

Emerging contaminants in recycled water

Final report

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EPA acknowledges Aboriginal people as the first peoples and Traditional custodians of the land and water on which we live, work and depend. We pay respect to Aboriginal Elders past and present and recognize the continuing connection to, and aspirations for Country.



Message from the Chief Environmental Scientist

The new EPA Act (2017) shifted our focus to the prevention of harm to the environment and human health. Given that EPA Victoria is a science-based regulator, we apply the most up to date *state of knowledge* available to make the best and highest decisions to protect human health and the environment. However, given the myriad of chemicals used by humans in both industrial and domestic activities not all emerging chemical risks are measured and assessed for. In this regard, EPA undertakes horizon scanning research to foreshadow emerging risks to better understand how we might manage for those into the future. This report examines a suite of unregulated contaminants of emerging concern that are not eliminated by wastewater treatment plants (WWTP) and are discharged into the environment.

The treatment of effluent moving through wastewater treatment systems has typically focussed on solids, microbial pathogens, nutrients, and salinity. However, international research has made it clear that a number of emerging and known pollutants, such as pharmaceuticals and personal care products (comprising human and veterinary medicines to treat diseases or illnesses), endocrine disrupting chemicals (EDCs) (hormone mimicking compounds), per- and polyfluoroalkyl substances (PFAS), along with industrial chemicals such as phenols, herbicides, pesticides, and disinfection byproducts, are present in effluent waters. Water treatment systems are variably effective at treating many of these compounds. International research has shown that WWTP effluent discharged into adjoining rivers or used for irrigation have been shown to include contaminants of emerging concern, many of which are designed to have adverse effects on humans and organisms at low concentrations.

This report establishes the prevalence of potentially harmless substances in recycled waters, which will form the basis of starting to close the gap setting ecological, human health and recycled water-specific guidelines for many of these chemicals. This work will form the basis for any future EPA assessment of risk that will apply an integrated tiered risk-based management framework approach to ensure harm to the environment and human health is eliminated or minimised to a level that is practical and achievable using best available technologies.

Professor Mark Patrick Taylor
Chief Environmental Scientist
Environment Protection Authority Victoria

Executive summary

The purpose of this project was to identify the prevalence and frequency of emerging and legacy contaminants in recycled water in Victoria. The collected data improves the state of knowledge on emerging contaminants and supports proponents in designing recycled water schemes and managing potential risks to human health and the environment. Overall, this project contributes toward enabling the Environment Protection Authority Victoria (EPA) to identify where there may be new and emerging priority areas for research, regulatory responses, and identify sectors to work with to prevent and reduce environmental pollution.

EPA analysed influent (raw sewage) and effluent water (treated wastewater) samples at 31 wastewater treatment plants (WWTPs) across Victoria for pharmaceuticals and personal care products, endocrine disrupting chemicals, industrial compounds, disinfection by-products, phenols, and per- and poly-fluorinated substances (PFAS)). Using a combination of passive samplers and spot sampling, the study investigated emerging contaminants in wastewater intended for reuse in agriculture, industry, and limited domestic use across a range of treatment trains and treatment classes (class A, B, C). Fourteen specific treatment trains (TT) were classified based on reduction and removal processes identified by O'Connor and Stevens (2020).

From 230 wastewater samples collected, a total of 413 emerging contaminants were analysed in each sample, of which 233 contaminants were below the limit of reporting. Overall, 180 emerging contaminants were detected in influent and effluent waters, of which 48 were pharmaceuticals and personal care products (PPCPs), 15 endocrine disrupting chemicals (EDCs), 21 PFAS, 34 herbicides, 8 insecticides, 7 fungicides, 12 industrial compounds, 7 phenols and 28 disinfection by-products (DBPs). To further measure concentrations of emerging contaminants in 30 WWTPs, 93 polar organic chemical integrative samplers (POCIS) were deployed.

For most of the emerging contaminants, concentrations were lower in effluent waters than in sewage influent. Reduction during treatment was found to be compound specific. Even if the highest concentrations ($\mu\text{g/L}$) substantially decreased during treatment, some compounds passed through treatment trains relatively untreated. Based on our study, treatment train TT12 removed the highest proportion of a range of emerging contaminants from the wastewater. TT12 uses a treatment combination of activated sludge process (ASP) with extended aeration, ultraviolet (UV) light disinfection, microfiltration, reverse osmosis (RO), and chlorine. Overall, our study found treatment percent reduction to be chemical group specific and vary between treatment trains.

For PPCPs, concentrations in sewage influent ranged from <0.2 up to $2,900 \mu\text{g/L}$, and in effluent waters from <0.02 up to $8.1 \mu\text{g/L}$. There are currently no guideline values for PPCPs in Australia (ANZG, 2021), and therefore risks associated with the detected concentrations are unknown.

For PFAS, concentrations in sewage influent ranged from <0.0002 up to $0.21 \mu\text{g/L}$, and in effluent waters from <0.0001 up to $0.065 \mu\text{g/L}$. The mean effluent concentrations of PFOS exceeded the ecological 99% species protection guideline for aquatic ecosystems ($0.0091 \mu\text{g/L}$, ANZG, 2023) at some of the sites, with exception of two sites treated by treatment trains TT12 and TT13 (ASP, lagoon, RO, chlorine). Only one of these two treatment trains (TT12) showed a percent reduction of $>99\%$ of PFOS from the influent waters. None of the effluent samples exceeded human-health based guidelines for PFAS.

For pesticides, concentrations in influent ranged from <1 up to $3.8 \mu\text{g/L}$ and in effluent from <0.1 up to $3.4 \mu\text{g/L}$, respectively. A few exceedances of 95% and 99% ecological species protection guidelines (ANZG, 2018) were detected for three compounds (metolachlor, diazinon and simazine).

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It is important to note that the presence of emerging contaminants in recycled water does not necessarily translate to risks to environment and human health. Risk assessment is a site-specific exercise and should be assessed through the lens of the general environmental duty (EP Act, 2017). Any assessment should be proportionate to the scale of the proposal and the magnitude of potential harm (Publication 1992, EPA 2021). For instance, assessment of recycled water should be conducted with the end use and disposal pathways in mind (e.g., car washing, garden watering, or irrigation of crops) (EPA 2021, Publication 1910; EPA 2022, Publication 168.3).

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Background

Given that recycled water is becoming an increasingly valuable resource in a drying climate (Radcliffe and Page 2020), it is critical to ensure that recycled water meets suitable criteria to ensure protection of human health and the environment.

Emerging contaminants are compounds that are newly introduced into the environment (e.g. pharmaceutical, industrial or agricultural compounds that have only recently been developed) or that, although possibly around for longer times, have only recently been detected in the environment due to advances in detection technologies (e.g. PFAS). They present a new regulatory challenge, as their prevalence and concentrations, and their potential risks, are not generally well understood (Lampard et al. 2010; Xu et al. 2015). Furthermore, many emerging contaminants do not have water quality reuse criteria or water quality guidelines and there is insufficient knowledge of the toxicological properties of individual chemicals along with the combined mixture effects (Kristiansson et al. 2021). Thus, a clearer risk-based management framework is needed for prioritising parameters for monitoring and assessment (O'Connor and Stevens, 2021).

Concerns, issues, and risks associated with emerging contaminants are a broad societal issue extending beyond the sewerage system and recycled water use. They are present in many of the products we rely on – from medication to common toiletry products, to a wide range of common household products (e.g. non-stick cookware, cleaning products, food packaging) (Kotthoff et al. 2015). Emerging contaminants are continuously detected throughout the environment, including in freshwater, soil, air, dust, biota and plants (e.g. Zenker et al. 2014; Kotthoff et al. 2020; Borsuah et al. 2020).

The Victorian Water Plan 'Water for Victoria' sets out to make better use of recycled water to improve water security and reduce the impact from climate change on Victorian waterways (Water for Victoria, 2016). These water sources are expected to make a greater contribution to water security as our climate changes and population grows. As Victoria's environmental regulator, EPA has a role to address issues associated with the reuse, disposal, and management of emerging contaminants in line with Victoria's environment protection regulations. Under the Environment Protection Act 2017 (the Act), duty holders have a general environmental duty (GED) and thus responsibility to minimise risks of harm to human health and the environment from pollution and waste, so far as reasonably practicable. To address this, a stronger scientific understanding on the prevalence and environmental risks from emerging contaminants in water and wastewater will help to improve wastewater management, duty holders' requirements for meeting the GED, water quality and food security, as well as inform future policy.

A current limitation of assessment of recycled water schemes is that they are focused only on the treatment objectives of pathogens reduction and conventional physical-chemical water quality parameters, such as organics, suspended solids nutrients pH, and salinity (EPA, 2021). There are no limits or targets set for emerging contaminants. However, 'Victorian guideline for water recycling' (EPA Publication 1910) promotes the use of the risk management framework used in the Australian Guidelines for Water Recycling (AGWR) within a Victorian context. Even if some of the water corporations already undertake risk-based approaches, classes of recycled water are based on organics, suspended solids, nutrients, and pH.

There are currently around 170 recycled water program approvals with EPA, including 22 approved Class A schemes. The majority of these rely on recycled water sourced from rural and urban municipal treatment plants. Municipal treatment plants primarily process domestic wastewater but also receive trade waste from a range of industries particularly in the Melbourne metropolitan area and major regional centres. Trade waste is more commonly processed in urban wastewater treatment plants and in

larger regional centres. Approximately 20 recycled water schemes utilise recycled water from other industrial processes, including abattoirs, and food processing plants.

The overall aim of this study was to determine the concentrations of emerging contaminants in influent and effluent waters across 31 WWTPs in Victoria, Australia. Based on a literature review (unpublished) and their chemical properties (e.g. solubility, hydrophilicity, sorption, partitioning, and polarity), the following compound groups were chosen for the study: PPCPs, EDCs, PFAS, herbicides, pesticides, industrial chemicals, phenols and DBPs.

The four specific aims were to:

- (1) quantify the number, type, and concentration of emerging contaminants in influent and effluent water;
- (2) estimate percent reduction of emerging contaminant by treatment technologies;
- (3) update risk assessment;
- (4) examine toxicity of recycled water using bioassays and a receptor localisation assays.

Methods

Sampling design

We sampled sewage influent and effluent water from 30 WWTPs across Victoria. Sites were selected based on the VicWater 2019 risk assessment (O'Connor and Stevens, 2020). Samples were collected on separate visits from Monday to Friday between 27 April and 22 July 2021. Influent samples were collected by autosamplers (ISCO, SIGMA, HACH) as a composite sample over a 24-hour period, with time weighted samples collected every 15-60 mins. Effluent samples were collected using a combination of POCIS deployed in treated wastewater effluent for 28 ± 1 days, coinciding with collection spot water samples on day 0 and on day 28, when passive samplers were retrieved. Each site was sampled for six groups of emerging contaminants: PPCPs, PFAS, EDCs, pesticides, herbicides, and DBPs. Methods for sample collection, handling, transport, storing, and quality assurance and control were consistent with EPA publication IWRG 701 (2009), PFAS National Environmental Management Plan (NEMP) (2018) and ISO-5667-23.

Sampling technique 1: spot water samples

Spot water samples were collected on day 0 and day 28 using a stainless-steel sampling pole. All samples were collected in either amber glass or HDPE bottles provided by the analytical laboratory (pre-rinsed with either solvent or ultra-pure water). Standard preservatives were added for pharmaceuticals and trihalomethanes, and sodium thiosulfate was added for EDCs, pesticides, and herbicides where chlorination was used.

