^-) antagonism	X		
Estrogen receptor (ER^+) agonism	X	X	X
Estrogen receptor (ER^-) antagonism	X		
Aryl hydrocarbon receptor (AhR) activation	X		X
Oxidative stress response (Nrf2 activity)	X	X	X
Immune response (Nfκβ activity)	X		

3.4.2 Reporter gene assay cell lines

As previously described in section 1.5.1 and 3.4.1, in vitro luciferase reporter gene assays were used to assess hormone-mediated effects, ER and AR activity, Aryl hydrocarbon receptor (AhR) activity and oxidative stress response (Nrf2 activation). Two of the reporter gene assays used here have been validated by the Organization for Economic Co-operation and Development (OECD) and official guidelines exists for the ER agonist activity assessment (OECD TB 455) and AR agonist assessment (OECD TG 458). The experiments was in principle conducted in accordance with above-mentioned guidelines, with minor modifications. For each assay, the associated mammalian cell line transfected with the assay specific response element is described as follows:

Androgen receptor (AR) activity was assessed using 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 with a glucocorticoid receptor knockout, commonly referred to as the AR-Ecoscreen GR-KO^- M1 cell line. Reference compound

was dihydrotestosterone (DHT). For antagonist mode reference compound was hydroxyflutamide (OHF). The cells were acquired from Hiro Biotec, Japanese collection of research bioresources cell bank (JCRB1761), National institute of biomedical innovation, health and nutrition (Osaka, Japan).

Aryl hydrocarbon receptor (AhR) activity was assessed using the mouse hepatoma Hepa1c1c7 cell line stably transfected with a reporter plasmid of seven copies of the dioxin response elements, commonly referred to as the DR Ecoscreen cell line. Reference compound was 2,3,7,8-tetra-chlorodibenzdioxin (TCDD). The cells were acquired from Hiro Biotec, JCBR cell bank (JCRB1630) as above.

Estrogen receptor (ER) activity was assessed using the human breast carcinoma cell line MCF-7, stably transfected with estrogen receptor luciferase plasmid, commonly referred to as the VM7Luc4E2 cell line. Reference compound was 17-β-estradiol (E2). For antagonist mode, Reference compound was raloxifene. The cells were donated by the late professor Michael Denison (University of California, USA).

Oxidative stress response (Nrf2 activity) was assessed using the human mammary MCF7-derived reporter cell line which contains eight copies of the rat glutathione-S-transferase (GST) antioxidant response element, commonly referred to as the MCF7AREc32 cell line. Reference compound was tert-butyl-hydroquinone (tBHQ). The cells were gifted from prof. Ronald Wolf (University of Dundee, Scotland).

Immune response (Nfκβ activity), (HepG2-nuclear factor κ-light-chain-enhancer of activated β) was assessed in the human hepatocellular carcinoma cell line, stably transfected with pTA-NFkB-luciferase reporter vector containing 4 repeats of NFkB binding sites, commonly referred to as HepG2-Nfκβ cell line. Reference compound was TNFα. Cells were acquired from Signosis Inc, Santa Clara, California.

3.4.3 MTS and ATPase based assays for cell viability assessment

For all papers, cell viability of all included samples was assessed in parallel to bioactivity assessments. The main purpose of this testing was to ensure that bioactivity analysis was performed under non-cytotoxic conditions as a reduction in viability of the cells could negatively affect the bioactivity output. The two cytotoxicity assays differ in their output, colorimetrics and fluorescence, but are both based on quantifying cellular metabolic activity as a measure of cell viability.

AR, AhR and Nrf2 assays were paired with a MTS based colorimetric assay (Cell Titer 96® Aqueous One Solution Cell Proliferation Assay, Promega, USA). This assay is based on the reduction of a tetrazolium compound, MTS. Viable cells would produce a coloured formazan product, which can be quantified by measuring absorbance at 490 nm. The amount of formazan product, is directly proportional to the amount of living cells.

The ER assay was paired with an APTase based luminescence assay (CellTiter-Glo® Luminescent cell viability assay, Promega USA). This assay determines viable cells by the amount of present ATP. Where ATP catalyses a reaction of luciferin into a luminescent product, oxyluciferin, that emits light at 550–570nm.

3.5 Data Handling

Data processing of bioassay output was principally the same for all three papers. Luminescence output was first adjusted for background activity by subtracting the luminescence raw data of the solvent control of all sample data and positive controls. Onward, the sample data was normalised to assay maximum by dividing the adjusted sample raw data by the adjusted maximum raw luminescence data of the reference compound, and multiplying by a factor 100. In this way, all data points were transformed from raw data to % of assay maximum for each specific experimental trial. This summary is true for all included assessments of ER agonistic activity, AhR activity, and AR agonistic activity. An alternative data processing was performed for all assessments of oxidative stress response, Nrf2 activity, in the three papers as well as Nfκβ assessment, and antagonistic assessments in

Paper I. The alternative data processing was not normalised to assay maximum, but to solvent control. By dividing the raw luminescence data of a sample, to the mean raw luminescence data of the solvent control, the sample data was converted into fold-change bioactivity as compared to solvent control. Handling raw data, data storage, and conversion into % of assay maximum and fold-change output was performed in Excel. Here follows a few key concepts for understanding the data output.

3.5.1 Relative enrichment factors - REFs

The starting point of an investigation of an environmental water sample is an unknown complex mixture of unknown compounds at unknown concentrations. The most fundamental concept in toxicology is that *it is the dose that makes the poison*. To translate the unknown part of an environmental sample into a quantifiable dose or concentration to assess, a nomenclature of relative concentration, or enrichment, of water samples is applied. As previously described in extraction section (3.2), analysing water samples for organic micropollutants using bioassays requires extraction of samples. The relative concentration of the water samples changes from the conditions in the original water sample – Relative enrichment factor 1, REF 1, to 1000 or 2000 times enriched through the SPE process, and finally a dilution step in the bioassay. The highest exposure concentrations was 100, 50 or 20 units of REF in the three papers. A unit of REF above 1 signifies that a sample has been concentrated or enriched as compared to the conditions prior SPE, a unit of REF below 1 signifies that a sample has been diluted as compared to sample conditions prior SPE. As defined by Escher *et al*. 2014 [42] (a-c).

$$REF = enrichment\ factor\ SPE * dilution factor\ Bioassay \quad \text{(a)}$$

$$Enrichment\ factor\ SPE = \frac{volume\ waster\ sample}{volume\ extract} \quad \text{(b)}$$

$$Dilution factor\ Bioassay = \frac{volume\ extract\ added\ to\ bioassay}{exposure\ volume\ of\ bioassay} \quad \text{(c)}$$

3.5.2 Dose-response analysis – sigmodal and linear relationships

Traditionally, dose-response or concentration-response analysis in toxicology involves testing a subject, sample or compound, in a concentration dependent manner to read out a specific effect, to a point where that effect is saturated. This concentration-response relationship can typically be displayed in a sigmodal log(x)-y relationship (left panel, Figure 6). Albeit, there are additional concentration-response curve shapes, but discussing them here exceeds the scope of this thesis. This sigmoidal way of displaying concentration-response relationships is best applied for endpoints that has a strong saturation, or maximum effects, as lethality assessments, or the binding to a receptor in a cell. Other endpoints of more fluent processes, cannot be attributed the same saturation effect, and can typically not be displayed in sigmoidal concentration-response curves. Examples of endpoints with a maximum response in this thesis are the sex hormone receptor mediated activation and Aryl hydrocarbon receptor activation, as there as a limited number of receptors in a cell. The sigmoidal concentration-response relationship can be found displaying the full range bioactivity of the reference compounds of these three endpoints throughout this thesis. However, the sample processing for these three saturation endpoints, the has not been displayed as in full sigmoidal curves, but rather focused on the lower end of the log(x)-y curve, which can typically be displayed in a linear x-y relationship [54]. Examples of a more fluent endpoint is the oxidative stress response (Nrf2 activity), which is not dependent of receptor activation and can therefore not be attributed the same celling of saturation and is typically described directly in a linear x-y relationship [33].

In summary, for all papers in this thesis, a linear concentration-response approach has been adopted to assess sample concentration-response analysis. This was done to avoid interference from cytotoxicity, which could affect the bioactivity output at higher concentrations, in accordance with Escher *et al.* 2018 [54]. For reference compound and determination of assay maximum, the sigmodal approach was applied, as dose-response linearity in not assumed at assay maximum. The concentration-response analysis was performed by plotting the concentrations of the assessed samples (in 2-fold

dilution series) and reference compounds against the normalised % of max luminescence output.

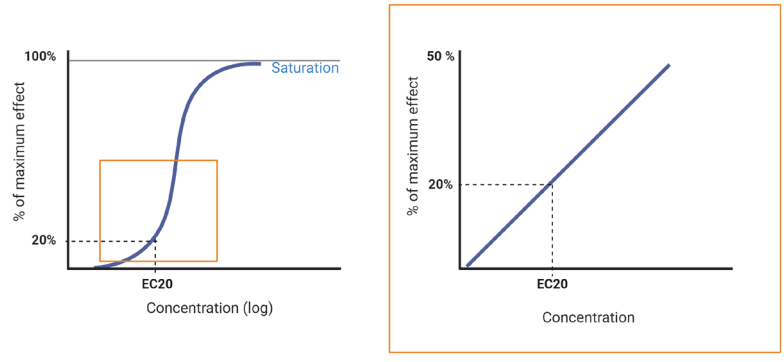

Figure 6: Traditional dose-response relationship displayed as a sigmoidal effect curve (left). Linear dose-response analysis focusing on the lower part of the sigmodal curve (right). Adopted & modified from Escher *et al.* 2018 [54] . Figure was created using biorender.com

3.5.3 Derivation of effect concentrations (EC values)

For all papers, data analysis included derivation of effect-concentrations, or EC values, as a stepping-stone towards achieving bioequivalent concentration (BEQ) values (more details in section 3.5.4 below). Effect-concentrations signifies at which concentration a sample elicits a specific level of effect, i.e. EC_{20} represents the concentration (REF) needed to achieve 20 % of assay maximum. In other contexts, outside this thesis, EC_x could represent the x % of the maximum effect elicited by the subject/compound/sample. However, because the data here is normalised to assay specific maximum (section 3.5), EC_x is x % of assay maximum and not x % of the maximum effect elicited by the sample. Here, EC_x is derived from the linear regression analysis found in the equations below (d-e).

For oxidative stress response (Nrf2 activity), as previously described in 3.5 and 3.5.2, no assay maximum was determined and the output of this endpoint was in fold-change as compared to solvent control. Due to the reactive nature of this endpoint, it is favourable to avoid interference of cytotoxicity, and typically, an induction ration of 1.5 fold change as compared to solvent

control ($EC_{IR.15}$) is utilized as point of departure for sample potency comparison for this endpoint [33].

$$y = kx + m, \quad x = \frac{(y-m)}{k} \quad \text{(d)}$$

$$ECx = \frac{(y-m)}{k} \quad \text{(e)}$$

3.5.4 Bioequivalent (BEQ) concentrations

Bioequivalent concentrations expresses that a given water sample has the equivalent effect of a specific concentration of a reference compound. BEQ values are a continuation of the effect-concentrations of the investigated water samples and are achieved by relating the bioactivity in a sample to that of the reference compound for each specific assay. The effect concentration, at for example 20 % of assay maximum, for the reference compound can be derived to a specific concentration using the plotted dose-response relationship previously explained in Figure 6, section 3.5.2. This EC_x is then divided by the same EC_x of the sample (Figure 7, equation f).

$$Bioequivalent\ (BEQ)\ concentration\ = \frac{EC_{X\ Reference\ compound}}{EC_{X\ Sample}} \quad \text{(f)}$$

Using this approach, without knowing the content or concentration of mixture components a water sample, the biological impact or effect, can be translated into a relatable output that can be readily compared to other water samples. Through the bioassays, we can for example state that a given water sample has the *estrogenic effect* equivalent to x moles of 17-β-estradiol.

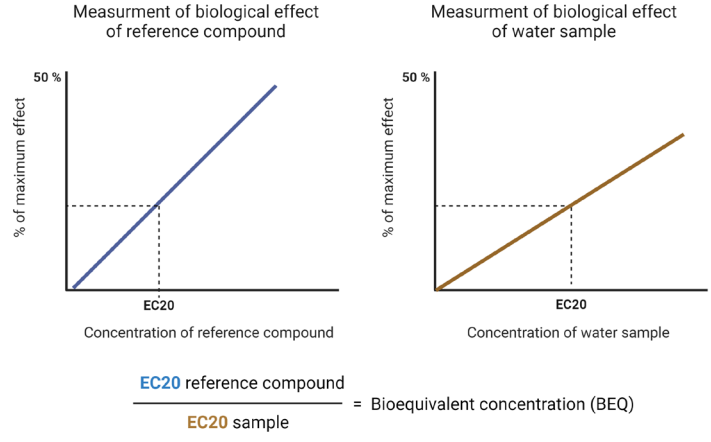

Figure 7: Relating the bioactivity in a water sample to that of the reference compound will derive bioequivalent concentration (BEQ) values. Assessing unknown components and unknown mixture effects of a water sample, through the bioassay, results in a bioactivity-value that signifies an effect equal to a specific concentration of the reference compound. The figure was created using biorender.com

3.5.5 Effect-recovery calculations (Paper II)

In Paper II, effect-recovery of spiked samples was calculated by dividing the BEQ of the spiked sample with that of the nominal spiked concentration prior SPE. The received quotient was then multiplied by 100 to receive a proportional recovery output (equation g).

$$Effect - recovery = \frac{BEQ_{Spiked\ sample}}{Spiked\ concentration\ prior\ SPE} * 100 \qquad \text{(g)}$$

3.5.6 Cumulative BEQ (Paper III)

In Paper III, the cumulative BEQ was calculated by adding the BEQ of bioactive sample fractions.

4. Results & Discussion

4.1 Paper I

4.1.1 Study area and sampling sites

In paper I, water quality was assessed in a full-scale trial of indirect wastewater reuse for drinking water production (Figure 8). As a mean of replenishing the supply for drinking water production, treated wastewater was transported upstream the intake for drinking water production in the city of Barcelona, Spain (Figure 8). To investigate the possibility of reducing the discharge of pathogenic microorganisms into the recipient river, a setup with chlorination of the treated wastewater prior to discharge was tested. Water samples from the full system, at seven sampling points, collected both with and without this additional chlorination treatment. Potential hazards in this type of wastewater reuse into drinking water production is triple. The first hazard is introducing micropollutants due to inadequate wastewater treatment. The second hazard is introducing pathogenic microorganisms due to lack of dilution effect between wastewater outlet and inlet for drinking water production. The final hazard is introducing disinfection by-products into drinking water production due to the additional chlorination treatment step.

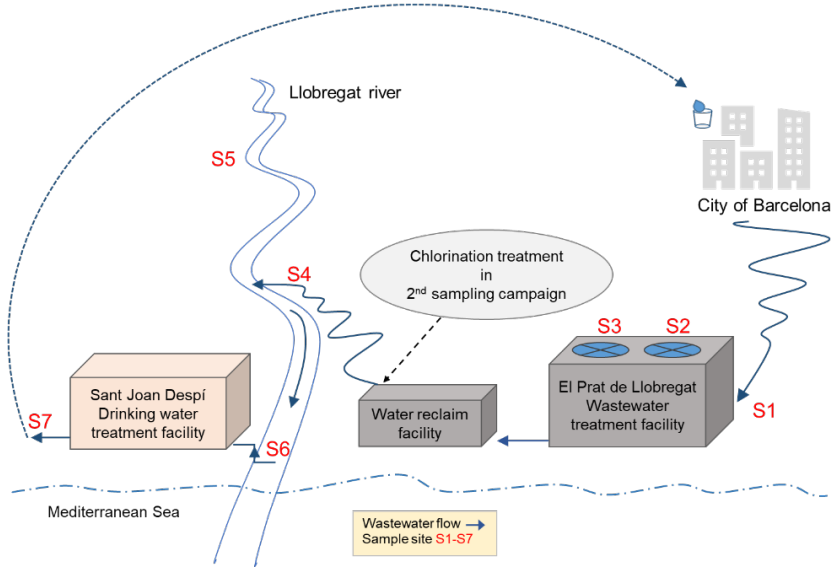

Figure 8: Schematic description of sampling sites of Paper I. The actual distance between S4 and S6 was 8.5 km. Copy from paper I.

The treatment techniques at El prat de Llobregat wastewater treatment facility was of tertiary measures, including biological degradation in nitrification/denitrification and UV-radiation followed by sand filtration. At Saint Joan Depsi drinking water treatment facility treatment steps included one treatment-line of ozonation followed by GAC and one treatment-line of ultrafiltration followed by reversed osmosis and a final disinfection of the joint treatment lines through chlorination.

The bioactivity assessment through the wastewater treatment showed that incoming waters were bioactive for most of the included endpoints in the two sampling campaigns (Figure 9). Subsequent treatment methods decreased the levels of bioactivity sequentially until the end of the treatment train (S1 through S3) for all endpoints except for oxidative stress response (Nrf2) that showed a small increase in bioactivity between S2 and S3 in both sampling campaigns (Table 4, Paper I) which could be an indication of reactive transformation products from the UV-radiation treatment. At the end of the pipeline transport of reclaimed wastewater, at sample site S4, we found three interesting changes in the measured bioactivity as compared to S3. These

changes were attributed to the pipeline transport itself, and to the additional chlorination treatment. These finding are described below in section 4.1.2 and 4.1.3.

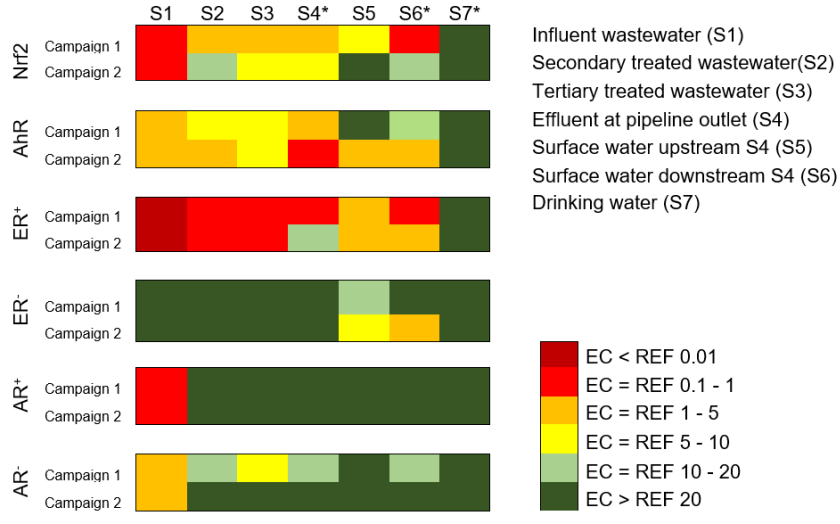

Figure 9: Heat map displaying effect concentrations (EC) as REF at $EC_{I.R1.5}$, EC_{30} (ER^+, AR^+), EC_{40} (AhR), and IC_{30} (ER^-, AR^-). In sampling campaign 2, sample S4, S6 and S7 were affected by chlorination treatment (*). The colour gradient was set between REF 0.01 and REF 20. Copy from Paper I.

4.1.2 Effects of chlorination of treated wastewater

First of all, we could not observe an increase in oxidative stress response after chlorination treatment (campaign 2, S3-S4 comparison, Figure 8-9) as the difference in oxidative stress between these sampling sites was also present and slightly more pronounced in sampling campaign 1, without disinfection. Previous research has shown the formation of certain disinfection by-products to increase with increasing chlorine dose [55]. To achieve actual disinfection of a treated water, there needs to be a residual amount of free chlorine to elicit disinfection (i.e. a dose beyond breakpoint chlorination). The dose applied in this study (13 mg Cl_2 L^{-1}) was below breakpoint of the effluent wastewater (experimentally measured at 30 mg Cl_2

L^{-1}) and we hypothesised that there might have been a stronger bioactivity response for oxidative stress, had the chlorine dose been higher. A parallel, co-current, study of the same wastewater reuse trial, not previously discussed in the published version of Paper I, shed some light on the microbial presence as well as disinfection by-products after the applied chlorination treatment [56]. Through wastewater treatment, this study showed that some microbial presence (*Escherichia coli* and somatic coliphages) was reduced by the applied chlorination treatment, while a third microbial (*Clostridium perfringens*) had better clearance through wastewater treatment without the additional disinfection step [56]. Once the treated effluent reached the recipient river, no difference in microbial presence could be detected between the samplings campaigns. The authors conclude that the microbial impact of the river water masked the effects of the additional chlorination treatment of effluent wastewater. In the same study, targeted chemical analysis was also performed, and it could be concluded that some pollutants were removed by the chlorination treatment, however it was also concluded that disinfection by-products was formed in the process [56]. The latter was also found in a study previously mentioned in Paper I [57]. However, we concluded that these by-products were seemingly not present in high enough concentration, or was not able, to activate an oxidative stress response through the Nrf2 pathway.

