Global Water
Research Coalition

Effect Based Monitoring in Water Safety Planning

PROJECT REPORT

Effect Based Monitoring in Water Safety Planning

WP3.3: Sampling strategies and sample pre-treatment options and decision-making tool for selection of sampling methods

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GWRC in brief

In 2002, twelve leading research organisations established an international water research alliance: the Global Water Research Coalition (GWRC). GWRC is a non-profit organisation that serves as a focal point for the global collaboration for research planning and execution on water and wastewater related issues.

The Coalition focuses on water supply and wastewater issues and renewable water resources: the urban water cycle. The function of the GWRC is to leverage funding and expertise among the participating research organisations, coordinate research strategies, secure additional funding not available to single country research foundations, and actively manage a centralised approach to global issues. GWRC offers its members the opportunity to leverage resources through cooperative planning and implementation of research.

The GWRC Members are: Canadian Water Association (Canada), KWR – Water B.V. (Netherlands), PUB – Public Utilities Board (Singapore), Stowa- Foundation for Applied Water Research (Netherlands), SUEZ - CIRSEE (France), TZW - Water Technology Center (Germany), UK Water Industry Research (UK), Veolia Research and Innovation (VERI) (France), Water Research Australia (Australia), Water Research Commission (South Africa), The Water Research Foundation (USA), and the Water Services Association of Australia.

The US Environmental Protection Agency has been a formal partner of the GWRC since 2003. The Global Water Research Coalition is affiliated with the International Water Association (IWA).

GWRC members represents the interests and needs of 500 million consumers and have access to research programs with a cumulative annual budget of more than €150 million. The research portfolio of the GWRC members spans the entire urban water cycle and covers all aspects of resource management.

Executive summary

In vitro bioassays are sensitive screening tools to detect complex mixtures of micropollutants in water samples, but micropollutants are often present at low concentrations, particularly in drinking water and clean source water. Therefore, there is a need to enrich samples prior to bioanalysis. This report summarizes commonly applied sampling strategies and sample preparation options and proposes a decision-making tool to assist users to select appropriate sampling methods.

The sampling strategy will depend on the purpose and objective of the sampling campaign, as well as the sample context. For example, if the purpose of a sampling campaign is to assess the product quality of a drinking water treatment plant, with the objective of comparing the effect in the treated product water to an effect-based trigger value, then only the product water needs to be collected. If we want to understand critical processes, it might be required to sample at different stages of a treatment chain. The type and volume required will depend on the sample, with composite sampling and smaller volumes recommended for wastewater influent and effluent. In contrast, grab sampling and larger sample volumes are more suitable for drinking water and recycled water.

Once a water sample has been collected, a number of decisions regarding sample pre-treatment need to be made. For example, samples should be processed within 48 hours of collection, while samples containing chlorine should be quenched immediately after collection. While many studies filter samples prior to enrichment, the type of filter and the filter pore size used varies widely in the literature. We recommend filtering water samples with a turbidity of 5 nephelometric turbidity units (NTU) or greater using glass fibre filters with a pore size between 0.7 to 1.5 µm.

Common sample extraction methods include solid-phase extraction (SPE), passive sampling and liquid-liquid extraction, with SPE the most frequently used method to enrich micropollutants prior to bioanalysis. Testing native or unenriched water samples is not recommended as it is not possible to differentiate the effect of organic micropollutants from other components in water. Different SPE sorbents have been used in the literature, with multilayer SPE containing different sorbents to capture a wider range of micropollutants increasingly being applied. However, blank effects have previously been detected, so it is important to select an extraction method with no blank effects as bioassays cannot differentiate between effects from a water sample and effects from impurities from sample processing. Few studies have evaluated effect recovery by SPE, with one study finding effect recovery to be within a factor of two of the optimal 100% recovery for most bioassays.

As there are a number of decisions to be made regarding sample collection, pre-treatment and enrichment a decision-making flow chart was developed to guide users through the key steps. Once the final sample pre-treatment and processing methods have been selected, it is important to use the same approach for all samples that you want to compare. It is important to note that the information used to support the sample processing decisions is often based on user experience and chemical analysis protocols, with few studies investigating the impact of different sample processing options on the biological effect. One of the least standardised, but very important, pre-treatment steps is sample filtration, with further experimental work required to validate the approach proposed in this report.

Abbrevations: AhR: aryl hydrocarbon receptor; AR: androgen receptor; BEQ: bioanalytical equivalent concentration; DBP: disinfection by-product; DF: dilution factor; DMSO: dimethyl sulfoxide; EEQ: 17β-estradiol equivalent concentration; EF: enrichment factor; ER: estrogen receptor; EROD: ethoxyresorufin-O-deethylase; GR: glucocorticoid receptor; LLE: liquid-liquid extraction; LVSPE: large volume solid-phase extraction; MTBE: methyl tert-butyl ether; NTU: nephelometric turbidity units; POCIS: polar organic chemical integrative samplers; PPAR: peroxisome proliferator-activated receptor; PR: progesterone receptor; PXR: pregnane X receptor; REF: relative enrichment factor; SPE: solid-phase extraction; SPM: suspended particulate matter; TR: thyroid receptor; WET: whole-effluent toxicity; WWTP: wastewater treatment plant

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1 Introduction

There is increasing interest in applying effect-based methods (i.e., *in vitro* bioassays and well plate-based *in vivo* assays) for water quality monitoring (Brack *et al.*, 2019). However, to ensure that bioassay results are meaningful, it is important to select an appropriate sampling strategy and use suitable sample pre-treatment and processing methods. While bioassays are sensitive screening tools to detect complex mixtures of organic micropollutants in water samples, micropollutants are often present at low concentrations (e.g., nanogram per litre) in drinking water and clean source waters (e.g., Glassmeyer *et al.*, 2017; Troger *et al.*, 2018), so water samples may need to be enriched up to 100 times in the assay before an effect can be detected. *In vitro* assays typically target complex mixtures of organic micropollutants but not inorganics and metals, which can be comprehensively analysed using chemical methods. Therefore, extraction methods also serve to separate the organic micropollutants from the matrix, inorganics and metals in a water sample. The literature reviewed in WP3.2 *Medium-to-high throughput bioanalytical tools and decision-making tool for selection of bioassays* indicated that three methods were used to enrich and isolate organic micropollutants from whole water samples: solid-phase extraction (SPE), passive sampling and liquid-liquid extraction (LLE). This report will summarize commonly applied sampling strategies and sample preparation options, including sample pre-treatment and extraction. The report focused on current methods, though it should be noted that new sample pre-treatment and extraction methods are continuously being developed. Finally, a decision-making tool will be developed to assist users with selecting appropriate sampling methods.

2 Sampling strategies

The sampling strategy will depend on the purpose, objectives and sample context (Figure 1). If the purpose of a sampling campaign is to assess the product quality of a drinking water treatment plant, with the objective of comparing the effect in the final water to an effect-based trigger value, then only the product water needs to be collected (with appropriate blank and control samples). In contrast, both source water and product water are required if the purpose is to evaluate treatment process efficiency. Samples can also be collected after intermediate steps throughout the treatment train, such as after advanced oxidation or disinfection, if the purpose of the sampling campaign is to understand critical processes. Composite samples are recommended for wastewater to capture the diurnal variation observed for some micropollutants (Nelson *et al.*, 2011; Petrie *et al.*, 2017), with many studies collecting 24 h composite influent and effluent samples (e.g., Korner *et al.*, 2001; Bicchi *et al.*, 2009; Macova *et al.*, 2010; Reungoat *et al.*, 2010; Jalova *et al.*, 2013; Bain *et al.*, 2014; Roberts *et al.*, 2015). Grab sampling is suitable for collecting drinking water or recycled water samples if little difference in quality over time is demonstrated. Water samples should be collected in solvent-washed and or burnt-out (500˚C, 2h) amber glass bottles with caps that do not contain polymer liners, with the bottles stored on ice and in the dark until returning the samples rapidly to the laboratory for further processing. Field and process blanks have to be included in any sampling strategy. Denison et al. (2020) also recommended matrix-spiked samples for recovery experiments.