Sampling technique 2: autosampler

Raw influent samples were collected as 24h-composite influent samples operated in time proportional mode (see Gallen et al. 2018). Only vinyl tubing was used, and all internal components were rinsed with analytical grade ethanol followed by triple rinse of certified PFAS-free water supplied by the analytical laboratory. Raw influent samples were composited over a 24-hour period between 6am Monday and 6pm Thursday, when industrial trade waste loads were likely to impact wastewater.

Sampling technique 3: polar organic chemical integrative sampler (POCIS)

Passive samplers were deployed in effluent, only. The passive sampler, POCIS, comprises a solid-phase sorbent enclosed between two hydrophilic microporous polyether sulfone (PES) membranes, held together by two stainless steel rings. Passive samplers offer distinct advantages to grab water sampling as they integrate temporal variations. Emerging contaminants accumulate over time in the receiving phase by diffusion, then sorption, as the integrative step (linear accumulation) prior to reaching equilibrium. This allows for time-weighted average concentrations to be determined using derived sampling uptake rates (R_s), which represents the volume of water (mL) cleared per unit of time (day) for a particular compound (Morin et al. 2012). Validation of passive samples in the field is recommended (e.g. Huckins et al. 2002), as *in situ* conditions can differ to those used in laboratory sampling rates (Zabiegala et al. 2010). Accordingly, in this study, field-based calibration was used to optimize the use of passive samplers.

Three types of POCIS sorbents were used to detect different groups of emerging contaminants:

- 1) POCIS-PFC (Affinisep) were analysed by LC-MSMS for 30 PFASs.
- 2) POCIS-Pharma (Affinisep) were analysed by LC-MSMS for 72 PPCPs.
- 3) POCIS-EDC (Affinisep) were analysed by GC-MSMS for 21 EDCs.

See Supplementary Information for further details. Please note that in the current study only PFAS, PPCPs and EDCs were measured with POCIS due to budget limitation. However, passive samplers can be used to sample a wider range of compounds over time.

Uptake rate derived from field calibration

Field calibration was conducted simultaneously by deploying POCIS (in duplicate), alongside autosamplers with hourly sampled (composited daily), providing a time weighted average water concentration. This was done across four sites (two TT4 and two TT7 WWTPs) for PPCPs EDCs, and PFAS, and as a pilot study for a limited subset of three sites (two TT4 and one TT7 TTWPs) for herbicides, insecticides and fungicides by LC-MSMS. Uptake rates (R_s) were derived from a linear sampling model (see Supplementary Info (SI), Equations 4 – 7; Alvarez et al. 2004) using Eq. 1:

$$R_s = C_s / C_w t \quad (\text{Eq. 1})$$

where C_s is the sampler concentration, C_w is the water concentration, and t is time (days).

We used the mean uptake rates (R_s) (L/day) derived from calibration to calculate time weighted average concentration for each compound for each wastewater effluent (C_{TWA}) using Eq. 2:

$$C_{TWA} = M_s / R_s t \quad (\text{Eq. 2})$$

Using time weighted average concentrations from four autosamplers and the amount (mass) of compound adsorbed to the POCIS receiving phase, uptake rates (R_s) were calculated for compounds detected in both POCIS and composite waters (Supplementary Information (SI), Table S2). Time weighted average water concentrations were estimated for a subset of compounds (SI, Table S3).

Laboratory Analysis

Detailed methods are provided in Supplementary information. Briefly, samples were transported at 4° C to the laboratories for analysis and analysed within 3-5 days of collection. Samples were analysed for 80 PPCPs (72 by liquid chromatography tandem mass spectrometry (LC-MSMS) and 8 by gas

chromatography tandem mass spectrometry (GC-MSMS), 21 EDCs by GC-MSMS, 30 PFAS by LC-MSMS, 17 industrial compounds by GC-MSMS, 17 phenols, 28 disinfection by-products (DBPs). For pesticides, two screens were completed using LC-MSMS and GC-MSMS to cover a larger range of compounds present in the environment. 220 pesticides were analysed, with 82 herbicides (20 by GC-MSMS and 62 by LC-MSMS), 122 insecticides (104 by GC-MSMS and 18 by LC-MSMS), and 16 fungicides (14 by GC-MSMS and 2 by LC-MSMS). Pesticides are separated in this report by mode of action (i.e. herbicides, insecticides and fungicides). Groups of emerging contaminants primarily follow the chemical screens provided by the analytical laboratories, and therefore in some instances do not accurately reflect the source or use of these compounds. For example, some are considered EDCs rather listed by their main use or principal source (e.g. the surfactant nonylphenol), while others, are included in PPCPs despite their main use as food additives (e.g. the artificial sweetener Acesulfame K). Other emerging contaminants are potential EDCs, such as fragrances (galaxolide and tonalide) and non-steroidal pharmaceuticals (paracetamol, ibuprofen, naproxen, diclofenac, and fluoxetine (Caliman and Gavirilescu, 2009)) but in this report were considered by their primary use. For conventional classifications by source and potential fate, please see Lapworth et al. (2012) and McCance et al. (2018).

Both laboratories were accredited for analytical tests by the National Association of Testing Authorities (NATA). Limits of reporting (LORs) for influent samples were typically raised by 10 times compared with effluent samples for PPCPs, EDCs, herbicides, insecticides, fungicides and industrial compounds due to matrix interferences from the raw sewage influent. Where the LOR for influent was higher than the mean effluent concentration for a site, we did not calculate percent reduction rate (%RR).

Totals (i.e. sum of compounds) are not presented in the report (e.g., PFAS Σ_{30} PFAS, Total Diuron (diuron + metabolites)) to avoid repetition and issues with variable LORs. A full list of the analytes is presented in Appendix A Tables 2 – 10.

Quality Control

Quality assurance and quality control procedures included collection of sample blind duplicates, interlaboratory duplicates, field, and trip blanks. The laboratories analysing the samples (Queensland Health Forensic Scientific Services (QHFSS) and EuroFins mgt) have ISO:17025 accreditation with analytical performance and quality assurance.

A variety of analytical techniques were used for sample analysis quality control. Spiked samples and blank samples were analysed. Method recoveries were determined by spike recoveries >20% and < 150% (checked). LORs were calculated based on signal-to-noise ratios (S/N). Laboratory and field duplicates and inter-laboratory results were generally consistent and within 30% agreement of each other. No blank subtractions were performed. The LOR for the analyte was raised if any of the analytes were present in the blank between 0.1 to 0.5 times LOR. If any analytes were present in blanks >0.5 times the LOR, results were investigated and either repeated or blank corrected.

Data analysis

If analysed concentrations were below LOR, for the calculation of mean values, a value \times 0.5 LOR was imputed. If the resulting mean value was less than the LOR, the reported concentration was referred to as less than the limit of quantification (EC Directive, 2009). Calculations for total sums for groups are not included, as the potential effects of individuals compounds within the broad groups are not yet fully understood. Summary results and statistical analysis were conducted using R (version 3.6.3, 2020). Mean, maximum, minimum, and standard deviations were calculated from all sites.

It is worth noting that matrix interferences caused LORs to be raised for some groups of analytes in the influent samples (as noted in Tables 2, 3, and 5 - 8). This included PPCPs, EDCs, herbicides, and pesticides. For example, for atrazine, influent water samples have LOR of 0.2 µg/L while it is 0.16 µg/L for effluent. This resulted in increased detection rates (effluent > influent), which could be misinterpreted as increased production of atrazine during treatment.

Percent reduction (%R)

To understand how different compounds behaved in each treatment train, we estimated the change in concentration for individual compounds and treatment trains. The term reduction refers to conversion of a parent compound to a degradation compound product (i.e. an unmeasured metabolite) (Jelic et al. 2011). Reduction depends on the loss (or absence based on analytical detection limits) of parent compounds via various treatment process. The potential reduction pathways from the treatment plants include physical and chemical degradation, transformation, biodegradation (through anaerobic and aerobic bacteria), photolysis, and adsorption to solids and settlement. Note that mixing pond detention time, the volume of influent, and the scale of each treatment process plays a role in reduction of chemicals. Given the nature of how influent (by autosampler) and effluent (by passive and spot samples) were collected, there are inherent uncertainties associated with the data set. Therefore, percent reduction should be taken as an estimate, rather than an absolute measurement. For the percent reduction (%R) estimates, we followed methods of Jelic et al. (2011) and Luo et al. (2014).

Percent reduction (%R) was estimated for a range of key compounds from each chemical group from the effluent and influent as the percent of a compound concentration remaining after moving through treatment (Luo et al. 2014). We acknowledge that this estimation of percent reduction does not incorporate a mass balance approach, which would consider both influent load versus effluent load with sorption and settling of sludge (e.g. Jelic et al. 2011; Gallen et al. 2018), incorporate metabolites or transformation of precursors (e.g. Eriksson et al. 2017; Lu et al 2020; Lenka et al. 2021), or changes in volume through evaporative loss, transpiration or diversion to recycled water over different seasons (e.g. Matamoros and Salvadó, 2012).

When estimating %R, we excluded sites where a compound was not detected in the influent (<LOR) unless the LOR was lower than the detected concentration in effluent. This was because influent sampling was more likely to detect a compound given the method of sampling (auto sampler). The mean effluent concentration (from duplicate day 0 and day 28 samples) and a single 24-hour composite influent sample (collected between day 0 and day 28) were used to estimate %R for key each compound through the treatment train process. Sewage influent consisted of one 24hr composite sample made up from hourly subsamples. It is noted that only collecting a single influent sample per site is a limitation of this study. The timing of the sampling of influent was chosen to correspond with the effluent sampling and duration for the detention time for the associated lagoon systems. However, these times do not necessary coincide with the movement of the same water through the treatment process, as some lagoons have longer retention times (> 1 month). Further sampling with replicate influent samples would provide a better estimate of percent reduction.

The %R from the concentrations in influent versus the effluent for key compounds were calculated following method in Luo et al. (2014). The percentage from the initial influent to final effluent concentration, based on the 24hr autosampler influent concentration (C_{inf}) minus the mean effluent concentration (C_{eff}) ($n = 2-3$), divided by the influent concentration (C_{inf}), expressed as a percentage (Eq. 7):

$$\text{Percent reduction (\%R)} = 100 \times \% \left(\frac{C_{inf} - C_{eff}}{C_{inf}} \right) \quad (\text{Eq. 3})$$

where a positive reduction indicates a decrease, and a negative percent reduction indicates a potential increase.

See Supplementary Information for further details about data handling for the percent reduction calculations.

Photobacteria bioassay

Photobacteria bioassays have been widely used to measure toxicity of chemicals and screen the whole toxicity of pollutants in a water sample (see Asami et al. 1996; Marinella et al. 2004). Briefly, the bioassay is based on the production of light per unit time by living luminescent bacteria (photobacteria), which reflects the rate at which a complex set of energy-producing reactions is operating. Chemical inhibition of any of the enzymes will alter this rate, consequently changing the amount of light produced. Chemical inhibition is determined by measuring the IC50 (the chemical concentration calculated to inhibit luminescence in 50% of the test organisms) (Allinson et al. 2015; Allinson et al. 2007). Full details of the methods are provided in Supplementary information.

The IC50 values are reported according to a concentration ratio (CR), which is the concentration ratio at 50% of inhibition against a control by performing a linear correlation after serial dilution. The number reported is effectively how much the sample would have had to be concentrated to inhibit luminescence in 50% of the photobacteria. The lower the IC50 (CR) reported, the higher the toxicity of the sample (and vice versa, i.e. the higher the IC50 (CR), the lower the toxicity). Any samples with an IC50 between 300 and 500 are shown as non-toxic with all others below this requirement are recommended for further and more specific investigation (Allinson et al. 2015; Allinson et al. 2007).