Secondly, a surprising change in bioactivity between S3 and S4 was the fact that chlorination treatment seemed to have reduced the estrogenic activity. There was a reduction in estrogenic bioactivity between sample site the S3 and S4 (Figure 9) following chlorination treatment. Previous studies has in line with our results, shown a reduction in estrogenic activity in wastewater post chlorination and/or UV-treatment [58-61]. Additionally, a more mechanistic study have shown that the structure of estrogenic compounds are susceptible for oxidation by chlorination treatment [62]. There was also a reduction in estrogenic activity between the same sample sites, S3 and S4, without chlorination treatment in the first sampling campaign. However, it was a comparably much less pronounced difference.

4.1.3 Effects at the end of the pipeline

A significant finding related to the pipeline transport of the treated wastewater was that AhR activity markedly increased at sample site S4 as

compared to S3 in sampling campaign 2, following chlorination treatment. The TCDD equivalent concentration even surpassed the bioactivity found in the untreated incoming wastewater (S1). In the published paper, we state that an increase in AhR activity after chlorination treatment had not previously been reported on. However, when writing this thesis, new information was achieved. A study from 2015 had found an increase in AhR activity post chlorination- and UV-treatment [63]. The authors suggests that a common compound found in wastewater, Tricolsan, could form dioxin-like structures during chlorination treatment, and could thus activate the AhR via transformation products, as proposed by Buth *et al.* 2011 [64]. Tricolsan has previously been detected in the Llobregat wastewater system [65] and in addition to what was previously discussed in Paper I, this could be an alternative explanation for the increase in AhR activity after chlorination treatment. In paper I, we hypothesised that the chlorine treatment triggered release of AhR inducers from biofilm within the pipeline. Another reason for the increase in AhR activity at the end of the pipeline could be that there was a diffuse source of AhR inducers with the pipeline system as there was also an increase in AhR activity between S3 and S4 without chlorination treatment in campaign I, albeit the increase was comparably not as pronounced.

4.1.4 Indirect reuse of wastewater for drinking water production

The final and overarching finding from this water quality assessment was that drinking water was not affected by reclaiming wastewater in this trial, nor by the additional chlorination treatment step. Despite differing levels of bioactivities in the incoming water to the treatment plant (Figure 9, S6), little remained once the water went through drinking water treatment (S7). There was detectable bioactivity of oxidative stress, AhR activity in both campaigns in the finished drinking water but at such high sample enrichment it was not a cause for concern.

4.1.5 Bioactivity in blank samples

Another important finding of Paper I, was the fact that SPE blank samples from the two campaigns was bioactive, primarily for ER agonistic activity and AhR activity, and just above the cut-off for bioactivity for oxidative stress response at the highest tested concentration (REF 20). One of the main

objectives of the study was to use Nrf2 activity as an indicator for disinfection by-products with and without chlorination treatment. Having bioactive blanks could mean that the sample preparation process contaminated the samples with compounds causing oxidative stress and thereby increase the risk of false positive results. However, as the bioactivity in the blanks was similar in the two sample sets, for oxidative stress response as well as ER and AhR activity, we concluded that the intra-study comparison of the samples could still be made. However, some caution needs to be applied when comparing our results with other studies.

Including procedural blanks in effect-based studies is important, to ensure that the results are reliable. One strategy to deal with bioactive blanks is to compensate the sample data by subtracting the activity in the blank. In a study like Paper I, where different water types with different sample volumes and maximum exposure concentrations was assessed, this strategy was not applicable. In retrospect, including one procedural blank per water type could have been a way to circumvent this issue, or having a blank sample representative of the highest sample enrichment included in the study. The bioactivity in the procedural blanks in paper I was onward, the seed of the investigation purpose of Paper II, as we found little was published on the matter of bioactive blanks in effect-based assessments.

4.2 Paper II

In Paper II, four SPE methods were chosen from literature of previously published studies using *in vitro* bioassays for water quality assessments. The aim was to investigate procedural blank effects, as well as recovery of bioactivity through the different methods. When assessing complex environmental samples with unknown content, the true recovery of bioactivity can of course not be known, but an intra-comparison of possible recovery differences between methods can be made. Additionally, a spiked sample using compounds with known bioactivity was included to compare how the different SPE methods would recover bioactivity from spiked compounds.

4.2.1 Bioactivity in blank samples

Blank experiments showed that two of the included SPE processes, LIC and ML, contaminated the extract with estrogenic compounds (Figure 10). The

estrogenic bioactivity for the Milli-Q water processed over the ML column was similar in Paper II, as was seen in Paper I. The spiked water sample from the LIC SPE process also greatly exceeded the bioactivity of was expected of the spiked 17-β-estradiol. There are several routes of explanation for these blank observations. Firstly, the bioactivity could stem from the sorbent material in the SPE column. Secondly, it could be that the migration of bioactive compounds are stimulated to a higher degree when processing ultrapure waters like Milli-Q water, as seen in LIC blank and Spiked sample (Figure 10). These two mechanisms could also work in chorus of course.

While it has been long custom to process ultrapure water samples alongside environmental samples as procedural control, for effect-based assessment of water quality as well as chemical analysis, discussions of what constitutes a true blank sample are being held in the community. A drawback of using pure water samples (as Milli-Q water or ultrapure water) as process blanks is that they differ in ionic strength as compared to environmental samples, and might interact differently with the sorbent material in the SPE cartridges. The interaction could occur in such a way where the pure water matrix is comparably too empty, that it could create a concentration gradient of impurities into the sample from the sorbent material. Contrary, a drawback of using any other type of water as process blank with higher ionic strength, containing more complex matrix, as tap-water for example, is that it is known to contain micropollutants, and could also trigger bioactivity in applied bioassays. To circumvent this problem, another option would be to use an ultrapure water but to balance it by adding salts, and evaluate that this additive would not cause bioactivity by testing the salt addition in cell-culture medium separately.

In studies investigating effect-recovery of known compounds through SPE processes, spiking is sometimes performed using environmental water sample as vessel or matrix. i.e. the bioactivity in an environmental sample could be compared to the bioactivity in the same sample that has added compounds of known concentrations. However, there are drawbacks of that kind of investigation too, as it is not certain how the unknown complex mixture in the environmental sample would interact with the spiked chemicals (more on this in paper III). As previously mentioned, an existing strategy for accounting for bioactive procedural blanks is to subtract or

correct the raw data of the sample, for the bioactivity in the blank. Controversy, then if correcting the data, one implies that whichever matrix one has chosen as a blank representative, pure-, tap- or other, is equal in its matrix as the environmental sample. To ponder about a true representative blank sample for effect-based assessments, the ideal process blank or vessel for spiking for complex environmental mixture assessments would perhaps be a site-specific effluent wastewater treatment sample that was known to be clear of micropollutants. However, I do not know such a water to exist.

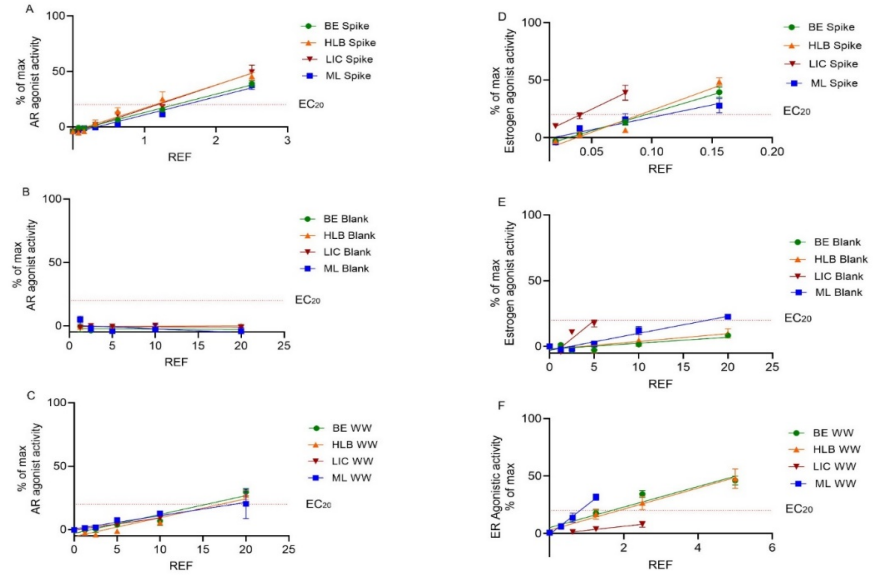

Figure 10: Concentration (REF) – response (% of max activity) assessment of the AR agonist (A, B, C) and ER agonist (D, E, F) bioassays. Bioactivities are shown of 3 samples: SpikeE2,DHT (A, D), Milli-Q water blank (B, E) and effluent wastewater (WW) (C, F) extracted using four SPE procedures BE, HLB, LIC and ML. Activities of samples (n=4 per concentration) are displayed as % of assay maximum (mean±SD), of reference compound for each assay (DHT and E2 respectively). Data points above ~40 % of max were removed to enable analysis through linear regression. Red dotted line shows the cut-off for bioactivity at 20% of assay maximum (EC_{20}).

Table 2: Summary of effect-concentrations, bioequivalent concentrations (BEQ) and effect recovery of assessment of AR-, and ER- agonist activity

SPE method		BE				ML				HLB				LIC			
Bioactivity output		EC_{20} (REF)	BEQ (pM)	BEQ (pg/L)	R (%)	EC_{20} (REF)	BEQ (pM)	BEQ (pg/L)	R (%)	EC_{20} (REF)	BEQ (pM)	BEQ (pg/L)	R (%)	EC_{20} (REF)	BEQ (pM)	BEQ (pg/L)	R (%)
AR Activity	Spiked Milli-Q	1.4	40		81	1.6	37		74	1.1	50		100	1.2	49		98
	Milli-Q Blank	> 20	< 2	< 580		> 20	< 2	< 580		> 20	< 2	< 580		> 20	< 2	< 580	
	Wastewater	15	3	870		19	2	725		17	3	790		> 20	< 2	< 680	
ER Activity	Spiked Milli-Q	0.09	26		132	0.11	23		113	0.09	28		139	0.04	61		307
	Milli-Q Blank	> 20	< 0.2	< 45		18	0.2	50		> 20	< 0.2	< 45		6	0.6	160	
	Wastewater	2	1.5	420		0.8	3.1	852		2	1.4	375		7	0.4	104	

Recovery of bioactivity (R) = (BEQ/Spiked concentration x 100)
Spiked concentration was 50 pM DHT and 20 pM E2

Table 3: Summary of effect concentrations, bioequivalent concentration (BEQ) and recovery in assessment of Nrf2 activity

SPE method		BE				ML				HLB				LIC			
Bioactivity output		$EC_{IR1.5}$ (REF)	$EC_{IR1.5}$ (µM)	BEQ (µM)	R (%)	$EC_{IR1.5}$ (REF)	$EC_{IR1.5}$ (µM)	BEQ (µM)	R (%)	$EC_{IR1.5}$ (REF)	$EC_{IR1.5}$ (µM)	BEQ (µM)	R (%)	$EC_{IR1.5}$ (REF)	$EC_{IR1.5}$ (µM)	BEQ (µM)	R (%)
Nrf2 activity	tBHQ spike	17		0.27	27	16		0.27	27	2.6		1.36	136	> 20		< 0.26	< 26
	tBHQ standard		4.6				4.6				3.5				5.2		
	Timeline tBHQ		4.3				4.7				4.3				5.9		
	Milli-Q Blank	> 20		< 0.26	< 26	> 20		< 0.26	< 26	> 20		< 0.26	< 26	> 20		< 0.26	< 26

Recovery (R) = ($EC_{IR1.5}$/Spiked concentration) x 100

4.2.2 Effect-recovery of wastewater and spiked sample

The assessment of spiked DHT showed similar recovery of AR activity for the included SPE methods, which was all within a 100 ± 30 % range (Figure 9, Table 2). The estrogenic effects of spiked E2 was also recovered successfully in three out of four SPE methods, at a range of 100 ± 40 % but was greatly exceeded, at 300 %, using the LIC SPE method. The assessment of wastewater showed similar estrogenic bioactivity in two of the included methods, HLB and BE. However, comparably the ML SPE process showed higher estrogenic activity, and LIC showed lower estrogenic activity (Figure 9).

4.2.3 Recovery of oxidative stress response and spiked tBHQ

In a separate investigation in paper II, effect recovery of spiked tBHQ was assessed along with blank sample evaluation for oxidative stress response. tBHQ has been claimed to be a poor choice of reference for oxidative stress response due to variability of potency. The critique has been that using it as reference, could yield unstable results that was sensitive to handling processes and process time from preparation of stocks to time of exposure [33, 66]. We could show that prepared tBHQ stocks (timeline tBHQ, Table 3) and spiked tBHQ SPE extracts had stable bioactivity up to 34 days after sample preparation, i.e. the spiked sample and the timeline tBHQ showed stable $EC_{IR1.5}$ after 34 days of storage (Paper II supplement figure S4). Out of the four SPE methods assessed, only one, HLB, could successfully recover bioactivity from spiked tBHQ.

4.2.4 Summary of effect-recovery in Paper II

In summary, out of the four included SPE methods the results from the whole AR assessment did not differ much in three (BE, ML, HLB) of the included methods. Thus using any of the methods appears feasible for the purpose of investigating AR activity. However, bioassays are usually performed in battery, and the results varied more for estrogenic activity assessment as well as oxidative stress assessment. Additionally, we have the previous experience of bioactivity in procedural blanks from the ML SPE method in Paper I. Our collected viewpoint from Paper II, was that HLB would be the most appropriate choice of sample preparation method for a bioassay battery set-up as was used here.

4.3 Paper III

Environmental water samples and wastewater effluents are known to contain complex mixtures of thousands known and unknown organic micropollutants. While single pollutants are most often present at very low concentrations, perhaps even below their limit of detection, the effect of the mixture they comprise can still be a cause for concern [67, 68]. In a mixture, chemicals can act independently and simply add to the effects caused by another chemical, or they can interact, and either inhibit the effect caused by another chemical (antagonism) or enhance (synergism) the effect caused by another chemical. When it comes to mixture assessment, many studies have investigated how artificial mixtures of a limited numbers of compounds interact [69, 70]. However, investigations on true complex mixtures as they appear in the aquatic environment is not as commonly published. In a typical effect-based assessment using reporter gene cell lines, the cumulative effect of the different components of a mixture, whether additive, antagonistic or synergistic, cannot be separated, but is displayed as a net effect. A strength of an effect-based assessment to reflect the net exposure as is in the environment. However, in order to reveal more details of the make-up of the detected sum bioactivity, it is necessary to reduce sample complexity. By splitting up environmental sample components using liquid chromatography refined mixture components can be made visible.

In paper III, extracted (SPE) wastewater effluent was fractioned into 54 sample fractions using high-performance liquid chromatography (HPLC or LC) and assessed for mixture toxicity by comparing the bioactivity in the whole sample to the bioactivity in the sample fractions (Figure 11). The whole sample and sample fractions were assessed for AR, ER, AhR and Nrf2 bioactivity. Samples was part of a collaboration project and was fractioned for bioanalysis parallel with LC-MS analysis, and was therefore assessed in pairs of two solvents, using two differing mobile phases for positive and negative ionisation mode in the LC-MS. Onward, the two version of samples are referred to as simply positive and negative mode solvents.

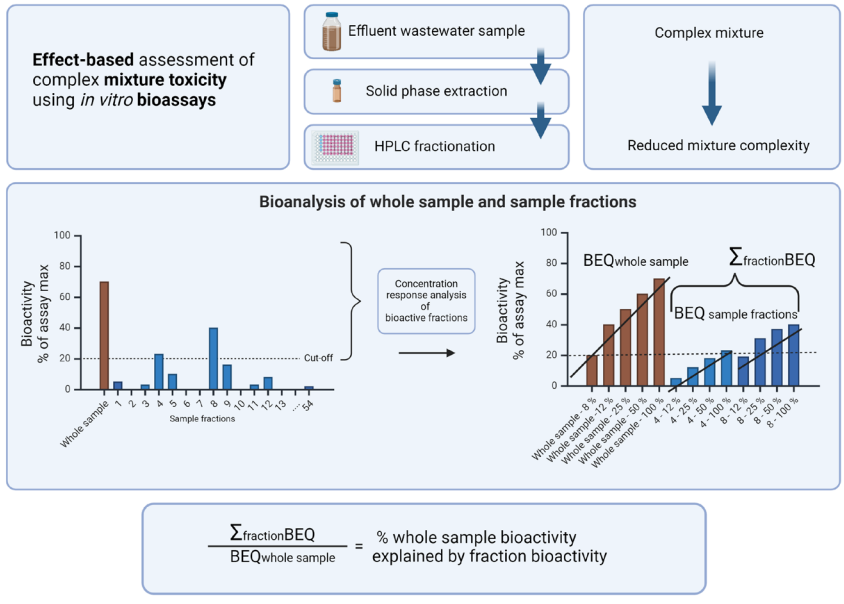

Figure 11. Graphical representations of the experimental set-up in Paper III. Treated wastewater effluent was extracted using solid-phase extraction and the extract was then fractionated using HPLC, into 54 fractions, to reduce sample complexity. Bioactivity was then assessed in the whole, unfractioned SPE extract, sample and in the sample fractions to evaluate if the sum bioactivity in the fractions could explain the bioactivity seen in the whole sample.

4.3.1 Estrogenic and Androgenic mixture toxicity

The assessment of Estrogen receptor activity analysis showed strong bioactivity for the whole sample, with a BEQ of 26 pM E2 equivalents. The sample fractions showed bioactivity above 20 % of assay maximum in two fractions in the positive mode solvent and five fractions in the negative mode solvent (figure 12). We hypothesised that the difference in number of active fractions could be due to a comparably lower resolution in chromatographic separation in the positive solvent. However, when comparing the cumulative BEQ of sample fractions with the BEQ of the whole sample, it was quite similar at 47 % in negative - and 61 % in positive solvents. We hypothesised that the overall loss of bioactivity in the sample fractions could be explained by synergistic mixture effects, giving the whole sample a stronger estrogenic potency when the mixture components are present together. Another

hypothesis for bioactivity loss in the sample fractions was the spread of low potency estrogenic compounds into several fractions, thus excluding them from detection in the sample fractions.

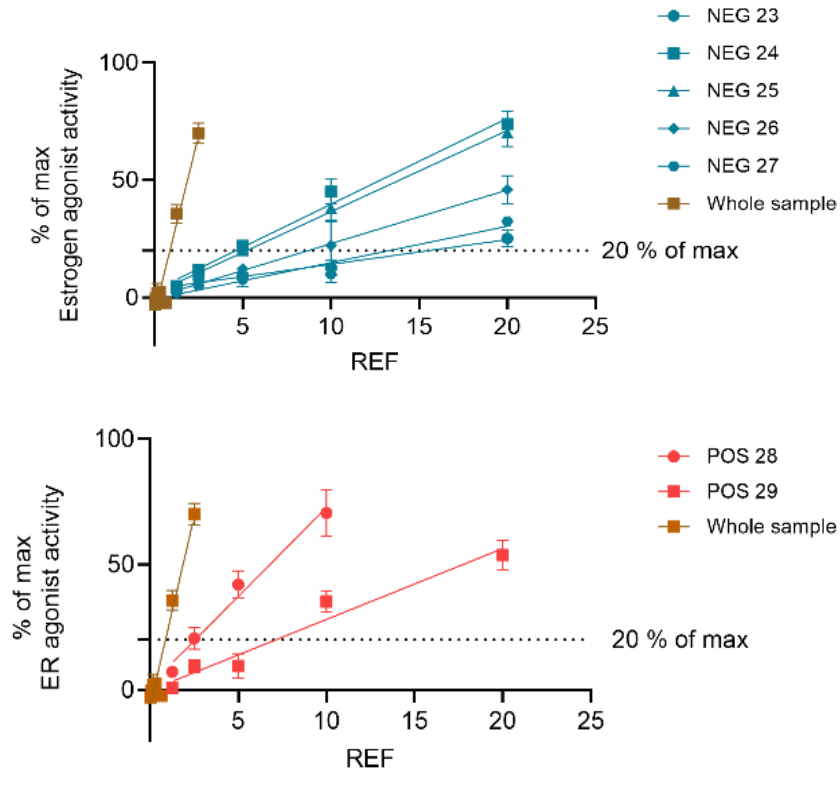

Figure 12: Concentration (REF) - response (% of assay maximum activity) assessment of ER agonist bioassay. Bioactivities are shown for negative solvent active fractions (top) and positive solvent active fractions (bottom). Activities of samples (n = 4 per concentration) are displayed as % of assay maximum (mean±SD) determined by 17-β-estradiol. Dotted line shows the cut-off for bioactivity at 20% of assay maximum.

