Evaluation of reverse osmosis drinking water treatment of riverbank filtrate using bioanalytical tools and non-target screening

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ABSTRACT

In The Netherlands, stand-alone reverse osmosis (RO) has been proposed to produce high-quality drinking water from raw riverbank filtrate impacted by anthropogenic activities. To evaluate RO's efficacy in removing organic micropollutants, biological analyses were combined with non-target screening using high-resolution mass spectrometry and open cheminformatics tools. The bank filtrate induced xenobiotic metabolism mediated by the aryl hydrocarbon receptor AhR, adaptive stress response mediated by the transcription factor Nrf2 and genotoxicy in the Ames-fluctuation test. These effects were absent in RO permeate (product water), indicating removal of bioactive micropollutants by RO membranes. In the

water samples, 49 potentially toxic compounds were tentatively identified with the in silico fragmentation tool MetFrag using the US Environmental Protection Agency CompTox Chemistry Dashboard database. 5 compounds were confirmed with reference standards and 16 were tentatively identified with high confidence based on similarities to accurate mass spectra in open libraries. Bioactivity data of the confirmed chemicals from Tox21 indicated that 2,6-dichlorobenzamide and bentazone in water samples can contribute to the activation of AhR and oxidative stress response, respectively. Bioactivity data of 7 compounds tentatively identified with high confidence indicated that these structures can contribute to induction of such effects. This study shows that riverbank filtration-RO could produce drinking water free of the investigated toxic effects.

1. INTRODUCTION

Natural drinking water sources are ubiquitously contaminated with polar organic micropollutants and their transformation products (TPs) (1–4). The chemical mixtures that threaten the quality of source waters and drinking water can vary widely, including persistent and pseudo-persistent, *i.e.* continuously emitted, mobile hydrophilic compounds (5). As the potential adverse effects to human health are not fully understood (6,7), it is preferred to maximise micropollutant removal from drinking water and to efficiently, comprehensively evaluate its quality.

Reverse osmosis (RO) has shown great potential to remove organic micropollutants from a variety of water matrices (8–10). RO uses semi-permeable membranes to separate solutes from water molecules under the driving force of an externally applied pressure (11). Chemical passage through RO membranes follows a solution-diffusion mechanism (12), with solvent and solutes independently transported to the permeate side along their transmembrane chemical potential gradient. Diffusion of organics is

mainly hindered by compound size and influenced by charge and hydrophobicity of solutes and membrane (12,13). As the baseline mechanism behind chemical removal by RO is physical separation, by-products are not expected unless membrane integrity is compromised or the feed water is disinfected (13). Although RO is considered as an energy intensive step when incorporated in conventional treatment trains (14), stand-alone RO applications to produce potable water from natural waters requiring minimum pre-treatment have emerged, representing a new scenario to achieve excellent removal of harmful chemicals and waterborne pathogens with low operational costs and environmental impact (15).

In The Netherlands, RO has been proposed as a single-step treatment to produce high-quality drinking water from riverbank filtrate. Riverbank filtration (RBF) is an energy-efficient process that occurs naturally or can be induced to increase source water quality in catchments areas impacted by anthropogenic activities (16–20). RBF can attenuate micropollutant concentrations as a result of biodegradation and sorption phenomena taking place mostly in the hyporheic zone (21,22) and to a lesser extent in the aquifer (23). The fate of polar organics largely depends on the biogeochemical conditions of RBF systems and on compound physicochemical properties (19). Typically, sorption is effective in retaining non-polar, moderately hydrophobic compounds, as well as cationic compounds by hydrophobic and electrostatic interaction mechanisms, respectively, whereas neutral hydrophilic substances and anionic organics can pass the hyporheic zone unchanged if not biodegraded (16,18).

To comprehensively assess water quality, a combination of chemical analysis and effect-based methods (EBM) has been proposed recently (24,25). EBMs relying on low-complexity *in vivo* or cell-based *in vitro* bioanalytical tools with specific endpoints can be employed to evaluate the

adverse effects of (organic) chemicals (26), emphasising mixture effects of water samples rather than single components (27). EBMs focussing on genotoxicity and cytotoxicity emerged in the 1970s (28,29), whereas reporter genes assays were introduced in the 1990s (30). Nowadays, EMBs are being increasingly integrated in routine applications to evaluate toxicity pathways with biological endpoints relevant for water quality. Sensitive test batteries covering specific and non-specific mode of actions are employed, including bioassays representative for receptor-mediated endocrine disruption, metabolism of xenobiotics and adaptive stress response indicated as minimum requirement (31).

Dissolved polar organics are typically characterised by liquid-chromatography coupled to tandem mass spectrometry (LC-MS/MS). The capabilities of recent high-resolution MS (HRMS) have set the basis for suspect screening and non-target screening (NTS), *i.e.* methodologies to elucidate the structures of unknown ions by tentative annotation of accurate mass full-scan spectra (HRMS1) and tandem mass spectra (HRMS2) without the need for reference standards (32–34), Suspect screening deals with the tentative annotation of compounds expected to occur in the samples. Typically, suspect chemicals have known structure, fragmentation behaviour and chromatographic retention time. Instead, NTS deals with the elucidation of structures for which *a priori* information of their occurrence in a sample is not available. State-of-the art NTS uses the high-throughput performance of open cheminformatics tools such as MetFrag and SIRIUS (35,36), *in silico* fragmenters that query a chemical database, *e.g.* PubChem (37), to retrieve candidate structures. These are scored on the basis of the fit of the *in silico*-generated MS fragments to the experimental HRMS2 data and on selected metadata associated to candidate structures. This approach has shown potential to increase chemical identification success rate (38). The U.S.