Receptor localisation assay

To examine overall toxicity of endocrine disrupting chemicals and chemicals with endocrine disrupting properties, receptor localisation assays are used. Current high throughput assays for endocrine disruptors have significant shortcomings that limit their sensitivity and specificity. For example, they are either cell-free biochemical assays, or employ cell lines that are not naturally responsive to endocrine disruption. Furthermore, they assess single mechanistic endpoints and therefore are not able to capture the true complexity of endocrine signalling, such as indirect actions on receptors through alterations in endocrine hormone metabolism. The receptor localisation assay uses an endogenously endocrine hormone sensitive cell line that expresses a broad range of endocrine receptors, mediators, target genes and hormone metabolic genes. By assessing receptor localization, we can simultaneously assess potential endocrine disruption action through multiple direct and indirect actions that more closely reflect the engagement of these endocrine pathways *in vivo*. In addition, this assay identifies actions on individual endocrine pathways, something that existing high throughput assays struggle with. The receptor localisation assay will provide a greater quantity of more biologically relevant data to make a high-throughput assessment of endocrine disruption in environmental water samples. In the cell-based immunolocalization assay, androgen (AR- red) and estrogen receptors (ER – green) in Michigan Cancer Foundation (MCF) MCF7 breast cancer cells were treated with influent and effluent water for 24 hours. Immunolocalization assay was conducted from two random sites and for a mixture sample made up from samples collected from four different sites. This was done to assess if the assay is sensitive enough to differentiate changes induced by cumulative/synergistic actions.

Cell culture and treatment

MCF7 breast cancer cells were maintained in Dulbecco's modified Eagle's medium (DMEM) plus 10% fetal bovine serum and 1x antibiotic/antimycotic (full medium), cultured in 5% CO₂ and ambient oxygen, and passaged every 7 days. At experimental setup, 250,000 cells were plated on sterilised 22mm² glass coverslips in 6-well plates using full medium. After 24 hours, cell medium was changed to DMEM plus 10% dextran-coated charcoal-stripped fetal bovine serum and 1x anti-biotic/antimycotic (stripped medium). Medium was changed each day for 2 days, after which control or wastewater samples were applied (using stripped medium). Control samples included vehicle (0.1% EtOH) or ultrapure water (25%), 17 β -estradiol (10nM in EtOH – estrogen receptor (ER) activator) or dihydrotestosterone (10nM in EtOH – androgen receptor (AR) activator). Wastewater-containing samples substituted 25% of stripped medium for wastewater, with medium components supplemented with powdered medium to ensure comparable osmotic balance and nutrient content to that of full medium. Cells were treated for 24 hours, before washing briefly in phosphate buffered saline (PBS) and subsequent fixation for 10 minutes using 4% paraformaldehyde in PBS.

Immunostaining and confocal imaging of cells on coverslips

Cell membranes were permeabilised for 30 minutes using 0.05% Triton-X100 in PBS before application of primary antibody for 1 hour at room temperature (mouse anti-AR, 1:50; rabbit anti-ER, 1:400) in PBS with 10% normal goat serum. Coverslips were washed PBS twice for 5 minutes, followed by addition of secondary antibodies (goat anti-mouse Alexa Fluor 555; goat anti-rabbit Alexa Fluor 488, 1:400) for 30 minutes at room temperature in PBS plus 10% normal goat serum. Subsequently, coverslips were washed twice for 5 minutes in PBS, after which 1nM DAPI in PBS was added for nuclear counterstaining. Coverslips were mounted on microscope slides using mounting medium containing 20mM Tris and 0.05% n-propyl gallate in glycerol. Cells were imaged on a Nikon A1R confocal microscope using sequential scanning and minimal Airy units. Four images at 20x magnification were acquired per coverslip. Image analysis applied a custom ImageJ plugin, with nuclear regions denoted by DAPI and the integrated mean fluorescence intensity determined for each channel (AR or ER) and each cell. The fluorescence intensity is converted to a binary measure (positive/negative) with cut-offs for each channel determined by data in vehicle versus positive control samples. This ensures the highest possible dynamic range to determine increases or decreases in protein expression.

qPCR analysis

Samples from two random sites were further tested with qPCR following a standard method (Hickey et al. 2021). Specifically, after removal of coverslips for immunostaining, remaining cells were lysed and RNA extracted using the Sigma GeneElute total RNA miniprep Kit, followed by Invitrogen TURBO DNase for DNA removal. 500ng of RNA was reverse transcribed using the Invitrogen SuperScript III first strand synthesis kit. qPCR was performed using the Thermo PowerTrack Sybr kit. All steps were performed as per manufacturer's instructions. Primer sequences for qPCR were derived from Hickey et al. (2021). Data was analysed using the delta-delta Ct method.

Risk Assessment

Due to the prohibitively expensive nature of testing for emerging contaminants, water corporations have generally adopted a tiered or structured risk-based approach to understanding and prioritising these chemicals (EPA 2009; EPA 2021; O'Connor and Stevens, 2020). In 2020, the water industry, via VicWater, developed a desktop-based risk assessment framework (RAF) (O'Connor and Stevens, 2020) to progress this approach. The framework assesses, categorises catchment risks and prioritises various priority

contaminants of concern to inform monitoring programs and potential controls. Since the development of this risk assessment framework approach, it has been followed by the water industry to address/evaluate emerging contaminants. Given that the 2020 risk assessment was mostly based on modelling, the sampling results of the current work have now been used to update the risk assessment framework. Please see attachment (O'Connor and Stevens, 2021) for a full updated risk assessment report.

Results

Pharmaceuticals and personal care products (PPCP)

Concentrations of PPCPs in influent ranged from $<0.2 \mu\text{g/L}$ up to $2,900 \mu\text{g/L}$, and in effluent from $<0.02 \mu\text{g/L}$ up to $8.1 \mu\text{g/L}$ (Figure 1). Of the 80 PPCPs analysed in waters, 48 were detected in spot samples. Of the 72 PPCPs analysed in POCIS, 66 were detected in passive samplers. More PPCPs were detected in POCIS versus spot samples because many PPCP concentrations (C_{TWA}) measured in POCIS were below limit of reporting for spot samples.

In influent, the highest concentration detected was $2,900 \mu\text{g/L}$ for codeine, from a wastewater treatment plant receiving water from a local pharmaceutical industry. The highest concentrations from conventional domestic mixed with some industrial wastewater influents were paracetamol (analgesic also known as acetaminophen, $470 \mu\text{g/L}$), caffeine ($99 \mu\text{g/L}$), acesulfame K (sweetener) ($72 \mu\text{g/L}$), salicylic acid ($70 \mu\text{g/L}$), for amidotrizoate (sodium) ($54 \mu\text{g/L}$) and triclosan (antibacterial and antifungal agent) ($16 \mu\text{g/L}$) (Table 2). The most frequently detected PPCPs were codeine, acesulfame K, atenolol, caffeine, gabapentin, galaxolide, lamotrigine, methamphetamine, naproxen, paracetamol, sulfamethoxazole, valsartan, and venlafaxine (Table 2). For example, codeine was detected in 100% of samples, and beta blocker atenolol, the polycyclic musk fragrance galaxolide, and the antidepressant venlafaxine in 97% of samples.

In effluent, the highest concentrations detected were acesulfame K ($8.1 \mu\text{g/L}$), amidotrizoate (sodium) ($4.2 \mu\text{g/L}$), gabapentin ($3.1 \mu\text{g/L}$), galaxolide ($2.5 \mu\text{g/L}$), hydrochlorothiazide ($2.0 \mu\text{g/L}$), and lamotrigine ($1.9 \mu\text{g/L}$). The most frequently detected PPCPs were carbamazepine (92%), lamotrigine (91%), amidotrizoate (sodium) (89%), oxazepam (89%), temazepam (89%), and acesulfame K (88%) (Table 2).

From 72 PPCP compounds analysed in POCIS samples from effluent, 66 were detected. There were 38 compounds for which uptake rates in POCIS sorbent were derived from effluent water concentrations simultaneously measured by autosamplers (Table S2). Based on estimated uptake rates for PPCPs, concentrations in POCIS showed that the highest time weighted average concentrations were acesulfame K (sweetener) ($14.8 \mu\text{g/L TWAC}$), gabapentin ($11.2 \mu\text{g/L TWAC}$), amidotrizoate (sodium) ($7.5 \mu\text{g/L}$), cefalexin ($8.8 \mu\text{g/L}$), furosemide ($2.76 \mu\text{g/L}$), and venlafaxine ($2.50 \mu\text{g/L}$) (Supplementary Information, Table S3). Mean and maximum time weighted average concentrations from POCIS showed close agreement with spot water samples, with concentrations generally less than two times higher or lower. Eight compounds were detected in 100% of POCIS samples (caffeine, carbamazepine, diethyltoluamide, methamphetamine, metoprolol, sulfamethoxazole, tramadol and venlafaxine). A similar detection frequency was observed in POCIS, compared to spot samples, for acesulfame K (79%), amidotrizoate (sodium) (65%), oxazepam and temazepam (97%).

Endocrine Disrupting Compounds (EDCs)

Note: that due to the low concentrations, this EDCs section uses ng/L units ($1 \text{ ng/L} = 0.001 \mu\text{g/L}$).

For EDCs, concentrations in influent waters ranged from <20 up to 12,000 ng/L and in effluent from <2 up to 5,400 ng/L (Table 3). From the 21 compounds measured, 13 EDCs were detected in influent, while 11 EDCs were detected in effluent.

In influent, the six highest EDC concentrations were nonylphenol (12,000 ng/L), etiocholanolone (4,600 ng/L), androsterone (1,400 ng/L), estriol (470 ng/L), testosterone (370 ng/L), and bisphenol A (260 ng/L). The maximum predicted estradiol equivalent (EEQ) was 83 ng/L. The six most frequently detected EDCs were etiocholanolone (83%), androsterone (80%), estriol (60%), estrone (60%), bisphenol A (30%), nonylphenol (27%), while the EEQ was calculated in 23% of samples.

In effluent, the six highest concentrations were nonylphenol (540 ng/L), bisphenol A (220 ng/L), estrone (29 ng/L), 4-t-octylphenol (21 ng/L), estriol (11 ng/L), and androstenedione (12 ng/L). The maximum predicted estradiol equivalent was 13 ng/L. The most frequently detected compounds in effluent samples were nonylphenol (42% of samples), bisphenol A (38%), estrone (35%), 17-estradiol (9%), androsterone (6%) and 17 α -estradiol and 4-t-octylphenol (3%), while the EEQ was calculated in 23% of samples (Table 3).

In POCIS samples, of the 21 EDC compounds, nine were detected. Across all sites, time weighted average concentrations were calculated for nonylphenol (<10 to 255 ng/L), estrone (<0.2 to 24 ng/L) and BPA (27 out of 34 detected; <34 to 261 ng/L). For the other six EDCs without field derived uptake rates, the highest nominal concentrations in POCIS were androstenedione (50 ng/POCIS), 4-t-octylphenol (30 ng/POCIS), 17 β -estradiol (6 ng/POCIS), estriol (5 ng/POCIS), equilenin (3 ng/POCIS), and 17 α -estradiol (2 ng/POCIS). The most frequently detected EDC compounds in POCIS were: nonylphenol (91%), BPA (79%), and estrone (70%), followed by 4-t-octylphenol (74%), 17 β -estradiol (29%), androstenedione and equilenin (12%), estriol (9%), and 17 α -estradiol (6%).

