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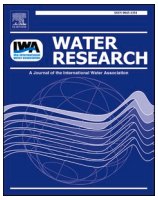

Effect-based evaluation of water quality in a system of indirect reuse of wastewater for drinking water production

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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

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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.

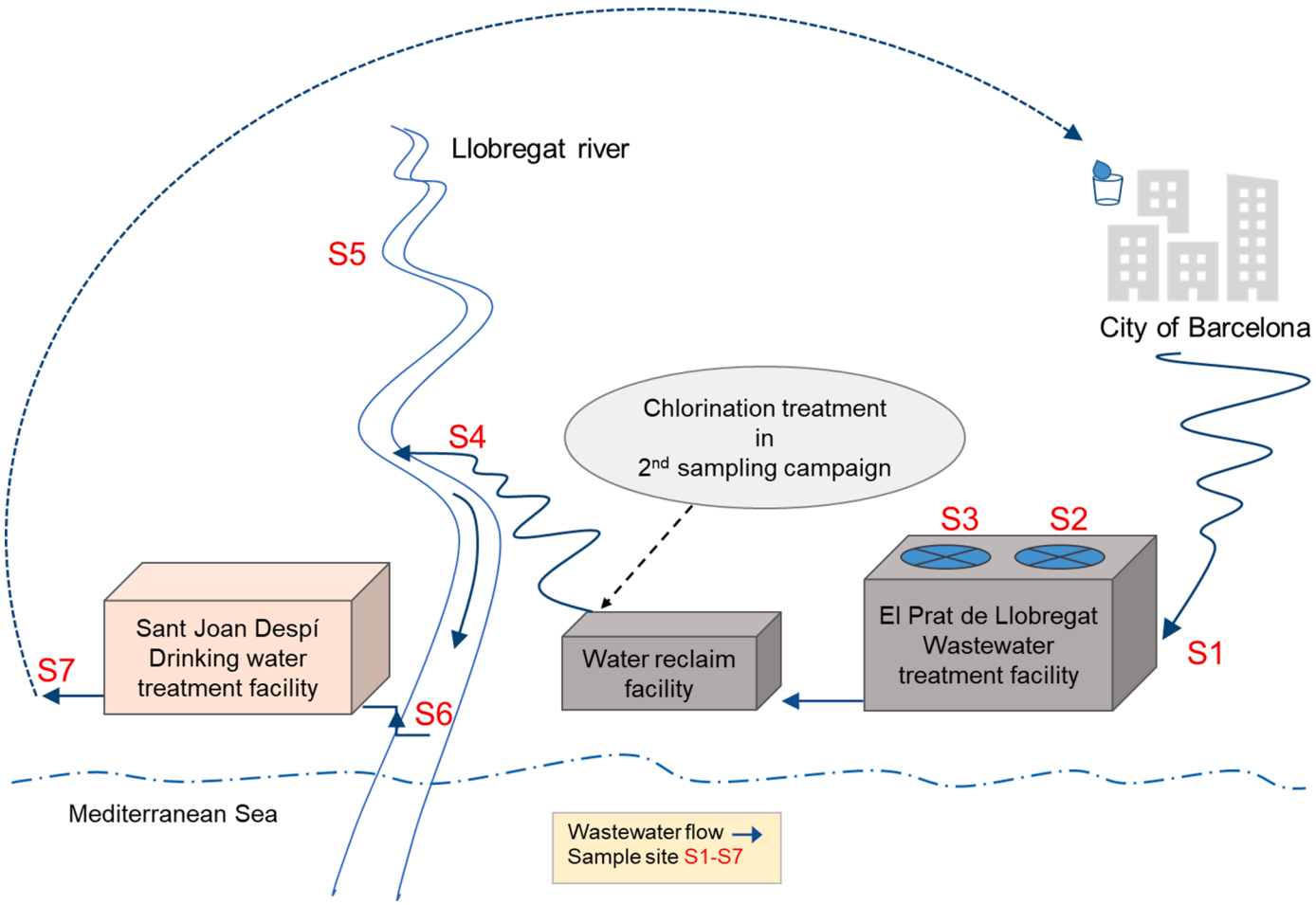

Fig. 1. Schematic description of sampling sites. The actual distance between S4 and S6 was 8.5 km.

