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

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ABSTRACT

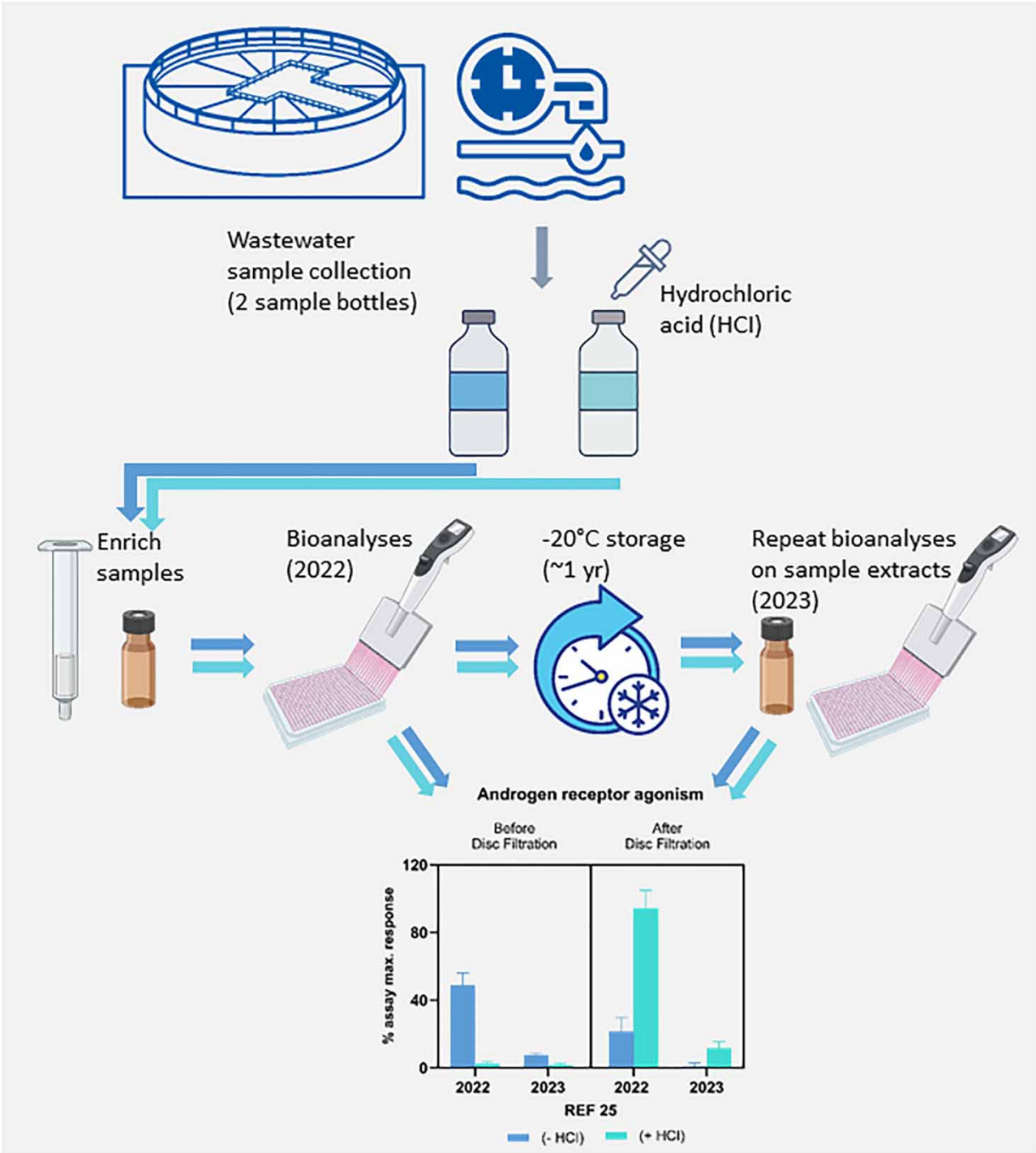
An underemphasized aspect of sampling strategies in effect-based *in vitro* testing is to determine suitable collection and preparation techniques. In the current study, the impact of sample acidification on bioactivities was assessed using *in vitro* bioassays for hormone receptor-mediated effects (estrogen receptor [ER] and androgen receptor [AR]) and the oxidative stress response (Nrf2 activity). Sampling was conducted at a recently upgraded Swedish wastewater treatment plant. Future plans for the treated wastewater include reuse for irrigation or as a potential drinking water source. In the AR and Nrf2 assays, acidification decreased bioactivities in the wastewater influent sample extracts, whereas acidification increased bioactivities following further treatment (disc filtration). In the ER assay, acidification had no impact on the observed bioactivities in the sample extracts. A secondary objective of the study was to assess the stability of the sample extracts over time. Lower activities were detected in the ER and AR assays in all extracts after storage for approximately 1 year. Nrf2 activities did not decrease over time, but rather increased in some of the acidified sample extracts. Overall, the findings suggest that sampling strategies involving acidification may need to be tailored depending on the selected bioassay(s) and the type of wastewater treatments being assessed.

Key words: effect-based methods, *in vitro* bioassays, sample acidification, wastewater treatment, water reuse

HIGHLIGHTS

- Sample acidification impacted *in vitro* bioactivities of wastewater extracts.
- Impact of acidification differed before versus after disc filtration treatment.
- Storage time of approximately 1 year reduced hormone receptor-mediated bioactivities.
- Findings suggest against a 'one-size-fits-all' approach involving sample acidification and storage.