Per- and Poly-fluoroalkyl Substances (PFAS)

Concentrations of PFAS in influent ranged from <0.0002 to 0.21 μ g/L, and in effluent from <0.0001 up to 0.065 μ g/L (Table 4). Of the 30 PFAS-compounds analysed, 19 were detected in the influent and 18 in the effluent water. Of the 30 PFAS compounds analysed, 15 PFAS were detected in POCIS.

In influent waters, the highest PFAS concentration detected was for PFOS (0.21 μ g/L), followed by 6:2 FTSA (0.18 μ g/L), PFHxS (0.16 μ g/L), PFHxA (0.058 μ g/L), PFOA (0.031 μ g/L), PFPeA (0.029 μ g/L), and PFPeS (0.021 μ g/L). The most frequently detected compounds were PFOS (93%), PFHxA (83%), PFPeA (77%), PFHxS (73%), PFOA (67%), PFBS (60%), and PFDA (53%).

In effluent, the highest concentrations detected were for 6:2 FTSA (0.065 μ g/L), followed by N-EtFOSA (0.051 μ g/L), PFOA (0.044 μ g/L), PFHxA (0.034), PFHxS (0.028 μ g/L), PFPeA (0.024 μ g/L), and PFOS was the seventh highest concentration with a maximum of 0.021 μ g/L. The most frequently detected compound was PFOS (98.5%), followed by PFOA (94%), PFHxA (94%), PFPeA (92%), PFHxS (85%), PFHpA (77%), and PFDA (66%).

In POCIS, 15 of the 30 target compounds were detected. The highest maximum concentrations detected were for 6:2 FTSA (120 ng/POCIS), PFOA (110 ng/POCIS), PFHxA (67 ng/POCIS), PFPeA (45 ng/POCIS), PFHxS (34 ng/POCIS), and PFOS (23 ng/POCIS), PFHpA (17 ng/POCIS). The most frequently detected compounds in POCIS were PFOA (97%), PFOS (94%), PFHxA (94%), PFHxS (91%), PFPeA (82%), PFBS (82%), and PFBA (76%). In effluent, six PFAS compounds were not detected in both POCIS and spot water samples (10:2 FTSA, N-EtFOSE, N-MeFOSA, N-MeFOSE, 4:2 FTSA, 8:2: FTSA). There were nine PFAS detected in effluent

spot samples, which were not detected in POCIS (N-EtFOSA, PFDS, PFDoS, PFDoA, PFHpS, PFNS, FOSA, PFTeDA, PFTrDA). See Supplementary Information for estimated sampling rates and examples of time weighted average concentrations for PFAS.

Pesticides

Concentrations of pesticides in influent ranged from <1 µg/L to 3.8 µg/L, and in effluent from <0.1 to 3.4 µg/L (Tables 5 - 7). Of the 220 pesticide compounds analysed 28 were detected in influent waters and 46 were detected in effluent waters. A further summary for each pesticide group is detailed below.

Herbicides

Concentrations of herbicides in influent ranged from <0.1 to 3.5 µg/L, and in effluent from <0.01 to 3.4 µg/L (Table 5). Of the 82 herbicide compounds analysed (62 by LC-MSMS and 20 by GC-MSMS), 19 were detected in influent and 36 were detected in effluent water. Of the 62 herbicides measured in POCIS, 29 were detected.

In influent, the highest concentrations detected were diuron (3.5 µg/L), MCPA (2.9 µg/L), simazine (1.8 µg/L), and triclopyr (0.81 µg/L). The most frequently detected compounds in influent were simazine (43%), MCPA (13%), diuron (10%), and mecoprop (7%). A further four herbicides were only detected in one of the 31 influent samples (3%), including dalapon (2,2-DPA), clomazone, metolachlor and triclopyr. More herbicides are likely to be present at lower concentrations in influent, however the LOR was 10x higher due to matrix interferences.

In effluent, the highest concentrations detected were hexazinone (3.4 µg/L), dalapon (2,2-DPA) (2.9 µg/L), simazine (2.3 µg/L), MCPA (0.91 µg/L), desisopropyl atrazine (0.48 µg/L), and triclopyr (0.4 µg/L). In effluent, the most frequently detected herbicide compounds include the triazine herbicide simazine detected in 82% of samples, followed by the diuron (79%), then the phenoxy-acid MCPA (71%), the chlorophenoxy-acid mecoprop (41%), the phenoxy acid 2,4-D (32%), the triazine metabolite desisopropyl atrazine (21%), and the triazine atrazine (26%).

Atrazine was not detected in any influent samples, despite being detected in 26% of effluent samples, which is likely an artifact of higher LORs for influent samples. Similarly, three primary triazine metabolites (2-hydroxy-, desethyl- and desisopropyl-atrazine) while not detected in influent, were present in 8 – 21% of samples in the effluent at concentrations ranging from <0.01 to 0.48 µg/L. The triazine herbicide simazine was detected in a smaller proportion of influent samples (43%) at a maximum concentration of 1.8 µg/L compared to effluent samples (82%) with a maximum concentration of 2.3 µg/L. Also, DCPMU, the primary metabolite of diuron, was not detected in either influent or effluent, indicating diuron is still present in the effluent, suggesting limited breakdown of this pesticide in wastewater.

A limited subset of POCIS were analysed for herbicide concentrations from three calibration sites (Ballarat North, Black Rock, and Warrnambool). The results showed 29 of the 62 herbicides measured by LC-MSMS were detected. Uptake rates were derived in POCIS from time weighted average concentrations for seven compounds and three metabolites (diuron, the phenoxy acids 2,4-D, mecoprop, and MCPA, the triazines atrazine, simazine, and all three metabolites of atrazine and simazine (desethyl atrazine, desisopropyl, and atrazine, 2-hydroxy), the sulfonyl urea trifloxysulfuron (Supplementary Information, Table S2).

Insecticides

Concentrations of insecticides from influent ranged from <0.1 µg/L to 1.8 µg/L, and in effluent from <0.01 µg/L to 1.4 µg/L (Table 6). Of the 41 insecticide compounds analysed, six were detected in influent and six were detected in effluent water. Of the 18 insecticides measured in POCIS, 11 were detected in POCIS.

In influent, the highest concentrations were for carbamate propoxur (1.8 µg/L), neonicotinoid imidacloprid (1.3 µg/L), and insecticide synergist piperonyl butoxide (1.6 µg/L). The most frequently detected insecticides were the neonicotinoid imidacloprid in 40% of samples, followed by piperonyl butoxide (27% of samples), metabolites of imidacloprid (7%), and the nitrile insecticide fipronil, the carbamate propoxur and the neonicotinoid thiamethoxam (3%).

In effluent, the highest concentrations detected were imidacloprid (1.4 µg/L), metabolites of imidacloprid (0.3 µg/L), the insecticide synergist piperonyl butoxide (0.1 µg/L), the neonicotinoid thiacloprid (0.07 µg/L), the nitrile insecticide fipronil (0.02 µg/L) and the organophosphate diazinon (0.04 µg/L) (Table 6). The most frequently detected insecticides were neonicotinoid imidacloprid (62%), metabolites of imidacloprid (17%), the neonicotinoid thiacloprid (8%), the organophosphate diazinon (5%), and the nitrile insecticide fipronil (3%), and the insecticide synergist piperonyl butoxide (3%).

POCIS were analysed for insecticide concentrations at three sites (Ballarat North, Black Rock and Warrnambool). Elevated of the 18 insecticides measured by LC-MSMS were detected. Time weighted average concentrations were calculated for seven compounds plus four metabolites (the neonicotinoids acetamiprid, clothianidin, imidacloprid and metabolites, thiacloprid, and thiamethoxam, the nitrile fipronil plus three metabolites fipronil desulfinyl, fipronil sulfide and fipronil sulfone, and the organophosphate chlorpyrifos) (Supplementary Information, Table S2).

Fungicides

Concentrations of fungicides in influent ranged from <0.2 µg/L to 3.8 µg/L, and in effluent from <0.02 up to 0.46 µg/L (Table 7). Of the 22 fungicide compounds analysed (2 by LC-MSMS, and 20 by GC-MSMS), two were detected in the influent and seven in the effluent. One of the two fungicides measured in POCIS was detected.

In influent, two fungicides were detected in one site only. The highest concentration was thiabendazole (3.8 µg/L), followed by flutriafol (0.52 µg/L).

In effluent, the highest concentrations detected were thiabendazole (1 µg/L), flutriafol (0.46 µg/L), propiconazole (0.3 µg/L), metalaxyl (0.2 µg/L), tebuconazole (0.2 µg/L), and triadimenol (0.2 µg/L). The most frequently detected fungicide compounds were flutriafol (16%), metalaxyl (8%), propiconazole (8%), tebuconazole (3%), thiabendazole (3%) and triadimenol (3%) (Table 7).

POCIS were analysed for fungicides at three sites (Tables S2 and S3). One of the two fungicide compounds was detected in all three sites, with a maximum concentration of flutriafol (515 ng/POCIS). Flusilazole was not detected in any POCIS, neither was it detected in any of the water samples ($n = 62$) (Supplementary Information, Table S2).

Industrial compounds

Concentrations of industrial compounds in influent ranged from <0.1 to 12 µg/L, and in effluent from <0.02 up to 1.5 µg/L (Table 8). Of the 17 industrial compounds analysed, five were detected in influent, and 11 were detected in effluent. The dominant compound in influent samples was 4-Chloro-3,5-dimethylphenol, also known as chloroxylenol (or para-chloro-meta-xylene), which is widely used as a household and medical disinfectant. Tris(chloropropyl) phosphate isomers were the dominant compound in effluent samples.

In influent samples, the highest concentrations of industrial compounds detected were 4-chloro-3,5-dimethylphenol (12 µg/L), tris(chloropropyl) phosphate isomers (7.9 µg/L), 1H-benzotriazole, 5-methyl (3.8 µg/L), benzenesulfonamide (2.4 µg/L), and N-butylbenzenesulfonamide (1 µg/L). The antimicrobial and disinfectant 4-chloro-3,5-dimethylphenol (12 µg/L) was the most frequently detected compound (87%). The organophosphorus flame retarding triphenyl compound tris(chloropropyl) phosphate isomers was the second most frequently detected compound (20%). The corrosion inhibitor metabolite 1H-benzotriazole, 5-methyl (3.8 µg/L) was the third most frequently detected compound (13%) in influent.

In effluent, 11 of the 17 compounds were detected in the effluent samples. The organophosphorus flame retarding triphenyl compounds (including tris(2-chloroethyl) phosphate) were the most frequently detected compounds (45 – 74%). Tris(2-chloroethyl) phosphate is often used as a flame retardant, a plasticiser, and to regulate viscosity in many plastics. The most frequently detected industrial compounds were tris(chloropropyl) phosphate isomers (74%), tris(chloropropyl) phosphate (55%), 1H-benzotriazole, 5-methyl (55%), tris(dichloropropyl) phosphate (50%), triethyl phosphate (45%), and N-butylbenzenesulfonamide (24%). The highest detected concentration of industrial compounds were tris(chloropropyl) phosphate isomers (1.5 µg/L), followed by tris(dichloropropyl) phosphate (0.3 µg/L), 1H-benzotriazole and 1H-benzotriazole, 4-methyl (0.9 µg/L), tris(chloroethyl) phosphate (0.4 µg/L), N-butylbenzenesulfonamide (0.4 µg/L), and triethyl phosphate (0.3 µg/L) (Table 8). Note: POCIS were not deployed or analysed for industrial compounds.