Environmental Protection Agency (EPA) hosts the CompTox Chemicals Dashboard,(39) an open database with high-quality, structure-curated data of ~875,000 substances (40). The structures deposited in the Dashboard are linked to human and ecological hazard data from various sources, including *in vitro* bioactivity data from ToxCast and Tox21 high-throughput screening programmes (41,42), predicted exposure data from the ExpoCast project,(43) and a variety of high-interest environmental lists of chemicals. A valuable and so far unique feature of the Dashboard is the accessibility to MS-ready form structures (44). The Dashboard is downloadable, giving the possibility of being used as local database in MetFrag (or other applications). Because of the health- and environment-relevant metadata, the Dashboard is a valuable tool for NTS of environmental contaminants with potential toxic effects (45).

The aim of this study was to evaluate the application of RO as stand-alone treatment step to produce high quality drinking water from a raw riverbank filtrate that originated from the lower Rhine in the Netherlands, using the biological and chemical methods mentioned above. The Rhine catchment area, despite regulatory actions and mitigation measures that substantially improved its ecological status (46), remains contaminated with anthropogenic organic micropollutants (7,47,48), so that their removal from the river water by RBF and RO requires continuous monitoring. We adopted a combined approach relying on (i) EBMs representative for endocrine disruption, xenobiotic metabolism, adaptive stress response and genotoxicity relevant for human health and (ii) NTS of LC-HRMS/MS data using open cheminformatics tools in connection with the EPA CompTox Chemistry Dashboard. The bioassay test battery provided a broad coverage of modes of action and represented toxicity pathways relevant for human health known to be triggered by micropollutants in environmental water samples

(24,31,49). To our knowledge this is the first effect-based monitoring study of a RO drinking water treatment plant fed with a raw natural freshwater where potentially toxic compounds were characterised by state-of-the-art NTS with open cheminformatics.

2. MATERIALS AND METHODS

2.1. Full-scale RO treatment plant and sampling

The full-scale RO system was operated for research purposes in the premises of an actual drinking water treatment plant located in the Dutch municipality of Woerden. The system consisted of a three-stage filtration series equipped with ten ESPA2-LD-4040 membrane modules (Hydranautics, Oceanside, CA) in 6:3:1 configuration. The ESPA2 is a thin-film composite with an active layer of cross-linked aromatic polyamide (50), currently considered the commercial standard RO membrane. Molecular weight cut-off (MWCO) values for this membrane range between 100 and 200 Da (51–53). It is noteworthy that RO membranes are considered non-porous and thus the MWCO principle may not be applicable since solute-membrane affinity interactions influence compound removal rather than only compound size (13). Each step was equipped with flow meters to monitor feed water, permeate and concentrate lines. The RO system was fed with ≈ 9 m^3/h of an actual drinking water source consisting of raw anaerobic riverbank filtrate with an average travel time of 30 years and freshly abstracted on site. The RO system was set at 70% productivity, resulting in a permeate flow of ≈ 6.3 m^3/h and implying that 30% of the feed water was discarded as RO concentrate. Feed water, RO permeate and RO concentrate samples (n=4) from the same water package were collected in one sampling event. As the quality of the RBF and the conditions of RO are stable throughout time, no variations were expected. The samples were taken from faucets built on the system, transferred to 10L polypropylene

bottles and stored in the dark at 2 °C for 12 days before enrichment by solid-phase extraction (SPE). From these samples, aliquots of different volumes and number of replicates were taken to comply with different enrichment protocols as indicated in section 2.2 and in the Supplementary Information (SI) S-1.

2.2. Sample enrichment by solid-phase extraction

To comply with pre-established extraction protocols and avoid problems with the biological and chemical analysis, three enrichment procedures relying on hydrophilic-lipophilic balance (HLB) sorbent material with solid-phase extraction (SPE) Oasis cartridges by Waters (Etten-Leur, The Netherlands) were used: one for the reporter gene assays, one for the Ames tests and one for chemical analysis, respectively. Details on the different procedures are given in the Supplementary Information (SI) section S-1. The enrichment protocols differed for the sample load and elution solvent composition. Although this may represent a drawback, the same broad range of organic compounds is expected to be covered by the three procedures as (i) there were no differences in the pH of water samples and wash solvents and (ii) organic eluents of comparable polarity were used in all cases. The SPE enrichment factor for the reporter gene assays procedure was 1,000x, that for the Ames test was 10,000x and that for chemical analysis was 100x (taking into account dilution in ultrapure water for the extracts to be compatible with the chromatographic mobile phase used for chemical analysis).

2.3. Bioanalysis

2.3.1. *In vitro* reporter gene assays

In vitro nuclear receptor reporter gene assays representative for seven endpoints were used to evaluate specific and non-specific toxicity. In these assays, chemicals with receptor affinity (*i.e.*, ligands) cause a ligand-receptor

complex to translocate into the nucleus, where expression of a reporter gene is induced by binding of the complex to a receptor-specific response element on the DNA (26). Endocrine disruption was assessed with a hormone receptor test battery consisting of four cell lines expressing the human estrogen receptor alpha (ERα-GeneBLAzer), the rat androgen receptor (AR-GeneBLAzer), the human glucocorticoid receptor (GR-GeneBLAzer) and the human progestagenic receptor (PR-GeneBLAzer), respectively. For these bioassays, ligand-receptor binding induced expression of a reporter gene encoding the enzyme β-lactamase. Further details including experimental procedures for activation of the nuclear receptor and cytotoxicity are described in the literature (54,55). Induction of xenobiotic metabolism was evaluated with two bioassays. The first assay was based on the rat cell line H4L1.1c4 expressing the aryl hydrocarbon receptor containing a chemical-activated luciferase reporter gene (AhR-CALUX). This assay is sensitive to compounds exhibiting dioxin-like activity, which induce the transcription of metabolic enzymes, *e.g.* the cytochrome P450, that can convert AhR ligands to reactive intermediates (56). Further details including the procedure adopted for the AhR assay can be found in the literature (49,54). The second bioassay to assess the xenobiotic metabolism was based on the human cell line HEK 293H expressing the peroxisome proliferator-activated receptor gamma (PPARγ-GeneBLAzer) with a reporter gene encoding for β-lactamase and followed a procedure previously described (49). This assay is representative for the induction of enzymes responsible for glucose, lipid and fatty acid metabolism. The adaptive stress response was evaluated with a methodology by Escher et al. (57) based on AREc32 (58), a stable antioxidant response element-driven Nrf2 reporter gene cell line derived from the human breast cancer MCF7 cells with the addition of a luciferase gene. Activation of the oxidative stress response in AREc32 can be triggered by electrophilic chemicals and reactive oxygen species (57,58).