GRAPHICAL ABSTRACT



INTRODUCTION

Effect-based methods (EBMs) using *in vitro* bioassays have been increasingly utilized in water quality monitoring (Escher *et al.* 2012; Altenburger *et al.* 2015; Busch *et al.* 2016; Neale *et al.* 2017; Tousova *et al.* 2017; Brack *et al.* 2019; Lundqvist *et al.* 2019) and to assess various water treatment schemes for wastewater, recycled water, and drinking water (Macova *et al.* 2011; Schenck *et al.* 2011; Conley *et al.* 2017; Leusch *et al.* 2018; Rosenmai *et al.* 2018; Barceló *et al.* 2020; Xu *et al.* 2020;

Oskarsson *et al.* 2021; Yu *et al.* 2021). EBMs are a complementary tool to chemical analysis in water quality monitoring as they can detect the biological effects of unknown chemicals and complex chemical mixtures in a given sample (Dingemans *et al.* 2019). An important but often overlooked aspect of water sampling strategies in EBMs is to develop a proper sampling design that includes the use of appropriate sample collection and preparation techniques (GWRC 2020). Sample collection and preparation are important to maintain the sample integrity and ensure accurate results in the subsequent bioanalyses such that the observed effects resulted from those compounds that were present in the water at the time of collection, which might otherwise become lost due to degradation of the bioactive compounds.

Sample acidification at the time of collection is one such sample preparation technique that can, among other purposes, limit the microbial activity in the sample, which can otherwise potentially cause the biodegradation or biotransformation of organic micropollutants (OMPs) (GWRC 2020). OMPs, by general definition, are anthropogenic-sourced compounds present in the environment in trace amounts (i.e., concentrations ranging from μg/L to less than ng/L) (Bacci & Campo 2022). The pH of a sample can be adjusted to a target pH at the time of collection by adding an acidifying agent to the sample volume. From an analytical chemistry perspective, the importance of proper collection and sample acidification techniques has already been emphasized (Ort *et al.* 2010; Vanderford *et al.* 2011). pH adjustments, for instance, are considered one of the critical factors in controlling the retention and elution of target analytes and can impact the recovery of some chemicals during the extraction process (Vulliet *et al.* 2008; Kuster *et al.* 2010). The process of reducing the pH of a sample via acidification can improve the extraction of certain weak acids, which in turn can improve the recovery of certain classes of pharmaceuticals (Escher *et al.* 2005). In addition, the removal of interfering compounds, such as dissolved organic matter, at different pHs may impact the extraction efficiency differently (Han *et al.* 2022). While sample acidification techniques have been utilized in certain studies involving *in vitro* bioassays, as summarized recently by Robitaille *et al.* 2022, only a few studies have specifically assessed the impact of sample acidification on bioactivities (Šauer *et al.* 2018; Abbas *et al.* 2019).

Other important aspects of study designs in EBMs are sample storage time and the effect storage has on bioactivities. Chemical analysis of certain pharmaceuticals and illicit drugs in aqueous environmental samples and solid-phase extracts, for instance, found that certain compounds do degrade over time and that stability was influenced by acidification (Castiglioni *et al.* 2006; Baker & Kasprzyk-Hordern 2011; Vanderford *et al.* 2011). Some studies that tested stored wastewater samples with *in vitro* bioassays reported storing the samples prior to extractions (Aerni *et al.* 2004; Cargouët *et al.* 2004; Fang *et al.* 2012; Kolkman *et al.* 2013; Könemann *et al.* 2018). Elsewhere, it has been reported that estrogenicities of wastewater effluent extracts remained similar in most of the samples extracted within 48 h versus 45 days after collection (Jarošová *et al.* 2014). However, to the best of our knowledge, there have been no studies that investigated the impact of storage time on bioactivities in solid-phase extraction (SPE)-extracted water samples.

In general, there remains a lack of standardized protocols for sample preparation techniques when using EBMs to assess water quality. Developing standardized protocols to harmonize not only the bioassay test methods themselves but also the associated sampling strategies is particularly important for the validation and acceptance of EBMs for regulatory testing purposes. In the current study, *in vitro* bioassays measuring hormonal receptor activity (estrogen receptor [ER] androgen receptor [AR]) and the oxidative stress response (Nrf2 activity) were used to assess the impact of sample acidification as well as long-term storage of water extracts on bioactivities in samples collected from a small-scale wastewater treatment plant (WWTP).

MATERIALS AND METHODS

Site description

The Kivik WWTP is located in southern Sweden in the Simrishamn municipality and services the town of Kivik. The WWTP was originally constructed over 50 years ago but was reconstructed and expanded in 2019/20 to meet future higher demands on wastewater treatment. The treatment process at this WWTP consists of pretreatment with cleaning grates, grease separation, and sand capture, followed by chemical precipitation and disc filtration. Disc filters are designed for solid removals and can separate solids as small as 30 μm up to 350 μm. This is then followed by a membrane bioreactor with two parallel ultrafilters and a mixing reservoir, followed by two parallel granular activated carbon filters. The treated water is then pumped into the adjacent Hanö Bay on the east coast of Skåne, South Sweden. This WWTP is designed for a flow of 180 m^3/h. An important consideration in the treatment design of the reconstructed plant was to implement new technologies to clean the wastewater from environmental micropollutants such as pharmaceutical residues. The aim of the facility's new

design is to be able to reuse the water for irrigation, swimming pools, and re-infiltration, and even potentially as a drinking water source, without unnecessary discharges to the sea.