For AR activity, the whole sample was only borderline bioactive and thus conclusions about mixture toxicity could not be made. Previous samples effluent samples from the same WWTP in Uppsala has shown similar

bioactivity for AR agonism. Another study on AR mixture toxicity fractioned effluent wastewater into 288 fractions, and while their whole sample was not bioactive, sample fractions were [2]. Highlighting the importance of antagonistic masking for this endpoint, as antagonistic bioactivity can be more prevalently found in wastewater as compared to agonistic effects [42]. The same point was made in yet another study where fractioned wastewater into 30 fractions and the cumulative DHT BEQ of the fractions exceeded that of the whole sample [71].

4.3.2 Oxidative stress and AhR mixture toxicity

For AhR activity assessment the whole sample showed bioactivity but no fractions showed any detectable bioactivity. The effect in the whole sample thus seems to be an additive or synergistic mixture effect of several active compounds that were separated into different fractions during LC.
For both AhR and Nrf2 activity, the bioactivity in the whole sample could not be detected in the sample fractions, indicating that both these endpoints are strongly dependent on mixture effects.

4.3.3 Summary of mixture toxicity assessment

It is known how pollution of the aquatic environment occurs through low-level pollution of countless chemical compounds. Many identified single compounds occur well below exposure limits and guidance values set out for human and animal health. Although the mechanisms of mixture toxicity in such complex matrixes as environmental water samples are difficult to disclose, the overarching image is that it really is the mixture of compounds, known and unknown, that threatens global water quality [67-70, 72]. Our results here are in line with the bulk of existing effect-based environmental mixture assessments. Where in some instances drivers behind specific effects are successfully narrowed down to certain degrees of explanation, mostly for endpoints of high specificity as sex hormone mediated effects. What seems more constant in the published literature is the unexplained mixture effects typically depicted in endpoints triggered by bigger groups of compounds as the oxidative stress response and aryl hydrocarbon receptor mediated effects.

5. General conclusions and future perspectives

In the papers comprising this thesis, effect-based methods have been a valuable tool to investigate unknown components and mixture effects of contemporary pollution of the aquatic environment. In Paper I, we were able to perform an evaluation of treatment efficiency in a full-scale trial of indirect potable reuse of treated wastewater. The effect-based assessment could contribute with novel insights into unforeseen effects from the included treatment processes. Firstly that chlorination treatment seemed to reduce estrogenic bioactivity and secondly that the transport system, the pipeline, seemed to be a source of AhR inducing compounds. A third highlight from paper I, was perhaps the most positive one, that reclaiming treated wastewater for drinking water production did not seem to negatively affect the drinking water quality. A hopeful insight into future work on sustainable water usage in the face of global climate change.

In Paper II, sample preparation methods commonly applied both for effect-based and chemical water quality analysis, evaluated for effect-recovery and bioactivity in procedural blanks. Sample preparation procedures plays a vital part in ensuring reliable results of any water quality assessment for micropollutants presence. We could show that, depending on the composition of the battery of bioassays used for water quality assessment, some sample preparation methods were more applicable than other was.

In Paper III, effect-based methods were applied to elucidate mixture-effects of a real life complex mixture. By fractionating treated wastewater effluent into 54 fractions using chromatographic separation and comparing the bioactivity in the whole sample with that of the fractions mixture, we could show that mixture effects due to sample complexity was a dominant feature for AhR activity, Oxidative stress response, and partly also for ER activity.

The load of micropollutants in the aquatic environment is often described as an iceberg, with known compounds with known effects depicted as the tip of the iceberg, and unknown compounds with unknown single and mixture effects as the submerged part of the iceberg (Figure 1). Legislation in the work towards a non-toxic environment is based on the tip of the iceberg and

is lacking in the sense that it is mostly based around single chemical exposures and is not considering a realistic complex mixture exposure. With advancing analytical methods and growing efforts to combine effect-based analysis with chemical analysis, the known part of the iceberg slowly grows bigger, and can occasionally lead to a lowered tolerance in legislation towards acceptable levels of specific substances. Kortenkamp 2023 discusses that levels that was considered "safe" only recently can be revaluated and drastically change with progressing research and highlights the radical 20 000-fold drop in tolerable exposure limits for bisphenol A (BPA, an estrogenic compound) suggested by the European Food Safety Authority in 2023. Another example, also brought to the public eye in Sweden is the lowered acceptable limits per- fluorinated alkyl (PFAS) substances in drinking water, that many drinking water treatment facilities will have trouble meeting. As Kortenkamp 2023 writes, I too would like to stress the fact that in the progression of developing legislation for a non-toxic environment there has never been an instance where advancing knowledge has led to a correction upwards, a softening of tolerance, for specific substances. Rather it seems that the more we learn about the existing low-level pollution of the aquatic environment and about complex mixture toxicity, the greater the consequences are attributed to this type of exposures for aquatic organisms and for human health aspects. Hopefully effect-based analysis can continue to gain weight as an valuable tool in assessing water quality and contribute to a more holistic approach in understanding and combating pollution of the aquatic environment.

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Popular science summary

Thousands of chemical pollutants can be found in the aquatic environment. Remains from pharmaceuticals that we ingest and excrete, are flushed down our wastewater drains along with many kinds of everyday chemicals that are used in our homes and in industrial settings. Water-repellent coating of textiles and clothes, flame retardants protecting various household electronics from catching fire, softeners in plastic materials, just to mention a few different classes of substances. The occurrence of man-made chemicals that were created to have a biological effect, like pharmaceuticals, are especially problematic when they end up in the aquatic environment, as organisms living in and around water are at risk of being exposed to unwanted levels of bioactive substances. Humans are also at risk of being exposed to unwanted levels of substances, as polluted surface water could be used as a source for drinking water production.
One source of pollutants in the aquatic environment is incomplete wastewater treatment. Conventional wastewater treatment was first designed to combat excessive nutrient influx, eutrophication, of waterbodies receiving the wastewater, and was therefore not designed to remove more things than excess nutrients from the incoming water. Today, it is well known that treated wastewater can cause negative effects in wildlife living in water bodies receiving the wastewater because of the pollutants that slip through treatment. More advanced treatment methods have been and are continuously being implemented within the EU and elsewhere, to combat the pollution that occurs through incomplete wastewater treatment.

In the face of climate change and increasing water scarcity, reusing treated wastewater for agricultural or drinking water purposes would be an act towards a more sustainable freshwater usage. However, that kind of

wastewater reuse can be problematic from a human health perspective in several ways. The biggest hazard is introducing illness via food or water intake due to microbial contamination from the wastewater. A second hazard can be because of the unknown impact of incomplete removal of pollutants in the treated wastewater.

Assessing the biological impact of the pollutant content of a water sample, cannot be done with chemical analysis alone, as chemical analysis only detects chemical components, but tells little about their effects. Additionally, there are many thousands of compounds known to be present in environmental water samples, making it an impossible task to target all of them using chemical analysis.

As a complement to chemical analysis, using an analytical method that focuses on the biological effect of the sum of all pollutants in a water sample, can be helpful when trying to unveil the consequences of the burden of pollutants in a water sample. These kinds of analyses are called effect-based methods. A specific type of effect-based method involves cultured cells that have been modified to produce a luminescent light signal, if a type of pollutant is present in a water sample. Due to the reporting light signal, the cells are described as carrying a reporter-gene. The reporter-gene cells are cultured in a lab and come in different variants, or cell lines, each made to detect different classes of pollutants. An example of a reporter gene cell line are cells made to detect estrogenic compounds, that is, compounds that can act on the estrogen-response system in living organism. Other examples of reporter gene cells are cells that can detect compounds that act on the androgen-response system, the oxidative stress response-system and on the aryl hydrocarbon receptor (a common receptor for detecting a broad range of environmental pollutants). By using a range of reporter-gene cell lines together when investigating one water sample, something can be learned about the total impact the different kind pollutants might have on an organism exposed to that water.

In this thesis, several reporter-gene cell lines were used to investigate how effectively different kinds of wastewater treatment methods could remove the effects detected by the cells, as a measure of how well pollutants were removed from the water. Reporter-gene cell lines were also used to compare

how different water sample preparation methods would affect the effects detected by the cells, as a measure of how well each method could collect, or extract, pollutants from a water sample. Lastly, reporter-gene cell lines was used to investigate how the effects of a complex mixture of many pollutants was changed when the sample was split into many new, less complex sample fractions.

In the first study, a Spanish water agency wanted to test how drinking water quality would be affected, when treated wastewater was to replenish the river where raw water for drinking water production was sourced. Samples were taken from untreated incoming wastewater, through several treatment steps, out in the receiving river, as well as in finished drinking water. We found that the quality of the finished drinking water was not negatively affected by reusing the wastewater in this specific water system. A hopeful result in future efforts to develop more sustainable water usage.

In the second study, four different sample preparation methods was compared by investigating a wastewater sample, a blank sample as a measure of process contamination, and a sample spiked with compounds known to trigger a response in the reporter-gene cells. From the blank sample investigation, we found that some methods showed too high background activation of the estrogenic reporter-gene cells, indicating that the sorption material in the specific method contaminated the blank sample with compounds that could act on the estrogen receptor. From the investigation of the spiked sample, we found that the same method that showed high blank sample activation of the reporter-gene cells also showed too high activation from the spiked estrogen, again indicating contamination of estrogenic compounds from the sample preparation process. From the investigation of the wastewater sample, we found that two methods showed similar estrogenic results, while the other two methods indicated lower and higher activation of the estrogenic reporter-gene cells, respectively. The results from the four methods in the androgenic reporter-gene cells showed similar results in all three sample types, indicating that the choice of sample preparation method was less important for achieving stable results.

In the third study, an unknown complex mixture of pollutants, a treated wastewater sample, was used to investigate how important the sum mixture

of pollutants could impact the biological effect of the pollutants present in the sample. By splitting the unknown constituents of the sample into 54 *new* samples using liquid chromatography, and comparing the bioactivity in the whole sample with that of the sample fractions, we could investigate if the sum of bioactivity in the fractions was equal to that of the whole sample. If the sum bioactivity is less as compared to the whole sample, it can be concluded that the sample constituents are not as potent alone as they are together, which means that it is the mixture of different compounds acting together that is creating the hazard. In this complex mixture study, four reporter-gene cell lines was used and it was only the estrogenic-reporter gene cells that was activated both by the sample fractions and the whole sample. The sum estrogenic bioactivity was roughly 50 % of what was found in the whole sample. This meant that to a 50 % degree, the bioactivity of few or single compounds in the sample fractions could account for the bioactivity in the whole sample. The rest of the bioactivity in the whole sample was attributed to compounds having a stronger effect as they appeared together in the mixture, i.e. dependent on mixture effects. For the other reporter cell lines included in this study, no bioactivity could be detected when the sample was split up into sample fractions. That meant that the effects seen in the whole sample when using the oxidative stress reporter cell line and the aryl hydrocarbon receptor cell line were strongly dependent of mixture effects. The bioactivity in the whole sample, when using the androgenic cell line, was too weak to conclude anything about the difference between the whole sample and the sample fractions.

In summary, the work in this thesis has highlighted that choosing an appropriate sample preparation method is important for gaining knowledge of pollution of the aquatic environment. This thesis has also highlighted that for several endpoints, it is really the mixture of many different pollutants that is the main challenge of water pollution. Finally, there was an example of how treated wastewater, could be reused for sustainable drinking water production without carrying hazards into the finished product.

Populärvetenskaplig sammanfattning

Tusentals kemiska ämnen förekommer i den akvatiska miljön. Läkemedelsrester som vi människor intar och utsöndrar spolas ned i avlopp tillsammans med många andra typer av kemikalier som används i hushåll och inom industrier. Kemikalier används för att täta och skydda kläder, material och elektronik, göra material mjuka och behagliga eller på många annat sätt optimera och förenkla vardagen i ett modernt samhälle. Spridningen av dessa kemikalier från dess tilltänkta syfte ut till vattenmiljön, blir särskilt problematisk för de klasser av ämnen som är tillverkade med avsikt att ha en biologisk effekt, till exempel läkemedel. Då djur och växter som lever i eller runtomkring förorenat vatten riskerar att bli exponerade för ämnen som kan ge oönskade biologiska effekter. Även människor riskerar att drabbas negativt av oönskade kemikalier då förorenat ytvatten kan användas för dricksvattenproduktion.

En stor källa till förorening av vattenmiljön är ofullständig rening av avloppsvatten. Traditionell rening av avloppsvatten blev en gång tillämpad för att främst råds bot på övergödning av sjöar och vattendrag som tog emot avloppsvatten. Det inflöde av näringsämnen från orenat avloppsvatten till vattenmiljön skapade rubbningar i ekosystemen och reningen av avloppsvatten ämnade främst att ta bort kväve och fosfor från det behandlade vattnet. Därmed är traditionell rening av avloppsvatten inte utformad att ta bort *fler* ämnen från det behandlade vattnet än just oönskade näringsämnen. Idag är problematiken kring utsläpp av förorenat avloppsvatten välkänd och synlig genom grön lagstiftning inom EU och i andra delar av världen.

I klimatförändringarnas spår och den i globalt ökande bristen på färskvatten vore återanvändning av renat avloppsvatten ett mer hållbart sätt att hushålla med begränsade vattenresurser. Återanvändning av renat avloppsvatten för jordbruksbevattning eller dricksvattenproduktion medför dock flera farhågor. Den första faran är att sprida sjukdomsframkallande mikroorganismer från avloppsvattnet till grödor eller in i dricksvatten. En annan fara kan vara att sprida oönskade biologiska effekter från kemiska ämnen som passerat igenom en ofullständig reningsprocess.

Att förstå betydelsen av föroreningen av vattenmiljön genom ofullständigt renat avloppsvatten går inte att göra med enbart kemisk analys. Riktad kemisk analys kan ge information om vilka typer av ämnen som finns i vattnet, men ger ingen ytterligare information om vilken biologisk eller toxikologisk betydelse den mängd eller sammansättning av ämnen som är närvarande ger. Som ett komplement till kemisk analys kan man använda en effektbaserad analytisk metod. Effektbaserade metoder används för att se till helhetsbilden av betydelsen, eller effekten, av summan de föroreningar som kan finnas i ett vattenprov. En specifik effektbaserad metod innefattar odlade celler som har modifierats att utge en ljussignal då de utsätts för ett specifikt ämne i ett vattenprov. På grund av denna ljussignals-rapportör benämns dessa typer av celler att bära på en reportergen och hela analysmetoden kallas reportergenanalys.

Reportergenanalyser finns i många olika odlande celltyper och kan vara designade att upptäcka och rapportera, olika typer av kemiska ämnen. Två exempel på reportergenanalyser är celler som är konstruerade att upptäcka östrogena ämnen, alltså ämnen som kan verka på det kvinnliga könshormonsystemet i en organism eller androgena ämnen, ämnen som verkar på det manliga könshormonsystemet i en organism. Andra exempel på reportergenanalyser är celler som är gjorda att upptäcka reaktiva ämnen som skapar oxidativ stress, eller ämnen som binder till dioxinreceptorn (en receptor som vanligtvis används som indikator för att upptäcka en viss typ av miljögifter). Genom att använda flera olika reportergenanalyser för ett vattenprov kan man försöka återge den sammanslagna effekten som en organism skulle utsättas för om exponerad för ett specifikt vattenprov.

I denna avhandling har reportergenanalyser används för att undersöka hur effektivt olika typer av vattenreningsmetoder har kunnat rena effekterna som detekterats av cellerna, som ett mått på hur väl föroreningar renats från vattnet. Reportergenanalyser har också används för att undersöka hur förberedande steg i processen att ta ett vattenprov från miljön till att exponera reportergen-celler kan påverka utkomsten av analysen, som ett mått på hur väl olika metoder kan samla upp de ämnen som finns i ett vattenprov. Slutligen har reportergenanalyser används för att undersöka hur negativa effekter av den komplexa blandningen av ämnen som finns in vattenmiljön är beroende av så kallade blandningseffekter.

I den första studien ville spanska vattenmyndigheter undersöka hur dricksvattenkvalliten kunde påverkas av att återanvända renat avloppsvatten till att fylla på den å, i vilken råvatten för dricksvattenproduktion drogs. Genom att renat avloppsvatten omdirigerades uppströms råvattenintaget för dricksvattenproduktion istället för att ledas ut i havet, var denna studie ett fullskaligt test av kopplad avloppsvattenrening och dricksvattenproduktionen i Barcelona. Vattenprover från hela systemet inkluderades i studien, från orenat avloppsvatten, genom olika reningssteg, ut i ytvattenmiljön och slutligen färdigt dricksvatten. Det största fyndet från denna studie var att vi inte kunde påvisa att dricksvattenkvaliteten påverkats negativt av att återanvända renat avloppsvatten i detta undersökta återbrukssystem. Ett hoppingivande resultat för framtida ansträngningar att upprätta mer hållbar vattenföring i områden med svår vattenbrist.

I avhandlingens andra studie undersöktes fyra olika extraktionsmetoder genom att analysera tre olika vattenprover. Renat avloppsvatten, ett blankprov och ett spikat prov innehållande ämnen kända att kunna upptäckas av cellerna. Genom undersökningen av blanka prover kunde vi se att några av extraktionsmetoderna verkade förorena proverna med östrogena ämnen. I underökning av de spikade proverna kunde vi se att samma metod som verkat förorena blankprovet också visade för hög signal från reportergen-cellerna, vilket sammanslagningsvis pekade på att det möjligen läckte östrogena ämnen från materialet i metoden. I undersökningen av avloppsvattnet kunde vi se att två extraktionsmetoder resulterade i liknande signal från cellerna, och de andra två metoderna gav respektive högre och lägre signal när det kom till analys av östrogena ämnen. I analysen av androgena ämnen var

resultaten likvärdiga för alla fyra extraktionsmetodera och de tre vattentyperna. Således verkar valet av extraktionsprocess spela större roll för reportergenanalys av östrogena ämnen än för androgena ämnen.

I avhandlingens tredje studie undersöktes hur blandningseffekten i ett renat avloppsvatten påverkades att minska komplexiteten genom att dela upp provet i många mindre komplexa fraktioner. Renat avloppsvatten fraktionerades genom vätskekromatografi till 54 fraktioner och hela provet undersöktes tillsammans med provfraktionerna i fyra olika reportergenanalyser för att jämföra effekten i hela provet med summan av effekten i provfraktionerna. Om summan av effekten i provfraktionerna skulle visa sig lägre än den i hela provet, skulle det innebära att ämnena tillsammans i blandning var mer potenta än enskilda ämnen, det vill säga, det är blandningen som skapar faran. Av fyra inkluderade reportergenanalyser var det bara den för östrogena ämnen i vilken vi kunde påvisa aktivitet i cellerna, i både hela provet och provfraktioner. Summan av östrogen effekt i provfraktionerna uppgick till ca 50 % av den i hela provet. Det betydde att effekten av enskilda ämnen enbart kunde förklara 50 % av effekten i hela provet och att resterande effekt berodde på att ämnen hade en starkare påverkan tillsammans i blandningen. För resterande analyser kunde vi enbart se effekt i hela provet, som sedan försvann då provet fraktionerades. Det betydde att de effekter som kunde upptäckas för oxidativ stress och för dioxinreceptorn var kraftigt beroende av blandningseffekter. För analysen av androgena ämnen hade vi för låg effekt i hela provet för att kunna säga något om skillnaden mellan hela provet och provfraktioner.

Sammanfattningsvis, kunde arbetet i den här avhandlingen påvisa att det är viktigt att välja rätt extraktionsmetod av vattenprover för att säkerställa rättvisa resultat. Arbetet här kunde också påvisa att för flera grupper av förorenande ämnen i den akvatiska miljön är det verkligen blandningseffekter som skapar faran i den förorening som finns i vattenmiljön. Slutligen innehöll denna avhandling även ett fullskaligt exempel på lyckad återanvändning av renat avloppsvatten i ett av vattenbrist hårdast drabbade områden i Europa.