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

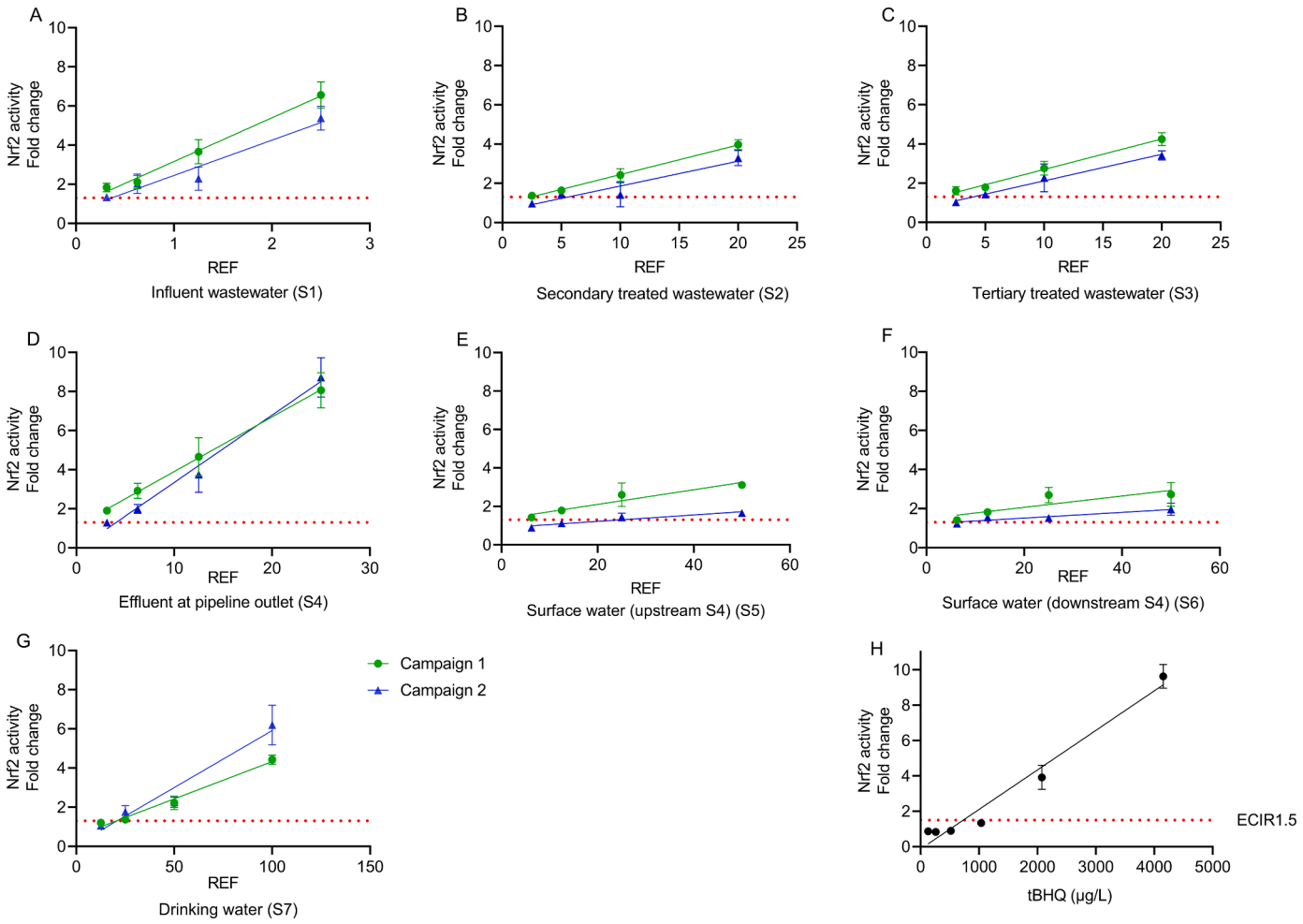

Fig. 2. Concentration-response of the Nrf2 bioassay in water samples collected at seven sites (A-G; S1 to S7) from two campaign events. Activities of water samples (n = 4 per concentration) and tBHQ as a reference compound (n = 4 per concentration) are displayed as fold change (mean±SD), compared to 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