Phenols

Concentrations of phenols in influent ranged from <0.00005 to 1.4 µg/L, and in effluent from <0.00005 up to 0.0006 µg/L (Table 9). Of the 17 phenols analysed, 6 were detected in the influent samples and 5 in effluent samples.

In influent, the highest concentrations of phenols were 3&4-methylphenol (m&p-cresol) (up to 1.4 µg/L), phenol (0.3 µg/L), 2,4-dimethylphenol (0.014 µg/L), 2-methylphenol (o-cresol) (0.0088 µg/L), 4-chloro-3-methylphenol (0.001 µg/L), and 2-chlorophenol (0.00044 µg/L). The most frequently detected phenols were phenol, which was detected in 77% of samples, followed by 3&4-methylphenol (m&p Cresol) (73% of samples), then 2-methylphenol (o-cresol) (30%), 4-chloro-3-methylphenol (13%), 2,4-dimethylphenol (10%), and 2-chlorophenol (3%).

In effluent, 5 of the 17 phenols analysed were detected, at much lower concentrations than influent with concentrations ranging from <0.00005 to 0.0006 µg/L. The maximum concentrations detected were 4-chloro-3-methylphenol (0.0006 µg/L). The most frequently detected compound was phenol (14%), while the other four compounds had fewer detections (2% or less), including 4-chloro-3-methylphenol, 2-chlorophenol, 2,4,6-trichlorophenol, 3&4-methylphenol (m&p-cresol). Note: POCIS were not deployed or analysed for phenols.

Disinfection by-products (DBPs)

Disinfection by-products are emerging contaminants that result from the treatment process itself. Concentrations of DBPs in effluent ranged from <1 µg/L up to 64 µg/L (Table 10). Of the 28 DBPs analysed, 17 were detected in spot samples. Of the 29 DBP compounds measured, 19 were detected in one or more spot water sample.

The most frequently detected DBPs in effluent were haloacetic acids (bromodichloroacetic acid (BDCAA) and trichloroacetic acid (TCAA)) and trihalomethanes (bromodichloromethane and chloroform). The highest effluent concentrations detected were TCAA (64 µg/L), chloroform (59 µg/L), bromodichloromethane (49 µg/L) and N-nitrosodimethylamine (33 µg/L).

Eight of the nine haloacetic acids were detected, of which most frequently detected was trichloroacetic acid (TCAA, 18%), which also was the highest concentration (64 µg/L) measured. Five of the eight haloacetonitriles were detected, of which most frequently detected was 1,1,1-trichloropropan-2-one and bromochloroacetonitrile (12%), and highest concentration measured for chloral hydrate (21 µg/L). Of the nitrosamines, only one of the seven compounds, N-nitrosodimethylamine, was detected in one sample (4%) at a concentration of 5.76 ng/L.

All four of the trihalomethane compounds were detected in more than one sample, most frequently were the compounds bromodichloromethane and chloroform (18%), with the highest concentration for these three ranging from 49 and 58 µg/L. Note: POCIS were not deployed or analysed for disinfection by-products.

Treatment trains and the estimated percent reduction (%R)

Based on our study, the estimated percent reduction (%R) of emerging contaminants across treatment trains demonstrated a wide range of reduction, with up to 99.997% reduction for some compounds, while conversely there was a change in concentration by 14 times for PFOS in effluent waters in one treatment system (Table 11). Near complete %R (elimination of >99.9%) of a single contaminant was rare, with exception of paracetamol (>99.9%), and caffeine (with >98% across all sites).

For PPCPs, across eight treatment trains, the percent reduction ranged from an increase of 1.8 times the influent concentration (for lamotrigine) to a reduction of 99.997% (paracetamol) (Table 11; Figure 1). Overall, the %R for PPCPs was found to be compound specific (see full details in Appendix A, Table 11). For example, there were high %R for paracetamol, caffeine and acesulfame K, which decreased at a rate of >99.99, >98.4 and 81.7%. The %R of galaxolide was substantial, with a range of 47% to 99.4% compared with the influent, with the highest %R for TT1, TT2, TT6, and TT12. Conversely, the %R for carbamazepine (increasing by 0.9 times to a decrease of 90.8%), and venlafaxine (increasing by 0.9 times 0.27 times to 99.3%) was variable, and in all but two treatment trains showed limited reduction (Figure 1). Furthermore, for lamotrigine, there was no evidence of %R, with influent concentrations being lower than effluent concentrations (Figure 1). With variable %R, only TT12 and TT13 clearly and consistently showed high %R of PPCPs (>98%) for venlafaxine, while the same was not true for lamotrigine. Our study suggests that that %R was greatest in Class A recycled water, however, estimated %R was still negligible or incomplete for lamotrigine and carbamazepine in two of the four Class A treatment sites. Future evaluation of effectiveness of treatment trains on compound specific removal, would benefit from adopting a mass balance approach. This evaluation was out of the scope of the study.

For EDCs, mean %R rates across treatment trains were >66% between the influent and effluent waters. Treatment efficiency across all treatment trains for androsterone, BPA, estriol, estrone, etiocholanolone and nonylphenol was above 66% in effluent compared with influent samples. Etiocholanolone, which is a metabolite of testosterone, was not detected in any effluent samples even if it was detected in 83% of influent waters, suggesting a significant reduction of this steroid from wastewater during treatment. The lowest %R was for estrone in TT9 (33%).

For PFAS, the %R was variable between treatment trains and compounds for effluent waters. Specifically, the longer-chain perfluoroalkane sulfonic acids (PFASs), including PFOS, PFHxS, as well as the short-chain PFBS, showed variable %R, with some sites showing an increase between influent and effluents waters. For example, for PFOS, one site had a high %R with a decrease of 99.9% (TT12), where the highest influent concentration of PFOS (0.21 µg/L; single 24hr composite sample) was subsequently treated to 0.0002 ± 0 µg/L (two 24-hour composite samples). However, at several other sites, PFOS showed an apparent increase by an order of magnitude (by up to 13.8 times). Likewise, PFHxS percent reduction was highly variable, ranging from an increase from influent to effluent by 150% (-150%) to a decrease of 99.7% (Table 11; Figure 2). Of the PFAS, the more readily soluble perfluoroalkyl carboxylic acids (PFCAs) showed similar variability, with both increases and decreases from influent to effluent. Negative, very low and higher %R were observed over various treatment trains, including PFOA (an increase of 600% to a decrease of 97%), PFHxA (an increase by 9.5 times to a decrease of 99.1%) and PFPeA (an increase in concentration by 6.5 times to a decrease of 98.3%). In terms of treatment train percent reduction, the lowest %R (and thus highest increase in concentration between influent and effluent) was shown for TT1, while the most efficient %R was for TT12 for five compounds, followed closely by TT13, as well as TT7 for two compounds. For both TT12 and TT13 these observations are limited to one sample with no replication. Previous studies have shown reverse osmosis (RO) to successfully remove PFAS, in particular PFOS, from wastewater (e.g. Tang et al. 2008). Treatment train TT12 includes RO treatment step, which could be one of the reasons for the improved %R in the current study.

For pesticides, the %R in the final effluent ranged from an increase of 10% to a reduction of 96.9% (Table 11). Of the 146 compounds, 9 were detected in both influent and effluent in more than one site to calculate %R efficiency. A further summary for each pesticide group is detailed below.

For herbicides, the %R could only be calculated in four compounds. Specifically, %R ranged from an increase by 6.35 times (simazine) to a decrease of 96.9% (triclopyr) between influent and effluent (Table 11). Simazine showed the most variable %R efficiency, ranging from an increase by 6.35 times higher than the influent concentrations to a reduction of 94%. Triclopyr had the highest %R, despite having only one site with concentrations detected in both influent and effluent samples. The most consistent %R was for MCPA, with four samples ranging between a %R of 83% to 88%. Three samples showed diuron %R ranged between 6 and 95%. Despite detects for some effluent samples, no %R could be calculated for atrazine or its metabolite desisopropyl atrazine or hexazinone, as there were no paired detections in influent samples (below LOR) for any site. In terms treatment trains for which estimations were possible (TT1, 2, 4, 7, 8, 9, 13, 14), the highest %R appeared to be compound specific, with the highest %R for diuron in TT2, the highest %R for simazine in TT13, and variable performance for all treatment trains.

For insecticides, the %R for effluent waters ranged between an increase of 10% to a decrease of 96%, both of which were calculated for imidacloprid (Table 11). The highest %R was imidacloprid, which was also the most variable. Only one site had detected fipronil concentrations in both influent and effluent, with a calculated %R of 70%. Despite variable %R, TT1 showed the highest %R for the insecticide imidacloprid (>90%), while TT2 had the second higher %R (Table 11).

For fungicides, the %R for the two compounds (flutriafol and thiabendazole) was calculated for effluent waters from two sites as 74% (Table 11).

For industrial compounds, %R ranged from 52% to 99.5% in effluent waters. The percent reduction rates of industrial compounds via water treatment were variable, with a high %R for 4-chloro-3,5-dimethylphenol (>89.6%) but lower %R for TCP (from 66% to 92%). TCP was detected in most samples, with the highest %R in TT4, suggesting a longer residence time, with photolysis, hydrolysis, and anaerobic degradation, may have an influence in higher %R of TCP in effluent waters.

For phenols, %R were only estimated for 3&4-Methylphenol (m&p Cresol) and phenol. High %R was shown for 3&4-Methylphenol (m&p-Cresol) which reduced by 88% to 99.99% across all sites and all treatment trains. Phenol was removed by up to 99.99% in some sites, while an increase in the effluent by 1.7 times the influent concentration was estimated in one instance (assuming a value of half the LOR for influent) (Table 11).

Guidelines

To provide a preliminary indication of risks to human health and the environment from emerging contaminants in water recycled from sewage, we compared concentrations of detected compounds with guideline values from Australian and other international sources. For example, Health-based guidelines have been determined for drinking water (Australian Drinking Water Guidelines, 2018) for a range of contaminants. However, ecological guidelines for water quality are less readily available, with fewer published guideline values derived for species protection. A comparison of detected maximum concentration in effluent with water quality guidelines is shown in Table 12.

Note: the application of the water quality guidelines above is conservative as the guideline values have been applied at the point of measurement as opposed to the point of exposure (i.e., where humans or aquatic organisms may come in to contact with the contaminant).

Guidelines used in this study are listed below for human-health and ecosystem protection. In the first instance, Australian guidelines have been selected. Where an applicable Australian guideline was not available, a suitably reliable guideline value from other international jurisdictions have been selected.

Human health-based guidelines for drinking water

Australian Guidelines:

- a) NHMRC, NRMMC (2011) Australian Drinking Water Guidelines

International Guidelines:

- b) NZ MoH (2018). Drinking Water Standards for New Zealand
- c) USEPA (2018) 2018 Edition of the Drinking Water Standards and Health Advisories
- d) WHO (2017) Guidelines for Drinking-Water Quality

Ecosystem based guidelines for water quality

Australian Guidelines

- a) ANZG (2021). Australian and New Zealand Guidelines for Fresh and Marine Water Quality.
 - a. with reference to: VicGov ERS (2021).

- b) ANZG (2023). Toxicant default guideline values for aquatic ecosystem protection. Perfluorooctane sulfonate (PFOS) in freshwater. Technical brief, May 2023.

International Guidelines

- c) CCME (2021). Summary table. Canadian Environmental Quality Guidelines.
 d) USEPA (2021). National Recommended Water Quality Criteria. Aquatic Life Criteria Table.
 e) USEPA (2018) Ecological risk assessment – supplemental guidance.