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Appendix I

Paper I
Effect-based evaluation of water quality in a system of indirect reuse of wastewater for drinking water production

Appendix II

Paper II
An effect-based evaluation of recovery and blank activities on solid-phase extraction processes used for water quality assessment

Appendix III

Paper III
Evaluation of mixture toxicity in treated wastewater using effect-based methods and sample fractionation assessment

I

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Effect-based evaluation of water quality in a system of indirect reuse of wastewater for drinking water production

Kim Frieberg [a,*], Pablo Gago-Ferrero [b,c], Lubertus Bijlsma [d], Lutz Ahrens [e], Karin Wiberg [e], Félix Hernández [d], Agneta Oskarsson [a], Johan Lundqvist [a]

[a] Department of Biomedical Sciences and Veterinary Public Health, Swedish University of Agricultural Sciences (SLU), Box 7028, Uppsala SE-750 07, Sweden
[b] Department of Environmental Chemistry, Spanish Council of Scientific Research (CSIC), Institute of Environmental Assessment and Water Research – Severo Ochoa Excellence Center (IDAEA), Jordi Girona, 18-26, Barcelona 08034, Spain
[c] Edifici H20 – Parc Científic i Tecnològic de Girona, Catalan Institute for Water Research (ICRA), Institut Català de Recerca de l'Aigua (ICRA), Carrer Emili Grahit, 101, Girona E-17003, Spain
[d] Environmental and Public Health Analytical Chemistry, Research Institute for Pesticides and Water, University Jaume I, Castellón E-12071, Spain
[e] Department of Aquatic Sciences and Assessment, Swedish University of Agricultural Sciences (SLU), Box 7050 SE, Uppsala 750 07, Sweden

A B S T R A C T

Indirect potable reuse of wastewater is a practice that is gaining attention, aiming to increase freshwater supplies to meet water scarcity. However, reusing effluent wastewater for drinking water production comes with a paired risk of adverse health effects, due to the potential presence of pathogenic microorganisms and hazardous micropollutants. Disinfection is an established method to reduce microbial hazards in drinking water, but it has been associated with formation of disinfection by-products (DBPs). In this study, we performed an effect-based assessment of chemical hazards in a system wherein a full-scale trial of disinfection by chlorination, of the treated wastewater was performed prior discharge to the reciepient river. The presence of bioactive pollutants was assessed along the entire treatment system, starting from incoming wastewater to finished drinking water at seven sites in and around the Llobregat River in Barcelona, Spain. Samples were collected in two campaigns, with and without applied chlorination treatment (13 mg Cl_2/L) to the effluent wastewater. The water samples were analysed for cell viability, oxidative stress response (Nrf2 activity), estrogenicity, androgenicity, aryl hydrocarbon receptor (AhR) activity and activation of NFκB (nuclear factor kappa-light-chain-enhancer of activated B cells) signaling using stably transfected mammalian cell lines. Nrf2 activity, estrogen receptor activation and AhR activation was detected in all investigated samples. Overall, removal efficiencies were high in both wastewater treatment and drinking water treatment samples for most of the studied endpoints. No increase in oxidative stress (Nrf2 activity) could be attributed to the additional chlorination treatment of the effluent wastewater. However, we found an increase in AhR activity and a reduction of ER agonistic activity after chlorination treatment of effluent wastewater. The bioactivity detected in finished drinking water was considerably lower compared to what was found in effluent wastewater. We could thus conclude that indirect reuse of treated wastewater for drinking water production can be possible without compromising drinking water quality. This study contributed important knowledge in efforts to increase the reuse of treated wastewater as a source for drinking water production.

1. Introduction

Safeguarding freshwater supplies from contamination by hazardous chemicals is of utmost importance to achieve the United Nations' sustainable development goal of universal access to safe drinking water. Climate change is expected to result in more frequently occurring droughts and other extreme weather events, which in many regions could severely jeopardize the availability of clean drinking water (Masson-Delmotte et al., 2021). Additionally, freshwater sources are under pressure due to urbanization, high demand for irrigation purposes as well as a ubiquitous increase in chemical usage. Altogether, these current and forthcoming societal challenges have increased the interest in drinking water supply systems that implement recycling of water (Gerrity et al., 2013,3).

Effluents from wastewater treatment (WWT) plants are major sources of chemical pollutants in their recipient water systems (Ternes et al., 2009; Konig et al., 2017; Schwarzenbach et al., 2006; Volker et al., 2019; Lopez et al., 2022). Pollution from WWT can be of concern both from an ecotoxicological perspective (Jobling et al., 2002; Englert et al., 2013; Stalter et al., 2013; Cavallin et al., 2021) as well as a human health perspective when surface water affected by WWT effluent is used for drinking water production (Schwarzenbach et al., 2010; WHO 2017). Assessing the presence of hazardous chemicals both in wastewater treatment and in drinking water treatment (DWT) processes is important

* Corresponding author.
E-mail address: kim.frieberg@slu.se (K. Frieberg).

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to secure adequate removal of the incoming load of pollutants.

Furthermore, it is important to monitor the presence of hazardous compounds that could potentially form during treatment processes (Neale et al., 2012; Muller et al., 2018; Hebert et al., 2018; Oskarsson et al., 2021). A treatment process that has been associated with introducing chemical hazards in the processed water is disinfection (Neale et al., 2012; Hebert et al., 2018). Disinfection is a well-established method to handle risks of microbial contamination but has been associated with the formation of disinfection by-products (DBPs) both in drinking water- and wastewater treatment processes (Neale et al., 2012; Hebert et al., 2018; Le Roux et al., 2017; Li and Mitch, 2018; J Lundqvist et al., 2019; Zhong et al., 2019). DBPs can form when disinfectants (such as chlorine, chloramine or ozone) react with dissolved organic matter (DOM) present in the water (Richardson and Postigo, 2015; Sanchis et al., 2020). DBP formation is dependent on the quality (e.g. DOM and ammonia content) of the source water and other details of the disinfection process, such as contact time, temperature, purity and dose of the disinfectant (Zhong et al., 2019; Singer, 1994; Hong et al., 2013). Previous studies have shown DBPs to induce oxidative stress, as determined by the activation of the Nuclear factor erythroid 2-related factor 2 (Nrf2) pathway (Lundqvist et al., 2019; Zhong et al., 2019; Escher et al., 2012; Neale et al., 2017). More than 700 DBPs have been identified (Richardson and Temes, 2018) but there is limited knowledge on the toxicity of most of these compounds (Postigo et al., 2021). Specific DBPs have however been linked to various cancers and other human health disorders (Srivastav et al., 2020).

A large proportion of adverse biological effects observed in water samples are caused by unknown chemicals or mixture effects (Neale et al., 2020; Escher et al., 2020). For some toxicity endpoints as little as 0.1 - 1% of observed effects could be linked to known chemicals, as demonstrated by bioanalytical methods (Escher et al., 2013). This highlights the need of using analytical methods that can integrate the effects of both known and unknown chemicals as well as mixture effects when studying hazardous compounds in aquatic systems. Effect-based methods (EBMs) such as *in vitro* bioassays based on genetically modified mammalian cell lines, have shown great promise in water quality assessments of waste- and drinking water (Escher et al., 2020; Escher et al., 2014; Brand et al., 2013).

In Catalonia, Spain, water shortages have become a more frequent problem over the last decades. In the highly urbanized area of Barcelona, the Llobregat River functions both as a recipient for treated wastewater as well as a source of drinking water production (Marcé et al., 2012). The Catalan Water Agency (ACA) set up a trial of reusing treated wastewater from the El Prat de Llobregat WWT facility to replenish the lower parts of the river. Rather than discharging into the Mediterranean Sea, tertiary treated wastewater effluent was redirected upstream via pipeline transport. The pipeline then discharged (up to 2 m^3/s) the effluent into the river upstream the surface water intake for one of the major DWT plants serving Barcelona and its metropolitan area. In times of drought, the fraction of water running in this part of the river can be 100% reclaimed wastewater effluent (Pérez et al., 2012). To reduce the risk of pathogenic contamination in the drinking water supply, while still replenishing the city's drinking water source, chlorination of the reclaimed wastewater effluent was tested in the summer of 2019.

The aim of this study was to perform an effect-based evaluation of the water quality in a full-scale trial-system for indirect reuse of treated wastewater for drinking water production. In addition, it was investigated whether chlorination of the treated wastewater would affect water quality, e.g. by formation of new chemical hazards. The overall objective of the present study was, thereby, to provide knowledge on the safe reuse of treated wastewater for drinking water production. Samples from the full water cycle, starting from untreated wastewater to treated drinking water, were analysed for seven toxicity endpoints, including oxidative stress response (Nrf2 activity), estrogen receptor (ER) activity, arylhydrocarbon receptor (AhR) activation, androgen receptor (AR) activity and immune response by nuclear factor kappa beta (NFκβ) activation.

2. Materials and methods

2.1. Water sampling

Grab water samples were collected at seven sample sites (S1-S7, Fig. 1 and Table 1) under the coordination of the ACA in June and July of 2019. Sampling sites were located in the lower part of the Llobregat River basin between the inlet of the DWT plant of Sant Joan Despí and a point 8.5 km upstream. Based on the hydraulic retention and residence time between the different sampling sites the samples were collected in scheduled timely accordance, aiming to collect the same bulk of water parcel along the distribution system. The difference between the two sampling campaigns (C1 and C2) was that chlorination of reclaimed wastewater effluent was applied between sites S3 and S4 in the second campaign, at a dose of 13 mg Cl_2/L (sodium hypochlorite, NaOCl) (Fig. 1). This dose of chlorine was set according to pump capacity limitations and was below breakpoint chlorination, which was experimentally measured at 30 mg Cl_2/L. The applied WWT methods at El Prat de Llobregat treatment facility include nitrification/denitrification (secondary treatment), membrane filtration and UV-treatment (tertiary treatment). At Sant Joan Despí DWT facility treatment methods include two parallel treatment lines. One treatment line consist of ozonation and GAC filtration and the other line consist of ultrafiltration followed by reversed osmosis. The two treatment lines are blended prior to final disinfection with chlorine. Water sample characteristics such as total organic carbon concentration (TOC) (mg C/L), pH and conductivity (μS/cm) are described in Table S1 in Supporting Information (SI).

2.2. Water sample extraction

Water samples (volumes presented in Table 1) were subjected to extraction within 24 h of collection along with MilliQ-water procedural blanks at Catalan Institute for Water Research (ICRA). Samples were filtered over 0.7 μm GF/F and GF/D and pH was adjusted to ≈ 6.5 using ammonia and formic acid. Solid phase extraction (SPE) was performed according to Gago-Ferrero et al. (Gago-Ferrero et al., 2015). SPE cartridges were prepared in-house using 6 mL SPE polypropylene tubes (Phenomenex, Torrance, USA) and four sorbents; Sepra ZT (Strata-X), Sepra ZT-WCX (Strata-X-CW), ZT-WAX (Strata-X-AW) (Phenomenex, Torrance, USA) and Isolute ENV+ along with frits (20 μL, 6 mL) (Biotage, Ystrad Mynach, UK). SPE extracts (500 μL 1:4 v/v MeOH:EtOH) were stored at -20 °C pending bioassay analysis. A detailed description of the SPE protocol is described in SI.

2.3. Bioassays

The water samples were analysed for seven toxicity endpoints of relevance for both human and ecological hazard identification (Table 2). All samples were tested in cell viability assessments, to ensure that bioactivities were studied under non-cytotoxic conditions. A detailed description of the applied bioassays is given in SI. The seven endpoints were assessed along with solvent control, reference compound and procedural blanks in stably transfected luciferase reporter gene assays in 384-well plate (Corning, USA) format. A TECAN (Infinite M1000) reader was used to measure luminescence after addition of luciferin. The concentrations of samples studied in the bioassays are expressed as relative enrichment factors (REF). The highest REF tested was calculated as enrichment factor at SPE x 0.01 (100-fold dilution with cell medium at bioassay). A REF>1 implies that the water sample has been enriched, as compared to the grab water sample, and a REF<1 that the sample has been diluted. All bioassays were conducted with a constant solvent concentration (1% 1:4 MeOH: EtOH v/v). Description of data evaluation and calculations of EC- and BEQ-values can be found in SI.

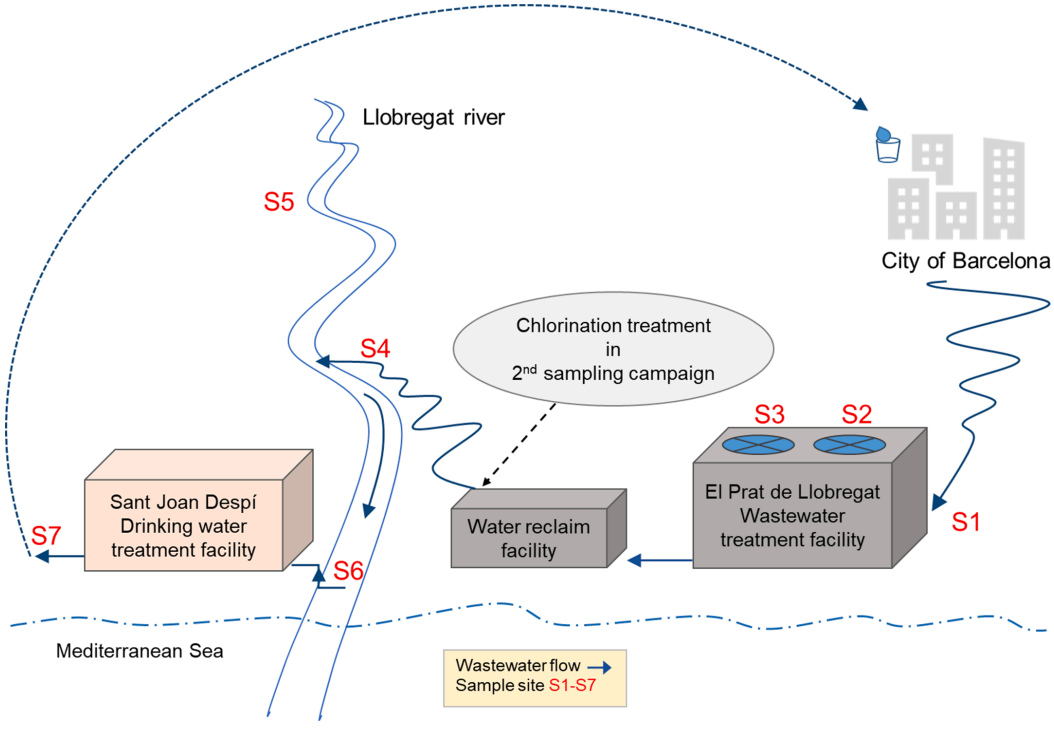

Fig. 1. Schematic description of sampling sites. The actual distance between S4 and S6 was 8.5 km.

3. Results and discussion

3.1. Cell viability

Samples were tested for cytotoxicity in AR-EcoScreen, VM7Luc4E2, MCF7AREc32, HepG2-NFκβ and DR-EcoScreen cell lines. Cytotoxicity was defined as cell viability of <80% compared to the solvent control. The highest tested REF values were 20 for wastewater, 50 for surface water and 100 for drinking water i.e. wastewater was tested at 20x enrichment, surface water at of 50x enrichment and drinking water at 100x enrichment as compared to grab water samples (Table 1). Most samples did not show cytotoxicity at the highest tested REF in any of the cell lines (Figs. S:1–5 in SI), except for influent wastewater which for most cell lines had to be diluted to REF 2.5, to reach non-cytotoxic conditions. The following samples were cytotoxic at the highest concentrations, and thus excluded from regression analyses: Sample C2S4, C2S5 and C2S6 were cytotoxic at REF 50 in DR-EcoScreen (Figs. S1, SI). Sample C2S4 was cytotoxic at REF 50 in VM7luc4ER (Figs. S2, SI) and sample C2S3 was cytotoxic at REF 10) in AR-EcoScreen (Figs. S4, SI). Additionally, sample concentrations that showed signs of potential masked cytotoxicity, i.e. displayed a negative trend of bioactivity with increasing REF were also excluded from regression analyses.

3.2. Procedural blanks

In three of the endpoints in this study, the procedural blanks, MilliQ water concentrated over SPE, showed some bioactivity (Table 3). In the Nrf2 assessment, the two blanks from C1 and C2 showed activity at REF 20. The bioactivity was just above the cut-off value in the two campaigns. At the next tested concentration, REF 10, no activity was detected. One of the objectives here was to investigate the effect of chlorination treatment between the campaigns using Nrf2 activity as an indicator of DBP formation. Since the background activity was borderline above cut-off and comparably equal between the two blanks in Nrf2 activity assessment, we argue that this comparison could still be made successfully. The two other endpoints were blanks showed bioactivity was AhR activation at REF 20 and ER agonist activity at REF 20 through REF 5 (Table 3). Samples S5-S7 were analysed at higher REFs in all assays as compared to the other samples. The samples analysed at the highest tested REF values were finished drinking water (S7). However, for ER Agonist activity, for example, the drinking water from both campaigns exhibited lower bioactivity at REF 20 than what the procedural blank did at REF 20, (at around 2% and 6% of assay maximum). Hence, the potential contamination of samples observed in the procedural blank did not seem to be as pronounced in the real samples. We hypothesize that this might be due to the low ionic strength of the deionized water used to prepare the procedural blank, which could make this blank sample extra susceptible to contamination from the SPE process. Actual samples, with a higher ionic strength, did not seem to be as susceptible to contamination from the SPE process. We cannot rule out the possibility of overestimation of the endpoints tested at concentrations higher than the active blanks. However, to claim the contrary, our ER agonist and AhR data are comparably low in relation to literature data (See Sections 3.4.2 and 3.5.2) We argue that the intra-sample comparison in this study can still confidently be made but some caution is advisable when comparing our data with other studies.

Table 1
Water sample description.

Sample site	Sample description	Grab sample volume (L)	Concentration factor of SPE extract	Highest relative enrichment factor (REF) tested in bioassays
S1	Influent wastewater	1	2000x	20
S2	Secondary treated wastewater (N/DN)	1	2000x	20
S3	Tertiary treated wastewater (Sand filter/UV)	1	2000x	20
S4*	Effluent at pipeline outlet	2.5	5000x	50
S5	Surface water upstream all other samples	2.5	5000x	50
S6*	Surface water at point of inlet to drinking water plant	2.5	5000x	50
S7*	Treated drinking water (O_3/GAC; UF/RO +Cl_2)	5	10 000x	100
	Procedural blank – MilliQ water	1	2000x	20

* = Samples affected by chlorination treatment in the second sampling campaign, N/DN = nitrification/denitrification, GAC = granular activated carbon filtration, UF = ultra-filtration, RO = reversed osmosis, REF = enrichment $factor_{SPE}$ x dilution $factor_{bioassay}$.

3.3. Oxidative stress response (Nrf2 activity)

Oxidative stress response, measured as Nrf2 activity, was observed in all analysed samples. Concentration-response relationships are presented in Fig. 2, $EC_{IR1.5}$ and BEQ values are presented in Table 4 and removal efficiencies in Table 5 and Table 6.

The highest detected Nrf2 activity was found in influent wastewater samples (S1) at 2900 and 1500 μg tBHQeq/L in C1 and C2 respectively. After secondary treatment (nitrification/denitrification) (S2) the Nrf2 activity was reduced compared to S1 with a removal efficiency of 93% in both campaigns. In tertiary treated wastewater (filtration / UV treatment) (S3) the Nrf2 activity was higher in C1 compared to C2 and there was an increase in activity in both campaigns compared to S2.

At the end of the pipeline (S4) there was an increase in Nrf2 activity in both campaigns as compared to S3 with a negative removal efficiency of -66% in C1 and -33% in C2. The increase was hence larger in C1 as compared to C2 with chlorination treatment. Thus, chlorination treatment could not be concluded to have a major impact on the Nrf2 activity, since there was an increase in activity both with and without chlorination treatment. Rather, the increase between sample sites S3 and S4 indicates some contaminating factor within the pipeline contributing to an increase in Nrf2 activity.

In the upstream river sample (S5), the Nrf2 activity was higher in C1 compared to C2 at 170 μg tBHQeq/L in C1 and 20 μg tBHQeq/L in C2. At the raw water intake to the DWT plant (S6), the Nrf2 activity increased compared to the upstream samples and was continuously higher in C1 at 1500 μg tBHQeq/L as compared to 40 μg tBHQeq/L for C2. Thus, the overall potency for oxidative stress in the river was higher in C1 as compared to C2. The reason for this difference in oxidative stress in surface water samples between the studied campaigns cannot easily be elucidated. Possible influencing factors during times of sampling include variations in river flow, contaminant concentration and precipitation. Hence, further research would be needed to explain these variations in oxidative stress in surface water.