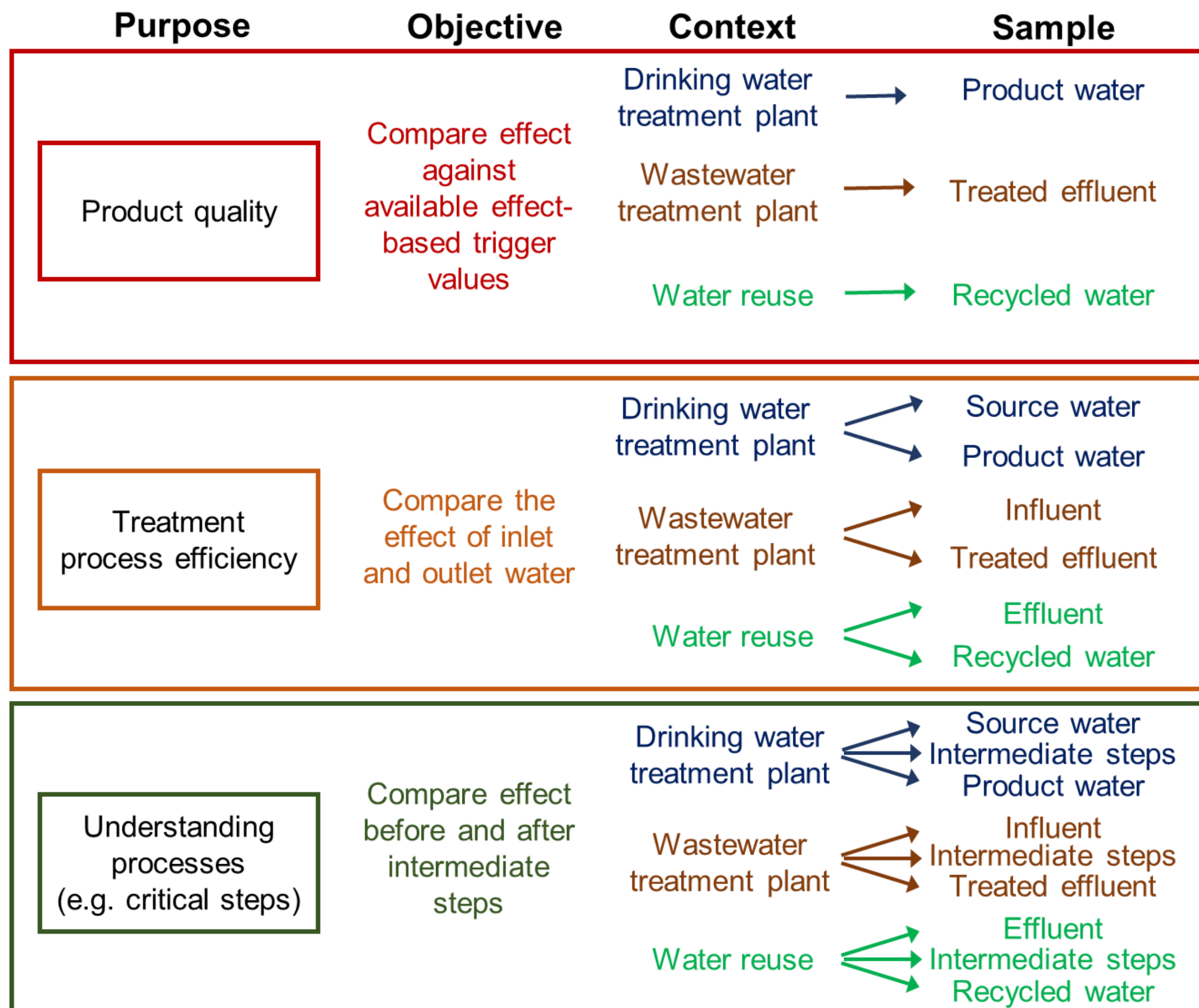

Figure 1: Examples of different sampling campaign purposes for drinking water, wastewater and water reuse with the required samples for each purpose indicated.

The required volume of water to be collected will depend on the expected level of chemical contamination and thus the need for concentration, with less sample required for wastewater influent and a larger volume needed for drinking water. Using the example of SPE, previous experience from the authors and the peer reviewed literature suggests that a common 200 mg/6 cc SPE cartridge can enrich 0.5 L of wastewater influent, 1 L of wastewater effluent or surface water and 2 L of drinking water, recycled water or clean surface water. Double the volume can be applied to larger SPE sorbent masses (e.g. 500 mg cartridges). Assuming the volumes applied to a 200 mg SPE cartridge and a final extract volume of 0.5 mL, this equates to an enrichment factor (EF) of 1000 for wastewater influent, 2000 for wastewater effluent or surface water and 4000 for drinking water, recycled water or clean surface water. Assuming a bioassay dilution factor (DF) of 100 (1% solvent) to 1,000 (0.1% solvent), this results in a maximum relative enrichment factor (REF) of 1 to 10 for wastewater influent, 2 to 20 for wastewater effluent or surface water and 4 to 40 for drinking water, recycled water or clean surface water. The number of assays that can be run from a single extract will depend on the number of repeats planned and how much extract is dosed, but typically only small extract volumes (microlitres) are required for 96 and 384-well plate assays meaning that a number of different assays can often be run.

If higher REF are required, samples can be extracted on multiple SPE cartridges and combined into one final extract (Escher *et al.*, 2014; Neale *et al.*, 2017). An alternative approach is to use large volume SPE (LVSPE), which has been applied to enrich between 6 L (influent) to 500 L (surface water) (Neale *et al.*, 2015; Valitalo *et al.*, 2017). LVSPE has been applied in a

limited number of studies compared to SPE and has primarily been applied to surface water (e.g., Konig *et al.*, 2017; Tousova *et al.*, 2017), but it is not expected to be relevant for drinking water. LVSPE allows onsite sampling, but also requires more equipment than other sample extraction methods, limiting its more widespread use.

While the conventional SPE volumes are recommended based on years of experience from the literature, Simon *et al.* (2019) explored the impact of sample volume on effect recovery, with 0.5 and 2 L of wastewater effluent and 1 and 4 L of surface water spiked with a mixture of four estrogenic compounds and 11 pesticides and enriched with SPE using LiChrolut EN/RP-18 (300 mg sorbent). The extracts were analysed in assays indicative of estrogenic activity (ERα CALUX), photosystem II inhibition and algal growth (combined algae assay) and bacterial toxicity using *Aliivibrio fischeri*. The average activity in the large volume extracts was between 79 to 104% of the activity in the lower volume extracts, showing sample volume did not have a significant impact on recovery.

Therefore, the recommended sample volumes in the report should not have a negative impact on recovery, though it should be noted that some chemicals, such as highly polar chemicals, may not be well recovered by conventional SPE and ideally recovery experiments should be performed with spiked samples during method development and validation.

3 Sample pre-treatment options

Once an appropriate water sample has been collected, there are a several decisions to be made regarding sample pre-treatment, including sample storage time and sample filtration. This section reviews common sample pre-treatment options based on a review of the studies included in WP3.2, with a focus on SPE as the most common sample enrichment option from WP3.2. However, it should be noted that the majority of examples have focused on estrogenic activity as it is the most commonly studied endpoint (e.g., Jarošová *et al.*, 2014; Konemann *et al.*, 2018), with little information about the impact of sample storage and pre-treatment on other relevant endpoints

3.1 Sample preservation and storage

Around 48 of 111 studies from WP3.2 that applied SPE adjusted the pH of the water sample to an acidic pH using concentrated acid, such as hydrochloric acid or sulfuric acid. Six of the studies had no pH adjustment or adjusted to a neutral pH, while the remainder of the studies did not provide any information about sample pH. An acidic pH can reduce microbial activity in the sample, which could potentially cause micropollutant biodegradation or biotransformation. Reducing the pH can also improve the extraction of weak acids, with improved recovery of a pharmaceutical cocktail in a bioluminescence inhibition assay at pH 3 compared to pH 7 observed for three different SPE sorbents (Escher *et al.*, 2005). In contrast, Sauer *et al.* (2018) found no difference in androgenic or anti-androgenic activity in wastewater influent in duplicate samples at pH 3 and pH 7.4, respectively. The samples were extracted using C18 SPE disks. In addition to pH adjustment, a small number of samples have used copper(II) sulfate pentahydrate (10 mg per litre) (Conley *et al.*, 2017a) or sodium azide (1 g per litre) (Mehinto *et al.*, 2015) for sample preservation.

In the case of chlorinated samples, the chlorine residual should be quenched immediately after sampling. While chlorine will not be extracted by SPE, quenching is important to prevent the formation of additional disinfection by-products (DBPs) and to prevent the chlorine from potentially reacting with the SPE sorbent. Many studies use sodium thiosulphate to quench the chlorine residual (Macova *et al.*, 2011; Escher *et al.*, 2014; Neale *et al.*, 2020), though some use ascorbic acid instead (Conley *et al.*, 2017b). Typically, 3.5 mg/L of sodium thiosulphate quenches 1 mg/L free Cl_2 and 5 mg/L ascorbic acid quenches 1 mg/L free Cl_2 (Farre *et al.*, 2013). Hebert *et al.* (2018) found no difference in toxicity of glass bottled Evian water controls with and without 20 mg/L sodium thiosulphate in assays indicative of the oxidative stress response, p53 response and NF-κB response. Similarly, sodium thiosulphate controls did not have any effects in assays indicative of hormone receptor-mediated effects (Neale *et al.*, 2020). Ascorbic acid also had no negative impact on hormone receptor reporter gene assays.

After collection, water samples are commonly stored for no longer than 48 h at 4°C before extraction (e.g., Aerni *et al.*, 2004; Cargouet *et al.*, 2004; Fang *et al.*, 2012; Daniels *et al.*, 2018). Alternatively, some studies froze water samples to store for a longer period prior to extraction (Konemann *et al.*, 2018). Jarošová *et al.* (2014) investigated the impact of sample storage time prior to extraction on estrogenic activity in wastewater effluent, with matching samples extracted 48 hours and 45 days after collection (stored at 4°C prior to extraction). Of the seven samples, the estrogenic activity at least doubled in two of the samples, but overall the difference in effect was small (e.g., 0.7 ng/L after 48 hours and 1.7 ng/L after 45 days).

3.2 Sample filtration

Around 70% of the reviewed studies in WP3.2 that applied SPE filtered samples prior to SPE, with glass fibre filters most commonly used. Glass fibre filters have previously shown to sorb only negligible amounts of estrogens, compared to cellulose acetate and nylon filters, with the latter found to adsorb a significant fraction of estrogens from solution (Walker and Watson, 2010). The filter pore size (i.e., the particle size retained with 98% efficacy) used in the literature varies widely from 0.1 µm to 11 µm, with the majority of studies using filters with a pore size of 0.7 to 2 µm (Figure 2). Several studies did not report the filter pore size, so these studies are not captured in Figure 2.

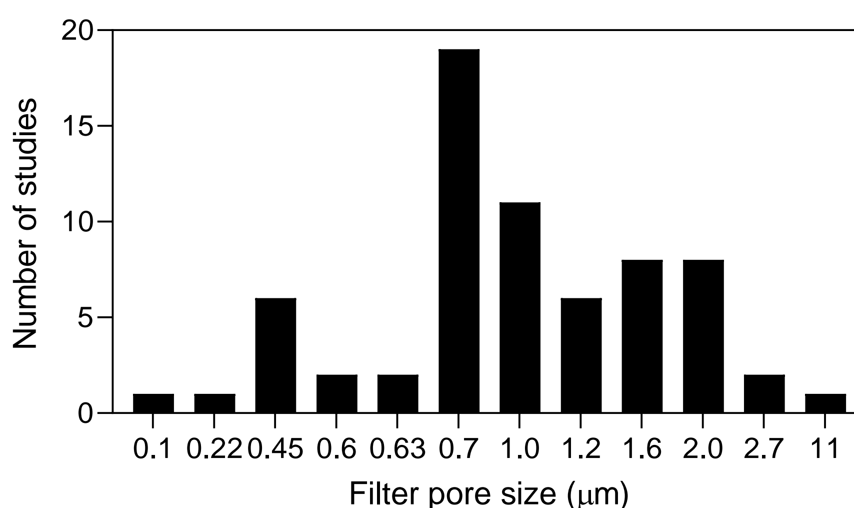

Figure 2: Overview of different filter pore sizes (µm) used for sample filtration prior to SPE based on WP3.2 literature review.