Agricultural Irrigation based guidelines for water quality

Australian Guidelines

- a) ANZECC, ARMCANZ, 2000. Volume 3: Primary industries

International Guidelines

- b) CCME (2021). Summary table. Canadian Environmental Quality Guidelines. Water Quality Guidelines for the Protection of Agriculture

The selected guideline values provide a preliminary indication of risk and could be used as trigger points for further investigation. The relevance of these guideline values is dependent on the end-use of the effluent (i.e. recycled water use in irrigation, toilet flushing, discharge to land, freshwater or marine water) and site-specific receptors and exposure scenarios. Further risk assessment based on an integrative tiered risk-based management framework approach should be done in accordance with EPA's Victorian guideline for water recycling (EPA 2021, Publication 1910.2).

For the purpose of this report, we have selected guideline values that have been published in official guidance documents which have gone through rigorous peer-review protected and thus are considered to have a known reliability. Due to limitations of the study, we have not used derived guidelines from published literature (i.e., outside of formal jurisdictional guidance documents), as the reliability and relative uncertainty of these make it difficult to quantify potential risks.

Ecotoxicity assays

Photobacteria bioassay

A total of 74 whole effluent samples were analysed using the photobacteria (yeast) bioassay. Briefly, almost all recycled water samples showed toxicity from some sort of chemicals contained in the recycled water. The test does not differentiate which of these chemicals are the most likely cause of toxicity. A few samples showed much weaker toxicity, including all samples from treatment train TT12, and two of the samples from treatment train TT8. The five most toxic samples and nine of the top 10 most toxic samples (when ranked from highest to lowest toxicity) were from treatment trains TT1 and TT2.

Receptor localisation assay

In the cell-based immunolocalization assay, androgen (AR- red) and estrogen receptors (ER – green) in MCF7 breast cancer cells were treated with influent and effluent water for 24 hours. The assay showed that influent sample from two sites and a mixture sample activated the estrogen receptor and induced expression of estrogen receptor target genes, demonstrating EDC activity. This activity was significantly reduced in effluent samples ($p < 0.05$). For example, a reduction of ER levels (green) indicates activation of the ER (surrogate of EDC action), therefore influent samples have a stronger impact (i.e. greater EDC

action). Overall, influent samples showed higher ER activation than effluent samples. ER activation is known to have strong adverse effects on physiology and development for both humans and the receiving aquatic environments (Deroo and Korach, 2006).

qPCR

A sub-set of influent and effluent samples (two sites) were analysed for mRNA levels for ER and AR target genes. qPCR showed that influent samples induce strong activation of the ER, and effluent samples induce a weaker activation. Interestingly for some target genes in effluent samples, ER activation is still higher than that of vehicle samples (distilled water controls), which means that residual ER activity exists in effluent samples. Work is underway to test physiological and developmental effects of effluent waters using a genital tubercle culture assay.

Discussion

Prevalence and frequency

The study analysed a total of 413 emerging contaminants. Specifically, 80 PPCPs, 21 EDCs, 30 PFAS, 82 herbicides, 122 insecticides, 16 fungicides, 17 industrial compounds, 17 phenols and 28 DBPs were analysed. Results show that 180 emerging contaminants were detected in both influent and effluent 48 PPCPs, 15 EDCs, 21 PFAS, 34 herbicides, 8 insecticides, 7 fungicides, 12 industrial compounds, 7 phenols and 28 DBPs. In POCIS samples, 126 emerging contaminants were detected (66 PPCPs, 9 EDCs, 14 PFAS, 29 herbicides, 8 insecticides, 1 fungicide (no industrial compounds, phenols or disinfection by-products were analysed in POCIS). There are several factors which can influence concentrations and composition of emerging contaminants in influent, such as the timing of industrial discharges, wet weather events, or daily fluctuations, which were not factored into influent sampling using a single 24-hour composite sampling approach.

The most frequently detected PPCPs in effluent were antiepileptic drugs lamotrigine and carbamazepine, a contrast agent used during X-ray imaging (amidotrizoate (sodium)), medication used to treat anxiety and acute alcohol withdrawal (oxazepam), medication used to treat insomnia (temazepam), and an artificial sweetener (acesulfame K). The highest effluent concentrations of PPCPs detected were for the acesulfame K, amidotrizoate (sodium), an opioid pain-relief medicine (codeine), anticonvulsant medication (gabapentin), high blood pressure medication (hydrochlorthiazide) and lamotrigine. This is consistent with our previous work (Saaristo et al. in prep). Lamotrigine is an antiepileptic drug, which has been detected at concentrations above 1 µg/L in water bodies and effluent waters around the world (Writer et al. 2013; Zonja et al. 2016; Goldstein et al. 2018). Carbamazepine is one of the most frequently prescribed antiepileptic drugs in the world (Hsia et al. 2010; Berman et al. 2016) and known to have low percent reduction and minimal degradability in wastewater treatment plants (Ternes et al. 1998; Miege et al. 2009; Wrtier et al. 2013).

The highest PFAS concentrations detected in effluent were 6:2 FTSA (0.065 µg/L), N-EtFOSA (0.051 µg/L), PFOA (0.044 µg/L), PFHxA (0.034 µg/L), and PFHxS (0.028 µg/L). Interestingly, PFOS was the seventh highest concentration detected (0.021 µg/L), not the highest, as we have seen in our previous work (Sardina et al. 2019: EPA Publication 1879). This is likely because our previous work collected environmental samples from streams and rivers. However, PFOS concentration in the effluent is dependent on the primary source of the influent (industrial and/or domestic water sources) and the degradation of precursors during the wastewater treatment train processes (Ahrens 2011; Lu et al 2020; Lenka et al. 2021). In this study, the most frequently detected compound was PFOS (98.5%), followed by PFOA (94%), PFHxA

(94%), PFPeA (92%), and PFHxS (85%). This is consistent with our previous work (Sardina et al. 2019; EPA Publication 1879).

Concentrations of pesticides in influent ranged from <1 µg/L to 3.8 µg/L, and in effluent from <0.1 µg/L to 3.4 µg/L. The urea herbicide diuron was the second most frequently detected compound in effluent samples (79%), followed by mecoprop (40% effluent samples). Mecoprop is often formulated as a salt in the active ingredients of weed killers for lawns. Following application, mecoprop dissociates to the metabolites mecoprop-P acid and dimethyl amine. Due to the limitations of the analytical screen mecoprop's metabolites were not analysed in the current study. Another frequently detected pesticide was atrazine (18% of effluent samples). Atrazine typically breaks down to three primary metabolites: hydroxy-2-atrazine (HA), desethyl atrazine (DEA) and desisopropyl atrazine (DIA), which were all detected in the current study. Further studies should include both parent compounds and metabolites to better understand the prevalence of pesticide use.

The highest concentration of an industrial compound detected was the compound 4-Chloro-3,5-dimethylphenol, also known as chloroxylenol (or para-chloro-meta-xyleneol), which is widely used as a household and medical disinfectant. The high frequency of detection of 4-chloro-3,5-dimethylphenol is not surprising during a pandemic, as it may have increase in use as an antimicrobial, along with various other antimicrobial compounds. Despite 4-chloro-3,5-dimethylphenol being detected in high frequency in this study, it was effectively removed during treatment (89.6 – 99.5%). This is consistent with previous studies showing higher percent reduction, such as from bioreactors (44 – 87%) via biological breakdown through dehalogenation and hydroxylation (Choi and Oh 2019). In addition, we detected tris(chloropropyl) phosphate isomers (TCPP, also referred to as tris(2-chloro-1-methylethyl) phosphate, tris(2-chloroisopropyl) phosphate and TCIPP; CASRN 6145-73-9). TCPP isomers are widely used organophosphorus flame retardant and frequently detected in environmental samples. TCPP has been detected in wastewaters (Woudneh 2015), indoor dust samples from homes, offices, and vehicle interiors (Meeker and Stapleton, 2010, Stapleton et al. 2009, Yao et al., 2021), in surface waters (Regnery and Püttmann, 2010; Reemtsma et al. 2008; Yao et al., 2021) and drinking water (Li et al., 2014, Rodil et al., 2012; Yao et al., 2021). TCPP was typically one of the highest concentrations of 13 organophosphorus flame retardants analysed in wastewater (raw influent, sludge, effluent, biosolids) (Woudneh et al. 2015). TCPP isomers can biodegrade, but are likely to remain in wastewater effluent, as it has a low affinity to adsorb to organic matter sediment or soil and a low potential to bioaccumulate in fish (EURAR, 2008).

Passive samplers (POCIS) had a higher detection rate for PPCPs, and a slightly lower detection rate for PFAS and EDCs, compared to spot sampling. For example, based on this study, POCIS was performing better than spot sampling for PPCPs (66 POCIS vs 48 spot), while spot sampling detected more PFAS compounds than POCIS (POCIS 14 vs 20 spot) and more EDCs than POCIS (POCIS 9 vs 11 spot). Higher detections rates for PPCPs are likely due to the lower LORs achieved by exposing the same sorbent used in the laboratory (HLB) to higher volumes of water. Due to the differences in chemical properties within the PFAS family, two types of POCIS in combination have been shown to provide better monitoring for the wide range of PFAS (Gobelius et al. 2019). As for the maximum concentrations, for PPCPs and EDCs, maximum time weighted average concentrations of POCIS were consistent with the highest detections with the spot sampling. It is good to note that fewer EDC compounds were detected in POCIS versus spot samples. On one hand, this could be due to the selective type of sorbent (molecularly imprinted polymer). On another hand, there are several factors that can influence the uptake rates of ECs in POCIS, such as flow rates, temperature and pH, which were not factored into calculations. All in all, an approach combining spot and passive sampling techniques, and calibration of POCIS for each site, is recommended to maximising sampling output.

Guidelines

Applying guideline values directly to recycled water use needs to be done with caution. Whilst the assessment of recycled water should be conducted with the end use and disposal pathways in mind, it is important to note that many emerging contaminants do not yet have water quality reuse criteria or water quality guidelines. Moreover, when guidelines exist for emerging contaminants, they are often set at concentrations much higher than those found in the environment (Saaristo et al. 2018). For instance, many pharmaceutical compounds are not directly toxic at the observed concentrations detected in the environment, but are known to induce sub-lethal changes in metabolism, physiology, behaviour of non-target terrestrial and aquatic organisms (e.g. Bertram et al. 2022), and alter ecosystem processes (e.g. algal production, and patterns of insect emergence, Richmond et al. 2016).

The relevance of these guideline values should be considered within the context of the risk management framework. Certain guidelines may provide a trigger for further investigation. In the context of recycled water, guidelines for ecological species protection could be used in relation to the point of exposure due to: (1) discharge are more relevant if effluent is released to freshwater or marine waters; (2) an incident of an emergency discharge of the recycled water (e.g. wet year); (3) incident and/or emergency. As an example, ANZG (2021) ecological species protection guidelines discussed below are most applicable for discharge to fresh and marine waters, but these guidelines can also be considered for irrigation water quality because recycled water used for irrigation purposes may end up leaching to groundwater or runoff to waterways.

Pharmaceuticals and personal care products (PPCPs)

There are currently no ecological and species-specific guideline values for pharmaceuticals and personal care products in Australia (ANZG, 2021). However, Kumar et al. (2016), using chronic endpoints of population relevance (biochemical, histological, or molecular) and following guideline derivation rules for Australia and New Zealand (Batley et al. 2014), derived 95% species protection guideline values for carbamazepine (9.2 µg/L), diclofenac (770 µg/L), fluoxetine (1.6 µg/L), and propanol (14 µg/L) (Kumar et al. 2016). The authors state: “these population-relevant values are significantly higher than the values derived for the European Commission, Switzerland, or Germany” (Kumar et al. 2016). One of the major challenges in developing better environmental reference standards and guideline values is that 88% of human pharmaceuticals detected in the environment are without environmental toxicity data (OECD, 2019). In order to effectively derive guidelines for PPCPs, future studies should investigate not only multigenerational sub-lethal effects of PPCPs on fitness-related endpoints (behavioural, reproductive) on Australian native species, but also evaluate overall ecosystem level effects (Richmond et al 2017).