Sample collection

Samples were collected in December 2021 and transported immediately to the laboratory where they were stored at −20 °C until sample extraction within 7 days. At the WWTP, samples were collected before the disc filtration unit (DF-in), after the disc filtration unit (DF-out), and at the outlet of the WWTP (TP-out) before the water is discharged into Hanö Bay. For each sampling point, grab samples were collected using a 12-L polyethylene bucket. From this bucket, two 1-L sterile PET bottles (VWR® collection) were filled. The pH of all sample volumes was recorded using pH test indicator strips. All samples were in the pH range of 6. To one of these bottles, approximately 3–4 mL of 1 M HCI was immediately added to a target pH range of 2–3. It should be mentioned that this specific type of sample bottle has previously been demonstrated not to contaminate water samples with any activity in the assays assessed in this study (Lundqvist *et al.* 2021). The bucket was rinsed three times with 99% ethanol (EtOH) and then three times with sample water prior to each sample collection. Two 1-L bottles of ultrapure water (Milli-Q®) sourced from the laboratory were also included as field controls, and one of these bottles was acidified with 1 M HCI onsite at the time of sample collection. Samples were also collected from a water tap located within the WWTP but not connected to the treatment process at this facility. This tap water is connected to the distribution network of the local potable water utility.

Sample preparation and storage

At the laboratory, all samples were first filtered using 0.45 μm polyethersulfone (PES) filters under vacuum. Following that, the samples (1-L volumes) were extracted via SPE using a SPE-03 8-Channel Automated SPE System (PromoChrom Technologies) and 6-mL HLB cartridges (6cc Oasis Prime HLB cartridge, sorbent weight 200 mg, Waters Corporation). The sample extraction process consisted of the following steps: preconditioning the cartridges with EtOH, loading of the samples, rinsing the sample bottles, then elution with EtOH, followed by evaporation. All sample extracts were resuspended with EtOH to obtain a final extract volume of 0.20 mL. Each water sample was thus enriched by a factor of 5,000. Additional information regarding the sample preparations is provided in the supplementary information (Section SI-1.1). A procedural control of ultrapure water (Milli-Q®) sourced from the laboratory was also included during the sample extractions. All concentrated water samples were transferred to 0.30 mL amber screw top fused insert vials and stored at −20 °C until bioanalysis (described further in the next subsection). Bioanalyses were conducted in 2022 soon after the completion of the sample extractions. The sample extracts were then stored in their original vials at −20 °C for approximately 1 year and reanalyzed in the same bioassays.

In vitro bioassays

The sample extracts along with the field and procedural (Milli-Q®) controls, vehicle (1% EtOH) controls, positive controls, and reference compounds were tested in luciferase reporter gene assays. Detailed descriptions of the cell lines tested are provided in the supplementary information (Section SI-1.2). The bioassays were selected based on their relevance to compounds commonly detected in wastewater extracts and represent different cellular toxicity pathways important to human health. The following endpoints were thus assessed: ER agonism, AR agonism and antagonism, and the oxidative stress response (Nrf2 activation). Cytotoxicity was initially assessed in all cell lines with cell viability assays (MTS for all assays except ER activity, where the ATP assay was used) and defined as <0.80 fold induction compared to the vehicle control. The main purpose of the cell viability testing was to ensure that the bioanalyses were performed under non-cytotoxic conditions.

For all bioassays, the sample extracts and controls were analyzed in quadruplicate, and all bioassays were repeated at least once to prove biological reproducibility. Detailed descriptions of the bioassays are provided in the supplementary information (Sections SI-1.3–1.5). In brief, all experiments were conducted in white 384-well cell culture plates with transparent bottoms (Corning Inc.). Cells were seeded in the plates and incubated for 24 h. The cells were then exposed to dilution series of the sample extracts and incubated for another 24 h. On the third day, bioactivities (i.e., luminescence signal) were measured on a TECAN Spark® Multimode Microplate Reader using the Luciferase® Reporter Assay System (Promega), according to the manufacturer's instructions. Vehicle controls and dilution series of the reference compounds were included on every experimental plate for each assay. For the ER assay, a weak positive control (p,p'-methoxychlor) was also included. Details of the bioassays, cell lines, and concentration ranges of the reference compounds are provided in Table 1. In all bioassays, the 5,000-fold enriched samples and controls were diluted 100-fold with cell medium to attain a final well concentration of 1% EtOH

Table 1 | Summary details of the selected bioassays and associated cell lines tested along with the reference compounds

Cellular endpoint	Cell line tested	Reference compound and conc. range
ER agonism	VM7Luc4E2	17ß-estradiol (E2) (0.36–370 pM)
AR agonism	AR-EcoScreen GR KO M1	Dihydrotestosterone (DHT) (0.001–1,000 pM)
AR antagonism	AR-EcoScreen GR KO M1	Hydroxyflutamide (OHF) (0.10–10,000 nM)
Oxidative stress response (Nrf2 activity)	MCF7AREc32	Tert-butylhydroquinone (tBHQ) (0.78–25 μM)

when incubated with the cells. The concentrations tested for the sample extracts were expressed in units of relative enrichment factor (REF). The enrichment and dilution of the samples together constitute the REF (Escher *et al.* 2014). For all bioassays, sample extracts were analyzed in two-fold dilution series from REF 6.25 to 50.