Despite the difference in Nrf2 activity at drinking water intake (S6), the Nrf2 activity was equal and low in both campaigns after drinking

Table 3
Bioactivity in procedural blanks.

Assay	Cut-off for bioactivity (1+3xSD of solvent control)	Blank activity					
		Campaign 1			Campaign 2		
		REF 20 (SD)	REF 10 (SD)	REF 5 (SD)	REF 20 (SD)	REF 10 (SD)	REF 5 (SD)
ER^+ % of max	7	20 (4)	15 (2)	13 (3)	19 (2)	13 (2)	11 (2)
Nrf2 Fold change	1.5	1.6 (0.2)	-	-	1.9 (0.3)	-	-
AhR % of max	3	11 (5)	4 (3)	-	15 (5)	4 (3)	-

+ = Agonistic activity, - = No activity detected, SD = Standard deviation.

Table 2
Endpoints, cell lines and reference compounds.

Endpoint	Cell line	Stimulant treatment	Reference compound	Concentration range	Calculated effect concentration	EC or IC of the applied reference compounds
Androgen receptor agonism	AR-EcoScreen GR-KO M1	–	Dihydrotestosterone (DHT)	0.03 - 300 000 pg/L	EC_{20}	164 pg/L
Androgen receptor antagonism	AR-EcoScreen GR-KO M1	DHT	Hydroxyflutamide (OHF)	0.03 – 3000 μg/L	IC_{30}	73 μg/L
Estrogen receptor agonism	VM7Luc4ER	–	17β-estradiol (E2)	0.1 - 100 ng/L	EC_{30}	0.2 ng/L
Estrogen receptor antagonism	VM7Luc4E2	17β-estradiol	Raloxifen	50 - 20 000 ng/L	IC_{30}	120 ng/L
Nrf2 activity (Oxidative stress response)	MCF7 AREc32	–	tert-Butylhydroquinone (tBHQ)	130 - 4 200 μg/L	$EC_{IR1.5}$	730 μg/L
NFkB activity (Inflammatory response)	HepG2-NFkB	–	Tumor necrosis factor-alpha (TNFα)	0.2 - 50 ng/mL	$EC_{IR1.5}$	0.5 ng/mL
Aryl hydrocarbon receptor activation	DR-Ecoscreen	–	2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD)	2.6 - 160 ng/L	EC_{40}	10 ng/L

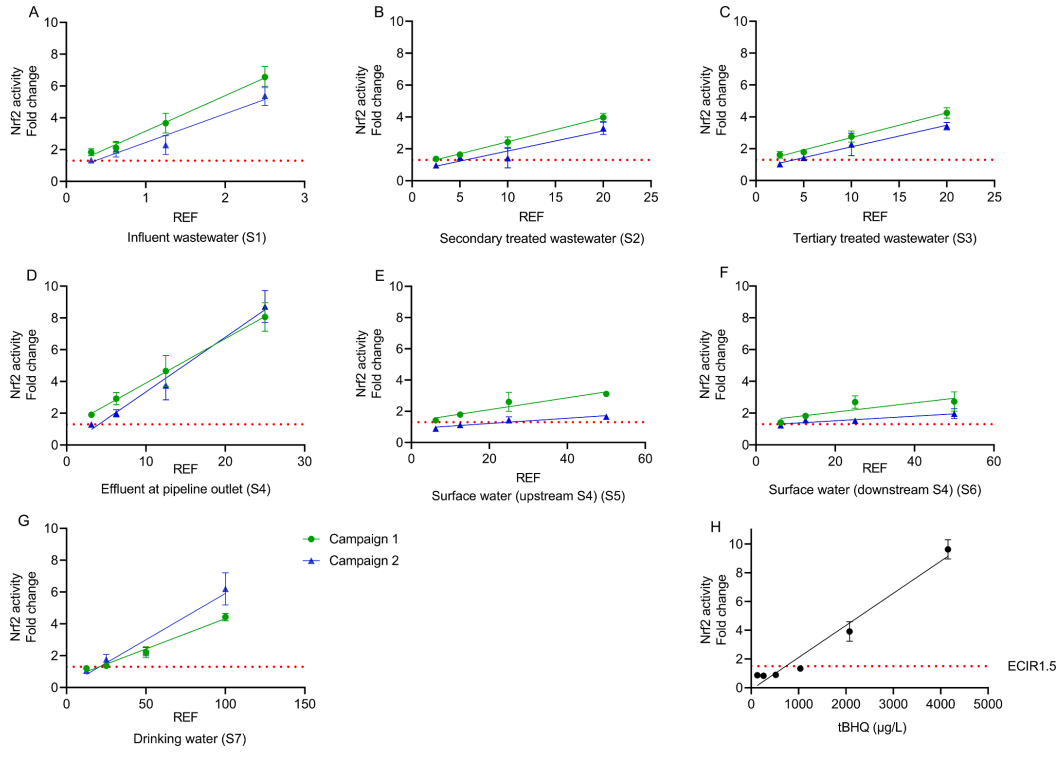

Fig. 2. Concentration-response of the Nrf2 bioassay in water samples collected at seven sites (A-G; S1 to S7) from two campaign events. Activities of water samples (n = 4 per concentration) and tBHQ as a reference compound (n = 4 per concentration) are displayed as fold change (mean±SD), compared to solvent control (n = 8) set to 1. The highest tested concentrations ranged from REF < 2.5 to 100 depending on the used enrichment factor and cytotoxicity of each sample (Figs. S3, SI). The red dotted line represents the cut-off for bioactivity at $EC_{IR1.5}$. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

water treatment (S7), at 33 µg tBHQeq/L. In summary, the oxidative stress response of the samples downstream of chlorination treatment in C2 was not higher compared to the unchlorinated equivalent samples in C1 (S4, S6, S7).

The applied dose of NaOCl in the chlorination treatment of effluent wastewater was 13 mg Cl_2/L. Breakpoint chlorination, after which a residual amount of chlorine exists to elicit disinfection, was experimentally measured at 30 mg Cl_2/L, thus the applied dose was below breakpoint. Reaching breakpoint chlorination when disinfecting drinking water is vital to secure adequate disinfection. In DWT, chlorination treatment is typically applied at the end of the treatment process where the oxidant demand of the water is low; normally at a dose of 0.5–2 mg Cl_2/L. Hence, a much lower dose of chlorine would be needed to reach breakpoint compared to wastewater effluent with a high oxidant demand. Previous studies on chlorination of wastewater found an increase of certain DBPs when dosing Cl_2 above breakpoint (Yang et al., 2005; Matamoros et al., 2007) and it could be speculated that our results would have been different if breakpoint conditions had been reached. A concurrent study of the same full-scale water reclamation trial in the Llobregat River, assessing alternations on DOM fingerprinting after chlorination treatment, did reveal formation of halogenated species downstream chlorination treatment at doses ranging from 10 to 14 mg Cl_2/L (Sanchis et al., 2021).

3.4. Comparison of Nrf2 activity with other studies

For incoming wastewater, we previously reported Nrf2 activities of 200 – 580 µg tBHQeq/L from Swedish WWT plants (Lundqvist et al., 2019) and Escher et al. (Escher et al., 2012) reported a range of 95–650 µg tBHQeq/L from Australian WWT plants, which are both lower than in the present study at 2900 and 1500 µg tBHQeq/L. In effluent wastewater, we found activities of 320 and 130 µg tBHQeq/L, which was higher compared to the Australian data of 50 µg tBHQeq/L (Escher et al., 2012). We previously reported on tBHQeq in Swedish wastewater effluent to be below LOD in several cases and at 180 µg/L in another case (Oskarsson et al., 2021; Lundqvist et al., 2019). In surface water affected by discharge from WWT plants, our present results were 1500 µg tBHQeq in C1 and 170 µg tBHQeq/L in C2. Reports from Germany and Australia on surface water affected by WWT discharge show lower activities of 5–16 µg tBHQeq /L and 24–29 µg tBHQeq/L, respectively (Muller et al., 2018; Escher et al., 2012). In summary, the Nrf2 activity in and around the El Prat de Llobregat WWT plant in this study was higher as compared to other published data. Notably, the anthropogenic pressure on the Llobregat river system, i.e. its surrounding population density, load of incoming pollutants and lack of dilution effect, is markedly different compared to some of the freshwater systems from the other studies mentioned above.

In drinking water treatment, increasing levels of tBHQeq have been

Table 4
Relative enrichment factor (REF) at effect concentrations EC_{20}, EC_{40}, IC_{30}, and $EC_{IR1.5}$ & corresponding bioequivalence (BEQ value) as compared to reference compound.

Endpoint:		Nrf2 activity		AhR activity		ER agonist		ER antagonist		AR agonist		AR antagonist		NfkB activity	
		$EC_{IR1.5}$ ± SE (REF)	tBHQ eq ± SE (μg/L)	EC_{40} ± SE (REF)	TCDD eq ± SE (ng/L)	EC_{30} ± SE (REF)	β-estradiol eq ± SE (pg/L)	IC_{30} (REF)	Raloxifen eq (ng/L)	EC_{20} ± SE (REF)	DHT eq ± SE (pg/L)	IC_{30} (REF)	OHF eq (ng/L)	$EC_{IR1.5}$ (REF)	TNFα eq (ng/mL)
Campaign 1	S1	0.3 ± 0.01	2860 ± 170	1.7 ± 0.4	5.8 ± 1.3	0.0002 ± 0.00002	1 077 000 ± 250 00	>20[a]	<60	0.04 ± 0.002	4300 ±340	1.2	61	>20[a]	<0.025
	S2	3.7 ± 0.2	200 ± 9	6.0 ± 0.8	1.6 ± 0.3	0.3 ± 0.04	630 ± 150	>20[a]	<60	>20[a]	<5.8	18	3.0	>20[a]	<0.025
	S3	2.3 ± 0.2	320 ± 29	4.9 ± 1.1	2.0 ± 0.5	0.1 ± 0.04	2240 ± 1000	>20[a]	<60	>20[a]	<5.8	5.4	15	>20[a]	<0.025
	S4	1.4 ± 0.1	530 ± 48	1.9 ± 0.8	5.2 ± 2.4	0.1 ± 0.04	2100 ± 900	>50[a]	<24	>50[a]	<1.4	11	5.8	>50[a]	< .01
	S5	4.3 ± 0.002	170 ± 0.2	23 ± 3.0	0.4 ± 0.1	1.1 ± 0.04	200 ± 40	14.6	140	>50[a]	<1.4	40	1.8	>50[a]	<0.01
	S6	0.5 ± 4.2	1500 ± 1296	13 ± 4.0	0.8 ± 0.3	0.6 ± 0.05	350 ± 80	>50[a]	<24	>50[a]	<1.4	16	4.7	>50[a]	<0.01
	S7	26 ± 0.7	30 ± 1	92 ± 11	0.1 ± 0.01	>100[a]	<2	>100[a]	<1.2	>100[a]	<30	>100[a]	<0.7	>100[a]	<0.005
Campaign 2	S1	0.5 ± 0.03	1540 ± 92	0.9 ± 0.5	11 ± 6.8	0.0001 ± 0.00004	1 350 000 ± 500 000	>20[a]	<60	0.04 ± 0.002	3900 ±270	2.5	29	>20[a]	<0.025
	S2	7.1 ± 0.5	100 ±8	4.7 ± 0.7	2.1 ± 0.3	0.5 ± 0.09	440 ± 130	>20[a]	<60	>20[a]	<5.8	>20[a]	< 3.6	>20[a]	<0.025
	S3	5.4 ± 0.4	130 ± 9	3.8 ± 1.4	2.6 ± 0.9	0.6 ± 0.06	360 ± 90	>20[a]	<60	>20[a]	<5.8	>20[a]	< 3.6	>20[a]	<0.025
	S4	4.6 ± 0.1	160 ± 4	0.3 ± 0.9	36 ± 12	8.5 ± 0.7	24 ± 5	>50[a]	<24	>50[a]	<1.4	>50[a]	< 1.4	>50[a]	<0.01
	S5	37 ± 4.3	20 ± 2	4.5 ± 2.4	2.2 ± 1.2	4.9 ± 0.4	40 ± 9	6.3	190	>50[a]	<1.4	>50[a]	< 1.4	>50[a]	<0.01
	S6	19 ± 6.5	40 ± 13	5.6 ± 2.2	1.8 ± 0.7	4.9 ± 3.0	40 ± 28	1.5	805	>50[a]	<1.4	>50[a]	< 1.4	>50[a]	<0.01
	S7	24 ± 0.8	30 ± 1	38 ± 3.8	0.3 ± 0.03	>100[a]	<2	>100[a]	<1.2	>100[a]	<30	>100[a]	<0.7	>100[a]	<0.005

[a] = EC higher than highest tested REF (which is stated); low bioactivity.
BEQ = EC reference compound / EC sample.
SE = Standard error, calculated according to Escher *et al.* 2018(65).

Table 5
Cumulative removal efficiency (% of BEQ).

	Treatment step		Nrf2 actvity	AhR activity	ER agonist	ER antagonist	AR antagonist	AR agonist
	Cumulative removal efficiency expressed as % of incoming wastewater (S1)							
Campaign 1	Secondary WW treatment (N/DN)	S2	93%	72%	99.9%		95%	> 99.8%
	Tertiary WW treatment (SF/UV)	S3	89%	66%	99.8%		75%	
	End of pipeline	S4	82%	10%	99.8%		91%	
Campaign 2	Secondary WW treatment (N/DN)	S2	93%	81%	99.9%		> 88%	> 99.8%
	Tertiary WW treatment (SF/UV)	S3	91%	76%	99.9%			
	End of pipeline + Cl_2 treatment	S4	89%	-227%	99.9%			
	Cumulative removal efficiency expressed as % of incoming water to DWT facility (S6)							
Campaign 1	Drinking water treatment (O_3/GAC; UF/RO +Cl_2)	S7	98%	87%	> 99%		> 85%	
Campaign 2	Drinking water treatment (O_3/GAC; UF/RO +Cl_2)	S7	0%*	83%	> 95%	99.9%		

A negative removal rate signifies an increase in BEQ as compared to incoming wastewater (S1).
* equal but low tBHQeq in incoming and outgoing water, see Table 4 for details.

Table 6
Removal efficiency of chlorination treatment (% BEQ of tertiary treated wastewater (S3)).

	Treatment step		Nrf2 actvity	AhR activity	ER agonist	ER antagonist	AR antagonist	AR agonist
Campaign 1	End of pipeline	S4	-66%	-160%	6%	n.a	61%	n.a
Campaign 2	End of pipeline + Cl_2 treatment	S4	-31%	-1284%	93%	n.a	n.a.	n.a.

Negative removal efficiency signifies an increase in BEQ as compared to tertiary treated wastewater (S3). n.a. = not applicable.

reported within the production line (Neale et al., 2012; Hebert et al., 2018; Oskarsson et al., 2021; Lundqvist et al., 2019; Escher et al., 2012). In one study, source water had 18 μg tBHQeq/L increasing to 42 μg tBHQeq/L in finished drinking water (Escher et al., 2012). In the present study, no increase in activity was seen, but rather, despite very differing incoming levels of activity at 1500 μg tBHQeq /L (C1) and 33 μg tBHQeq/L (C2), the activity in finished drinking water (S7) was equal in the two campaigns at 33 μg tBHQeq/L. Additionally, in the previously mentioned concurrent study on effects of chlorination on the reclaimed effluent in the Llobregat River (Sanchis et al., 2021), it was found that certain halogenated features persisted in the final drinking water. Our results, however, indicate that these formed features were not present at high enough concentrations or could not trigger oxidative stress response via the Nrf2 pathway.

3.4.1. *Aryl hydrocarbon receptor (AhR) activity*

We observed AhR activity in all tested samples (Fig. 3, Table 4). Concentration-response relationships are presented in Fig. 3, EC_{40} and BEQ values are presented in Table 4 and removal efficiencies in Tables 5 and 6.

In influent wastewater (S1) the AhR activity was slightly higher in C2 compared to C1 at 11 and 6 ng TCDDeq/L. During secondary treatment (S2), the removal efficiency was 72% and 81% in C1 and C2, respectively. Following tertiary treatment (S3), the removal efficiencies were 66% and 76% respectively for C1 and C2, and TCDDeq remained roughly the same between S2 and S3 in both campaigns.

At the end of the pipeline (S4), the AhR activity increased in both campaigns as compared to within the treatment plant (S3). The increase was most pronounced in C2 (including chlorination treatment), surpassing the TCDDeq seen in the incoming water with 227% in C2. The AhR activity in C2S4 was the highest found among all the samples at 41 ng TCDDeq/L. In the river, the upstream samples (S5) showed lower AhR activity in C1 as compared to C2. Further downstream, at raw water intake to the DWT plant (S6), the AhR activity was equal in both campaigns at a range of 0.8–1.8 ng TCDDeq/L. The removal efficiency for drinking water treatment was 87% and 83% as compared to incoming

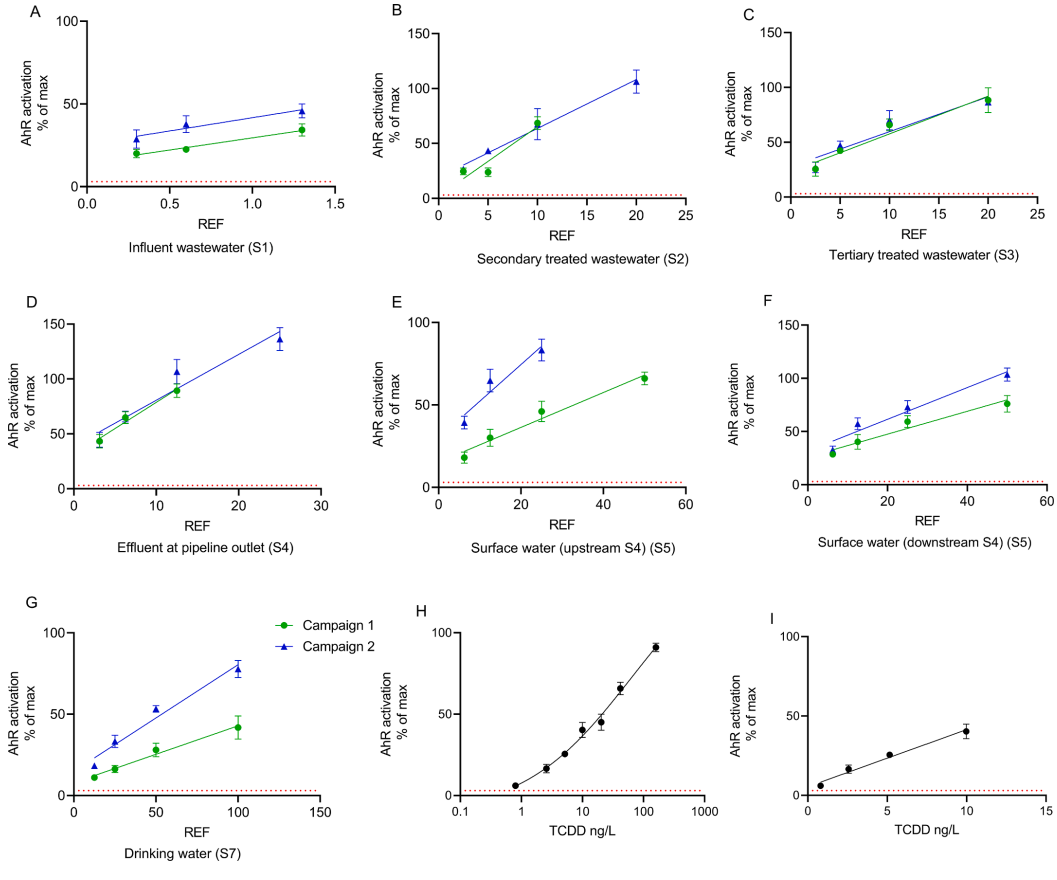

Fig. 3. Concentration-response of AhR activity in water samples collected at seven sites (A-G; S1 to S7) from two campaign events. Activities of water samples (n = 4 per concentration) and TCDD as a reference compound (n = 4 per concentration) are displayed as% of assay maximum (mean±SD), as compared to reference compound. The highest tested concentrations ranged from REF 1.25 to 100 depending on the used enrichment factor and cytotoxicity of each sample (Figs. S4, SI). The red dotted line represents the cut-off for bioactivity at mean+3xSD of solvent control (n = 8). The linear portion of the reference compound curve (panel I) was used to calculate standard errors for EC- and BEQ-values according to Escher et al. 2018. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

water. A remaining AhR activity was found below 1 ng TCDDeq/L in both campaigns.