The European Union has started implementing policy instruments to control pharmaceuticals in the environment (European Commission, 2020). For example, the recent OECD policy report (OECD, 2019) on pharmaceutical residues in freshwater provides guidance to governments and recommends overcoming the pharmaceutical problem by using a collective, life cycle approach to managing pharmaceuticals in the environment. The report lists the following recommendations: 1) design and implement a policy mix of source-directed, use-orientated and end-of-pipe measures; 2) target stakeholders throughout the life cycle of pharmaceuticals; and 3) use a combination of voluntary, economic and regulatory instruments (OECD, 2019).

Endocrine disrupting chemicals (EDCs)

There are currently few ecological guideline values for endocrine disrupting chemicals (EDCs) in Australia (ANZG, 2021). By contrast, the European Union has introduced legislative obligations aimed at phasing out endocrine disruptors in water, industrial chemicals, plant protection products and biocides (EU 1107/2009; 528/2012). For example, in REACH (Registration, Evaluation, Authorisation and Restriction of Chemicals), which is the European Union regulation for the production and use of chemical substances and their potential impacts on both human health and the environment, endocrine disrupting chemicals are considered substances of very high concern (EC 1907/2006). Concentrations of nonylphenol exceeded low-reliability ecological guidelines for nonylphenol in freshwaters (0.1 µg/L, ANZECC & ARM CANZ 2000) in 13 sites (18 samples), however, not all these sites discharge water to freshwater. All concentrations were below the moderate-reliability international guideline for freshwaters (1 µg/L) and marine ecosystem protection (1 µg/L; ANZG, 2021 and 0.7 µg/L, CCME) (Table 12). Human health guidelines for addendum for drinking water (AGWC, 2008) were also considered but no exceedances were detected.

PFAS

Currently, there are no recycled water-specific guidelines for PFAS. Therefore, we are using drinking water and recreational water guidelines. Human health-based guidance values exist for the sum of PFOS and PFHxS and PFOA, which are divided into drinking water (PFOS+PFHxS: 0.07 µg/L) and recreational water (PFOS+PFHxS: 0.7 µg/L) guidelines (HEPA, 2018). Draft freshwater and marine water ecosystem guidelines exist for PFOS and PFOA, and they are divided into 99%, 95%, 90% and 80% species protection exposure scenarios (PFAS NEMP, 2018). It is worth noting that more than 4,700 PFAS compounds have been created, with many manufactured for use in diverse applications, of which hundreds have been detected in environmental samples around the world (OECD, 2018). Despite this, Australia currently only has guidelines for three PFAS compounds. Because there is growing evidence that industry is shifting to unregulated PFAS compounds (Brendel et al. 2018), researchers are proposing a class-based approach for managing PFAS instead of individual chemicals (Kwiatkowski et al. 2020).

In the current study, none of the human health-based guidelines were exceeded. The most stringent 99% species protection guideline for PFOS (0.0091 µg/L) (ANZG, 2023) for the protection of fresh and marine water ecosystems was exceeded in effluent (<0.0001 – 0.021 µg/L, mean concentration = 0.0057 µg/L, n = 65, Table 4) in some of the sites, with exception of two sites from treatment train TT12 and TT13. Because we analysed pure effluent samples, not environmental samples, further work is required to enable assessment of risk for PFAS in the receiving waterways.

Pesticides

Health-based guidelines for drinking water (NHMRC–NRM MC 2011) and for recreational water (Guidelines for Managing Risks in Recreational Water, NHMRC 2008) exist for a range of pesticides, such as atrazine, diuron, iprodione, metolachlor, propargite, simazine, and tebuthiuron. Freshwater and marine water ecological guidelines also exist for a range of pesticides (ANZG, 2021). Below we have listed some examples of current ecological guideline values for pesticides (but see full details in <https://www.waterquality.gov.au/anz-guidelines/guideline-values/default/water-quality-toxicants/search#tox-180>). However, sites where exceedances were detected may not be discharging to surface water, thus ecological guidelines may not be relevant to certain reuse. Furthermore, in moderately to highly disturbed agricultural or urban waterways, concentrations of certain pesticides might already be present, and/or waterways may be afforded a lower level of species protection (i.e. 90%, ANZG, 2021).

In the current study, there were a small number of exceedances to the ANZ (2018) guidelines, as follows:

- 1) Sulfonyl-urea herbicide: metolachlor was detected in 10% (6/62) effluent samples and maximum concentration (0.37 µg/L) exceeded 99% species protection (0.0084 µg/L) but not the 95% level (0.46 µg/L). Metolachlor is used to control grass and broadleaf weeds. Its use is prohibited in areas where livestock graze (Jennings et al. 2017) and U.S. EPA is listing it as a possible carcinogen (USEPA/IRIS 2016).
- 2) Organophosphorus pesticides: diazinon was detected in small number of effluent samples (3/62, 5%) but when it was detected (max 0.04 µg/L), the concentrations exceeded both 99% (0.00003 µg/L) and 95% (0.01 µg/L) species protection levels. As diazinon is not a bioaccumulating toxicant, then the 99% species guidelines would not apply unless the waterway had a high conservation value. Diazinon is used for controlling sucking and chewing insects and mites on a wide variety of crops, for fruit flies, flies, cockroaches, and other household pests. In 1986, U.S. EPA banned its use on open areas such as sod farms and golf courses because it posed a danger to migratory birds (Aggarwal et al. 2013). In Australia, diazinon use as a sheep dip or broad spray for lice has been banned since 2007 (APVMA, 2007), and Australian Pesticides and Veterinary Medicines Authority (APVMA) is currently reviewing its use for targeted applications on cattle, sheep, goats and horse (e.g. swab, hand spraying), dog and cat collars, and other agricultural applications such as onions and mushrooms (APVMA, 2022).
- 3) Phenoxy acid herbicides: MCPA was the third most frequently detected compound in effluent samples (47/66, 71%) and highest concentration detected was 0.91 µg/L, which is below the current ecological water quality draft guideline (1.4 µg/L).
- 4) Triazine herbicides: simazine had the highest effluent detection of 1.8 µg/L, which exceeds the 99% species protection level (0.2 µg/L) but not the 95% (13 µg/L)
- 5) Urea herbicides: diuron the highest effluent detection was 0.19 µg/L, which is close to the recommended default guideline value (0.2 µg/L). It is worth noting that this (0.2 µg/L) guideline value is considered as of 'unknown' reliability due to insufficient data, and sublethal chronic exposure could have adverse effects at a lower dose. Based on the findings of the current study (79% detection rate in effluent and 3.5 µg/L influent concentration), this compound is one to be watched. Also, please see APVMA's revised guidance for diuron use (APVMA, 2012). Metsulfuron-methyl is an herbicide that sits within the sulfonylurea group of the urea family of herbicides. The highest effluent detection was 0.12 µg/L, which exceeds the default freshwater guideline value for 95% species protection of 0.018 µg/L (moderate reliability, ANZG, 2021). Metsulfuron-methyl is extensively used in agriculture to control broadleaf weeds and some annual grasses.

Disinfection by-products (DBPs)

Australian Drinking Water Guidelines (NHMRC–NRMCC 2011) list typical DBPs from oxidation and disinfection processes, and provide guideline values for some of them (e.g. chloroform, dichloroacetic acid, and N-nitrosodiethylamine) (AGWR, 2008). For example, advanced oxidation processes are used to remove problematic compounds, such as N-nitrosodimethylamine (NDMA), where treatment process (e.g. membrane filtration, reverse osmosis) may not have a substantial percent reduction. In the current study, the maximum concentration of bromodichloromethane (49 µg/L) exceeded guidelines for drinking water (6 µg/L), and N-nitrosodimethylamine guideline value of 10 ng/L was exceeded at one site (33 ng/L) (see details in Table 10). It is important to note that none of the sites with exceedances supplied effluent water for drinking water purposes.

Contaminant percent reduction

Contaminant reduction is dependent on the nature of the treatment system. Please see details of the different treatment systems covered in the current study in Table 1 (Appendix A).

For most of the emerging contaminants, concentrations were lower in effluent than in influent, and some of the highest concentrations ($\mu\text{g/L}$) in effluent showed substantial percent reduction during treatment. Conversely, some compounds pass through the treatment trains relatively unchanged, and a few appear to increase. This is a pattern which is commonly observed around the world (Zhang et al. 2008; Alvarino et al. 2018)

PPCPs showed a wide range of percent reduction, which were compound specific. For example, paracetamol, caffeine and acesulfame K were all removed with an efficiency of >99.99, >98.4 and >81.7% respectively, while percent reduction ranged from very poor to even negative (i.e. concentration increased from influent to effluent) for carbamazepine (an increase in concentration of 0.92 times to a %R of 91%), lamotrigine (an increase in concentration of 1.8 times to a %R of 98.7%) and venlafaxine (an increase of 27% to a percent reduction of 99.3%). Increased effluent concentrations for venlafaxine and lamotrigine could be due to reactivation from the human metabolite to the active pharmaceutical compound (Lacey et al., 2012). One treatment train (TT12) completely removed PPCPs from the final effluent through a combination of ASP, UF, MF/RO and chlorine. Higher concentration in effluent than in the influent indicates that treatment process is breaking down the parent compound. This is consistent with previous studies (Ternes, 1998; Radjenovic et al. 2007; Roberts et al. 2016) reporting up to 5-7 times higher concentration of venlafaxine in the effluent than in the influent after activated sludge treatment, and negative percent reduction of propranolol and carbamazepine (K'oreje et al. 2020; Gruchlik et al. 2018). It is important to note that percent reduction could not be evaluated for all the PPCPs detected in the effluent, as the LORs in influent were higher due to matrix interferences.

Sorption to sludge is one percent reduction mechanism. For instance, polycyclic musk fragrances galaxolide and tonalide, have been shown to undergo some degradation during treatment, around 20 to 40%, became sorbed to sludge (Carballa et al. 2007). Meanwhile, ibuprofen, naproxen and sulfamethoxazole were shown to degrade in aeration tanks by 50-70%, while the fraction associated with sludge was less than 1% (Carballa et al. 2007).

For EDCs, mean percent reduction was >66% between the influent and effluent waters. In influent, bisphenol A (260 ng/L) was one of the four highest EDC concentrations detected, and second highest concentration detected in the effluent (220ng/L). For bisphenol A (BPA), which is a widely known endocrine disruptor (e.g. Huang et al. 2012, Michalowicz 2014), percent reduction were >89% for bisphenol A (BPA) in the current study. Previous studies have reported variable percent reduction for BPA (Guerra et al. 2015) because it is known to be sorbed to particulates (Zielinksa et al. 2016), and thus strongly linked to the treatment train process applied (Guerra et al. 2015, Wang et al. 2019).