Data evaluation

Concentration-effect curves (CECs) were generated from the datasets for the sample extracts in all assays. To generate CECs of the datasets for the ER and AR agonism assays, the mean activities of the vehicle controls were first subtracted from all sample replicates; all adjusted values were then normalized to the mean activities of the vehicle controls and then to the maximum mean activities of the highest concentration of the respective reference compound (assay maximum, set to 100%). The normalized datasets were then fitted to a four-parameter sigmoidal nonlinear regression model. The concentrations causing a 10% effect (EC_{10}), expressed as REF, were then extrapolated from the CECs. For the AR antagonism assay: the mean activities of the unspiked vehicle control were first subtracted from the sample replicates; all adjusted values were then normalized to the mean activities of the unspiked vehicle control and then to the mean activities of the spiked vehicle control. The concentration causing a 30% inhibitory effect (IC_{30}), expressed as REF, was then interpolated from the CEC. For the Nrf2 assay where no maximum effect can be reached, fold inductions at each test concentration were normalized to that of the vehicle control and then fitted to a linear regression model. The concentration causing a 1.5-fold induction ($EC_{IR1.5}$) was estimated from the model. For some samples tested in 2022, their CECs did not follow an increasing REF concentration-effect pattern (i.e., bioactivities were lower at the highest REF) in the ER and AR agonist assays. Thus for the sake of consistency, CECs for all samples in the 2022 analysis were obtained based on the next highest REF of 25 and subsequent lower dilutions down to REF 6.25. For the 2023 analysis, as activities were assumed to decrease over time, CECs for all samples included the highest REF of 50.

The EC/IC values obtained from the CECs were further translated into bioanalytical equivalent concentrations (BEQ) in units of ng/L or μg/L, using the EC_{10}/IC_{30} values of the samples ($EC_{10}/IC_{30,\ sample}$) and the respective reference compounds ($EC_{10}/IC_{30,\ ref}$) of the particular assay using Equation (1). All data analyses as well as graphical presentations were performed using GraphPad Prism (v. 10.0.0).

$$BEQ = \frac{(EC_x \text{ or } IC_x)_{ref}}{(EC_x \text{ or } IC_x)_{sample}} \quad (1)$$

RESULTS

Cell viabilities

Results of the cell viability assays conducted in 2022 are provided in Figure SI-1 in the supplementary information. With the exception of the highest REF in the unacidified DF-in sample in the AR-EcoScreen cells, no other samples were cytotoxic. To confirm that the dose of the acidifying agent itself (HCI) did not cause any effect on cytotoxicity, the cell viabilities between the unacidified and acidified samples for each sampling point were assessed via pair-wise comparison tests for each REF concentration. HCI had no effect on the cell viabilities in any of the samples.

Treatment process

As the primary purpose of this study was to investigate the impact of sample acidification on bioactivities, the removal effects of the various treatment steps at the WWTP were not assessed in detail. However, some general comments are presented

herein. Treatment with disc filtration reduced the ER activities of the incoming pre-treated wastewater, as demonstrated in the 2022 samples both with and without HCI. The TP-out samples (i.e., finished water) had reduced activities in all bioassays compared to the samples taken at the start of the treatment process before and after disc filtration (Table 2). As such, the fact that the activities in the sample collected from the outlet of the WWTP were low or below the limit of detection (LOD) and similar to the activities in the tap water sample (TAP) demonstrates the efficient treatment process at this facility. A recent study evaluated the water reuse potential of this WWTP for irrigation or as a drinking water source (Takman *et al.* 2023). That study concluded that while drinking water quality could not be achieved, based on concentrations of *Escherichia coli* and total coliforms, the treated wastewater generally achieved quality criteria for irrigation.

Bioactivities

A summary of the BEQ values for all sample extracts in each of the assays in both the 2022 and 2023 analyses is presented in Table 2. No AR antagonist activities were detected in any samples in either the 2022 or 2023 analyses; as such, this endpoint is not included in Table 2. The CECs of the sample datasets are presented in Figures 1–3. In cases where the samples did not cause effects above the LODs, no BEQ values were calculated. A summary table of the LODs for each assay is provided in the supplementary information (Table SI-1).

Effect of sample acidification

Acidification resulted in lower AR and Nrf2 activities in DF-inlet samples and higher activities in DF-outlet samples. Acidification had little effect in the ER assay. Observations for each of the assays are described in further detail in the following sections.

AR activity

Interestingly, the most prominent finding in the AR assay was that acidification resulted in considerably higher AR activities in the DF-out samples compared to the unacidifed samples in both 2022 and 2023 (Figure 1(b) and 1(d)). In contrast, acidification reduced AR activities (i.e., below LOD) in the DF-in samples in both analyses. For the TP-out samples, AR activities were approximately the same in the acidified versus unacidified samples in the 2022 analysis. For the tap water samples, AR activities were detected in the acidified sample but not in the unacidified sample in 2022. No AR activities were detected in either the TP-out or tap water samples in the 2023 analysis. In this assay, in the absence of acidification, the DF-inlet samples exhibited the highest % of the maximum response in both 2022 and 2023. However, in the presence of acidification, the DF-outlet samples exhibited the highest % of the maximum response in both analyses.