An increase in AhR activity after chlorination treatment, as detected in C2S4, has not been reported in previously published studies. The removal efficiency for chlorination treatment in C2 was -1284% comparing samples S4 to S3. It seems the increase in activity was either due to transformation products created by the addition of chlorine to the tertiary treated wastewater or the addition of chlorine triggered a release of AhR-inducing compounds from within the pipeline. As an increase in AhR activity was also seen in C1 without chlorination treatment, perhaps a combination of the two mechanisms occurred. A previously published study, using a different cell line, found an increase in CYP1A1 expression (downstream AhR activation) after chlorination treatment of sediment from a drinking water reservoir (Wu et al., 2020). However, further studies did not confirm this finding (Liang et al., 2022). The increase in bioactivity between C1S3 and C1S4 is also depicted in the negative removal efficiency of -160%, even though no treatment occurred in this campaign (only water transport). This suggests some unknown source of AhR active compounds within the pipeline.

3.4.2. Comparison of AhR activity with other studies

Several studies report complete or partial removal of AhR activity when comparing influent wastewater versus effluent water (Lundqvist et al., 2019; Chou et al., 2014; Nivala et al., 2018). There are also reports of higher AhR activity in outgoing water as compared to untreated wastewater (Muller et al., 2018). We previously reported AhR activities, up to 400 ng TCDDeq/L in influent and up to 200 ng TCDDeq/L in effluent wastewaters (Lundqvist et al., 2019). Studies with considerably lower activities have also been reported with around 0.3 ng TCDDeq/L in wastewater (Nivala et al., 2018) and in the range of 0.009–0.16 ng TCDDeq/L in surface waters affected by wastewater discharge (Konig et al., 2017; Muller et al., 2018). Regardless of the peak in AhR activity in C2S4 sample, our results here are in the lower range compared to

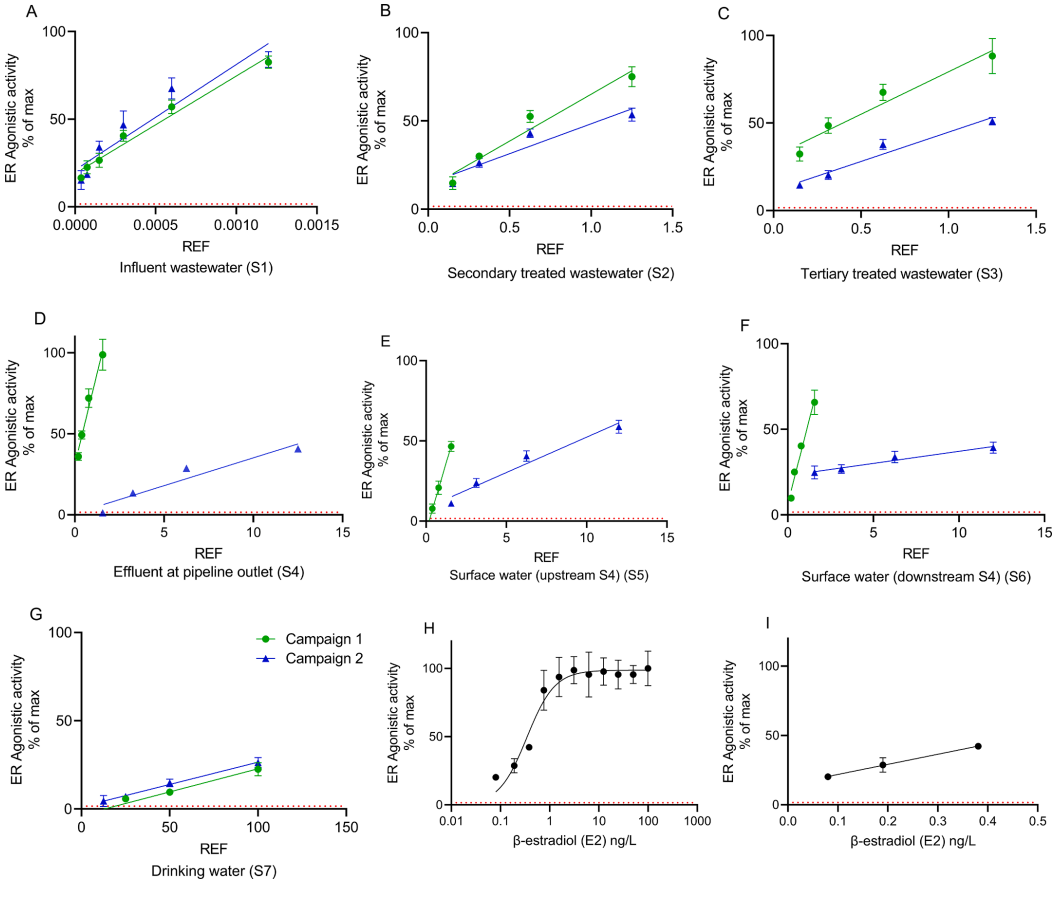

Fig. 4. Concentration-response of activation of the estrogen receptor (ER) in water samples collected at seven sites (A-G; S1 to S7) from two campaign events. Activities of water samples (n = 4 per concentration) and 17β-estradiol (E2) as reference compound (n = 4 per concentration) are displayed as% of assay maximum (mean±SD) as compared to reference compound. Highest tested concentrations ranges from REF below 1 to 100 depending on the used enrichment factor and cytotoxicity of each sample (Figs. S5, SI). The red dotted line represent the cut-off for bioactivity at mean+3xSD of solvent control (n = 8). The linear portion of the reference compound curve (panel I) was used to calculate standard errors for EC- and BEQ-values according to Escher et al. 2018. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

literature data. An effect-based trigger value (EBT) for potable reused water was suggested by the California Water Boards of 0.5 ng TCDDeq/L, (monitoring trigger limit [MTL]) (NORI 2020).

The AhR activity in drinking water samples in this study was the lowest of all tested samples at 0.1 and 0.3 ng TCDDeq/L in C1 and C2 respectively, which was below the proposed MTL value of 0.5 ng TCDDeq/L for potable reused water. Following the California Water Boards guideline on this trigger value, action is suggested to be taken when measured values exceed ten times the suggested trigger value, which the data here did not.

3.5. Estrogen receptor activity

3.5.1. ER agonistic activity

Estrogen receptor (ER) agonistic activity was detected in all tested samples. Concentration-response relationships are presented in Fig. 4, EC_{30} and BEQ values are presented in Table 4, and removal efficacies in Tables 5 and 6. The most potent activity was found in influent samples (S1) equal to 1 077 000 pg E2eq/L and 1 350 000 pg E2eq/L in C1 and C2 respectively. After secondary treatment (S2) the activity decreased with a removal efficiency of 99.9% in both campaigns leaving a remaining activity of 630 and 440 pg E2eq/L in C1 and C2 respectively.

At the end of the pipeline (S4) the ER activity was lower in C2 compared to C1. In C1, the activity was relatively unchanged between S3 and S4 with 6% removal going from 2240 to 2100 pg E2eq/L. In C2 however, after chlorination treatment, the ER activity in S4 was reduced by 94% as compared to S3 from 360 to 24 pg E2eq/L.

Here, despite an overall lower activity in C2 wastewater samples compared to C1, it seems chlorination treatment had a reducing effect on the estrogenic activity of the wastewater. Furthermore, previous studies have indicated that chlorination may reduce estrogenic activity. It has been hypothesized that the phenolic ring (found in BPA, E2 and EE2) can be susceptible to oxidation by chlorine (Lee et al., 2004; Wu et al., 2009; Lee et al., 2008; Li et al., 2017; Li et al., 2016). Consequently, chlorination treatment could reduce estrogenic activity in water treatment as well as reduce microbial contamination (Lee et al., 2004; Wu et al., 2009; Lee et al., 2008; Li et al., 2017; Li et al., 2016).

In surface water (S5 & S6) the ER activity was continuously less potent in C2 as compared to C1 samples; upstream (S5) discharge of WWT effluent as well as downstream discharge of WWT effluent (S6). The ER activity in drinking water (S7) was the lowest of all tested samples with a removal efficiency greater than 95% in both campaigns. The remaining activity in drinking water was below LOD at < 2 pg E2eq/L in both campaigns.

3.5.2. Comparison of estrogenic agonistic activity with other studies

The ER activity, expressed as E2eq, in incoming wastewaters was determined to 1 077 000 pg E2eq/L and 1350 000 pg E2eq/L. Other reports on ER agonist activity in incoming wastewater in the range of 800–250 000 pg E2eq/L (Lundqvist et al., 2019; Nivala et al., 2018; Valitalo et al., 2017). In effluent wastewater (S3), we found bioactivity of 24–1990 pg E2eq/L which was lower compared to other studies on effluent wastewater with activities in the range of 1000 – 40 000 pg E2eq/L (Lundqvist et al., 2019; Nivala et al., 2018; Valitalo et al., 2017). In effluent wastewater and surface water downstream wastewater effluent discharge, bioactivities have been reported in the range of 10 - 300 pg E2eq/L in a Serbian river system (Konig et al., 2017), 400 pg – 2000 pg E2eq/L in a German river system (Muller et al., 2018) and 800 – 6000 pg E2eq/L in an Australian river system (Bain et al., 2014). It should, however be noted that these studies have been conducted with different cell lines than ours, which might differ in sensitivity. Our findings range between 40 – 2000 pg E2eq/L at sample sites S3, S4 and S6 in the two campaigns and appear lower as compared with previously published data.

In surface water, Kase et al. (2018) proposed an EBT of 400 pg E2eq/L for environmentally safe levels of ER agonists (derived from n = 5 different ER assays).The estrogenic activity observed in the surface water samples (S5, S6) in this study was below this proposed value, but it should be noted that the proposed EBT is assay specific. In drinking water, the World Health Organization (WHO) suggested a benchmark value of 1 ng E2eq/L in drinking water for assessment of occurrence and treatment efficiency during the revision of the EU drinking water directive 2020 (EU, 2022). The European Commission included this value in the Watch List of endocrine disrupting substances of concern to the public in 2022 (EU, 2022). Previously Brand et al. (2013) suggested an EBT value for drinking water for safe human consumption of 3.8 ng E2eq/L and more recently the California Water Board recently suggested a MTL value of 3.5 ng E2eq /L in potable reused water (NORI 2020). The estrogenicity observed in drinking water in this study was < 2 pg E2eq/L in the two campaigns and well below all the above-mentioned trigger values.

3.5.3. ER antagonistic activity

ER antagonistic activity assessment revealed some presence of antagonistic compounds in three of the surface water samples (Fig. S6 in SI, Table 4). Since no ER antagonist activity could be detected in wastewater samples, the source of ER antagonistic activity in the river probably originated separate from El prat de Llobregat WWT facility. Although no wastewater samples showed activity in this study, a WWT study from Germany reported on low removal of ER antagonist activity (Wolf et al., 2022) and similarly a recent review showed low removal efficacy of bioactivities in WWT and DWT (Enault et al., 2023). Compared to estrogenic agonistic activity the ER antagonistic mode as an endpoint is not as widely studied and comparable data for surface water is sparse.

3.6. Androgen receptor activity

3.6.1. AR agonistic activity

Concentration-response relationships of AR agonistic activity are presented in Fig. S7 (SI). AR agonistic activity was only observed in influent wastewater with an activity of 4 DHTeq/L in the two campaigns. The removal efficiency following secondary treatment (S2) was 99.8%. Similarly, Leusch et al. (2014) reported high androgenic activity in influent wastewater and no observed androgenic activity (below LOD) for effluent wastewater. In general, the removal rate for androgenic compounds seems to be high across different WWT systems. Several studies from different countries report similar results as in this study with low or no activity in WWT systems (Lundqvist et al., 2019; Nivala et al., 2018; Valitalo et al., 2017; Van der Linden et al., 2008) as well as in DWT systems (Brand et al., 2013; Leusch et al., 2018).

3.6.2. AR antagonistic activity

Concentration-response relationships of AR antagonist activity are presented in Fig. S8 (SI). BEQ and removal efficacies can be found in Tables 4–6. AR antagonistic activity was observed in most of the samples in C1 but only in the influent wastewater sample (S1) in C2. The removal efficiency of secondary treatment was 95% in C1 and 88% in C2. In C1, there was a slight increase in activity after tertiary treatment from 3 to 15 ng OHFeq/L. At the end of the pipeline (S4), the activity decreased again, to 6 ng OHFeq/L indicating degradation of AR antagonists within the pipeline. In surface water, at the point of drinking water intake (S6) there was only a marginal difference in activity to that of the activity in the pipeline, indicating little dilution of the activity compared to the raw effluent. In drinking water (S7), the remaining bioactivity was below detection limit in both campaigns. Previous studies on wastewater effluent and drinking water have reported data in line with this study (Lundqvist et al., 2019; Leusch et al., 2018; Rosenmai et al., 2018). However, we have previously found cases of AR antagonistic activity in treated drinking water at 0.9 μg OHFeq/L (Oskarsson et al., 2021).

3.7. NFκβ activation

The assessment of NFκβ activation, in the HepG2-NFκβ cell line with Tumor necrosis factor-alpha (TNFα) as reference compound did not reveal any detectable activity in any of the samples (Figs. S9, SI). Though none of the samples showed activity in this study, previous assessments of wastewater, surface water and drinking water samples reported bioactivity for this endpoint (Konig et al., 2017; Hebert et al., 2018; Neale et al., 2017; Nivala et al., 2018). Overall result of the occurrence of chemical hazards in the water samples

3.8. Summarised effect concentrations

The effect concentrations, expressed as REF, are summarised in a heat map (Fig. 5). The heat map illustrates that effects observed in incoming untreated wastewater decreased in the subsequent samples throughout the wastewater treatment process (S1 through S3) for most of the studied endpoints. (See SI.7 section for further discussion of removal efficiencies.) Due to the operational strategy of the full-scale trial and our chosen sampling strategy (grab samples), our results represent a snap-shot of the pollutant pressure in this specific system at the time of sampling. Further research is needed to evaluate seasonal or temporal trends.

We can summarise three major findings in this study. Firstly, at the end of the pipeline (S4), as well as further downstream (S6 and S7), no increase in Nrf2 activity could be attributed to the additional chlorination treatment of effluent wastewater in the second campaign. Rather, there was an increase in both campaigns with a larger increase in the absence of applied chlorination treatment. Secondly, we detected an increase in AhR activity at the end of the pipeline, in both campaigns, but a stronger increase after chlorination treatment. This could be due to a source of AhR agonists between sample points S3 and S4 or that chlorination treatment of wastewater effluent might cause AhR activating by-products (see Section 3.4.) Thirdly, we found a decrease in ER agonistic activity after chlorination treatment indicating degradation of ER agonists following chlorination treatment (see Section 3.5.). Despite varying bioactivity in the incoming water to the DWT plant (S6), there were generally equal and low residual activities in the finished drinking water in the two campaigns. This shows that the treatment methods at Sant Joan Despí DWT plant have high removal efficiencies irrespective of the load of bioactivities observed in the untreated water. Additionally, it demonstrates that this kind of wastewater reclaim set-up for collecting source water for drinking water production can be made successfully without compromising drinking water quality, for the health-relevant parameters included in this study.

4. Conclusions

Our results indicate that, for the endpoints studied, indirect reuse of wastewater into drinking water sources can be successful without introducing chemical hazards in the finished potable water. Wastewater samples affected by chlorination treatment did not reveal a higher potency for oxidative stress, as determined by the Nrf2 pathway, in surface water nor in drinking water as compared to their equivalent unchlorinated samples. We detected an increase in AhR activity after chlorination treatment, which has not been reported previously. Further research is needed to clarify the mechanism behind this finding. Additionally chlorination treatment seems to have reduced ER agonist activity. This study provides important knowledge relevant to the advancement of climate change adaptation efforts. By applying an effect-based evaluation of this system of freshwater distribution, we have shown that intentional redistribution of treated wastewater into drinking water production could be an applicable and useful approach in safeguarding future water supplies.

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CRediT authorship contribution statement

Kim Frieberg: Data curation, Writing – original draft, Writing – review & editing. **Pablo Gago-Ferrero:** Conceptualization, Visualization,

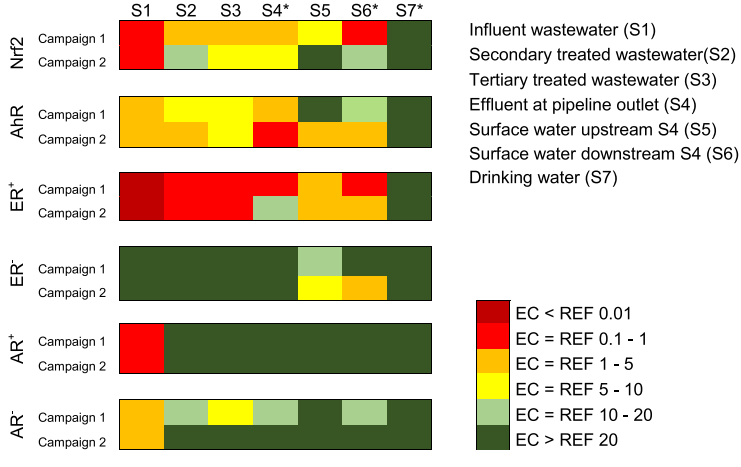

Fig. 5. Heat map displaying effect concentrations (EC) as REF at $EC_{IR1.5}$ (Nrf2), EC_{30} (ER^+, AR^+), EC_{40} (AhR) and IC_{30} (ER^-, AR^-). In sampling campaign 2, sample S4, S6 and S7 were affected by chlorination treatment (*). The color gradient was set between REF 0.01 and REF 20.

Writing – original draft, Writing – review & editing. **Lubertus Bijlsma:** Conceptualization, Visualization, Project administration, Methodology, Writing – original draft, Writing – review & editing. **Lutz Ahrens:** Conceptualization, Visualization, Writing – original draft, Writing – review & editing. **Karin Wiberg:** Conceptualization, Visualization, Data curation, Writing – original draft, Writing – review & editing. **Félix Hernández:** Conceptualization, Visualization, Writing – original draft, Writing – review & editing. **Agneta Oskarsson:** Writing – original draft, Writing – review & editing. **Johan Lundqvist:** Conceptualization, Visualization, Writing – original draft, Writing – review & editing.

Declaration of Competing Interest

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. All other authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.watres.2023.120147.

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Effect-based evaluation of water quality in a system of indirect reuse of wastewater for drinking water production

Frieberg Kim[1,*], Gago-Ferrero Pablo[2, 3] Bijlsma Lubertus[4], Ahrens Lutz[5], Wiberg Karin[5], Hernández Félix[4], Oskarsson Agneta[1], Lundqvist Johan[1]

[1]Department of Biomedical Sciences and Veterinary Public Health, Swedish University of Agricultural Sciences (SLU), Box 7028, SE-750 07 Uppsala, Sweden. kim.frieberg@slu.se agneta.oskarsson@slu.se johan.lundqvist@slu.se

[2] Present address: Department of Environmental Chemistry, Institute of Environmental Assessment and Water Research – Severo Ochoa Excellence Center (IDAEA), Spanish Council of Scientific Research (CSIC), Jordi Girona 18-26, 08034-Barcelona, Spain. pgfqam@cid.csic.es

[3]Catalan Institute for Water Research (ICRA), Institut Català de Recerca de l'Aigua (ICRA), Edifici H20 – Parc Cientific i Tecnològic de Girona, Carrer Emili Grahit, 101, E-17003 Girona, Spain.

[4]Environmental and Public Health Analytical Chemistry, Research Institute for Pesticides and Water, University Jaume I, E-12071 Castellón, Spain. bijlsma@uji.es hernandf@uji.es

[5]Department of Aquatic Sciences and Assessment, Swedish University of Agricultural Sciences (SLU), Box 7050, SE-750 07 Uppsala, Sweden. Lutz Ahrens lutz.ahrens@slu.se Karin.Wiberg@slu.se

*Corresponding author:

Kim Frieberg
Department of Biomedical Sciences and Veterinary Public health, Swedish University of Agricultural Sciences (SLU), Box 7028, SE-750 07 Uppsala, Sweden

Email: kim.frieberg@slu.se

Appendix A:
Supporting information

Table S1: Water sample characteristics

	Sample site	TOC (mg C/L)	pH	Conductivity (µS/cm)
Campaign 1	S2	15.1	7.2	2144
	S3	9.4	6.9	1951
	S4	10.5	7.2	2131
	S5	6.1	8.4	1513
	S6	8.7	8.0	1758
	S7	0.6	7.8	554
Campaign 2	S2	8.0	7.4	2248
	S3	8.1	6.9	2226
	S4	7.9	7.5	2259
	S5	5.7	8.0	1197
	S6	6.3	8.3	1481
	S7	2.0	7.4	866

All endpoints measured at ICRA

SI. Method

SI.1 Solid Phase Extraction (SPE)

Water samples were subjected to extraction within 24 h of collection along with MilliQ-water procedural blanks at Catalan Institute for Water Research (ICRA). SPE cartridges were conditioned without vacuum using 6 mL methanol (MeOH) followed by 6 mL MilliQ water. Samples were loaded under vacuum at a rate of ≈ 1 drop per second. After sample loading, the cartridges were dried under vacuum for 20 min. The samples were then eluted without vacuum using 4 mL of MeOH:ethyl acetate (1:1 v/v) (containing 2% ammonia). Subsequently, the cartridges were dried for 1 min under vacuum and 2 mL of MeOH:ethyl acetate (1:1 v/v) (containing 1.7% formic acid) followed by 1 min of vacuum drying. Extracts were evaporated under a gentle nitrogen stream to a volume of 100 µL. After transport to Sweden the extracts were reconstructed to a final volume of 500 µL (1:4 v/v MeOH:EtOH). Samples were stored at -20^{o}C pending bioassay analysis.