All sample concentrations were expressed in units of relative enrichment factor (REF), which take into account the SPE enrichment factor and the dilution factor in the bioassay (31). The maximum REF used in this study was 100, *i.e.* the highest enrichment factor in the bioassays was 100 times higher than the water samples. This could be accomplished by evaporating an aliquot of the extracts in a glass vial and re-solubilising the dried extract in bioassay medium, so that the reporter gene assays did not contain any solvent. For all assays, cell viability was assessed by a cell imaging method (59). To ensure that cytotoxicity would not mask the observed effects, all concentrations above the inhibitory concentration IC10 causing 10% cytotoxicity were not included in the concentration-response curves of the activation. For hormone receptor-mediated effects and xenobiotic metabolism, the concentrations (in REF) causing 10% of the maximum effect (EC_{10}) were derived. For the adaptive stress response there is no maximum of effect, so that the concentration causing an induction ratio of 1.5 ($EC_{IR1.5}$) was derived instead. All data were evaluated using linear concentration-effect curves as outlined in detail recently (60).

2.3.2. Ames fluctuation assays

The Ames-fluctuation test based on genetically modified *Salmonella typhimurium* strains TA98 and TA100 was performed to assess the potential of water samples to induce frame-shift mutations and base-pair substitution, respectively (29). The test was performed as reported previously with minor modifications (61). These modifications regarded the *Salmonella typhimurium* strains (TA100 was used here instead of TAmix), and the data treatment (chi-square test was used here instead of cumulative binomial distribution). Concentrated water samples and procedure controls were tested in duplicate with and without S9 enzyme mix, in two independent experiments. Solvent control (DMSO) and positive controls (in DMSO) were

tested in triplicate. The REF in the Ames test was 200, resulting from diluting 6 µL aliquots of water extracts in a final volume of 300 µl assay medium. Results were expressed as number of cell culture wells in which a colour change of a pH indicator in the medium was observed. Maximum (10) and minimum (25) average numbers of colour-changed wells were considered for the solvent controls and positive controls, respectively. A chi-square-test was used to determine statistically significant differences ($p<0.05$). Test conditions were compared to solvent and SPE blanks (procedure controls) for potential false positive results. Samples were considered mutagenic if a statistically significant response was repeated within independent experiments in at least one of the test conditions.

2.4. Chemical analysis followed by non-target screening

The SPE extracts were analysed with an ultrahigh-performance LC system (Nexera Shimadzu, Den Bosch, The Netherlands) coupled to a maXis 4G high resolution quadrupole time-of-flight HRMS (q-ToF/HRMS) upgraded with HD collision cell and equipped with a ESI source (Bruker Daltonics, Leiderdorp, The Netherlands). Further details on the LC-HRMS method are given in the SI (S-2).

NTS of HRMS data was entirely performed with the software *patRoon* executed within the R statistical environment (62,63). *patRoon* is a comprehensive platform that combines openly available cheminformatics tools for NTS and selected vendor software. Further documentation is available on the GitHub repository (62). An essential description of the workflow is given in this section, whereas the terminology used can be consulted elsewhere.(34) The raw LC-HRMS analysis files were converted to centroided *mzML* format by using an algorithm available in the HRMS system vendor software DataAnalysis (Bruker Daltonics, Wormer, The Netherlands). Processing of the non-target features, *i.e.* peak-picking,

grouping and retention time (t_R) alignment, was performed using the *OpenMS* algorithm within *patRoon* (64). An absolute intensity threshold of 10,000 was considered for peak picking. Feature groups were defined as unique *m/z* (comprehensive of carbon isotopes signals) and t_R pairs occurring in the different sample matrices. A tolerance window of 5 ppm mass accuracy and 20 sec t_R was considered. Only features present in all replicates and with intensities at least five times greater than in procedural blanks were kept for further processing. Protonated ($[M+H]^+$) and deprotonated ($[M-H]^-$) ions were considered for post processing of positive and negative electrospray ionisation mode datasets, respectively. The best molecular formula fitting precursor and product ions was calculated using the *GenForm* algorithm.(65) The MetFrag approach was chosen for tentative annotation of the non-target features (36). Candidate structures having neutral monoisotopic mass within ± 5 ppm from that of the non-target ions were retrieved from the EPA CompTox Chemistry Dashboard, which was used as local database (66). The structures were fragmented *in silico* and the fragments fitted to the experimental HRMS2 spectra. All candidate structures were scored based on the following scoring terms: (i) *FragScore:* fit of the *in silico* fragments to the experimental HRMS2 spectra; (ii) *MetFusionScore*: spectral similarities to MassBank of North America (MoNA) built within MetFrag with the MetFusion approach;(67,68) (iii) *individualMoNAscore*: spectral similarity by candidate structure InChIKey lookup in MoNA; (iv) *ExpoCast:* median exposure prediction (in mg per kg-body weight per day); (v) *ToxCastPercentActive*: percentage of active hit calls in ToxCast database; (vi) *pubMedReferences*: number of literature references in PubMed; (vii) *DataSources*: data sources on the Dashboard; (viii) *CPDatCount*: number of consumer products based on the EPA's Chemicals and Products database. These eight scoring terms were individually normalised by the highest value found among the proposed