The European Union Water Framework Directive (WFD) recommends sample extraction methods that capture the whole-water sample, which includes both the dissolved and suspended particulate phases (European Commission, 2009). The suspended particulate matter (SPM) captured by sample filtration is often discarded, but several studies have shown considerable biological activity associated with particulate matter (e.g., Legler *et al.*, 2003; Hamers *et al.*, 2015; Schulze *et al.*, 2015). For example, Dagnino *et al.* (2010) evaluated the estrogenic and aryl hydrocarbon receptor (AhR) activity in both the dissolved and particulate phases from three wastewater treatment plants (WWTPs), with both phases contributing to the estrogenic and AhR load discharged from the plants. A higher fraction of AhR activity was found on suspended particulate matter (Dagnino *et al.*, 2010). Similarly, Wolz *et al.* (2008) found that SPM from the Neckar River induced AhR-mediated activity in the ethoxyresorufin-O-deethylase (EROD) assay while the corresponding extracted water samples had no effect. These studies suggest that it is important to consider SPM to gain a better understanding of the bioactivity, but the decision to collect SPM will depend on the objectives of the sampling campaign and the studied endpoints, with SPM likely to be important for non-specific toxicity, activation of AhR and binding to the peroxisome proliferator-activated receptor (PPAR).

If intending to capture the whole-water sample, the options are to not filter before SPE or to filter prior to SPE and extract the captured SPM separately using solvents (Ademollo *et al.*, 2012). Some studies only filtered samples that were expected to block the SPE cartridge due to the high particle content, such as wastewater influent or river water (Xiao *et al.*, 2016; Gehrmann *et al.*, 2018). Similarly, many studies evaluating drinking water do not apply a filtration step (e.g., Van Zijl *et al.*, 2017; Hebert *et al.*, 2018; Valcarcel *et al.*, 2018). Further, Konemann *et al.* (2018) found no significant difference in estrogenicity for filtered and unfiltered surface water samples. To provide guidance on whether to filter or not, US EPA Method 1694 (Pharmaceuticals and Personal Care Products in Water,

Soil, Sediment, and Biosolids by HPLC/MS/MS) recommends that aqueous samples containing visible particles should be filtered prior to SPE (US EPA, 2007). Water samples with a turbidity of 5 nephelometric turbidity units (NTU, typically used in the US)or similarly Formazin Nephelometric Units (FNU, typically used in Europe) will visually appear slightly milky or cloudy, while crystal clear water usually has turbidity less than 1 NTU, with the turbidity only detected by instrumental analysis (NHMRC & NRMMC, 2011). Consequently, water samples with a turbidity of 5 NTU or greater should be filtered prior to SPE. In general, drinking water is typically below 1 NTU, recycled water can range from less than 1 NTU to around 2 NTU depending on treatment processes and secondary effluent is generally less than 2 NTU but can increase when sludge is poorly settled (D Middleton, pers. comm). The turbidity of river water can vary greatly, while the turbidity of lakes tends to be more stable. As an example, the turbidity of treated drinking water from Paris ranged from 0.02 to 0.04 NTU, while the water feeding these plants ranged from 2 to 14.3 NTU (Neale *et al.*, 2020). Further, the turbidity of Canadian surface waters ranged from 0.5 to 50 NTU (Cantwell and Hofmann, 2011).

Based on low sorption capacity, glass fibre filters are recommended for filtration of samples with a turbidity of 5 NTU or greater. As discussed above, a wide range of filter pore sizes are used within the literature, with glass fibre filters between 0.7 to 1.5 µm recommended for filtration prior to SPE for chemical analysis (International Organisation for Standardization, 1997; US EPA, 2007; Furlong *et al.*, 2008).

3.3 Testing of native water samples

Few studies have run unenriched or native water sample in yeast reporter gene and mammalian reporter gene assays (e.g., Niss *et al.*, 2018; Abbas *et al.*, 2019; Brettschneider *et al.*, 2019). This is equivalent to whole-effluent toxicity (WET) testing and would incorporate the effect from different components in water including salts, metals and other inorganics, as well as organic micropollutants. Consequently, the effect of organic micropollutants could not be differentiated from other components in water. This approach is used for wastewater samples but is unlikely to be able to detect an effect in cleaner water samples, such as highly treated wastewater, drinking water and surface water. As a minimum pre-treatment for testing native water samples, water must be filtered as described above to remove any particles. For mammalian cell-based bioassays it is also important to sterile filter the sample using a filter with a 0.22 µm pore size (Niss *et al.*, 2018).

As the current project is focused on the effect of organic micropollutant mixtures and applying bioassays to drinking water, running native water samples in bioassays is less applicable and the following section will focus on commonly applied sample extraction methods.

4 Sample extraction options

4.1 Solid-phase extraction

SPE is the most commonly used method to enrich micropollutants from water prior to bioanalysis, with 89% of reviewed studies in WP3.2 applying SPE. SPE cartridges or disks contain a sorbent that retains the analytes of interest (e.g., organic micropollutants), while other components present in water, such as metals, salts and other inorganics, pass through the cartridge or disk, thus simplifying the matrix (Poole, 2003). The sorbed analytes can then be eluted with solvents, creating a concentrated extract that can be run in bioassays. SPE has a number of advantages including good recovery of a wide

range of contaminants and ability to be automated, though cartridges can clog with samples with a high particulate content (Ademollo *et al.*, 2012). Given the wide use of SPE, Section 6 will provide more information about SPE sorbents and extraction procedures.

4.2 Passive sampling

Under 10% of reviewed studies in WP3.2 applied passive sampling (e.g., Creusot *et al.*, 2014; van der Oost *et al.*, 2017; Tousova *et al.*, 2019), with most studies focusing on surface water. Passive samplers collect micropollutants from the water environment over a longer period of time and allows chemical and bioassay analysis of very low concentrations of chemicals. However, uncertainties regarding the volume of water sampled need to be considered and parameters such as temperature and flow velocity can affect the uptake of chemicals into the sampler (Novak *et al.*, 2018). Further, the composition of the chemical mixture taken up into the sampler may differ from the chemical mixture in the water as different chemicals will have different uptake rates into the passive sampler. A number of different types of passive samplers have been applied in the literature, including silicone rubber, Empore disks and polar organic chemical integrative samplers (POCIS), in order to target chemicals with different hydrophobicities. For example, increased biological activity in Empore disk extracts was found compared to silicone rubber (Novak *et al.*, 2018), while Hamers *et al.* (2018) typically observed greater effect in Speedisk passive samples, which contain styrene divinylbenzene sorbent, compared to silicone rubber. Based on the chemicals extracted by different passive samplers, De Baat *et al.* (2019) applied non-polar silicone rubber extracts to assays indicative of activation of AhR, oxidative stress response and pregnane X receptor (PXR), while polar POCIS extracts were tested in assays indicative of hormone receptor-mediated effects. All studies included endpoints recommended in WP3.2 for water quality monitoring, namely activation of AhR, estrogenic activity and oxidative stress response.

4.3 Liquid-liquid extraction

Less than 2% of reviewed studies in WP3.2 applied LLE (Van der Linden *et al.*, 2008; Brand *et al.*, 2013), with ethyl acetate used to extract organic micropollutants from water samples. Three hundred millilitres of solvent were required per litre of water, which is around 7.5 to 10 times more than required for SPE (6 cc/500 mg cartridge). Due to the high solvent use, time consuming nature and lack of case studies, LLE is not recommended for sample enrichment of water samples, unless the focus is on total extraction of water plus SPM to capture also particle-bound water pollutants. Note that this can also be achieved with SPE by extracting the SPM retained on the filter.

4.4 Capturing volatile chemicals

Any solvent extraction, passive sampling and conventional SPE sample processing involves a blow down step, meaning that volatile chemicals, such as many solvents and some DBPs (e.g., trihalomethanes), will not be retained in the final extract. Further, mammalian cell-based assays are incubated at 37°C for often 16 to 24 h, also potentially resulting in the loss of volatile chemicals, though some bioassays can be adapted to be run without a headspace to prevent the loss of volatile chemicals (Stalter *et al.*, 2013). Stalter *et al.* (2016) developed a purge and cold-trap method to capture and concentrate volatile DBPs from drinking water. However, the method is tedious and requires extraction onsite or within a very short period of time. Therefore, it is not recommended for routine monitoring, but instead for research purposes. Importantly, volatile DBPs appear to only have a minor contribution to the overall effects (Stalter *et al.*, 2016), which suggests that we can capture the majority of DBP-associated toxicity with simpler common SPE methods.

5 Solid-phase extraction (SPE)

As SPE is the most commonly applied extraction method, this section provides further information on SPE, including different SPE sorbent options and common SPE procedure.