Nrf2 activity

Similar to what was observed for AR activities, acidification resulted in higher Nrf2 activities in the DF-out samples in the initial 2022 analysis as well as in the later 2023 analysis (Figure 2(b) and 2(d)). For the DF-in samples, on the other hand, acidification reduced Nrf2 activities (i.e., below LOD) in both analyses. For the TP-out and tap water samples, no Nrf2 activities were detected in either 2022 or 2023. As observed in the ER assay, the acidified DF-outlet sample exhibited the highest fold induction in both the 2022 and 2023 analyses.

ER activity

The ER activities were similar in the acidified versus unacidified samples in the initial 2022 analysis at all sampling points (Table 2). However, the acidified DF-outlet sample in the 2022 analysis reached a higher degree of the assay's maximum response (90%) compared to the other samples, wherein responses were less than 40% of the maximum response. The same pattern was observed in the 2023 analysis wherein the acidified DF-outlet sample exhibited the highest percent of maximum response, albeit lower than in 2022, compared to the responses in other samples (Figure 3(a) and 3(b)).

Detection of bioactivities in extracts after storage

A secondary objective of the current study was to assess the detectability of bioactivities in stored extracts over time. As previously mentioned, following the initial analysis in 2022, all sample extracts were stored in their original vials at −20 °C and re-tested in 2023. Interestingly, the Nrf2 activities in the unacidified DF-out and DF-in samples remained fairly stable and a higher Nrf2 activity was even detected in the acidified DF-out sample in 2023 versus in 2022. In the ER and AR agonist assays, the bioactivities were either lower or no longer detectable in the 2023 analyses compared to the initial 2022 results

Table 2 | Summary of bioanalytical equivalent concentrations (BEQ) of the samples

	DF-in				DF-out				TP-out				Reference sample (TAP)			
	2022		2023		2022		2023		2022		2023		2022		2023	
Assay	Without HCl	With HCl	Without HCl	With HCl	Without HCl	With HCl	Without HCl	With HCl	Without HCl	With HCl	Without HCl	With HCl	Without HCl	With HCl	Without HCl	With HCl
ER activity (ng E2eq/L)	2.14 (1.25, 5.53)	2.62 (1.87, 12.1)	0.019 (0.01, 0.03)	0.013 (0.01, 0.02)	0.71 (0.003, 1.41)	0.81 (0.28, 1.90)	<0.010	0.044 (0.03, 0.06)	0.11 (0.01, 0.24)	0.11 (0.02, 0.23)	<0.010	<0.010	0.17 (0.01, 0.35)	0.22 (0.01, 0.46)	<0.010	<0.010
AR agonist activity (ng DHTeq/L)	4.85 (2.75, 6.95)	<0.97	0.06 (0.03, 0.09)	<0.061	4.43 (3.20, 5.66)	16.7 (5.15, 28.3)	<0.061	0.23 (0.10, 0.60)	2.82 (0.65, 4.98)	3.36 (1.33, 5.38)	<0.061	<0.061	<0.97	3.52 (0.31, 11.4)	<0.061	<0.061
Nrf2 activity (µg tBHQeq/L)	16.2 (11.9, 20.5)	<6.57	12.1 (10.4, 13.8)	<6.98	10.4 (9.04, 11.7)	15.9 (14.1, 17.6)	9.30 (3.52, 15.1)	25.6 (23.5, 27.8)	<6.57	<6.57	<6.98	<6.98	<6.57	<6.57	<6.98	<6.98

Note: For each sampling point, presented are the BEQ values for the unacidified (without HCl) and acidified (with HCl) samples from the 2022 and 2023 analyses. Presented below each BEQ value are the respective 95% confidence intervals (LL, UL) in parentheses.