candidates and equal weighting of 1 was used. An additional score of 1 was added for hits in the following lists: (i) *SUSDAT*: merged list of >40,000 structures from the NORMAN Suspect List Exchange; (ii) *MASSBANK*: list of NORMAN compounds on the European MassBank; (iii) *TOXSL21*: list of substances included in the TOXSL21 programme*;* (iv) *ToxCast:* list of substance included in the ToxCast programme*.* Finally, a formula score was assigned to candidate structures for which consensus between formulas derived by MetFrag and calculated by GenForm was reached. The formula consensus approach was adopted as GenForm performs an algebraic calculation of the best formula fitting precursor and fragment ions accurate masses, whereas MetFrag finds the best candidate structure matching the (de)protonated monoisotopic mass used as query, *de facto* back-calculating formulas of the *in silico* fragments. Therefore, the two approaches are complementary and their combination can enhance spectra interpretation.

As the main aim of this NTS was to identify, with the highest possible confidence, micropollutants that could have been responsible for observed effects in the bioanalytical tools, prioritisation of the tentatively annotated features involved filtering out candidate structures that were not present in the *MASSBANK* list or for which an individual MoNA score could not be assigned. Evaluation of the results included visual assessment of chromatographic peaks and plots of de-noised HRMS2 spectra, as well as inspection of the MetFrag scores. All tentatively annotated structures were assigned identification confidence levels based on the scale proposed by Schymanski et al. (69). Whenever possible, this process was aided by calculation of spectral similarity to records in MoNA or MassBank with the R package *OrgMassSpecR* (70). Spectral matches were reviewed manually by at least three co-authors for plausibility.

3. RESULTS AND DISCUSSION

3.1. Reporter gene assays

Only AhR-CALUX and AREc32 showed activity, while none of the hormone receptor-mediated effects were induced by the feed water and RO samples. Concentration-effect curves limited to the assays that showed sufficient activity to allow the derivation of EC_{10} or $EC_{IR1.5}$ are provided in the SI (S-3), whereas inhibitory concentrations for cytotoxicity (IC_{10}) and effect concentrations for reporter gene activation (EC_{10} and $EC_{IR1.5}$) are reported in Table S-4.1. The results depicting the bioassays in which receptor-mediated effects were observed, limited to the water matrices that were active, are shown in Figure 1.

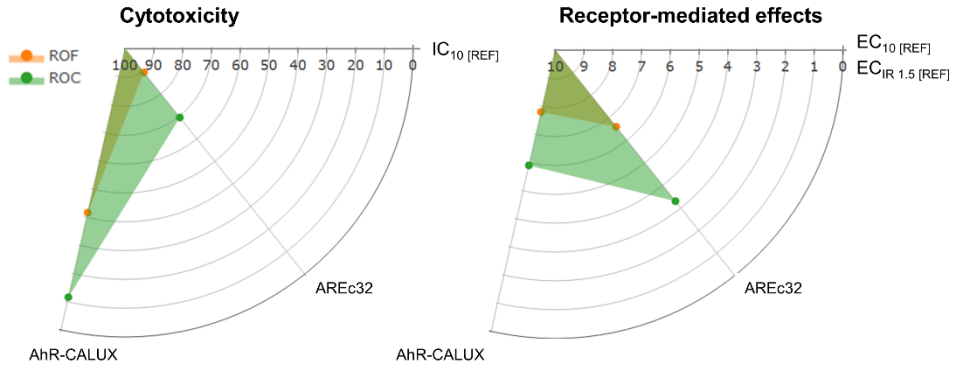

Figure 1. *Radar plots of cytotoxicity (left) and receptor-mediated effects (right) expressed as IC_{10} and EC_{10} and $EC_{IR1.5}$ in units of REF, respectively, depicting the gene reporter assays where effects were induced. RO permeate not plotted for graphic purposes as it did not induce cytotoxicity nor effects up to REF 100. ROF = reverse osmosis feed, i.e. riverbank filtrate; ROC = reverse osmosis concentrate.*

Lack of induction of hormone receptor-mediated effects could be rationalised based on the chemistry of the agonists of these receptors in relation to the investigated water matrices. Hormones, despite featuring polar functional groups along their structures, are mostly hydrophobic and thus they are expected to be retained in RBF systems by sorption phenomena (71).

Nevertheless, compounds other than hormones have shown the ability of inducing androgenic and estrogenic effects (49), thus it should be assumed that such chemicals were not present in the bank filtrate (RO feed water) or that they occurred at non-active concentrations within the tested REF range. A recent study observed that RBF could not fully remove estrogenic activity (72), nevertheless in that study a bank filtrate having a travel time of ≈ 20 days was tested, whereas in our case the travel time of the RBF was on average 30 years. We assumed that a much longer travel time could have maximised hormone removal or dilution to undetectable concentrations.

For RO feed water (ROF), the average $IC_{10\,was}$ ≈ 42 REF, whereas in AREc32 the IC_{10} was ≈ 89 REF. This indicated that the ROF needed to be enriched 42 and 89 times in order to cause 10% decrease in viability of the AREc32 and AhR cell lines, respectively. While the IC_{10} values of ROF were lower in AhR by a factor of 2 compared to AREc32, the greatest difference was observed when the cells were exposed to RO concentrate (ROC). In this case, an IC_{10} of ≈ 12 REF was quantified for the AhR cell line, whereas for AREc32 the IC_{10} was ≈ 70 REF. In line with previous literature (57), the AREc32 cell line was more robust and less prone to disturbance by non-specific toxicity. In all cases, the RO permeate (ROP) was not cytotoxic within the tested REF range, except in one ambiguous case discussed later in this section, where also receptor-mediated effects were induced. Overall our results indicated that ROP was not cytotoxic within the tested REF range up to REF 100.