SI.2 Cell culture

Nrf2 stably transfected cells, MC7AREc32, were cultured in Modified Eagle Medium (with 4.5 g/L glucose) (DMEM) (Lonza, Basel, Switzerland) supplemented with 2 mM L-glutamine (Lonza, Basel, Switzerland) with a final concentration of 100 U mL^{-1} penicillin, 100 µg mL^{-1} streptomycin, 0.25 µg mL^{-1} amphotericin B) (Gibco, Thermo Fisher Scientific), 10 % fetal bovine serum (FBS) (Gibco, Thermo Fisher Scientific) and 100 µg mL^{-1} of Hygromycin B (InvivoGen, USA).

VM7Luc4E2 cells, stably transfected to assay ER activity, were cultured in RPMI 1640 (Gibco, Thermo Fisher Scientific) supplemented with 8% fetal bovine serum (FBS) (Gibco, Thermo Fisher Scientific) and 0.9% penicillin-streptomycin (100 U mL^{-}

^{1} penicillin, 100 µg mL^{-1} streptomycin, Lonza). 0.55 mg/ml Gentamicin (Gentamicin Sulfate, 50mg mL^{-1}, Lonza) was used as positive selector. VM7Luc4E2 cells, were grown in experimental medium consisting of DMEM medium (with 4.5 g L^{-1} glucose) (Lonza), 4.5 % dextran-charcoal treated fetal bovine serum (Thermo Scientific), 2 % L-glutamine (Lonza, Basel, Switzerland), 0.9% penicillin-streptomycin (100 U mL^{-1} penicillin, 100 µg mL^{-1} streptomycin, Lonza) and 0.38 mg mL^{-1} Gentamicin (Gentamicin Sulfate, 50 mg mL^{-1}, Lonza) was used as positive selector.

Stably transfected HepG2-cells, DR EcoScreen, were used for aryl hydrocarbon receptor activation (AhR) activity assay. Cells were cultured in Minimum Essential Medium (α MEM) (Gibco), 5 % FBS (Gibco,Thermo Fisher Scientific), 1 % penicillin-streptomycin (100 U mL^{-1} penicillin, 100 µg mL^{-1} streptomycin, Lonza) and 1 % L-Glutamine (Lonza, Basel, Switzerland) and 150 µg/mL Hygromycin B (InvivoGen, USA) was used as selector.

The AR-EcoScreen cell line was used for the AR reporter gene assay. The AR-EcoScreen cells were cultured in DMEM F12 (Sigma) medium supplemented with 5 % FBS (Gibco), Penicillin/Streptomycin with a final concentration of 100 U mL^{-1} penicillin, 100 µg mL^{-1} streptomycin, (Lonza) 2 mM L-glutamine (Lonza, Basel, Switzerland), 100 µg mL^{-1} Hygromycin B (InvivoGen, USA), and 200 µg mL^{-1} Zeocin (Invitrogen, CA, USA). Experimental medium consisted of DMEM F12 medium (Sigma) supplemented with 5 % dextran-charcoal treated fetal bovine serum (Thermo Scientific), 4 mM L-glutamine (Gibco, Thermofisher Scientific) and Penicillin/Streptomycin 100 U mL^{-1} penicillin, 100 µg mL^{-1} streptomycin, (Lonza).

NFkB activity was assessed in stably transfected HepG2- NFkB cells. These cells were cultured in DMEM (Eagle,s Minimum Essential Medium LONZA), with 10 % FBS (Gibco,Thermo Fisher Scientific), 1 % pen-strep (penicillin-streptomycin 100 U mL^{-1} penicillin, 100 µg mL^{-1} streptomycin)(Lonza) and Hygromycin B 100 microgram/mL (InvivoGen, USA) was used as positive selector.

The cells were cultured in an incubator with humidified atmosphere at 37˚C containing 95% air and 5% CO_2. Medium was changed every 2-3 days. Trypsin-EDTA (Gibco, Thermofisher Scientific) was used for sub-culturing of cells.

SI. 3 Cell treatment

All water samples were tested in cell viability assay and reporter gene assays in quadruplicates. In all experiments, solvent control was included (elsewhere named control), consisting of 1% 1:4 ethanol:methanol (v/v), equivalent to the solvent concentration in the samples. Solvent controls were tested in eight replicates.

SI. 4 Cell viability assessment

The MTS-based colorimetric assay (Cell Titer 96® Aqueous One Solution Cell Proliferation Assay) (Promega) was used for all cells lines to assess the cell viability except VM7Luc4E2 cells where CellTiter-Glo® Luminescent Cell Viability Assay (Promega) was used.

Cells were seeded at respective density (Table S2) in transparent 384-well plates (Costar® Corning Incorporated), VM7Luc4E2 cells were seeded in white 384-well plates (Costar® Corning Incorporated), and were left to incubate for 24 hours. Water sample extracts were prepared at 5x exposure concentration in a transparent 96-well plate (Costar® Corning Incorporated) and then transferred to 5x dilution into the 384-well plate using a multichannel pipette. The cells were then exposed for 24 hours before termination of the experiment.

At experiment termination, 10 µL cell viability assay reagent, was added to each well and cells were incubated for approximately 30 minutes. Absorbance and luminescence for the MTS and ATP-based assay, respectively, was measured on a TECAN microplate reader (Infinite® M1000, PerkinElmer, Waltham, MA, USA).

SI.5 Reporter gene assays

Table S2: Detailed endpoints, Cell lines & Assays

Endpoint	Cell line	Seeding density (cells/mL) In 40 µL culture medium /well	Sample exposure volume /well (µL)	Total exposure volume in 384-well plate (µL)	Sample preparation volume in 96-well plate (µL)
Androgen receptor agonism	AR-EcoScreen	$1.5x10^5$	10	50	60
Androgen receptor antagonism	AR-EcoScreen	$1.5x10^5$	10	50	60
Estrogen receptor agonism	VM7Luc4E2	$4x10^5$	40	80	200
Estrogen receptor antagonism	VM7Luc4E2	$4x10^5$	40	80	200
Nrf2 activity	MCF7c32ARE	$1.3x10^5$	10	50	60
NFkB activity	HepG2-Signosis	$1x10^5$	10	50	60
Aryl hydrocarbon receptor activation	DR EcoScreen	$1x10^5$	10	50	60

As in the cell viability assessment all samples were prepared at 5x exposure concentration in 60 µL of experimental medium in a 96-well plate (3 µL sample to 57 µL medium for a final 1 % solvent exposure concentration). 10 µL of each sample dilution was transferred in four replicates into a pre-seeded (40 µL) 384-well plate using a multichannel pipette. Samples for ER activity were prepared at 2x exposure concentration in 200 µL in a 96-well plate and then 40 µL was transferred to each replicate 384-plate well.

All reporter gene experiments were conducted in white clear-bottomed 384-well plates (Corning, NY, USA) over a three-day period. Seeding of cells on day 1, exposure to water samples and reference compounds on day 2, and luciferase measurement on day 3.

At experiment termination, cells were lysed with passive lysis buffer (PLB) (Promega), 10 µL per well, for 20 minutes. Luciferase activity was measured using the Luciferase® Reporter Assay System (Promega) according to the manufacturer's instructions. Luminescence was measured on a TECAN plate reader (Infinite® M1000, PerkinElmer, Waltham, MA, USA) equipped with an automatic injection syringe. The injection volume for the Firefly luciferase reagent was 10 µL per well.

Luminescence measurement was conducted over a 5 s period, 2 s after reagent was automatically injected with Firefly luciferase reagent. White opaque adhesive stickers were attached to plate bottom before measurement.

tertButylhydroquinone (tBHQ) (Sigma Aldrich, USA) was used as reference compound in the Nrf2 reporter gene assay and tested in the range of 0.78-25 µM. 2,3,7,8-Tetrachlordibenso-p-dioxin (TCDD) was used as reference compound for AhR reporter gene assay and was tested in the range of 0.01-31 pM. Tumor necrosis factor alpha (TNFα) was used as reference compound for NFκB reporter gene assay in concentrations ranging from 0.2-50 ng mL^{-1}.

The ER and AR reporter gene assays were conducted in agonist and antagonist modes. 17β-estradiol (Sigma-Aldrich, USA) was used as reference compound in the ER agonist reporter gene assay and tested in concentrations in the range of 0.4 x 10^{-13} M to 4 x 10^{-10} M. Methoxychloride was used as positive control at 9.06 µM. In the ER antagonistic mode, 9.18 x 10^{-11} M of 17β-estradiol was added together with the water samples and standards to activate the ER. Raloxifene was used as reference compound in the range of 2.45 x 10^{-8} M to 9.57 x 10^{-11} M and Tamoxifen at 3. 36 x 10^{-6} M was used as positive control.

In the AR agonist reporter gene assay, reference compound for activation of the receptor was dihydrotestosterone (DHT) (Sigma-Aldrich, USA) in the range of 10^{-10} M to 10^{-16} M. In the AR antagonistic mode, 200 pM DHT was added together with the water samples and standards to activate the AR and reference compound was hydroxyflutamide (OHF) (Sigma-Aldrich, USA) in the range of 1 x 10^{-5} to 10 µM.

SI. 6 Data Evaluation

The bioactivity data for ER agonistic activity, AhR activity and AR agonistic activity were normalized to assay maximum activity. The data was first normalized for background activity by subtracting the mean activity of the solvent control (*n*=8) and then related to the maximum activity of the reference compound set to 100 %.

Cut-off values for classification of bioactivity were based on the limit of detection (LOD), calculated for AhR, ER and AR agonist activities as one plus three times the standard deviation (SD) of the solvent control. For Nrf2 the cut-off value was set as an induction ratio of 1.5 compared to the solvent control, as no true induction maximum applies for this endpoint, as proposed by Escher *et al*.(1). Based on the same principle as for Nrf2 induction, the cut-off for NFkB induction was also set at an induction ratio of 1.5 compared to the solvent control.

For ER and AR antagonistic activities, the bioactivity data for water samples and reference compounds were normalized to solvent control spiked with E2 and DHT respectively (set to 1). The LOD was calculated as one minus three times the SD. Any sample with a response equal to or above the cut-off was defined as bioactive

for agonistic endpoints. Any sample with a response equal to or below the cut-off was defined as bioactive for antagonistic endpoints. For all assays except Nrf2 and NFκβ standard curves of the positive control were produced by fitting data to a four-parameter sigmoidal curve model using GraphPad prism 8.3.0. Nrf2 and NFκβ reference compounds were fitted to a linear model. Regression analysis of concentration-response relationships of the water samples was performed in GraphPad Prism 8.3.0. Effect concentrations (EC_x) or inhibitory concentrations (IC_x), represent x % of the maximum effect of the reference compound. EC_{20}, EC_{40}, IC_{30} and $EC_{IR1.5}$ values were calculated for the water samples and reference compounds. Standard error was calculated for EC values of bioactive samples according to Escher *et al*. 2018 (2) based on standard error propagation. This was calculated for AhR activity, AR- and ER agonistic activity where a linear portion (up to 40 % of assay maximum) of the log logistic curve of the reference compound could be extracted. Standard errors were also calculated for EC values of active samples in the Nrf2 assay in the same way.

Bioanalytical equivalent concentrations (BEQs) were calculated according to Escher *et al*.(3) (equation 1). Standard errors for BEQ values were calculated according to Escher *et al.* 2018.(2) For wastewater samples, removal efficiencies were calculated as % of BEQ as compared to incoming wastewater (S1) for each treatment step. For drinking water samples, removal efficiencies were calculated as % of BEQ as compared to incoming water to the drinking water treatment facility (S6). Additionally, the removal efficiency of the chlorination treatment of effluent wastewater was calculated as % of BEQ at the end of the pipeline (S4) as compared to tertiary treated wastewater (S3).

(1) $$\text{BEQ} = \frac{(\text{EC}_x)\text{Reference compound}}{(\text{EC}_x)\text{Sample}}$$

SI. Results & discussion

SI. 7 Removal efficiency

Wastewater treatment

The removal efficiencies of current wastewater treatment methods as compared to the incoming wastewater (Table 5 in manuscript) ranged between 89 to 93 % for Nrf2 activity (-216% after chlorination), 65 to 82 % for AhR activity, 99.8 to 99.9% for ER agonistic activity, 75 to 95 % for AR antagonistic activity and was above 99.8 % for AR agonistic activity.

According to a review from 2019 (4), the removal of estrogenic activity assessed across different WWTPs (median, *n*=35) was around 92 % which is coherent with our results here. Furthermore, Völker *et al*. (4) also reported removal of androgenic effects at 98 % (median, *n*=10) across different WWTPs. The broad overview that removal of estrogenic and androgenic agonistic activity is generally high is also supported in a more recent review by Enault *et al*. 2023 (5). Though they highlight that despite high numbers of removal efficiency (median 5%) for estrogenic activity, remaining activity in outlet water could potentially pose a problem. While AR agonistic activity is typically not detected in outlet water, we previously reported on removal of AR agonistic activity ranging from 50 to >99% and removal of ER agonistic activity in the range of 77- >99% (*n*=5 for both endpoints) (6).

Both Völker (4), and Enault (5) as well as our previous data on removal of AhR activity and oxidative stress describe removal for these two endpoints as highly variable and site specific. Median removal of AhR activity from Völker *et al.* was 74.5 % (*n*=8), 4% (*n*=7) from Enault *et al*. and our data of 16-60% (*n*=5) (6).Median removal for oxidative stress was reported at 64 % (*n*=6) (5), and 87% (*n*=2) (4) and or on data on oxidative stress removal ranging from 25 to 56% (*n*=2)

Drinking water treatment

The removal efficiency of drinking water treatment was high in this study 98% for Nrf2 activity, 80-90 % for AhR activity, >83-99% for ER agonistic activity, 99.9 % for ER antagonistic activity and >85 % for AR antagonistic activity (Table 5 in manuscript).

Similarly, Enault *et al*. 2023 reported high removal for ER agonistic activity (5). Onward removal of AhR activity was reported with a median removal of 100 % and oxidative stress with low or no removal (5).

SI. 8 Viability assessment

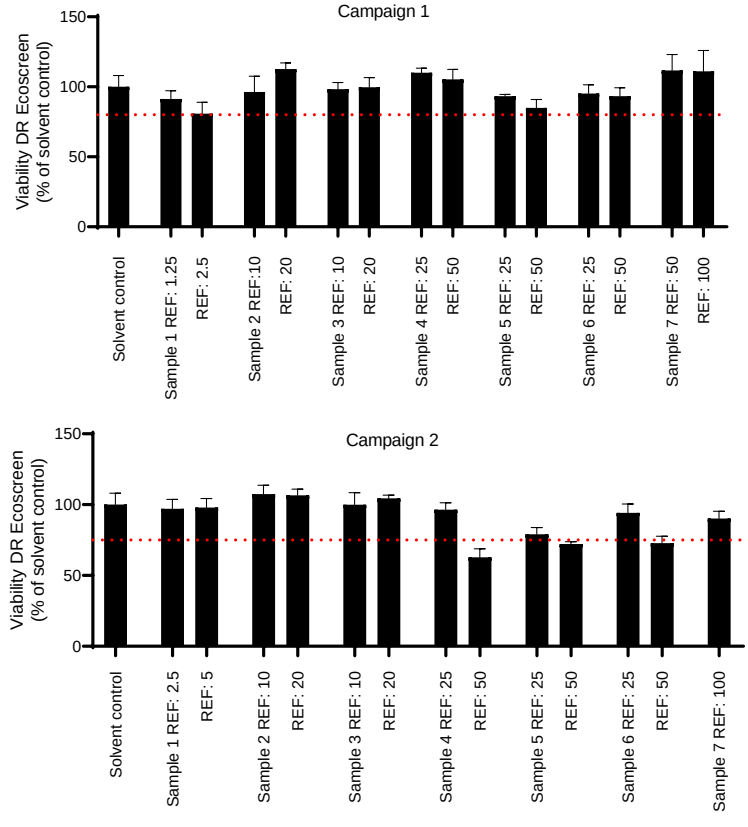

Figure S1: Viability assessment in DR Ecoscreen. MTS production (measured as absorbance) normalised to solvent control, set at 100%. The red line signifies cut-off at 80 % of solvent control, mean±SD; *n*=8 for control and *n*=4 for samples.

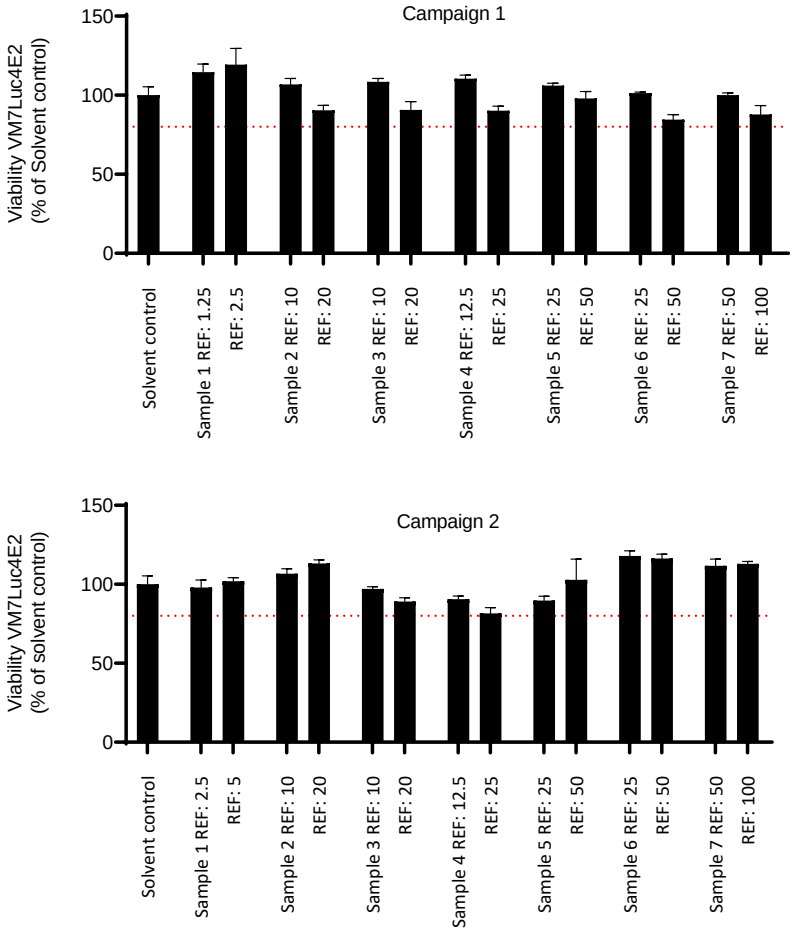

Figure S2: Viability assessment (ATPase) in VM7Luc4ER. ATPase production (measured as luminescence) normalised to solvent control, set at 100%. The red line signifies cut-off at 80 % of solvent control, mean±SD; *n*=8 for control and *n*=4 for samples.