5.1 SPE sorbents

A wide range of sorbents have been used for SPE, with Oasis HLB (Waters) the most commonly used (48% of reviewed studied in WP3.2 that applied SPE used Oasis HLB). Other common SPE sorbents include Chromabond HR-X (Macherey-Nagel), StrataX (Phenomenex) and octadecyl silica C18. Most of the commonly used sorbents contain a copolymer mix, such as poly(divinylbenzene-co-N-vinylpyrrolidone), with a hydrophilic monomer to capture polar chemicals and a lipophilic monomer to capture hydrophobic chemicals. However, these sorbents tend to recover a lower fraction of charged chemicals compared to neutral chemicals (Neale *et al.*, 2018; Osorio *et al.*, 2018). Consequently, some studies have applied combinations of multiple sorbents, such as reverse-phase sorbents with ion-exchange materials, in order to capture a wider range of micropollutants, including very polar chemicals and charged chemicals (Aerni *et al.*, 2004; Tousova *et al.*, 2017; Osorio *et al.*, 2018). Other studies have applied multilayer SPE with Oasis HLB and coconut charcoal to improve the recovery of highly polar compounds (Escher *et al.*, 2014; Leusch *et al.*, 2014b). However, a multilayer SPE cartridge with Oasis HLB, Strata-X-CW, Strata-X-AW and Isolute ENV+ (1:1:1.5) and Supelclean EnviCarb was found to show blank effects after around 20 times enrichment (Neale *et al.*, 2018). Bioassays are not able to differentiate between effects from a sample and effects due to impurities from sample processing, so it is important to select an extraction method with no blank effects. In any case, it is important to always include processing controls with ultrapure water or glass bottled water when enriching water samples to confirm that the observed effects are due to micropollutants in the sample and not related to the SPE sorbent or solvents.

In addition, a number of studies have compared the influence of different SPE sorbents on bioactivity. For example, Rosenmai *et al.* (2018) applied both Oasis HLB (poly(divinylbenzene-co-N-vinylpyrrolidone) and Bond Elut ENV (modified styrene divinylbenzene) sorbents to extract wastewater and drinking water samples, with no consistent difference in effect observed. Abbas *et al.* (2019) compared three SPE sorbents, Oasis HLB, Telos C18/ENV and Supelco ENVI-Carb+, at pH 2.5 and pH 7 and found that Telos C18/ENV at pH 7 was the most effective for wastewater effluent and groundwater, though considerable cytotoxicity was observed, which can mask the effect.

5.2 SPE procedure

Prior to extracting the water sample by SPE, it is necessary to condition the SPE cartridge or disk to wet and activate the sorbent bed. Water miscible methanol, followed by ultrapure water is commonly used for conditioning (e.g., Bain *et al.*, 2014; Alygizakis *et al.*, 2019; Lundqvist *et al.*, 2019). However, if other less polar solvents are used for eluting the cartridge, such dichloromethane or ethyl acetate, then these solvents must also be used for conditioning. After conditioning is finished, the cartridge must not run dry and the water sample should be immediately percolated through the SPE cartridge.

Once the sample is sorbed on the SPE sorbent, the cartridge must be completely dried in a vacuum or nitrogen stream. This might take up to 2h. The dried cartridge can be sealed with parafilm and kept from light with aluminium foil and stored at -20°C until elution (Tang *et al.*, 2014). Other studies have stored dried SPE cartridges for up to 2 weeks at 4°C (Scott *et al.*, 2014). Dried cartridges can also be sent to bioassay laboratories for elution, which is simpler and cheaper than sending litres of unenriched water.

To elute a wider range of polar and nonpolar chemicals, multiple solvents are often used for elution, such as methanol and 1:1 hexane:acetone (e.g., Scott *et al.*, 2014; Jia *et al.*, 2015) or methanol and ethyl acetate (e.g., Houtman *et al.*, 2018; Muller *et al.*, 2018). Other solvents used in the literature in different combinations include acetonitrile, dichloromethane and methyl tert-butyl ether (MTBE). It should be noted that impurities in the solvents can potentially cause blank effects in the

bioassays. Consequently, it is important to use high purity (e.g. HPLC grade) solvents for conditioning and elution and limit the volume of solvent used. Based on 500 mg (6 cc) Oasis HLB SPE cartridge, 10 mL of each solvent is often used for conditioning and 10 mL of each solvent is used for elution (Scott *et al.*, 2014; Muller *et al.*, 2018). Smaller solvent volumes can be used with smaller SPE sorbent beds.

Several studies have compared the effect of conditioning and elution solvents on bioactivity. For example, Leusch *et al.* (2014a) found no significant difference in the bioanalytical results when using 1:1 hexane:actone and methanol compared to methanol alone for conditioning and elution. Further, Prochazkova *et al.* (2018) compared the effect of two different solvent conditioning and elution combinations on estrogenic activity in surface extracts. The first method targeted estrogenic compounds by conditioning and eluting with methanol, while the second method targeted less polar compounds and used ethyl acetate, methanol and 20% 2-propanol for conditioning and ethyl acetate for elution. The different solvents often resulted in different 17β-estradiol equivalent concentrations (EEQ) values for the matching samples, but no systematic difference in estrogenicity was observed.

After elution, the elution solvent is blown to dryness under nitrogen gas. Rotatory evaporators are to be avoided due to contamination issues (Y. Levy, personal communication) The dried residue or largely reduced volume is then resuspended in a final solvent, such as methanol, dimethyl sulfoxide (DMSO) or ethanol. Murk *et al.* (2002) compared the effect of storage conditions on the same extract dissolved in ethanol and DMSO in the ERα CALUX assay. Initially, there was no difference in effect, but ethanol was found to evaporate quickly when stored at room temperature or 4˚C and even evaporated at -20˚C within 6 weeks. In contrast, the DMSO stock did not significantly change in activity over the 6-week period when stored at 4˚C and -20˚C. Alcohols have some advantage because they can be evaporated prior to the experiments, avoiding any solvent in the actual bioassay. If volatile solvents are used for storing extracts, weight control of the vials and stocking up on solvent volume if indicated, is time-consuming but recommended. See also Section 6 below for further consideration on the elution and reconstitution solvent.

5.3 Effect recovery by SPE

The recovery of individual chemicals by SPE has been well studied (e.g., Schulze *et al.*, 2017; Osorio *et al.*, 2018), but less is known about effect recovery for bioassays. Further, unlike chemical analysis where an internal standard can be added to correct for chemical recovery by SPE, internal standards should not be used for bioanalysis as they may induce an effect in the bioassay that cannot be distinguished from other micropollutants in the sample. There are a number of approaches that have been applied in the literature to assess recovery, with most involving spiking a cocktail of chemicals into the water matrix prior to SPE enrichment. As it can be difficult to measure the effect of the water alone, many studies compare the effect in the extract, often expressed as a bioanalytical equivalent concentration from bioanalysis ($BEQ_{bio,extract}$) to the predicted effect based on the concentration of chemicals detected in the extract ($BEQ_{chem,extract}$) or the nominal concentration of spiked chemicals ($BEQ_{chem,nominal}$) (e.g., Leusch *et al.*, 2010; Kunz *et al.*, 2017). The $BEQ_{chem,extract}$/ $BEQ_{bio,extract}$ and $BEQ_{chem,nominal}$/$BEQ_{bio,extract}$ ratio can be used as proxy for effect recovery, with examples of studies that applied this approach in

Table 1. Further, Abbas *et al.* (2019) attempted to assess SPE recovery by comparing the effect of the native water and SPE extracts in unspiked wastewater and groundwater. However, other components in the native water sample, such as salts, metals and other inorganics, could also have an effect in the bioassay in addition to organic micropollutants, making comparison difficult.

To truly evaluate effect recovery by SPE, it is necessary to consider the effect of the spiked mixture alone, the effect of the extracted sample and the effect of the unspiked water alone. Neale *et al.* (2018) evaluated the effect recovery of a mixture of 579 micropollutants spiked into pristine surface water using a suite of bioassays indicative of xenobiotic metabolism, hormone receptor-mediated effects and adaptive stress responses. LVSPE with HR-X sorbent was used. Effect recovery was calculated using the effect of the spiked water ($BEQ_{bio,extract}$ (water + mix) minus the effect of the unspiked water ($BEQ_{bio,extract}$ (water) divided by the effect of the mixture stock solution (BEQ_{bio} (mix)). Effect recovery ranged from 35% for the activation of the estrogen receptor (ER) assay to 236% for the oxidative stress response assay, with one extreme value of 1300% for activation of PXR. This was expected to be due to the small and variable effect of BEQ_{bio} (mix). Effect recovery was within a factor of two of the optimal 100% recovery for most assays, which suggests that LVSPE is suitable for capturing the majority of active chemicals.

As a quality control, one can also spike a sample with labelled surrogates and measure the recovery of these analytes, which is recommended when the matrix changes a lot. It has to be assured that the spike does not cause any effect in the bioassay or a separate recovery sample must be enriched.

Table 1: Studies that have determined the $BEQ_{bio,extract}/BEQ_{chem,extract}$ and the $BEQ_{bio,extract}/BEQ_{chem,nominal}$ ratio to assess chemical SPE recovery expressed as effect in a water matrix (taken from Neale et al. (2018)).