RO samples and SPE procedural blanks induced xenobiotics metabolism mediated by the AhR. Procedural blanks were active with an average EC_{10} of ≈ 72 REF, whereas the ROP samples displayed an average EC_{10} of ≈ 69 REF. As these values were comparable, activity of the ROP was attributed to impurities enriched during sample preparation and not to micropollutants

391 that were able to pass the RO membranes. EC_{10} values of ≈ 8 REF and ≈ 6
392 REF were quantified for ROF and ROC, respectively, indicating comparable
393 bioactivity of these matrices at low enrichment factor. A recent study on
394 groundwater impacted by sewage exfiltration found that deep aquifers used
395 as negative controls were equally active as water from shallow groundwater
396 wells in a AhR assay (73), indicating that some micropollutants caused
397 effects at levels below the limit of detection of their analytical methods. This
398 highlights the importance of obtaining adequate controls and blank samples
399 as well as the ability to discern between the sensitivity of the bioassays and
400 that of the detector used for targeted chemical analysis. In the cited study the
401 same results were obtained for ERα and GR, whereas in our study no
402 estrogenic and glucocorticoid activities were observed. These results
403 highlight the importance of applying robust barriers against organic
404 micropollutants during drinking water treatment and our study indicates that
405 RO filtration is a suitable barrier to remove potential precursors of
406 carcinogenic compounds.

407 The toxicity pathway representative for oxidative stress response was
408 induced by ROF and ROC, $EC_{IR1.5}$ values of ≈ 6.6 REF and ≈ 3.3 REF were
409 calculated, respectively. Procedural blanks and ROP samples were not
410 active, except for a single ROP replicate, which gave ambiguous results and
411 caused ≈ 10% reduction in cell viability with a very wide standard error at
412 REF ≈ 100. This sample induced the Nrf2 factor with an $EC_{IR1.5}$ of ≈ 60 REF.
413 This effect resulted from an unclear interference, as the remaining three
414 replicates did not induce oxidative stress. Escher et al. (57) used the reporter
415 gene assay AREc32 to investigate water recycling in an Australian advanced
416 water treatment plant (AWTP), which included RO filtration in the treatment
417 train (57). ROF and ROC from that AWTP displayed higher effects with
418 $EC_{IR1.5}$ of 0.89 REF for ROF and 0.38 REF for ROC higher compared to our

samples. This was not surprising as in their case RO was applied to a wastewater pre-treated with ultrafiltration, a membrane process effective for macromolecules with molecular weight ≥ 1 kDa (74), thus not suitable against micropollutants, whose size usually does not exceed 300 - 400 Da, thus it is conceivable that the ROF had a higher load of chemicals.

3.2. Ames tests

The results of the Ames-fluctuation tests for *S. typhimurium* strains TA98 and TA100 with and without the S9 mix are summarised in Table 1, with plots given in the SI (S-5). ROF was genotoxic to strain TA98-S9, indicating mutagenicity of micropollutants occurring in the bank filtrate non-mediated by the S9 enzyme mix. One ROF replicate induced genotoxicity in strain TA98+S9, indicating that enzyme-mediated chemical activation resulted in frame-shift mutations in the genome of this particular strain. However, we consider ROF to be non-genotoxic in this condition given the disagreement between replicate tests. Additionally, in condition TA98+S9 (and TA100+S9), a decrease of ≈ 25% viability compared to the control was observed when the strain was exposed to ROF, indicating non-specific cytotoxicity of organic components enriched from the bank filtrate that may have resulted in false negative results. In all these cases, genotoxicity was removed by RO as exposure to ROP extracts did not result in *S. typhimurium* revertants. For condition TA100-S9, genotoxicity of ROF was observed in both duplicate experiments, however this result might be a false positive given the mutagenic effects induced by one of the procedural blanks while negative controls were not mutagenic. One of the replicate ROP samples was also genotoxic to strain TA100-S9, however the effect could not be replicated and may result from impurities introduced during the extraction procedure. It was concluded that while direct genotoxic potential may be present in ROF, ROP was not mutagenic in any of the tested conditions. Supporting literature

indicating mutagenicity of groundwater to *S. typhimurium* strain TA98 without the S9 enzyme mix was found (75), although in that study activity was attributed to natural compounds and not anthropogenic pollutants. Another study on drinking water prepared from Dutch groundwater found that, when present, mutagenic activity was predominantly indirect for strain TA98, *i.e.* without S9, and that in some cases even drinking water was mutagenic to strain TA98-S9 (76).

Table 1. Ames test results of RO samples

Test conditions	ROF		ROP	
	Viability (%)	Genotoxicity	Viability (%)	Genotoxicity
TA98 (-S9)	122±1	positive (++)	130±15	negative (--)
TA98 (+S9)	75±20	negative (-+)	75±19	negative (--)
TA100 (-S9)	107±1	positive (++)[a]	110±6	negative (-+)[b]
TA100 (+S9)	75±1	negative (--)	93±16	negative (--)

ROF = RO feed water (riverbank filtrate); ROP = RO permeate; + = genotoxic; - = non genotoxicy; [a.] One out of two procedural blanks was genotoxic in one replicate experiment, but negative controls were not; [b] One out of two procedural blanks was genotoxic in one replicate experiment, but negative controls were not.

3.3. Non-target screening

An overview of the features detected in the ROF (bank filtrate), ROC and ROP is provided in Figure 2.