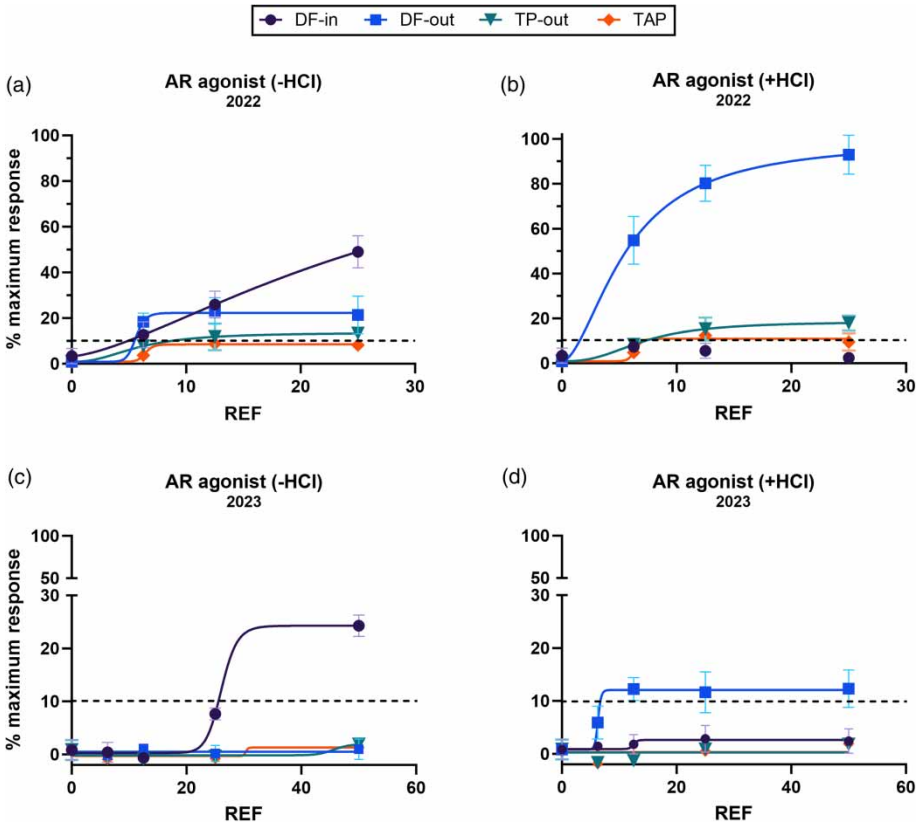


Figure 1 | CECs for AR agonist-active samples without and with acidification in the 2022 analysis (a, b) and the 2023 analysis (c, d). Treatment groups ($n = 4$) were normalized to the vehicle control ($n = 8$), and then to the maximum experimental response of the highest concentration of the reference compound (DHT) set to 100%. Data were fitted to a four-parameter sigmoidal regression model. The dotted line indicates 10% activity of the maximum response. In cases where the samples did not cause effects above the cut-off levels, no effect concentration values were calculated. Data are presented as mean ± SD.

in all samples. This demonstrated that, for these two particular assays, the activities had decreased considerably after storage for 1 year, regardless of acidification.

DISCUSSION

The most prominent results in the 2022 analysis were that acidification resulted in lower AR and Nrf2 activities in DF-inlet samples and higher activities in DF-outlet samples. In comparison to the findings of our study, another similar study that assessed the impacts of sample preparation techniques (e.g., acidification, filtration, and SPE) on the outcomes of multiple *in vitro* bioassays reported that sample acidification (with sulphuric acid [H_2SO_4]) significantly altered the endocrine activities (and mutagenicity) of their aqueous samples (Abbas *et al.* 2019). Samples were collected from various locations representing the water cycle, including from the influents and effluents of several municipal WWTPs. They utilized several different recombinant yeast-based reporter gene assays for endocrine activities (ER, anti-ER, AR, and anti-AR). In their study, androgenic activity was detected in the neutral (i.e., unacidified) untreated wastewater influent sample, while a significantly lower androgenic activity was detected in the acidified influent sample in comparison. Interestingly, while no androgenic activities were detected in any of the neutral wastewater effluent samples, significant androgenic activities were observed in all of the acidified wastewater effluent samples. These patterns of changes due to acidification are similar to our findings for the DF-inlet and DF-outlet samples in the AR (and Nrf2) assays. A possible explanation in the case of our study may be the higher presence of suspended solids in water before the disc filtration (DF-inlet) compared to after the disc filtration (DF-out) treatment. Further to this, it has been reported that acidification of water samples may influence partitioning between the aqueous phase and suspended matter for different bioactive compounds (Baker & Kasprzyk-Hordern 2011). Hence, the decrease

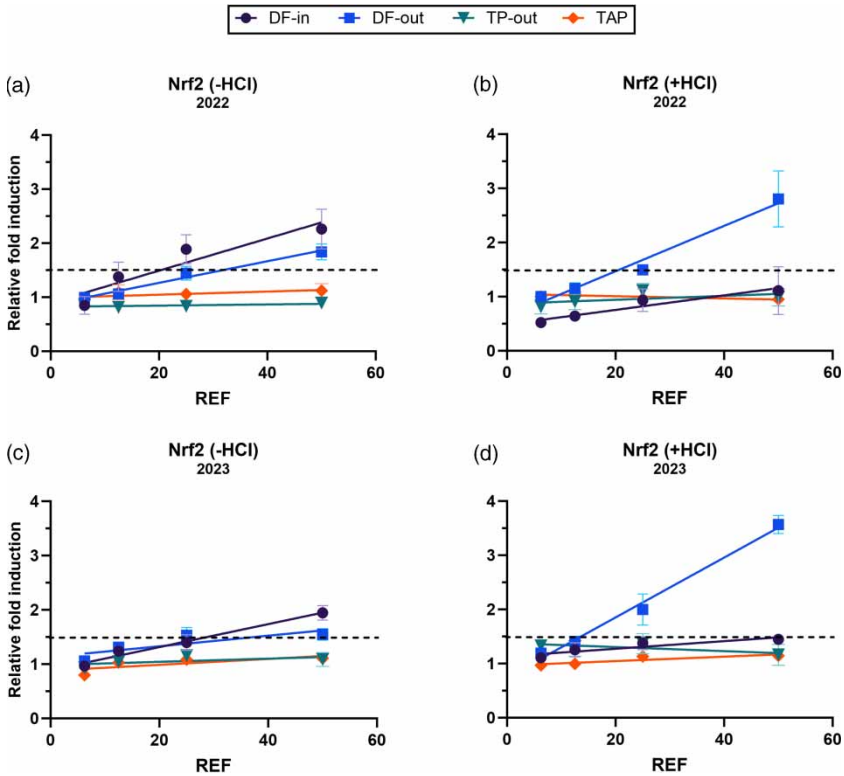


Figure 2 | CECs for Nrf2-active samples without and with acidification in the 2022 trial (a, b) and the 2023 trial (c, d). Data were fitted to a linear regression model using a REF range from 6.25 to 50. tBHQ was used as a reference compound. The dotted line represents the cut-off level of 1.5-fold induction. Treatment groups ($n = 4$) were normalized to the vehicle control ($n = 8$). In cases where the samples did not cause effects above the cut-off level, no effect concentration values were calculated. Data are presented as mean ± SD.

in AR and Nrf2 activity in the DF-inlet sample following acidification could be that the compounds causing these activities are partitioning toward the suspended matter phase due to the acidification and then removed from the sample in the later 0.45 μm PES filtering step. In the downstream sample (DF-out), where the level of suspended solids can be expected to be lower, this effect was not seen. Instead, an increase in activity following acidification was observed, which might be due to increased recovery of weak acids in the SPE procedure (discussed further below) and/or inhibited microbial degradation of the compounds causing these activities.