Study	Extraction Sorbent	Water Matrix	Spiked Chemical Mixture	Bioassay	Endpoint	$BEQ_{bio,extract}/BEQ_{chem,extract}$
Leusch *et al.* (2010)	Oasis HLB	Ground water, raw wastewater, treated wastewater, river water	Eight estrogenic compounds (17β-estradiol, estrone, estriol,17α-ethinylestradiol, 4-t-octylphenol, 4-nonylphenol, bisphenol A, benzyl butyl phthalate)	YES	Activation of ER	0.3-0.79
				ER CALUX	Activation of ER	0.98
				MELN	Activation of ER	0.46
				KBluc	Activation of ER	1.64
				E-SCREEN	Cell proliferation	0.68-0.97
Kolkman *et al.* (2013)	Oasis MCX	Surface Water	39 chemicals, including hormones, pesticides, pharmaceuticals and industrial compounds	ER CALUX	Activation of ER	0.44 (BEQ_{bio} 2.2 ng/L; BEQ_{chem} 5 ng/L)
				AR CALUX	Activation of AR	0.05 (BEQ_{bio} 8.2 ng/L; BEQ_{chem} 177 ng/L)
				GR CALUX	Activation of GR	1.06 (BEQ_{bio} 110 ng/L; BEQ_{chem} 104 ng/L)
				PR CALUX	Activation of PR	0.02 (BEQ_{bio} 0.91 ng/L; BEQ_{chem} 53 ng/L)
				TRβ CALUX	Activation of TRβ	0.38 (BEQ_{bio} 19 ng/L; BEQ_{chem} 50 ng/L)

Study	Extraction Sorbent	Water Matrix	Spiked Chemical Mixture	Bioassay	Endpoint	$BEQ_{bio,extract}/BEQ_{chem,nominal}$
Thorpe *et al.* (2006)	C18	Wastewater	Four estrogenic compounds (17β-estradiol, estrone, 17α-ethinylestradiol, nonylphenol)	Recombinant yeast estrogen screen	Activation of ER	1.13 to 1.24
Neale and Escher (2014)	Oasis HLB	Treated wastewater	Six herbicides (atrazine, diuron, fluometuron, hexazinone, simazine, terbutryn)	Combined algae assay	2 h photosystem II inhibition	0.91 (BEQ_{bio} 2.03 μg/L; BEQ_{chem} 2.24 μg/L)
Kunz *et al.* (2017)	LiChrolut EN-RP18	Ultrapure water	Four estrogenic compounds (17β-estradiol, estrone, 17α-ethinylestradiol bisphenol A)	YES	Activation of ER	1.38 (high mix) (BEQ_{bio} 4.4 ng/L; BEQ_{chem} 3.2 ng/L) 0.76 (low mix) (BEQ_{bio} 0.24 ng/L; BEQ_{chem} 0.32 ng/L)
				ERα CALUX	Activation of ER	0.96 (high mix) (BEQ_{bio} 1.3 ng/L; BEQ_{chem} 1.3 ng/L)

Study	Extraction Sorbent	Water Matrix	Spiked Chemical Mixture	Bioassay	Endpoint	$BEQ_{bio,extract}/BEQ_{chem,nominal}$
						0.98 (low mix) (BEQ_{bio} 0.13 ng/L; BEQ_{chem} 0.13 ng/L)
				T47D-KBluc	Activation of ER	0.32 (high mix) (BEQ_{bio} 1.8 ng/L; BEQ_{chem} 5.6 ng/L) 9.59 (low mix) (BEQ_{bio} 5.4 ng/L; BEQ_{chem} 0.56 ng/L)
				MELN	Activation of ER	0.27 (high mix) (BEQ_{bio} 0.4 ng/L; BEQ_{chem} 1.3 ng/L) 0.34 (low mix) (BEQ_{bio} 0.04 ng/L; BEQ_{chem} 0.13 ng/L)
				ERα GeneBLAzer	Activation of ER	0.54 (high mix) (BEQ_{bio} 2.1 ng/L; BEQ_{chem} 3.8 ng/L) 0.64 (low mix) (BEQ_{bio} 0.24 ng/L; BEQ_{chem} 0.38 ng/L)

6 Dosing into bioassays

After elution, the sample extract can be dosed directly into the bioassay or solvent exchanged to a less toxic solvent by blowing down the elution solvent and resuspending in a final solvent, such as DMSO or methanol. This is also relevant for passive sampling and LLE extracts. Methanol and DMSO are the most commonly used solvents for bioassay dosing. DMSO is able to dissolve a wider range of compounds than methanol, but it is non-volatile, meaning that the extract cannot be further enriched by blowing down. In contrast, methanol is volatile, meaning it can be blown down further to increase the enrichment factor. DMSO is also more toxic than methanol, with a final DMSO concentration of 0.1% recommended in the bioassay. In contrast, up to 1% of methanol can be added to some mammalian reporter gene assays (Leusch *et al.*, 2017). This equates to a DF in the assay of 1,000 for DMSO extracts compared to 100 for methanolic extracts, meaning extracts in DMSO need to be enriched 10 times more than methanolic extracts to give the same REF.

Further, the REF can be increased by exchanging the methanolic extract with cell culture media. This is achieved by adding a volume of methanol to a glass vial (i.e. 2 mL HPLC vial), blowing down to dryness and resuspending in cell culture media, which can be directly transferred to the cells. This increases the REF in the assay without inducing any solvent effects. This approach has been applied recently to drinking water extracts to help detect effects in relatively clean samples (Hebert *et al.*, 2018; Neale *et al.*, 2020). It must be assured that the sample is well dissolved in the bioassay medium. This is rarely a problem for water extracts but may occur if extracts from suspended solids are tested. It is also important to include solvent controls in the assay to ensure that the solvent itself is not inducing a response in the assay.

7 Decision-making tool

As discussed above, there are a number of decisions to be made regarding sample collection, pre-treatment and enrichment. Consequently, a decision-making flow chart (Figure 3) has been developed to guide users through some of the key decisions, with further information about each step provided in the report. Once the final sample pre-treatment and processing methods have been selected, it is important to use the same approach for all samples that you want to compare. It is not possible to truly compare changes over time or differences between sites if different sample pre-treatment and processing methods are used as this can affect the chemical mixture in the final extract. Further, where possible, the same bioassay and chemical analysis pre-treatment and sample processing methods should be used to allow greater comparability between the results.

As outlined above, the information used to support sample pre-treatment and processing decisions is often based on user experience, with few studies investigating the impact of different sample processing options on the biological effect. The majority of these studies focus on estrogenic activity, with little known about other endpoints. Further, some of the advice is based on chemical analysis protocols, rather than being specific for bioassays. One of the least standardised, but very important, pre-treatment steps is sample filtration. A wide range of filter pore sizes are used in the literature, which will affect the amount of SPM retained on the SPE cartridge. However, to our knowledge, the decision regarding which filter size to select or whether to filter or not is not based on scientific studies, but rather user experience. In the current study, we suggest a uniform approach of filtering samples with a turbidity of greater than 5 NTU using glass fibre filters, though further experimental work is required to validate this approach.

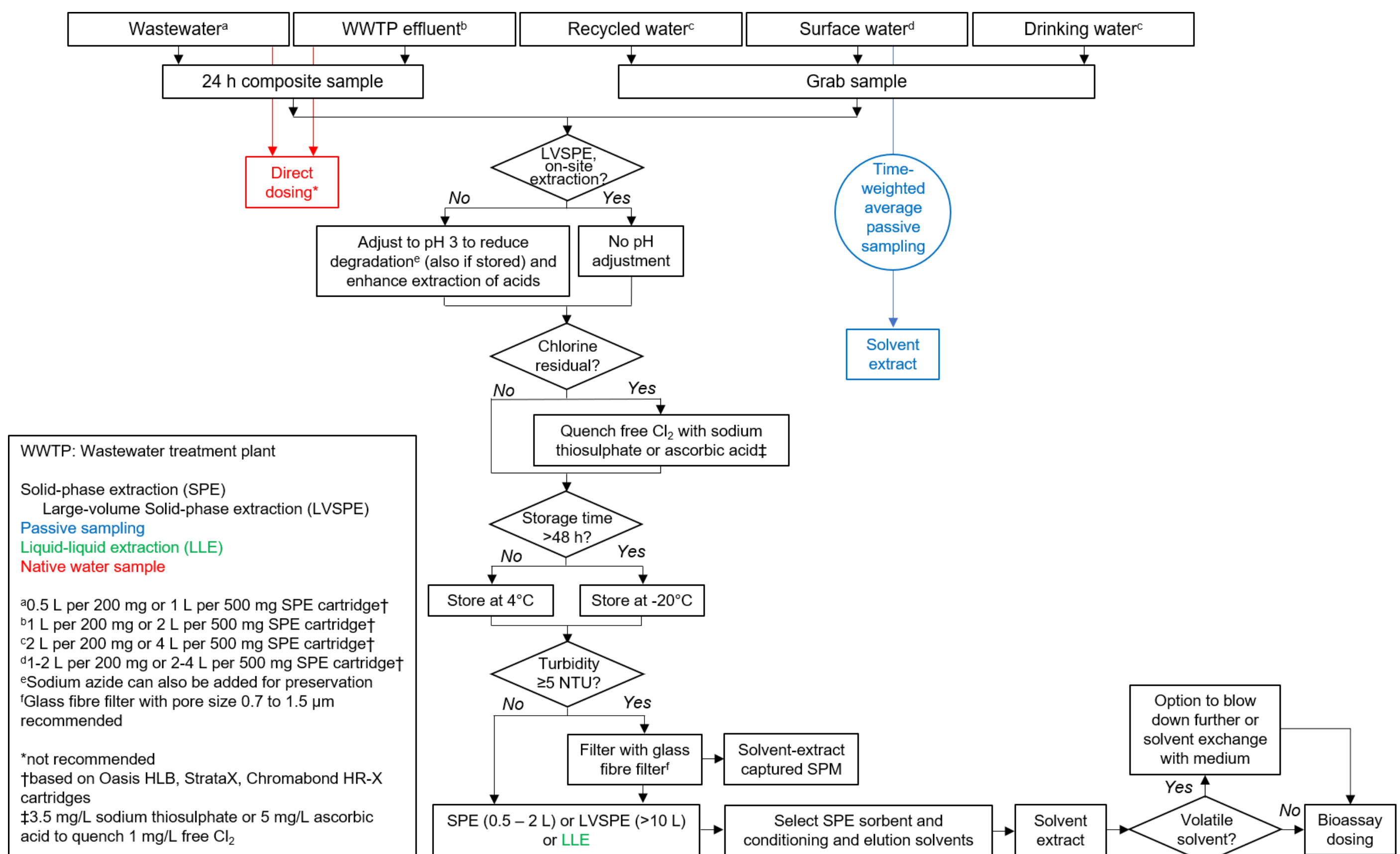

Figure 3: Sample pre-treatment and processing decision-making flow chart.

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References

Abbas, A., Schneider, I., Bollmann, A., Funke, J., Oehlmann, J., Prasse, C., Schulte-Oehlmann, U., Seitz, W., Ternes, T., Weber, M., Wesely, H. and Wagner, M. (2019). What you extract is what you see: Optimising the preparation of water and wastewater samples for *in vitro* bioassays. *Water Research*, **152**: 47-60.