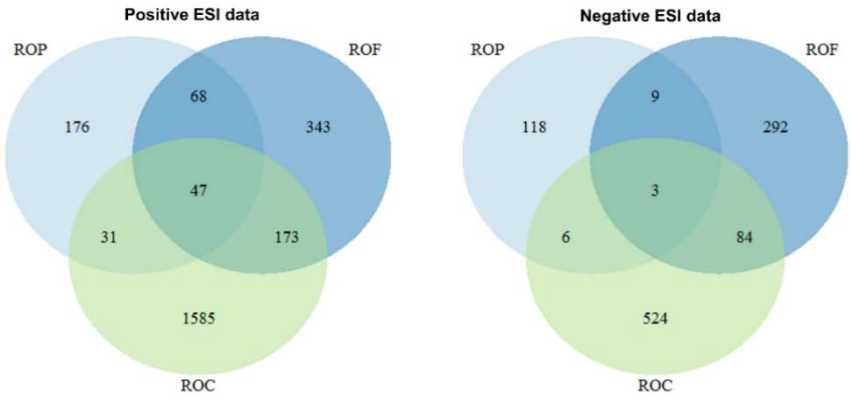

Figure 2. *Venn diagrams of non-target features in samples from the RO drinking water treatment plant detected in positive (left) and negative (right) electrospray ionisation (ESI) datasets. ROF: RO feed water; ROP: RO permeate; ROC: RO concentrate.*

In total, 2423 and 1036 features were detected in positive and negative electrospray ionisation (ESI), respectively, and considered for post processing. The distribution of positive and negative features among the RO water matrices was generally comparable in number except for ROC, in which 1836 and 617 positive and negative features were detected, respectively. In general, a higher number of features was expected in ROC as in this matrix the concentrations of solutes would reach levels up to 3.3 times higher than ROF assuming near-full rejection by RO. The lower number of negative features in ROC might result from ion suppression caused by dissolved organic matter, naturally occurring in this bank filtrate at concentrations around 7-8 mg/L and that might have been carried through the extraction to some extent (77). In addition, ionisation in negative ESI mode might have been suppressed by the acetic acid added to the LC mobile phase as a modifier. Lastly, as excellent rejection of inorganic ions can be achieved by RO,(50) different adducts could have formed in the ROC samples analysed in positive ESI mode, possibly explaining the higher number of positive features in this matrix. As shown in Fig. 2, only about 2/3 and 1/3 of the features detected in ROF were found also found in the positive and negative ROC data, respectively. This might result from matrix effects, such as ion suppression, which might have affected both ionisation or extraction efficiency in ROC. Additionally, in ROC we encountered some instances in which early eluting features fell out of the 20 sec tolerance window used to group features amongst water matrices, resulting in a given *m/z* being assigned to two different feature groups and thus not overlapping between ROF and ROC. This behaviour was not investigated further as these features were nonetheless considered for tentative identification if they

complied with the prioritisation criteria. Based on the physicochemical properties behind incomplete chemical removal by RO, it could be assumed that most features detected in ROP, which were overall comparable between the positive and negative datasets, were either small and hydrophilic uncharged compounds, small cationic compounds or uncharged (moderately) hydrophobic compounds exhibiting polar groups ionisable by HRMS (13). Features occurring only in ROP might have been undetectable elsewhere due to matrix effects or some of them might have even leached from the RO the system. An overview of the *m/z* values and retention time of the features detected in the different water matrices is provided in the SI (S-6).

Among the detected features, 1528 positive and 833 negative ions from all sample matrices were assigned a tentative structure by MetFrag. In the positive data, 53 tentatively annotated structures were present in the MassBank list, 24 of which were similar to spectra in MoNA. Additionally, 13 structures not present in the MassBank list were similar to records in MoNA. In the negative data, 28 candidate structures were similar to records in MoNA, 2 of which were also present in the MassBank list. All other structures were not found in spectral libraries and did not have associated bioactivity metadata. The InChIKey identifiers of candidates that exhibited good-quality chromatograms, plausible HRMS2 annotation and that would likely ionise in ESI-HRMS analysis (*e.g.*, neutral polar and ionic organics) were used to query MoNA and the European MassBank. Similarities to relevant spectra were calculated. This approach resulted in the tentative identification of 25 and 24 candidate structures in the positive and negative data, respectively. Analysis of reference standards led to confirmation of 2,6-dichlorobenzamide, phenazone and trimethyl phosphate in the positive ESI data, whereas bentazone and acesulfame were confirmed in the negative

ESI data. Supporting spectral library evidence, shown in the SI (S-8) and indicated here in parenthesis next to compound name, was found for the 16 structures. In the positive data 2-phenylethylamine (Fig. S-8.1), benzisothiazolinone (Fig. S-8.4), diethyl phosphate (Fig. S-8.5), diphenylphosphinic acid (Fig. S-8.9), triphenylphosphine oxide (Fig. S-8.10) were assigned identification confidence level 2a, the highest possible without reference standards. Anthranilic acid (Fig. S-8.2), 4-hydroxybenzoic acid (Fig. S-8.3) and fusaric acid (Fig. S-8.6) despite good match with library spectra could not be identified with confidence higher than level 3 as other isomers could not be ruled out. In the case of the triazine TPs 2-hydroxysimazine (Fig. S-8.7) and 2-hydroxyatrazine (Fig. S-8.8), despite good spectral similarity, level 3 was assigned due to (quasi-)isobaric interferences in the experimental HRMS2 data. In the negative data, acamprosate (Fig. S-8.13), saccharin (Fig. S-8.14) and mecoprop (Fig. S-8.16) were assigned level 2a, whereas catechol (Fig. S-8.11), mandelic acid (Fig. S-8.12) and 2-naphthalenesulfonic acid (Fig. S-8.15) could not be assigned a higher level than 3 as other isomers could not be ruled out. All level 2a were assigned based on matching spectra available on MoNA or MassBank, except diphenylphosphinic acid and saccharin for which spectra measured in house were used instead. For compounds identified as level 3 with supporting library spectra, it is important to stress the benefits of establishing a harmonised LC method for NTS in order to use a retention index, which could have increased confidence in the identification of isomers. The chemicals (tentatively) identified with the highest confidence having bioactivity metadata matching the endpoints covered by the bioassay test battery are listed in Table 2. In the SI (S-7) the complete lists of (tentatively) identified structures in the positive (Table S-7.1) and negative ESI datasets (Table S-7.2) are provided.