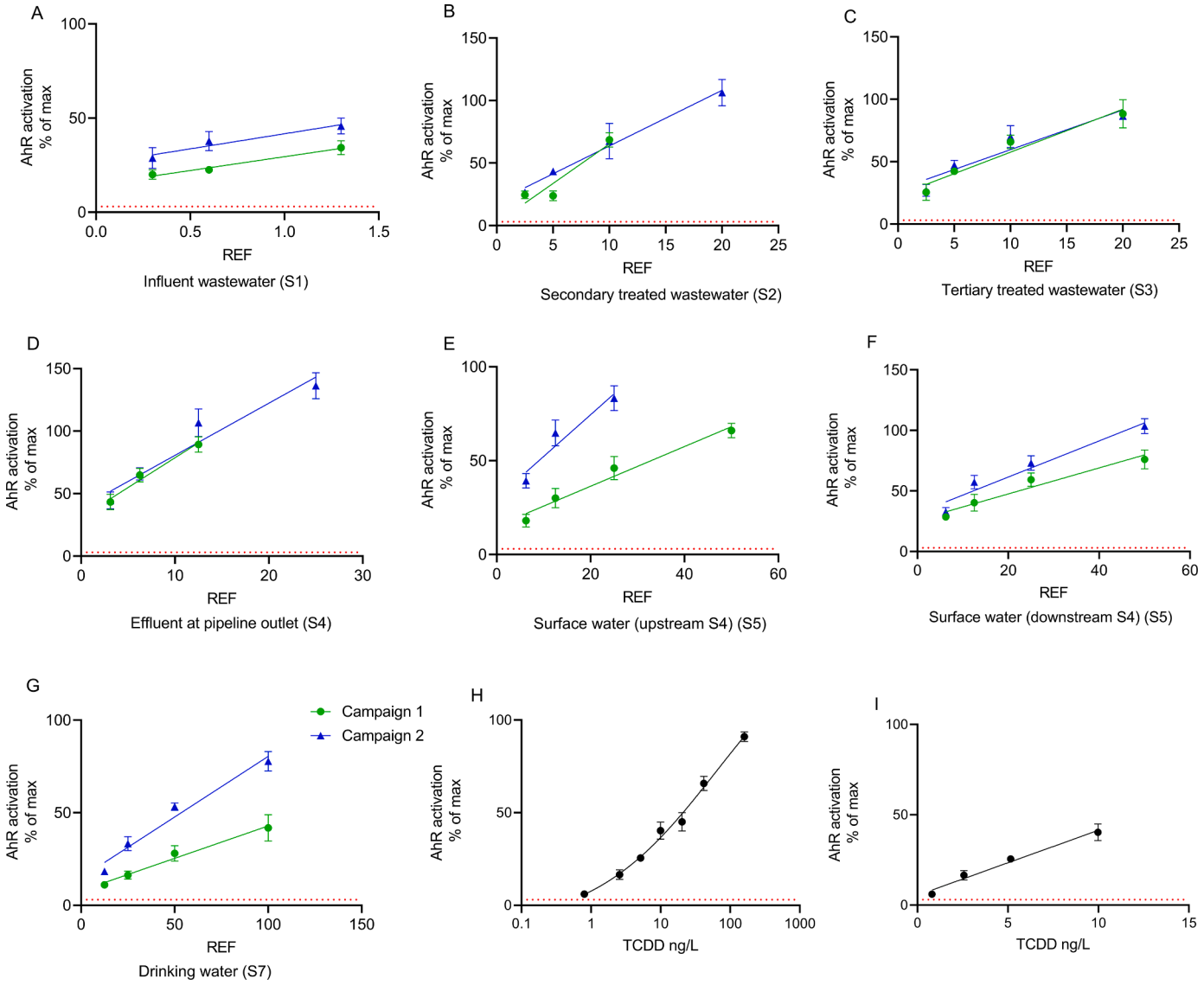

Fig. 3. Concentration-response of AhR activity in water samples collected at seven sites (A-G; S1 to S7) from two campaign events. Activities of water samples (n = 4 per concentration) and TCDD as a reference compound (n = 4 per concentration) are displayed as% of assay maximum (mean±SD), as compared 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