Ademollo, N., Patrolecco, L., Polesello, S., Valsecchi, S., Wollgast, J., Mariani, G. and Hanke, G. (2012). The analytical problem of measuring total concentrations of organic pollutants in whole water. *Trac-Trends in Analytical Chemistry*, **36**: 71-81.

Aerni, H.R., Kobler, B., Rutishauser, B.V., Wettstein, F.E., Fischer, R., Giger, W., Hungerbuhler, A., Marazuela, M.D., Peter, A., Schonenberger, R., Vogeli, A.C., Suter, M.J.F. and Eggen, R.I.L. (2004). Combined biological and chemical assessment of estrogenic activities in wastewater treatment plant effluents. *Analytical and Bioanalytical Chemistry*, **378**(3): 688-696.

Alygizakis, N.A., Besselink, H., Paulus, G.K., Oswald, P., Hornstra, L.M., Oswaldova, M., Medema, G., Thomaidis, N.S., Behnisch, P.A. and Slobodnik, J. (2019). Characterization of wastewater effluents in the Danube River Basin with chemical screening, *in vitro* bioassays and antibiotic resistant genes analysis. *Environment International*, **127**: 420-429.

Bain, P.A., Williams, M. and Kumar, A. (2014). Assessment of multiple hormonal activities in wastewater at different stages of treatment. *Environmental Toxicology and Chemistry*, **33**(10): 2297-2307.

Bicchi, C., Schiliro, T., Pignata, C., Fea, E., Cordero, C., Canale, F. and Gilli, G. (2009). Analysis of environmental endocrine disrupting chemicals using the E-screen method and stir bar sorptive extraction in wastewater treatment plant effluents. *Science of the Total Environment*, **407**(6): 1842-1851.

Brack, W., Ait Aissa, S., Backhaus, T., Dulio, V., Escher, B.I., Faust, M., Hilscherova, K., Hollender, J., Hollert, H., Muller, C., Munthe, J., Posthuma, L., Seiler, T.B., Slobodnik, J., Teodorovic, I., Tindall, A.J., Umbuzeiro, G.D., Zhang, X.W. and Altenburger, R. (2019). Effect-based methods are key. The European Collaborative Project SOLUTIONS recommends integrating effect-based methods for diagnosis and monitoring of water quality. *Environmental Sciences Europe*, **31**.

Brand, W., de Jongh, C.M., van der Linden, S.C., Mennes, W., Puijker, L.M., van Leeuwen, C.J., van Wezel, A.P., Schriks, M. and Heringa, M.B. (2013). Trigger values for investigation of hormonal activity in drinking water and its sources using CALUX bioassays. *Environment International*, **55**: 109-118.

Brettschneider, D.J., Misovic, A., Schulte-Oehlmann, U., Oetken, M. and Oehlmann, J. (2019). Detection of chemically induced ecotoxicological effects in rivers of the Nidda catchment (Hessen, Germany) and development of an ecotoxicological, Water Framework Directive-compliant assessment system. *Environmental Sciences Europe*, **31**: 7.

Cantwell, R.E. and Hofmann, R. (2011). Ultraviolet absorption properties of suspended particulate matter in untreated surface waters. *Water Research*, **45**(3): 1322-1328.

Cargouet, M., Perdiz, D., Mouatassim-Souali, A., Tamisier-Karolak, S. and Levi, Y. (2004). Assessment of river contamination by estrogenic compounds in Paris area (France). *Science of the Total Environment*, **324**(1-3): 55-66.

Conley, J.M., Evans, N., Cardon, M.C., Rosenblum, L., Iwanowicz, L.R., Hartig, P.C., Schenck, K.M., Bradley, P.M. and Wilson, V.S. (2017a). Occurrence and *in vitro* bioactivity of estrogen, androgen, and glucocorticoid compounds in a nationwide screen of United States stream waters. *Environmental Science & Technology*, **51**(9): 4781-4791.

Conley, J.M., Evans, N., Mash, H., Rosenblum, L., Schenck, K., Glassmeyer, S., Furlong, E.T., Kolpin, D.W. and Wilson, V.S. (2017b). Comparison of *in vitro* estrogenic activity and estrogen concentrations in source and treated waters from 25 US drinking water treatment plants. *Science of the Total Environment*, **579**: 1610-1617.

Creusot, N., Ait-Aissa, S., Tapie, N., Pardon, P., Brion, F., Sanchez, W., Thybaud, E., Porcher, J.M. and Budzinski, H. (2014). Identification of synthetic steroids in river water downstream from pharmaceutical manufacture discharges based on a bioanalytical approach and passive sampling. *Environmental Science & Technology*, **48**(7): 3649-3657.

Dagnino, S., Gomez, E., Picot, B., Cavailles, V., Casellas, C., Balaguer, P. and Fenet, H. (2010). Estrogenic and AhR activities in dissolved phase and suspended solids from wastewater treatment plants. *Science of the Total Environment*, **408**(12): 2608-2615.

Daniels, K.D., VanDervort, D., Wu, S.M., Leusch, F.D.L., van de Merwe, J.P., Jia, A. and Snyder, S.A. (2018). Downstream trends of *in vitro* bioassay responses in a wastewater effluent-dominated river. *Chemosphere*, **212**: 182-192.

De Baat, M.L., Kraak, M.H.S., Van der Oost, R., De Voogt, P. and Verdonschot, P.F.M. (2019). Effect-based nationwide surface water quality assessment to identify ecotoxicological risks. *Water Research*, **159**: 434-443.

Escher, B.I., Allinson, M., Altenburger, R., Bain, P.A., Balaguer, P., Busch, W., Crago, J., Denslow, N.D., Dopp, E., Hilscherova, K., Humpage, A.R., Kumar, A., Grimaldi, M., Jayasinghe, B.S., Jarosova, B., Jia, A., Makarov, S., Maruya, K.A., Medvedev, A., Mehinto, A.C., Mendez, J.E., Poulsen, A., Prochazka, E., Richard, J., Schifferli, A., Schlenk, D., Scholz, S., Shiraish, F., Snyder, S., Su, G.Y., Tang, J.Y.M., van der Burg, B., van der Linden, S.C., Werner, I., Westerheide, S.D., Wong, C.K.C., Yang, M., Yeung, B.H.Y., Zhang, X.W. and Leusch, F.D.L. (2014). Benchmarking organic micropollutants in wastewater, recycled water and drinking water with *in vitro* bioassays. *Environmental Science & Technology*, **48**(3): 1940-1956.

Escher, B.I., Bramaz, N., Maurer, M., Richter, M., Sutter, D., von Kanel, C. and Zschokke, M. (2005). Screening test battery for pharmaceuticals in urine and wastewater. *Environmental Toxicology and Chemistry*, **24**(3): 750-758.

European Commission (2009). Common Implementation Strategy for the WFD, Guidance Document No. 19, Guidance on Surface Water Chemical Monitoring.

Fang, Y.X., Ying, G.G., Zhao, J.L., Chen, F., Liu, S., Zhang, L.J. and Yang, B. (2012). Assessment of hormonal activities and genotoxicity of industrial effluents using *in vitro* bioassays combined with chemical analysis. *Environmental Toxicology and Chemistry*, **31**(6): 1273-1282.

Farre, M.J., Day, S., Neale, P.A., Stalter, D., Tang, J.Y.M. and Escher, B.I. (2013). Bioanalytical and chemical assessment of the disinfection by-product formation potential: Role of organic matter. *Water Research*, **47**(14): 5409-5421.

Furlong, E.T., Werner, S.L., Anderson, B.D. and Cahill, J.D. (2008). Determination of human-health pharmaceuticals in filtered water by chemically modified styrene–divinylbenzene resin-based solid-phase extraction and high-performance liquid chromatography/mass spectrometry. In: US Geological Survey Techniques and Methods (ed.), USGS, pp. 56.

Gehrmann, L., Bielak, H., Behr, M., Itzel, F., Lyko, S., Simon, A., Kunze, G., Dopp, E., Wagner, M. and Tuerk, J. (2018). (Anti-)estrogenic and (anti-)androgenic effects in wastewater during advanced treatment: Comparison of three *in vitro* bioassays. *Environmental Science and Pollution Research*, **25**(5): 4094-4104.

Glassmeyer, S.T., Furlong, E.T., Kolpin, D.W., Batt, A.L., Benson, R., Boone, J.S., Conerly, O., Donohue, M.J., King, D.N., Kostich, M.S., Mash, H.E., Pfaller, S.L., Schenck, K.M., Simmons, J.E., Varughese, E.A., Vesper, S.J., Villegas, E.N. and Wilson, V.S. (2017). Nationwide reconnaissance of contaminants of emerging concern in source and treated drinking waters of the United States. *Science of the Total Environment*, **581**: 909-922.

Hamers, T., Kamstra, J.H., van Gils, J., Kotte, M.C. and van Hattum, A.G.M. (2015). The influence of extreme river discharge conditions on the quality of suspended particulate matter in Rivers Meuse and Rhine (The Netherlands). *Environmental Research*, **143**: 241-255.

Hamers, T., Legradi, J., Zwart, N., Smedes, F., de Weert, J., van den Brandhof, E.J., van de Meent, D. and de Zwart, D. (2018). Time-Integrative Passive sampling combined with TOxicity Profiling (TIPTOP): an effect-based strategy for cost-effective chemical water quality assessment. *Environmental Toxicology and Pharmacology*, **64**: 48-59.

Hebert, A., Feliers, C., Lecarpentier, C., Neale, P.A., Schlichting, R., Thibert, S. and Escher, B.I. (2018). Bioanalytical assessment of adaptive stress responses in drinking water: A predictive tool to differentiate between micropollutants and disinfection by-products. *Water Research*, **132**: 340-349.