Table 2. Structures (tentatively) identified, identification confidence level (ICL) and relevant bioactivity metadata

Compound [a]	Formula	Class	ESI mode [b]	ICL[c]	Endpoints with AC50 (μM) [d]	ToxCast active (%)	Sample matrix [e]
Benzisothiazolinone	C_7H_5NOS	Herbicide	+	2a	Nrf2 induction (5.82)	30.6	ROF,ROC, ROP
2,6-dichlorobenzamide	$C_7H_5Cl_2NO$	Herbicide metabolite	+	1	AhR induction (60.6)	1.8	ROF, ROC
4-hydroxybenzoic acid	C_7H6O_3	Natural and industrial	+/-	3[1]	AhR induction (49.2); ERα induction (57.2)	1.3	ROF, ROC
Triphenylphosphine oxide	$C_{18}H_{15}OP$	Industrial	+	2a	Nrf2 induction (40.3)	1.8	ROF,ROC, ROP
Acamprosate	$C_5H_{11}NO_4S$	Pharmaceutical	-	2a	Nrf2 induction (43.6)	1.8	ROF, ROC
Bentazone	$C_{10}H_{12}N2O_3S$	Herbicide	-	1	Nrf2 induction (32.1)	3.3	ROF, ROC
Catechol	$C_6H_6O_2$	Natural and industrial	-	3[1]	Nrf2 induction (12.4); AhR induction (57.2); ERα induction (71–84)	14.1	ROF, ROC
Mecoprop	$C_{10}H_{11}ClO_3$	Herbicide	-	2a	AhR induction (30.3); PPARγ induction(85.3)	0.6	ROF, ROC
Naphthalene-2-sulfonic acid	$C_{10}H_8O_3S$	Industrial	-	3[1]	AhR induction (40.3)	2	ROF, ROC
Saccharin	$C7H_5NO_3S$	Sweetener	-	2a[2]	AhR induction (43.4)	1.3	ROF, ROC

[a] Hyperlink to compound bioactivity data on the EPA CompTox Chemistry Dashboard; [b] Detected adduct: + = $[M+H]^+$; - = $[M-H]^-$;
[c] Identification Confidence Level (69); [d] Data from EPA Chemistry Dashboard, limited to the reporter gene assays that were similar to those included in the test battery used for this study. AC_{50}: active concentration in μM causing 50% of the effects; [e] Sample matrix in which the compound was (tentatively) identified. ROF: reverse osmosis feed water (riverbank filtrate); ROC: reverse osmosis concentrate; ROP: reverse osmosis permeate; [1] Supporting library evidence found, but insufficient to rule out other isomers; [2] Reference spectrum previously measured in house.

3.4. Bioactivity of the (tentatively) identified micropollutants

ToxCast data in the EPA Dashboard indicated that 2,6-dichlorobenzamide (BAM) activated a similar AhR bioassay with an AC_{50} (active concentration causing 50% of the effects) of 60.6 µM. Based on a concentration of 39±2 ng/L quantified in a bank filtrate from the same RBF system that fed the full-scale RO treatment plant (78), only a minor contribution to the activation of AhR observed in the present work may be considered, if any. As chlorobenzamides are potentially mutagenic (79,80), BAM might have contributed to the genotoxicity characterised in ROF with the Ames tests. This chemical was not detected in ROP, which is in line with previous studies from our group (53), where BAM displayed less than 1% passage in pilot-scale RO drinking water treatment. Amongst the compounds tentatively identified with supporting library evidence, ToxCast data showed that 4-hydroxybenzoic acid, catechol, mecoprop, naphthalene-2-sulfonic acid and saccharin (all detected in ROF and ROC) can activate a similar assays based on the AhR gene reporter. Based on the acid dissociation constant (pK_a) of 4-hydroxybenzoic acid (pK_a = 4.6), mecoprop (pK_a = 3.7) and naphthalene-2-sulfonic (pK_a < 1), these chemicals would occur in ROF as dissociated acid as the pH value of this water matrix is ≈ 7, additionally supporting their occurrence in bank filtrate(16) and their lack of detection in ROP (13). Mecoprop was identified with highest possible confidence without a reference standard, *i.e.* lev. 2a, based on matching spectral records on MoNA and presence of distinctive isotopic peaks in both HRMS1 and HRMS2 experimental data. ToxCast data indicated that mecoprop elicited effects in a PPARγ assay with an AC_{50} nearly 3 times higher, thus less toxic, than that of AhR. Although we did not measure environmental concentrations of micropollutants, it would be plausible that mecoprop would not occur at levels high enough to induce PPARγ-mediated effects. This compound is a

household herbicide that has been frequently detected in European WWTP effluents at concentrations up to 2.2 µg/L (81). Mecoprop is not retained by RBF systems, leaving biodegradation as sole option of attenuation. Although evidence of degradation in oxic RBF system exist (82), mecoprop is persistent in anoxic conditions (83). Its lack of detection in ROP is in line with the high removal efficiency by RO reported in literature, which was higher than 97% (84). Mecoprop was found to be non-mutagenic to *S. typhimurium* strains TA98 and TA100 with and without the S9 enzyme (85). Saccharin is an artificial sweetener ubiquitously detected along with acesulfame (confirmed in ROF and ROC), both indicators of the impact of domestic wastewater on natural waters as they are added in high amounts to food and beverages (86). As these sweeteners occur in anionic form at pH values of natural waters, they have high mobility potential in the sub-surface (87). Their negative charge can explain detection in the RBF system and lack of detection in RO permeate. The latter is in line with literature data, which reported more than 90% removal by RO for both compounds (53,88). ToxCast data indicated that saccharin induced effects in an AhR assay with an AC_{50} of 43.4 µM, whereas data for acesulfame were not found. Both sweeteners were not genotoxic to *S. typhimurium* strain TA100 with and without the S9 enzyme (89).