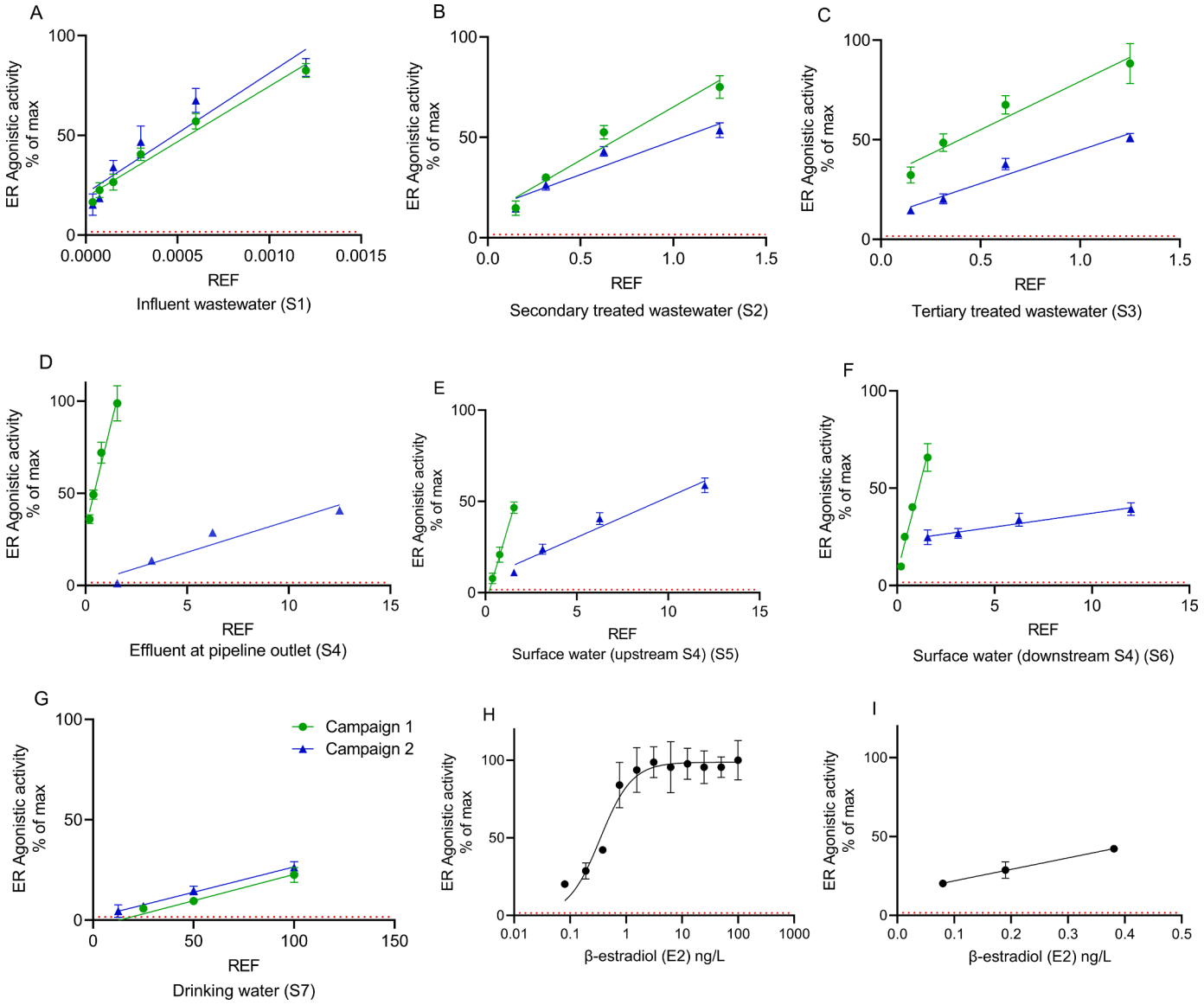

Fig. 4. Concentration-response of activation of the estrogen receptor (ER) in water samples collected at seven sites (A-G; S1 to S7) from two campaign events. Activities of water samples (n = 4 per concentration) and 17β-estradiol (E2) as reference compound (n = 4 per concentration) are displayed as% 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

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

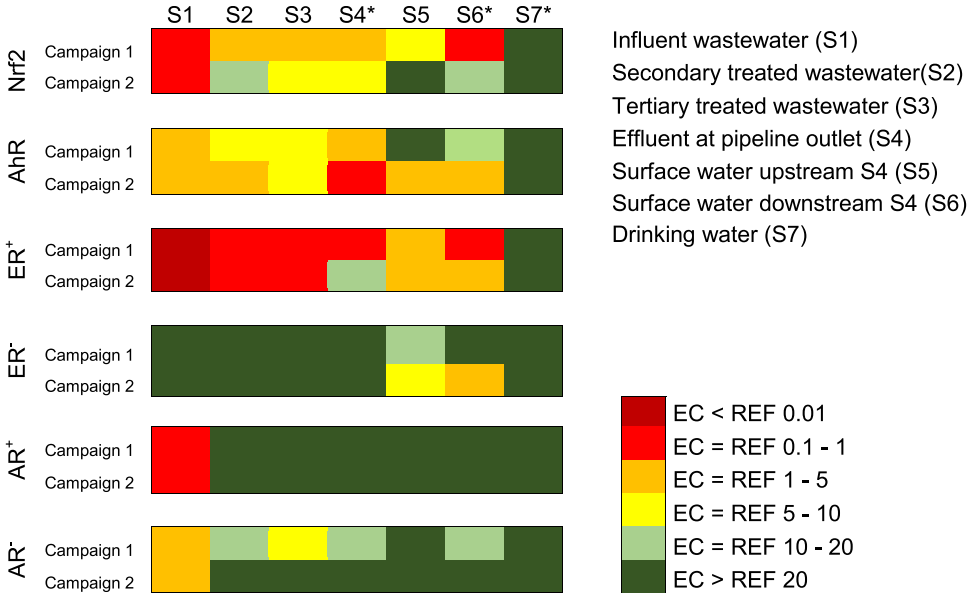

Fig. 5. Heat map displaying effect concentrations (EC) as REF at $EC_{IR1.5}$ (Nrf2), EC_{30} (ER^+, AR^+), EC_{40} (AhR) and IC_{30} (ER^-, AR^-). In sampling campaign 2, sample S4, S6 and S7 were affected by chlorination treatment (*). The color gradient was set between REF 0.01 and REF 20.

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