In contrast to AR and Nrf2 activities, the ER activity was not affected by acidification, which may be because the estrogenic compounds did not bind significantly to suspended matter and thus were not affected by the disc filtration. Elsewhere, estrogenic activity has been reported to be influenced by the suspended solids concentrations in WWTP influent and on the plant's suspended solid removal efficiency (Dagnino *et al.* 2010). Another aspect to consider is the influence of conjugation/deconjugation on the occurrence and fate of certain pharmaceuticals and hormones (Gewurtz *et al.* 2022). Conjugated forms present in the untreated influent to WWTPs, which can be identified by chemical analysis but not by *in vitro* bioassays, can become deconjugated to their free forms by enzymes and/or microbially mediated during the biological processes in the WWTP, thereby becoming detectable (Ting & Praveena 2017). Further, it has been reported that acidification with HCI or H_2SO_4 may cleave conjugated steroids, whereas microbial degradation of deconjugated steroids occurred in unpreserved samples (Havens *et al.* 2010). In our study, there was no main difference in estrogenic activity due to acidification, and the estrogenic compounds in the samples may not have been conjugated steroids but rather other estrogenic chemicals.

In another study involving analytical chemistry and *in vitro* bioassays, sodium azide (NaAz) adequately preserved the progestogens and most of the estrogens spiked into surface water runoff samples from cattle manure–amended fields in terms of analyte recoveries; however, it did not adequately preserve many of the spiked androgens (Havens *et al.* 2010). In that same study, the estrogenic and androgenic activities, as measured by the E-screen and A-screen assays, respectively, were stable in

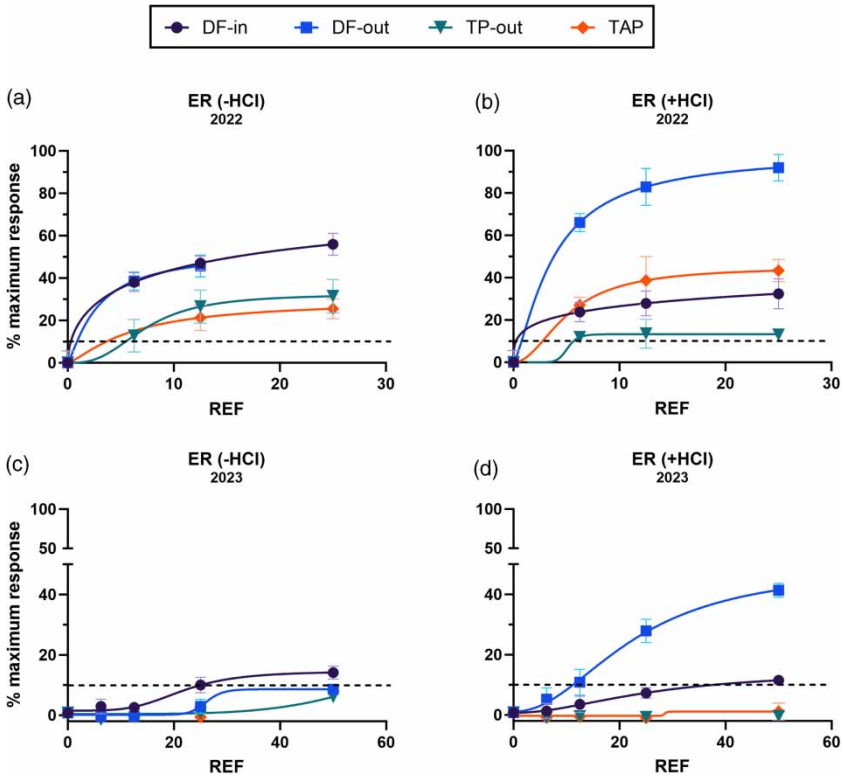


Figure 3 | CECs for estrogenic activities in samples without and with acidification in the 2022 analysis (a, b) and the 2023 analysis (c, d). Treatment groups ($n = 4$) were normalized to the vehicle control ($n = 8$) and then to the maximum experimental response for the highest concentration of the reference compound (E2), set to 100%. Data were fitted to a four-parameter sigmoidal regression model. The dotted line indicates 10% activity of the maximum response. Data are presented as mean ± SD.

H_2SO_4-preserved samples after 14 days of storage; however, the estrogenic and androgenic activities in the unpreserved and, to a lesser extent, NaAz-preserved samples were significantly decreased after 14 days (Havens *et al.* 2010).