ToxCast data for bentazone indicated its ability to induce transcription of Nrf2 with an AC_{50} of 32.1 µM. In line with literature data (53,84), this chemical is well removed by RO as it was not detected in ROP. Bentazone was identified in 32% of European groundwater and is currently approved for use in the EU (2). Bentazone was not mutagenic to *S. typhimurium* strains TA98 and TA100 with and without the S9 enzyme mix (85). Amongst the tentatively identified chemicals, benzisothiazolinone, acamprosate, catechol and triphenylphosphine oxide induced transcription of Nrf2. Benzisothiazolinone was the tentatively identified compounds with lowest AC_{50} (5.82 µM in Nrf2

assay) and the highest ToxCast percent active (31%). In a previous study with the AhR-CALUX variation used here this chemical was not active below cytotoxic concentrations (49). This biocide is removed by wastewater sludge (90), nevertheless indications of its high groundwater contamination potential were found (91), further supporting its tentative identification in the RBF system. Triphenylphosphine oxide is a persistent and toxic industrial chemical released in surface waters via wastewater effluents (92). A monitoring study on groundwater from various sources in The Netherlands found that triphenylphosphine oxide was more frequently detected in bank filtrate and confined groundwater, corroborating its tentative identification in the RO feed water (93). Acamprosate is the active ingredient of a pharmaceutical product to treat alcohol dependence, so far not detected in the environment, but indicated as potential drinking water contaminant (94). This chemical is anionic at any natural pH value and is excreted unchanged following therapeutic administration (95). This suggests that acamprosate may be released in surface water via domestic wastewater effluents and may pass the riverbank, reaching groundwater and exhibiting mobility in the sub-surface if not biodegraded. Given the lack of further environmentally relevant information, its inclusion in future suspect screenings is recommended.

It is noteworthy that although neither effects nor genotoxicity were observed for ROP, benzisothiazolinone, trimethyl phosphate and triphenylphosphine oxide were the only (tentatively) identified in the RO permeate. Benzisothiazolinone (151.18 Da), trimethyl phosphate (140.02 Da) and triphenylphosphine oxide (278.29 Da) are compounds whose physicochemical properties confer critical behaviour in RO filtration. Benzothiazolinone has a pK_a of 9.5, thus occurred as a neutral species in ROF, whereas trimethyl phosphate is always uncharged as its structure has no atoms that can be ionised. Benzisothiazolinone has a predicted log

octanol-water partition coefficient (logK_{ow}) of 1.02, whereas trimethyl phosphate has an experimental logK_{ow} of -0.65. Thus, both chemicals are hydrophilic, exhibit no affinity for the aromatic polyamide of which the separation layer of RO membranes is made of and remain dissolved in water, being able to pass through the RO membranes due to their small size. Triphenylphosphine oxide, instead, is also uncharged but exhibits a logK_{ow} of 2.83. Despite its larger size, this relatively hydrophobic chemical displays affinity for the aromatic polyamide active layer and likely undergoes adsorption-solution-diffusion onto-through polyamide RO membranes, resulting in breakthrough to the permeate side. Based on ToxCast data, it can be assumed that the concentrations of benzisothiazolinone and triphenylphosphine oxide were too low to trigger oxidative stress even after enrichment of the ROP samples. Nevertheless, as these chemicals were not fully removed they should be closely monitored in RO drinking water treatment processes as higher feed water concentrations might result in potentially toxic concentrations in ROP.

4. CONCLUSIONS

RO filtration directly applied to a raw riverbank filtrate in full-scale drinking water treatment was capable of producing potable water that did not induce any detectable adverse effects in the applied EBM battery. Toxicity pathways representative of xenobiotic metabolism, adaptive stress response and genotoxicity were activated by enriched bank filtrate. For the gene reporter assays, it would take no more than 6- to 8-fold concentration of this ROF to induce cellular toxicity pathways. The possible role of RBF in attenuating endocrine disrupting compounds was shown based on the lack of hormone receptor-mediated effects observed when RO feed water was tested. The water investigated in this study originated from anthropogenically impacted surface waters (*i.e.*, the lower Rhine), and the suitability of RBF as drinking

water pre-treatment seems confirmed. The bioanalytical tools used in this study indicated that RO is highly effective in removing chemicals that can induce specific and non-specific potentially toxic effects. Applying non-target screening relying on open cheminformatics tools and on an openly accessible chemical database aided the (tentative) identification of these micropollutants, while health-relevant chemical metadata could explain the biological activity observed with effect-based methods for a subset of (tentatively) identified structures. Further confirmation activities and quantification to link chemical and bioassay results will be the scope of follow-up work. As for quantification of compound concentrations in water samples, a complete validation study of the SPE method should be conducted for all investigated matrices to obtain recovery values, which are currently unknown. Testing the individual chemicals with a new test bioassay battery covering the same endpoints investigated in this study would then be necessary to confidently determine the contribution of each confirmed structure to the total observed effects. The tentatively identified structures could/should be monitored actively in future studies, for which reference standards should be obtained for higher confidence. Overall, identification confidence and success rate could be improved increasing the number of accurate mass spectra deposited in open libraries. Although the approach undertaken in this study is not meant to replace the use of reference compounds in both biological and chemical analysis, it demonstrates the potential of the employed methods to generate useful, real-world data about drinking water quality, increasing the knowledge about occurrence of chemicals in the environment and their behaviour in drinking water treatment. Additionally, the potential of elucidating chemical structures behind biological activities by non-target screening can be useful to derive cause-effect relationships.

CONFLICTS OF INTEREST

There are no conflicts of interest to declare.

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