For PFAS compounds, concentration of longer-chain perfluoroalkane sulfonic acids (PFASs), including PFOS, PFHxS, and PFBS, showed variable percent reduction (low or even negative percent reduction), with exception of treatment train TT12 which showed a percent reduction close to 99.99% for all PFAS through a combination of ASP, UF, MF/RO and chlorine. This is despite the propensity of these longer-chained compounds to bind more readily to solids. Variable percent reduction might be due to a limited number of replicates WWTPs per treatment train, higher limits of reporting for some compounds in the influent, sampling methods or a combination of these factors. Indeed, Tran et al. (2018) argues negative percent reduction and variable percent reduction for certain emerging contaminants could be an artifact of sampling methods, timing or both (i.e., timing of 24hr autosamplers and duplicate spot samples) (Tran et al. 2018). This is not consistent with findings from a study by Nguyen et al. (2019) who reported that over a

period of four years, there were no significant differences in daily PFAS load between weekdays and weekends, and little seasonal differences using 7-day composite influent samples (Nguyen et al. 2019). However, it is supported by Moodie et al. (2021) and Coggan et al. (2021) who demonstrated diurnal and daily variations in PFAS concentration in raw influent samples over a week, with higher concentrations measured during weekdays and business hours compared to the after-hours and the weekend. Many of the traditional wastewater treatment processes have shown low percent reduction or even observed an increase of PFAS, as wastewater treatment processes are generally ineffective in removing many PFAS compounds (Eriksson et al. 2017). For example, PFOS, PFOA, PFHxA and PFHxS all appeared in some instances to increase in concentration (up to 13.8 times) possibly due to biotransformation of precursor compounds (Eriksson et al. 2017). Percent reduction by reverse osmosis (RO) was shown to provide high percent reduction for PFAS (Thompson et al. 2011). However, RO is typically used when salinity is a point of concern, and the percent reduction of emerging contaminants is an additional benefit. The rejection of almost all dissolved contaminants by RO membrane mean that emerging contaminants will not be destroyed by RO process, rather it will concentrate these into a brine solution for disposal (Romeyn et al. 2016; Panagopoulos et al. 2019).

It is worth noting that in the current study, in some instances, percent reduction could not be calculated between influent and effluent as the limit or reporting for influent samples was raised in the influent due to matrix interferences.

As various factors can influence efficiency of reduction, such as chemical properties, level of treatment, retention times, pH, temperature, salinity, and UV (K'Oreje et al. 2020), further studies are needed to improve understanding of compound group specific reduction from wastewater (Tran et al. 2018).

Treatment trains and percent reduction

Based on our percent reduction estimates, TT12 was the most efficient in removing a range of emerging contaminants from the wastewater. TT12 uses treatment combination of ASP with extended aeration, UF, MF/RO1, MF/RO2, and chlorination. Overall, our study found percent reduction to be chemical group specific and differ between treatment trains.

For PPCPs, treatment trains TT13, TT7, TT2 and TT1 were estimated to be the most efficient overall in removing pharmaceuticals from influent. For instance, treatment trains TT1, TT2, TT6, TT12 and TT13 showed the highest percent reduction for lamotrigine (medication for epilepsy and bipolar disorder), carbamazepine (medication for epilepsy and neuropathic pain) and venlafaxine (medication for major depressive disorders). Whilst the treatment train TT13 (ASP, Lagoon, RO, Chlorine) showed the best percent reduction for lamotrigine, carbamazepine and venlafaxine, the poorest percent reduction was for treatment trains TT9 (ASP, lagoon), TT4 (aerated lagoon, lagoon) and TT7 (ASP) (Figure 1; Appendix A).

For EDCs, all TTs showed high percent reduction for at least two (estrone and etiocholanolane) and up to five compounds (plus 17 β -estradiol, BPA, nonylphenol). Out of the 12 treatment trains, TT7, TT4 and TT12 appeared slightly more efficient in removing EDCs from influent.

For PFAS, TT12 and TT7 (ASP) were the most efficient at removing PFAS compounds. Based on our study, treatment train TT12, which uses ASP extended aeration UF and reverse osmosis, showed >99% percent reduction of PFOS, PFHxS, and PFHxA. Interestingly, treatment train TT7 seemed to be only efficient in removing 6:2 FTSA (71%) and N-EtFOSA (87%) but not PFOS (15%) nor PFHxS (11%). It is important to note that in this study we only had one site, which used TT12 treatment and one site for TT7. Therefore, further work is required to confirm our results. Overall, based on our study, PFAS compounds were poorly removed from the wastewater by treatment trains TT1, TT2, TT4, TT6, TT8, TT9 (Table 11).

For pesticides, percent reduction was varied, and calculations were limited by the number of detected concentrations. For herbicides, TT2 showed better percent reduction for diuron and MCPA, while TT4 and TT8 showed better percent reduction for simazine. For insecticides, TT1 showed the best percent reduction, and TT8 the lower percent reduction for imidacloprid. There were insufficient detects to determine which treatment trains were more efficient in removing fungicides. For industrial compounds, TT6 and TT12 showed the best percent reduction. For phenols, all treatment trains were efficient in their percent reduction of compounds.

Based on ERA 2021 (O'Connor and Stevens, 2021), chlorination and use of reverse osmosis (membrane nanofiltration) improves the log percent reduction of emerging contaminants. For example, for venlafaxine, estimated log percent reduction were highest in treatment trains TT5, TT10, TT11, and TT12. These treatment trains all use a combination of treatment technologies: TT5 = ASP with extended aeration and RO; TT10 = ASP, Lagoon and RO-water; TT11 = ASP, lagoon, and chlorination, and TT12 = ASP, UV, UF, RO and chlorination. The lowest log percent reduction was estimated for treatment train TT7, which uses treatment of ASP, only. Based on this study, for venlafaxine, the lowest calculated percent reduction was TT4 (ASP and extended aeration), followed by TT7, while the highest was TT12. It is worth noting that we did not collect samples from sites that use TT3, TT5, TT10 and TT11 treatment technologies and thus a complete comparison between percent reduction between O'Connor and Stevens (2021) risk assessment and our percent reduction calculations cannot be done.

Conclusions

Our study shows that a wide range of PPCPs, some EDCs, PFAS, phenols, herbicides, pesticides, and disinfection byproducts are present in effluent used as recycled water. The study shows that for most of the emerging contaminants detected, concentrations were lower in effluent than in influent. Even if the highest concentrations ($\mu\text{g/L}$) are drastically reduced during treatment (>90%), some compounds pass through certain treatment trains relatively untreated.

Based on our percent reduction estimates, TT12 (ASP + UF + RO1 + RO2 + chlorination) was the most efficient in removing a range of emerging contaminants from influent. In addition, one of the sites sampled in TT8 showed high percent reduction for most compound groups. This percent reduction, however, was not consistent across all sites in TT8. Indeed, some sites within the same treatment train had low or even negative percent reduction rates for emerging contaminants in some sites, while good percent reduction in one or two other sites. In addition, treatment train TT7 showed higher percent reduction for two PFAS compounds. However, it is important to note that we only sampled one TT12, one TT6 site and at one time point (duplicates 28-days apart for effluent and a 24-hr composite for influent). Future evaluation would benefit from including measurements of wastewater (influent and effluent), brine, sludge and biosolids to gain a full picture of the risks of emerging contaminants.

Within a single treatment train, there can be different classes of recycled water with higher levels of treatment (i.e. WTP class A versus WTP class B). Further temporal data (e.g. sampling in spring, summer, autumn and winter) would help account for variation in the initial concentrations in the raw influent. For example, in the current study, there was substantial variation between sites within treatment trains, which may be due to a range of factors, such as the initial influent concentration, class, biological percent reduction systems, retention time and pH. Further work is required to establish the processes using a mass balance approach, which includes measurement of contaminants in sludge, as well as determine the efficiency for percent reduction of different emerging contaminants at each stage, and over multiple sampling occasions.

Photobacteria bioassay and receptor localisation assay provide valuable information on overall toxicity but should be used in combination with more traditional, quantitative techniques (e.g. LC-MSMS).

It is important to note that the presence of emerging contaminants in recycled water does not necessarily translate to risks to environment and human health. Risk assessment is a site-specific exercise and should be assessed through the lens of the general environmental duty (EP Act, 2019).

All in all, the results provided in this study enhance the state of knowledge for the Victorian water sector by providing a better understanding and baseline data on the prevalence of emerging contaminants in recycled water. In addition, the data shows how detected emerging contaminants respond to various treatment methods, and ultimately, improve our understanding of the likelihood of potential risks associated with end uses of recycled water.

Future directions

This project assists EPA in applying science-based and preventative regulation to the recycled water sector. Further, the project provides insights into the prevalence of emerging contaminants and percent reduction of treatment technologies. More broadly, the collected data provides insights into potential future priorities and activities regarding emerging contaminants in recycled water.

By contributing to updating the current desktop risk assessment (O'Connor and Stevens 2020), this study contributes to water corporations' state of knowledge regarding assessment and management of emerging contaminants in recycled water and informing future policy approaches.

EPA will continue to support the water sector to:

- progress a proportional, risk-based approach to emerging contaminants
- work with industry to identify priority areas to enhance state of knowledge

Sampling method guidance

EPA will develop further guidance on the use of time-integrated sampling approaches (such as passive samplers and auto-samplers) in surface water to inform industry and align academic and government practices. We recommend that future sampling approach for emerging contaminants in wastewater consider using a combination of multiple or integrated spot samples, auto-samplers and/or passive samplers to determine time-weighted average concentrations. Whilst using passive samplers, it is important to note the following:

- For each site, any sampling should integrate measurements by collecting samples over ≥ 1 day in influent and effluent
- Flow and sedimentation rates and concentrations in sludge should be measured to accurately calculate percent reduction and mass balance (Tran et al. 2018)
- Some advantages of passive sampling include unattended operation, no power requirements, low costs and integrated concentrations over time (Tan et al. 2007)
- Passive samplers require an initial sampling campaign alongside spot sampling or auto-samplers to calibrate the rate of accumulating emerging contaminants in different systems. It is then possible to use passive samplers for ongoing integrated monitoring of emerging contaminants in surface waters (for example see Allinson et al. 2023; Kaserzon et al. 2014).
- Further investigation should ensure time-weighted average sampling rates are determined (i.e. by using calibrated passive samplers, auto-samplers and/or time-integrated spot samples collected over ≥ 1 day in influent and effluent and measuring flow rates.)

Limitations of the study

- The study timeframe is limited, with the sampling program conducted between 27 April and 22 July 2021.
- Due to the overlap with COVID-19, use patterns and the potential influence of state-wide restrictions might have influenced the detected chemicals and concentrations in this study, especially with the restrictions specific to industrial and commercial businesses.
- Variations in the prevalence and concentrations of specific chemicals may be apparent due to:
 - Seasonal patterns of chemical use (e.g., herbicides).
 - Event based situations: e.g., increased repellent use from increased mosquito populations (e.g., DEET, picaridin)
 - Temperature influences on wastewater treatment efficacy and environmental fate
 - Diurnal influences for specific chemicals

Future work and priorities

- EPA is developing guidance for emerging contaminants in recycled water and conducting further priority testing to ensure recycled water proponents and users to assess and manage risks and meet their responsibilities under the general environmental duty (GED).
- EPA's guidance will include case studies for different end users of recycled water, including scenarios for site-specific recycled water use, and guideline value derivation for a selected group of priority contaminants.
- A field study, led by EPA and funded by Department of Energy, Environment and Climate Action (DEECA), on emerging contaminant uptake into Victorian crops irrigated with recycled water. The study aims to generate regionally relevant data that supports the development of guidance and guideline values for industry.
- EPA is conducting further research with Victoria's water sector to further build community confidence in the safe and suitable use of recycled water.
- EPA is currently partnering with Federation University and Barwon Water on a PhD-project that is looking at pharmaceutical pollution in the Yarrowee/Barwon River system and Lake Colac.

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