Houtman, C.J., ten Broek, R. and Brouwer, A. (2018). Steroid hormonal bioactivities, culprit natural and synthetic hormones and other emerging contaminants in waste water measured using bioassays and UPLC-tQ-MS. *Science of the Total Environment*, **630**: 1492-1501.

International Organisation for Standardization (1997). ISO 11369:1997: Water quality—determination of selected plant treatment agents—method using high-performance liquid chromatography with UV detection after solid-liquid extraction.

Jalova, V., Jarosova, B., Blaha, L., Giesy, J.P., Ocelka, T., Grabic, R., Jurcikova, J., Vrana, B. and Hilscherova, K. (2013). Estrogen-, androgen- and aryl hydrocarbon receptor mediated activities in passive and composite samples from municipal waste and surface waters. *Environment International*, **59**: 372-383.

Jarošová, B., Ersekova, A., Hilscherova, K., Loos, R., Gawlik, B.M., Giesy, J.P. and Blaha, L. (2014). Europe-wide survey of estrogenicity in wastewater treatment plant effluents: The need for the effect-based monitoring. *Environmental Science and Pollution Research*, **21**(18): 10970-10982.

Jia, A., Escher, B.I., Leusch, F.D.L., Tang, J.Y.M., Prochazka, E., Dong, B.F., Snyder, E.M. and Snyder, S.A. (2015). *In vitro* bioassays to evaluate complex chemical mixtures in recycled water. *Water Research*, **80**: 1-11.

Kolkman, A., Schriks, M., Brand, W., Bauerlein, P.S., van der Kooi, M.M.E., van Doorn, R.H., Emke, E., Reus, A.A., van der Linden, S.C., de Voogt, P. and Heringa, M.B. (2013). Sample preparation for combined chemical analysis and *in vitro* bioassay application in water quality assessment. *Environmental Toxicology and Pharmacology*, **36**(3): 1291-1303.

Konemann, S., Kase, R., Simon, E., Swart, K., Buchinger, S., Schlusener, M., Hollert, H., Escher, B.I., Werner, I., Ait-Aissa, S., Vermeirssen, E., Dulio, V., Valsecchi, S., Polesello, S., Behnisch, P., Javurkova, B., Perceval, O., Di Paolo, C., Olbrich, D., Sychrova, E., Schlichting, R., Leborgne, L., Clara, M., Scheffknecht, C., Marneffe, Y., Chalon, C., Tusil, P., Soldan, P., von Danwitz, B., Schwaiger, J., Becares, M.I.S., Bersani, F., Hilscherova, K., Reifferscheid, G., Ternes, T. and Carere, M. (2018). Effect-based and chemical analytical methods to monitor estrogens under the European Water Framework Directive. *TRAC-Trends in Analytical Chemistry*, **102**: 225-235.

Konig, M., Escher, B.I., Neale, P.A., Krauss, M., Hilscherova, K., Novak, J., Teodorovic, I., Schulze, T., Seidensticker, S., Hashmi, M.A.K., Ahlheim, J. and Brack, W. (2017). Impact of untreated wastewater on a major European river evaluated with a combination of *in vitro* bioassays and chemical analysis. *Environmental Pollution*, **220**: 1220-1230.

Korner, W., Spengler, P., Bolz, U., Schuller, W., Hanf, V. and Metzger, J.W. (2001). Substances with estrogenic activity in effluents of sewage treatment plants in southwestern Germany. 2. Biological analysis. *Environmental Toxicology and Chemistry*, **20**(10): 2142-2151.

Kunz, P.Y., Simon, E., Creusot, N., Jayasinghe, B.S., Kienle, C., Maletz, S., Schifferli, A., Schonlau, C., Ait-Aissa, S., Denslow, N.D., Hollert, H., Werner, I. and Vermeirssen, E.L.M. (2017). Effect-based tools for monitoring estrogenic mixtures: Evaluation of five *in vitro* bioassays. *Water Research*, **110**: 378-388.

Legler, J., Leonards, P., Spenkelink, A. and Murk, A.J. (2003). *In vitro* biomonitoring in polar extracts of solid phase matrices reveals the presence of unknown compounds with estrogenic activity. *Ecotoxicology*, **12**(1-4): 239-249.

Leusch, F.D.L., De Jager, C., Levi, Y., Lim, R., Puijker, L., Sacher, F., Tremblay, L.A., Wilson, V.S. and Chapman, H.F. (2010). Comparison of five *in vitro* bioassays to measure estrogenic activity in environmental waters. *Environmental Science & Technology*, **44**(10): 3853-3860.

Leusch, F.D.L., Khan, S.J., Gagnon, M.M., Quayle, P., Trinh, T., Coleman, H., Rawson, C., Chapman, H.F., Blair, P., Nice, H. and Reitsema, T. (2014a). Assessment of wastewater and recycled water quality: A comparison of lines of evidence from *in vitro*, *in vivo* and chemical analyses. *Water Research*, **50**: 420-431.

Leusch, F.D.L., Khan, S.J., Laingam, S., Prochazka, E., Froscio, S., Trinh, T., Chapman, H.F. and Humpage, A. (2014b). Assessment of the application of bioanalytical tools as surrogate measure of chemical contaminants in recycled water. *Water Research*, **49**: 300-315.

Leusch, F.D.L., Neale, P.A., Hebert, A., Scheurer, M. and Schriks, M.C.M. (2017). Analysis of the sensitivity of *in vitro* bioassays for androgenic, progestagenic, glucocorticoid, thyroid and estrogenic activity: Suitability for drinking and environmental waters. *Environment International*, **99**: 120-130.

Lundqvist, J., Andersson, A., Johannisson, A., Lavonen, E., Mandava, G., Kylin, H., Bastviken, D. and Oskarsson, A. (2019). Innovative drinking water treatment techniques reduce the disinfection-induced oxidative stress and genotoxic activity. *Water Research*, **155**: 182-192.

Macova, M., Escher, B.I., Reungoat, J., Carswell, S., Chue, K.L., Keller, J. and Mueller, J.F. (2010). Monitoring the biological activity of micropollutants during advanced wastewater treatment with ozonation and activated carbon filtration. *Water Research*, **44**(2): 477-492.

Macova, M., Toze, S., Hodgers, L., Mueller, J.F., Bartkow, M. and Escher, B.I. (2011). Bioanalytical tools for the evaluation of organic micropollutants during sewage treatment, water recycling and drinking water generation. *Water Research*, **45**(14): 4238-4247.

Mehinto, A.C., Jia, A., Snyder, S.A., Jayasinghe, B.S., Denslow, N.D., Crago, J., Schlenk, D., Menzie, C., Westerheide, S.D., Leusch, F.D.L. and Maruya, K.A. (2015). Interlaboratory comparison of *in vitro* bioassays for screening of endocrine active chemicals in recycled water. *Water Research*, **83**: 303-309.

Muller, M.E., Escher, B.I., Schwientek, M., Werneburg, M., Zarfl, C. and Zwiener, C. (2018). Combining *in vitro* reporter gene bioassays with chemical analysis to assess changes in the water quality along the Ammer River, Southwestern Germany. *Environmental Sciences Europe*, **30**.

Murk, A.J., Legler, J., van Lipzig, M.M.H., Meerman, J.H.N., Belfroid, A.C., Spenkelink, A., van der Burg, B., Rijs, G.B.J. and Vethaak, D. (2002). Detection of estrogenic potency in wastewater and surface water with three *in vitro* bioassays. *Environmental Toxicology and Chemistry*, **21**(1): 16-23.

Neale, P.A., Ait-Aissa, S., Brack, W., Creusot, N., Denison, M.S., Deutschmann, B., Hilscherova, K., Hollert, H., Krauss, M., Novak, J., Schulze, T., Seiler, T.B., Serra, H., Shao, Y. and Escher, B.I. (2015). Linking *in vitro* effects and detected organic micropollutants in surface water using mixture-toxicity modeling. *Environmental Science & Technology*, **49**(24): 14614-14624.

Neale, P.A., Brack, W., Ait-Aissa, S., Busch, W., Hollender, J., Krauss, M., Maillot-Marechal, E., Munz, N.A., Schlichting, R., Schulze, T., Vogler, B. and Escher, B.I. (2018). Solid-phase extraction as sample preparation of water samples for cell-based and other *in vitro* bioassays. *Environmental Science-Processes & Impacts*, **20**(3): 493-504.

Neale, P.A. and Escher, B.I. (2014). Does co-extracted dissolved organic carbon cause artefacts in cell-based bioassays? *Chemosphere*, **108**: 281-288.

Neale, P.A., Feliers, C., Glauch, L., König, M., Lecarpentier, C., Schlichting, R., Thibert, S. and Escher, B.I. (2020). Application of *in vitro* bioassays for water quality monitoring in three drinking water treatment plants using different treatment