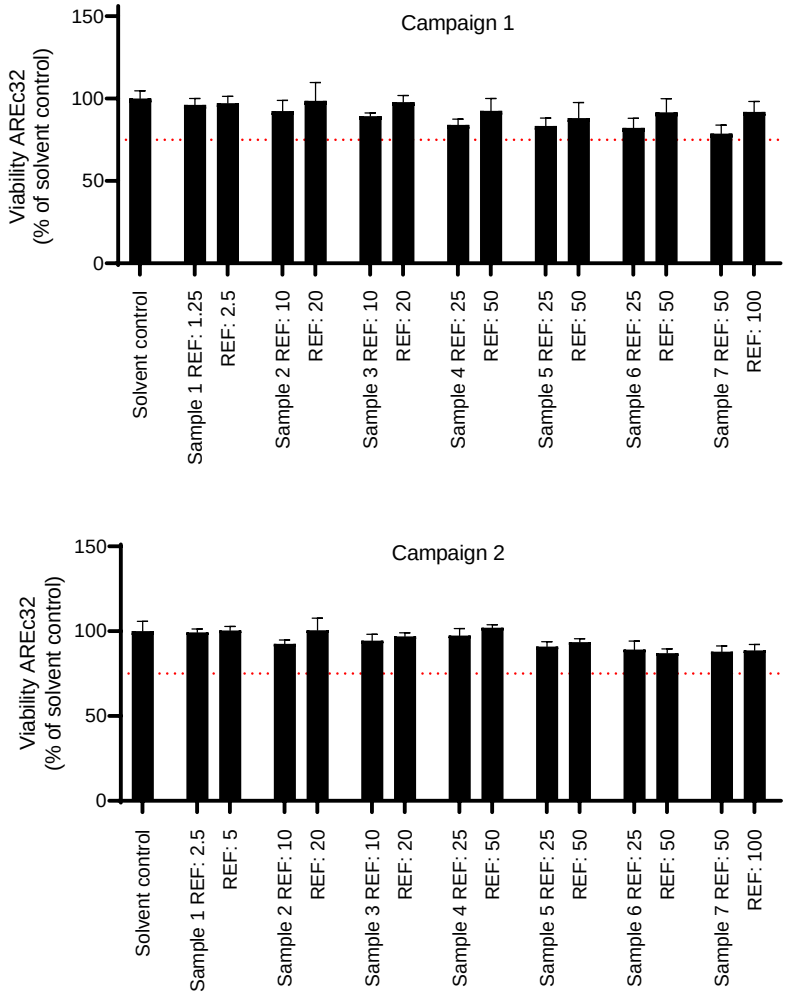

Figure S3: Viability assessment in AREc32, as MTS production (measured as absorbance) normalised to solvent control, set at 100%. The red line signifies cut-off at 80 % of solvent control, mean±SD; *n*=8 for control and *n*=4 for samples.

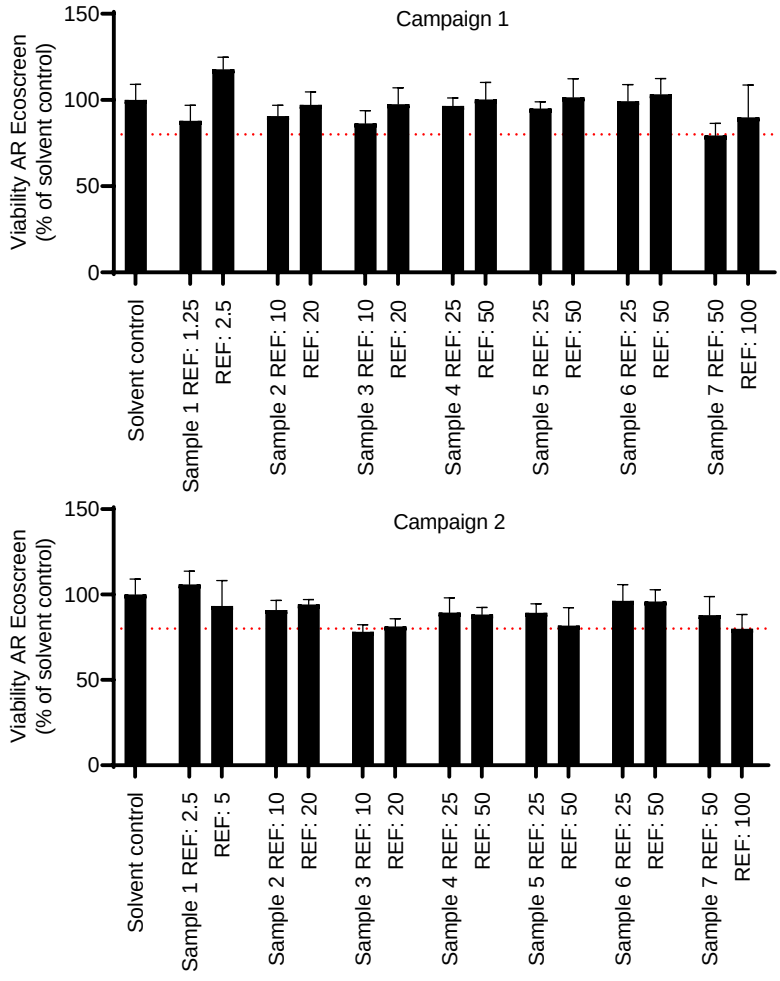

Figure S4: Viability assessment in AR EcoScreen as MTS production (measured as absorbance) normalised to solvent control, set at 100%. The red line signifies cut-off at 80 % of solvent control, mean±SD; *n*=8 for control and *n*=4 for samples.

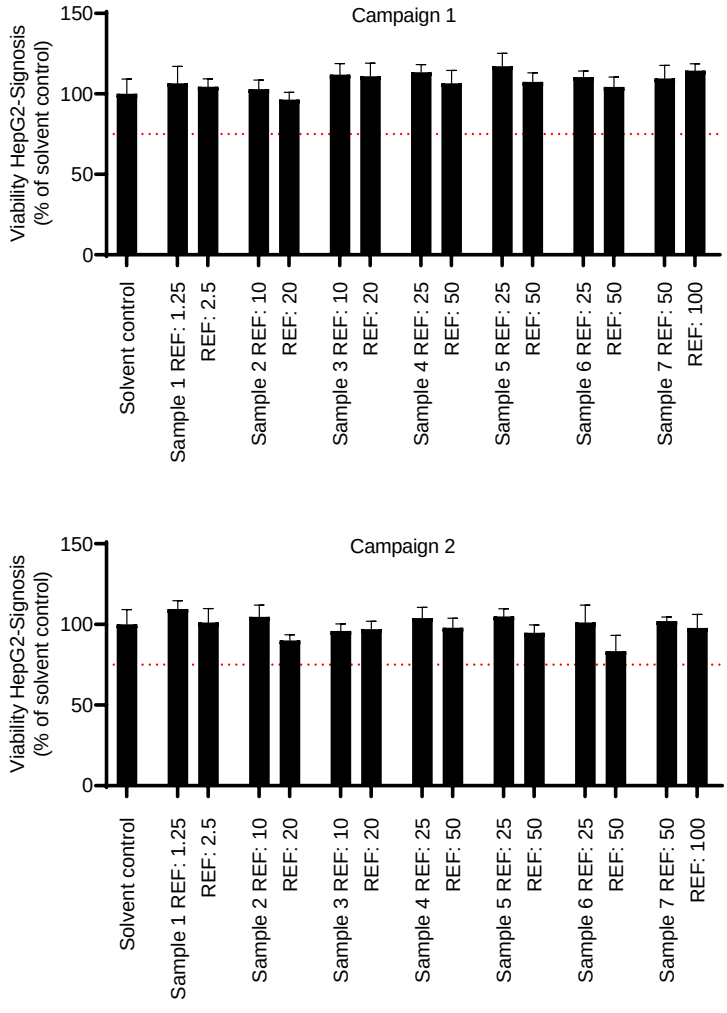

Figure S5: Viability assessment in HepG2-Signosis. MTS production (measured as absorbance) normalised to solvent control, set at 100%. The red line signifies cut-off at 80 % of solvent control, mean±SD; *n*=8 for control and *n*=4 for samples.

SI. 9 Bioactivity assessment

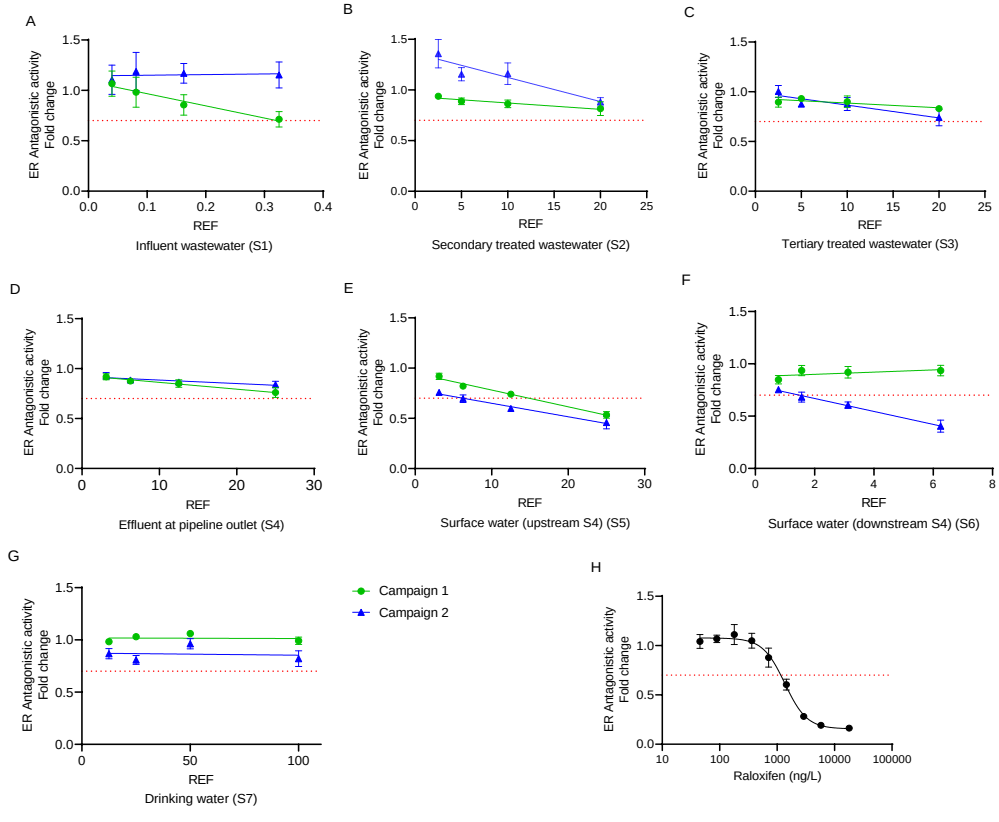

Figure S6: Concentration-response of Estrogen receptor (ER) antagonistic activity in water samples collected at seven sites (A-G; S1 to S7) from two campaign events. Activities of water samples (*n*=4 per concentration) and Raloxifene as a reference compound (*n*=4 per concentration) are displayed as fold change (mean±SD), compared to solvent control (*n*=8) set to 1. The highest tested concentrations ranged from below 1 to 100 depending on the used enrichment factor and cytotoxicity of each sample (Figure S4).). The red line signifies cut-off for bioactivity at 0.7 fold inhibition compared to control.

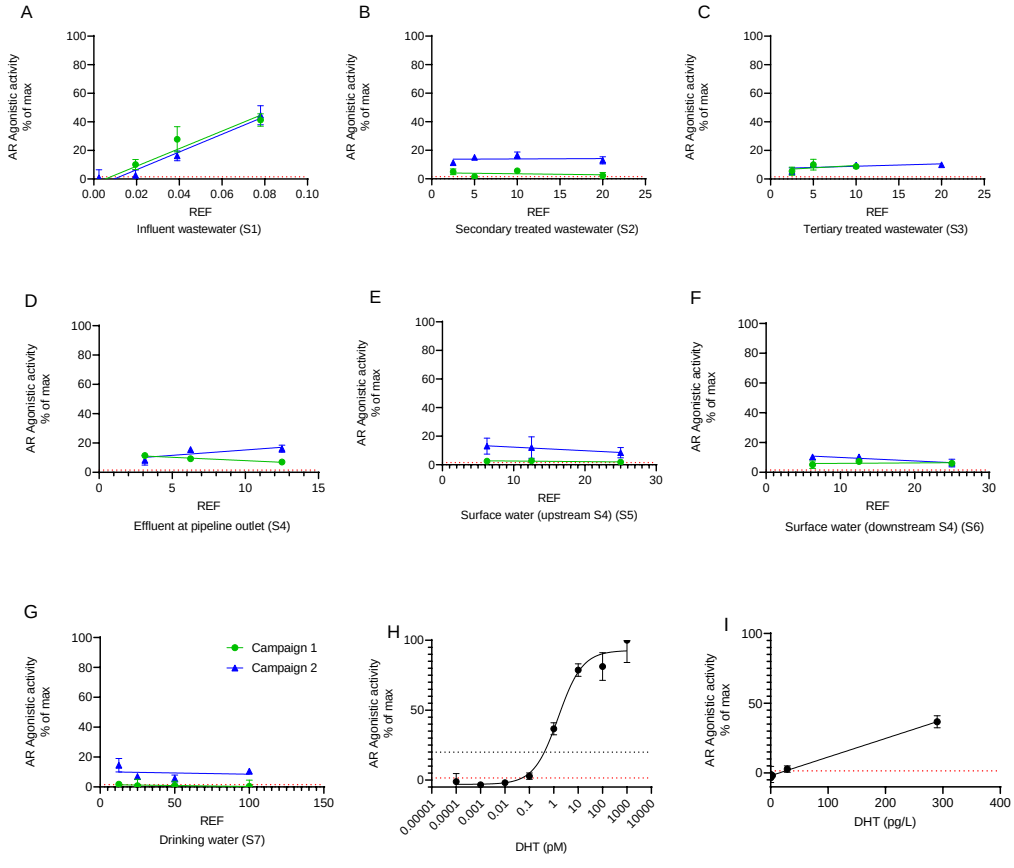

Figure S7: Concentration-response of Androgen receptor (AR) activation in water samples collected at seven sites (A-G; S1 to S7) of two campaigns. Activities of water samples (*n*=4 per concentration) and DHT as a reference compound (*n*=4 per concentration) are displayed as % of assay maximum (mean±SD) as compared to reference compound. The highest tested concentrations ranged from below 1 to 100 depending on the used enrichment factor and cytotoxicity of each sample (Figure S4). The red dotted line represents the cut-off for bioactivity at 1+3xstdev of solvent control (*n*=8). The linear portion of the reference compound curve (panel I) was used to calculate standard errors for EC- and BEQ-values according to Escher *et al.* 2018.

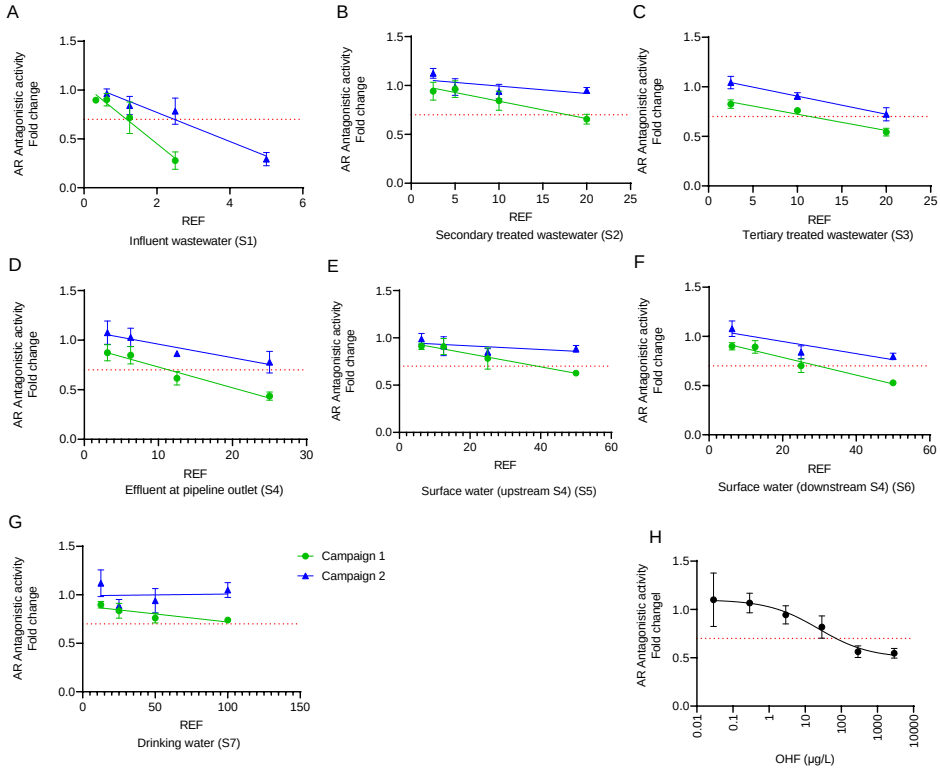

Figure S8: Concentration-response of Androgen receptor (AR) antagonism in water samples collected at seven sites (A-G; S1 to S7) from two campaign events. Activities of water samples (*n*=4 per concentration) and OHF as a reference compound (*n*=4 per concentration) are displayed as fold change (mean±SD), compared to solvent control (*n*=8) set to 1. Highest tested concentrations ranges from 1.25 to 100 depending on the used enrichment factor and cytotoxicity of each sample (Figure S4). The red line signifies cut-off for bioactivity at 0.7 fold inhibition compared to control.

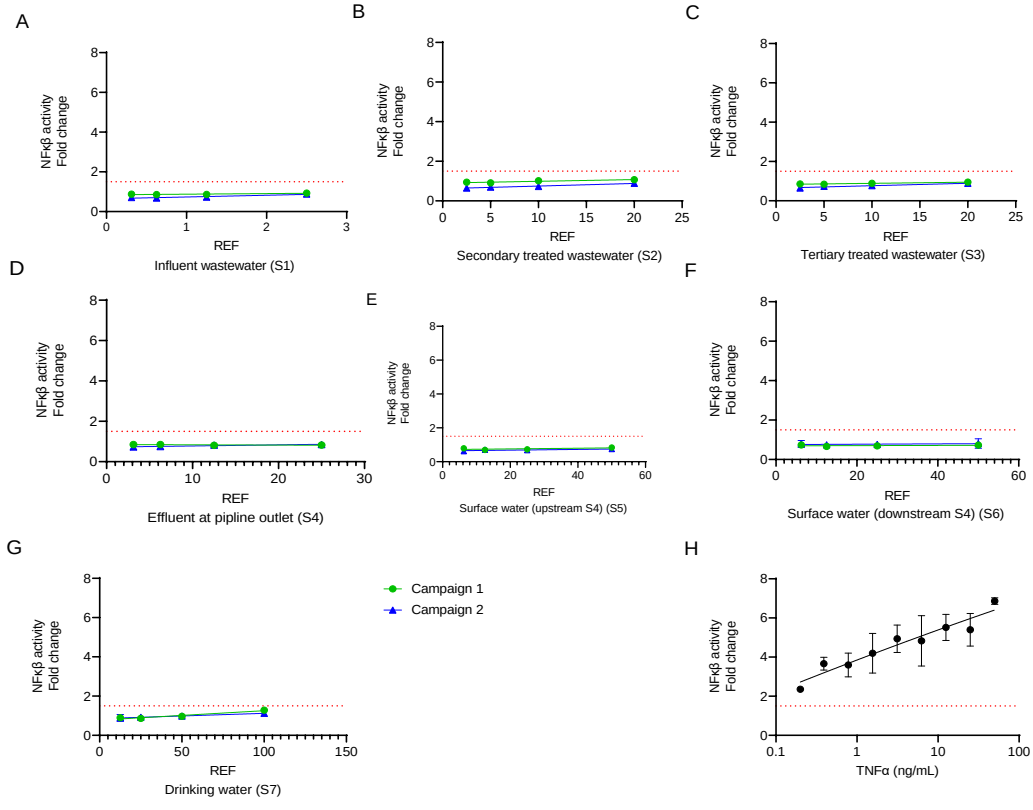

Figure S9: Concentration-response of NfkB activation in water samples collected at seven sites (A-G; S1 to S7) from two campaign events. Activities of water samples (*n*=4 per concentration) and tNFα as a reference compound (*n*=4 per concentration) are displayed as fold change (mean±SD), compared to solvent control (*n*=8) set to 1. The highest tested concentrations ranged from 1.25 to 100 depending on the used enrichment factor and cytotoxicity of each sample (Figure S4). The red line signifies cut-off for bioactivity at 1.5 fold induction compared to solvent control.

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It is known that pollution of the aquatic environment occurs through insufficient wastewater treatment. This thesis aimed to investigate the occurrence of pollutants in different water samples, using effect-based methods as tool for evaluating changing levels of pollutants through wastewater and drinking water treatment methods, sample preparation methods and for a complex mixture assessment.

Kim Frieberg received her doctoral education at the Department of Animal Biosciences, Swedish University of Agricultural Sciences. Her BSc and MSc degrees were obtained from Uppsala University.

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