It should also be mentioned that the ER activity observed at the WWTP outlet sample in the current study does not exceed the benchmark value of 1 ng E2/L proposed under the first watch list of the European Commission's (EC) 2022 Drinking Water Directive (European Commission 2022). As the treated wastewater at this facility is currently discharging into Hanö Bay, but potentially will be used in the future for irrigation purposes, the results can also be compared to the Annual Average Quality Standards (AA-QS) for 17β-estradiol of 0.18 ng/L for inland surface waters and 0.009 ng/L for other surface waters, proposed by the European Commission (SCHEER 2022). The estrogenic activity in the WWTP outlet sample detected in the 2022 analysis (0.11 ng E2 eq/L) would exceed the proposed AA-QS for other surface waters. A recent study evaluated the removals of 12 detected OMPs over the entire treatment process at this WWTP to assess the potential production of source water for drinking from the facility. One of these OMPs, estrone, was detected at a concentration of 7.1 ng/L in the influent but not in the effluent samples collected from the WWTP on the same day as the sample collection of the current study (Takman *et al.* 2023). Estrone has an approximate relative estrogenic activity of 0.01 compared to 1 for estradiol (Gutendorf & Westendorf 2001), which means that estrone only contributes a few % of the estrogenic activity observed in the inlet samples. The estrogenic activities thus detected in the inlet and outlet samples in the current study are likely attributed to additional estrogenic compounds, most probably mainly estradiol, that were not detected by the chemical analyses. It is well known that the detection limit for estradiol by chemical analysis is one to two orders of magnitude higher than what can be detected in *in vitro* bioassays as estrogenic activity from estradiol (Könemann *et al.* 2018).

The effects of acidification involving *in vitro* bioassays appear to be otherwise understudied, as there remains a lack of studies that purposely addressed this factor. The highlight of our study would be that acidification decreased certain

bioactivities in wastewater before disc filtration but increased activities following this treatment. The considerable increases in bioactivities in the acidified samples following disc treatment are also quite striking. As such, our findings suggest that in addition to microbial degradation of compounds, the water treatment process itself should be considered concerning sample acidification as this may affect the outcome of certain bioassays. Still, others have suggested to not employ sample acidification to better simulate real environmental conditions (Šauer *et al.* 2018). Given that OMPs, such as endocrine-disrupting compounds, are composed of various classes of compounds, the effects of acidification on the recovery of extraction eluates should also be studied further with bioassays.

With respect to the effect of storage time of SPE-extracted samples on the observed bioactivities, bioactivities were either lower or no longer detectable (i.e., below LOD) in the ER and AR assays in 2023 versus 2022. Other studies have demonstrated, mainly by chemical analyses, that endocrine compounds degrade over time, particularly in unpreserved samples. The recoveries of certain androgens (e.g., androstenedione, testosterone, progesterone) spiked into unpreserved extracts of surface waters, for instance, have been shown to decline quickly over time (14 days) (Vanderford *et al.* 2003). Elsewhere, severe losses were also reported in the recoveries of certain estrogens (e.g., estriol, estrone, and estradiol) stored for 7 days in unpreserved river water extracts (Baronti *et al.* 2000). The degradation of certain compounds over time can also depend on temperatures and the acidifying agents used (Baronti *et al.* 2000; Vanderford *et al.* 2003, 2011; Labadie & Budzinski 2005; Havens *et al.* 2010). In one study, a dramatic loss in progestogens to 14% of the initial concentration was observed in the dissolved phase of WWTP effluent extracts stored in the dark at 4 °C even after only 24 h, whereas concentrations were markedly higher at around 73% of the initial concentration after the same time period in samples preserved with either HCI or formaldehyde (Labadie & Budzinski 2005). In another study, testosterone and progesterone spiked into unpreserved aqueous surface water samples were partially degraded after storage at −20 °C for 28–35 days and completely degraded after storage at 25 and 4 °C experiments for 28–35 days, which the authors attributed to bacterial degradation (Vanderford *et al.* 2011). Regarding the results in our study from the Nrf2 assay, on the other hand, the increased activities over time may be because the Nrf2-inducing compounds did not degrade over time, while evaporation of the vehicle solvent (EtOH) may have occurred, which resulted in the increases. This evaporation of EtOH has been reported in another *in vitro*-based study that investigated sample preparation and storage of environmental extracts with a focus on estrogenic potency (Murk *et al.* 2002).

CONCLUSIONS

In the current study, the incorporation of sample acidification with HCI was assessed in samples collected from a small-scale WWTP using *in vitro* bioassays measuring hormonal receptor activity and oxidative stress response. The main finding of this study was that the incorporation of acidification decreased bioactivities in certain bioassays in wastewater influent (following pretreatment only), whereas acidification increased bioactivities following further treatment (e.g., disc filtration) for solids removal. The impact of acidification may thus be related to (1) a pH-dependent increase in the partitioning of bioactive compounds to solids in the influent water samples (which were subsequently removed in the SPE process before bioanalysis) before the disc filtration treatment; and (2) inhibition of microbial degradation of bioactive compounds in samples following disc filtration treatment. Another finding was the limited stability of hormonal effects and the increased activity of oxidative stress in some of the stored water extracts after approximately 1 year. It would thus seem preferable to conduct the bioassay testing as soon as possible after sample extraction, rather than storing extracts for prolonged periods of time (in EtOH solvent). All these findings suggest against a 'one-size-fits-all' approach involving acidification and sample storage. Rather, sampling strategies may need to consider that certain treatment processes may impact the resulting chemical compositions and the outcomes of certain bioassays.

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DECLARATIONS

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

DATA AVAILABILITY STATEMENT

All relevant data are included in the paper or its Supplementary Information.

CONFLICT OF INTEREST

Agneta Oskarsson, Elin Lavonen, Geeta Mandava, and Johan Lundqvist are affiliated with a company providing effect-based testing services to the water sector (BioCell Analytica Uppsala AB).

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