processes including biological treatment, nanofiltration and ozonation coupled with disinfection. *Environmental Science: Water Research & Technology*, **6**: 2444-2453.
Neale, P.A., Munz, N.A., Ait-Aissa, S., Altenburger, R., Brion, F., Busch, W., Escher, B.I., Hilscherova, K., Kienle, C., Novak, J., Seiler, T.B., Shao, Y., Stamm, C. and Hollender, J. (2017). Integrating chemical analysis and bioanalysis to evaluate the contribution of wastewater effluent on the micropollutant burden in small streams. *Science of the Total Environment*, **576**: 785-795.
Nelson, E.D., Do, H., Lewis, R.S. and Carr, S.A. (2011). Diurnal variability of pharmaceutical, personal care product, estrogen and alkylphenol concentrations in effluent from a tertiary wastewater treatment facility. *Environmental Science & Technology*, **45**(4): 1228-1234.
NHMRC & NRMMC (2011). Australian Drinking Water Guidelines 6, Version 3.5.
Niss, F., Rosenmai, A.K., Mandava, G., Orn, S., Oskarsson, A. and Lundqvist, J. (2018). Toxicity bioassays with concentrated cell culture media-a methodology to overcome the chemical loss by conventional preparation of water samples. *Environmental Science and Pollution Research*, **25**(12): 12183-12188.
Novak, J., Vrana, B., Rusina, T., Okonski, K., Grabic, R., Neale, P.A., Escher, B.I., Macova, M., Ait-Aissa, S., Creusot, N., Allan, I. and Hilscherova, K. (2018). Effect-based monitoring of the Danube River using mobile passive sampling. *Science of the Total Environment*, **636**: 1608-1619.
Osorio, V., Schriks, M., Vughs, D., de Voogt, P. and Kolkman, A. (2018). A novel sample preparation procedure for effect-directed analysis of micro-contaminants of emerging concern in surface waters. *Talanta*, **186**: 527-537.
Petrie, B., Proctor, K., Youdan, J., Barden, R. and Kasprzyk-Hordern, B. (2017). Critical evaluation of monitoring strategy for the multi-residue determination of 90 chiral and achiral micropollutants in effluent wastewater. *Science of the Total Environment*, **579**: 569-578.
Poole, C.F. (2003). New trends in solid-phase extraction. *TRAC-Trends in Analytical Chemistry*, **22**(6): 362-373.
Prochazkova, T., Sychrova, E., Vecerkova, J., Javurkova, B., Otoupalikova, A., Pernica, M., Simek, Z., Smutna, M., Lepsova-Skacelova, O. and Hilscherova, K. (2018). Estrogenic activity and contributing compounds in stagnant water bodies with massive occurrence of phytoplankton. *Water Research*, **136**: 12-21.
Reungoat, J., Macova, M., Escher, B.I., Carswell, S., Mueller, J.F. and Keller, J. (2010). Removal of micropollutants and reduction of biological activity in a full scale reclamation plant using ozonation and activated carbon filtration. *Water Research*, **44**(2): 625-637.
Roberts, J., Bain, P.A., Kumar, A., Hepplewhite, C., Ellis, D.J., Christy, A.G. and Beavis, S.G. (2015). Tracking multiple modes of endocrine activity in Australia's largest inland sewage treatment plant and effluent- receiving environment using a panel of *in vitro* bioassays. *Environmental Toxicology and Chemistry*, **34**(10): 2271-2281.
Rosenmai, A.K., Lundqvist, J., le Godec, T., Ohisson, A., Troger, R., Hellman, B. and Oskarsson, A. (2018). *In vitro* bioanalysis of drinking water from source to tap. *Water Research*, **139**: 272-280.
Sauer, P., Borik, A., Golovko, O., Grabic, R., Stanova, A.V., Valentova, O., Stara, A., Sandova, M. and Kroupova, H.K. (2018). Do progestins contribute to (anti-)androgenic activities in aquatic environments? *Environmental Pollution*, **242**: 417-425.
Schulze, T., Ahel, M., Ahlheim, J., Ait-Aissa, S., Brion, F., Di Paolo, C., Froment, J., Hidasi, A.O., Hollender, J., Hollert, H., Hu, M., Klolss, A., Koprivica, S., Krauss, M., Muz, M., Oswald, P., Petre, M., Schollee, J.E., Seiler, T.B., Shao, Y., Slobodnik, J., Sonavane, M., Suter, M.J.F., Tollefsen, K.E., Tousova, Z., Walz, K.H. and Brack, W. (2017). Assessment of a novel device for onsite integrative large-volume solid phase extraction of water samples to enable a comprehensive chemical and effect-based analysis. *Science of the Total Environment*, **581**: 350-358.
Schulze, T., Ulrich, M., Maier, D., Maier, M., Terytze, K., Braunbeck, T. and Hollert, H. (2015). Evaluation of the hazard potentials of river suspended particulate matter and floodplain soils in the Rhine basin using chemical analysis and *in vitro* bioassays. *Environmental Science and Pollution Research*, **22**(19): 14606-14620.
Scott, P.D., Bartkow, M., Blockwell, S.J., Coleman, H.M., Khan, S.J., Lim, R., McDonald, J.A., Nice, H., Nugegoda, D., Pettigrove, V., Tremblay, L.A., Warne, M.S.J. and Leusch, F.D.L. (2014). An assessment of endocrine activity in Australian rivers using chemical and *in vitro* analyses. *Environmental Science and Pollution Research*, **21**(22): 12951-12967.
Simon, E., Schifferli, A., Bucher, T.B., Olbrich, D., Werner, I. and Vermeirssen, E.L.M. (2019). Solid-phase extraction of estrogens and herbicides from environmental waters for bioassay analysis-effects of sample volume on recoveries. *Analytical and Bioanalytical Chemistry*, **411**(10): 2057-2069.
Stalter, D., Dutt, M. and Escher, B.I. (2013). Headspace-free setup of *in vitro* bioassays for the evaluation of volatile disinfection by-products. *Chemical Research in Toxicology*, **26**(11): 1605-1614.
Stalter, D., Peters, L.I., O'Malley, E., Tang, J.Y.M., Revalor, M., Farre, M.J., Watson, K., von Gunten, U. and Escher, B.I. (2016). Sample enrichment for bioanalytical assessment of disinfected drinking water: Concentrating the polar, the volatiles, and the unknowns. *Environmental Science & Technology*, **50**(12): 6495-6505.
Tang, J.Y.M., Busetti, F., Charrois, J.W.A. and Escher, B.I. (2014). Which chemicals drive biological effects in wastewater and recycled water? *Water Research*, **60**: 289-299.
Thorpe, K.L., Gross-Sorokin, M., Johnson, I., Brighty, G. and Tyler, C.R. (2006). An assessment of the model of concentration addition for predicting the estrogenic activity of chemical mixtures in wastewater treatment works effluents. *Environmental Health Perspectives*, **114**: 90-97.
Tousova, Z., Oswald, P., Slobodnik, J., Blaha, L., Muz, M., Hu, M., Brack, W., Krauss, M., Di Paolo, C., Tarcai, Z., Seiler, T.B., Hollert, H., Koprivica, S., Ahel, M., Schollee, J.E., Hollender, J., Suter, M.J.F., Hidasi, A.O., Schirmer, K., Sonavane, M., Ait-Aissa, S.,

Creusot, N., Brion, F., Froment, J., Almeida, A.C., Thomas, K., Tollefsen, K.E., Tufi, S., Ouyang, X.Y., Leonards, P., Lamoree, M., Torrens, V.O., Kolkman, A., Schriks, M., Spirhanzlova, P., Tindall, A. and Schulze, T. (2017). European demonstration program on the effect-based and chemical identification and monitoring of organic pollutants in European surface waters. *Science of the Total Environment*, **601**: 1849-1868.

Tousova, Z., Vrana, B., Smutna, M., Novak, J., Klucarova, V., Grabic, R., Slobodnik, J., Giesy, J.P. and Hilscherova, K. (2019). Analytical and bioanalytical assessments of organic micropollutants in the Bosna River using a combination of passive sampling, bioassays and multi-residue analysis. *Science of the Total Environment*, **650**: 1599-1612.

Troger, R., Klockner, P., Ahrens, L. and Wiberg, K. (2018). Micropollutants in drinking water from source to tap - Method development and application of a multiresidue screening method. *Science of the Total Environment*, **627**: 1404-1432.

US EPA (2007). Method: 1694, Pharmaceuticals and personal care products in water, soil, sediment, and biosolids by HPLC/MS/MS. In: EPA-821-R-08-002, Washington DC.

Valcarcel, Y., Valdehita, A., Becerra, E., de Alda, M.L., Gil, A., Gorga, M., Petrovic, M., Barcelo, D. and Navas, J.M. (2018). Determining the presence of chemicals with suspected endocrine activity in drinking water from the Madrid region (Spain) and assessment of their estrogenic, androgenic and thyroidal activities. *Chemosphere*, **201**: 388-398.

Valitalo, P., Massei, R., Heiskanen, I., Behnisch, P., Brack, W., Tindall, A.J., Du Pasquier, D., Kuster, E., Mikola, A., Schulze, T. and Sillanpaa, M. (2017). Effect-based assessment of toxicity removal during wastewater treatment. *Water Research*, **126**: 153-163.

Van der Linden, S.C., Heringa, M.B., Man, H.Y., Sonneveld, E., Puijker, L.M., Brouwer, A. and Van der Burg, B. (2008). Detection of multiple hormonal activities in wastewater effluents and surface water, using a panel of steroid receptor CALUX bioassays. *Environmental Science & Technology*, **42**(15): 5814-5820.

van der Oost, R., Sileno, G., Janse, T., Nguyen, M.T., Besselink, H. and Brouwer, A. (2017). SIMONI (Smart Integrated Monitoring) as a novel bioanalytical strategy for water quality assessment: Part II-field feasibility survey. *Environmental Toxicology and Chemistry*, **36**(9): 2400-2416.

Van Zijl, M.C., Aneck-Hahn, N.H., Swart, P., Hayward, S., Genthe, B. and De Jager, C. (2017). Estrogenic activity, chemical levels and health risk assessment of municipal distribution point water from Pretoria and Cape Town, South Africa. *Chemosphere*, **186**: 305-313.

Walker, C.W. and Watson, J.E. (2010). Adsorption of estrogens on laboratory materials and filters during sample preparation. *Journal of Environmental Quality*, **39**(2): 744-748.

Wolz, J., Engwall, M., Maletz, S., Takner, H.O., van Bavel, B., Kammann, U., Klempt, M., Weber, R., Braunbeck, T. and Hollert, H. (2008). Changes in toxicity and Ah receptor agonist activity of suspended particulate matter during flood events at the rivers Neckar and Rhine - a mass balance approach using *in vitro* methods and chemical analysis. *Environmental Science and Pollution Research*, **15**(7): 536-553.

Xiao, S.H., Lv, X.M., Lu, Y., Yang, X.M., Dong, X.R., Ma, K.P., Zeng, Y.F., Jin, T. and Tang, F. (2016). Occurrence and change of estrogenic activity in the process of drinking water treatment and distribution. *Environmental Science and Pollution Research*, **23**(17): 16977-16986.