Which chemicals drive biological effects in wastewater and recycled water?

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Abstract

Removal of organic micropollutants from wastewater during secondary treatment followed by reverse osmosis and UV disinfection was evaluated by a combination of four *in-vitro* cell-based bioassays and chemical analysis of 299 organic compounds. Concentrations detected in recycled water were below the Australian Guidelines for Water Recycling. Thus the detected chemicals were considered not to pose any health risk. The detected pesticides in the wastewater treatment plant effluent and partially advanced treated water explained all observed effects on photosynthesis inhibition. In contrast, mixture toxicity experiments with designed mixtures containing all detected chemicals at their detected concentrations demonstrated that the known chemicals explained less than 3% of the observed cytotoxicity and less than 1% of the oxidative stress response. Pesticides followed by pharmaceuticals and personal care products dominated the observed mixture effects. The detected chemicals were not related to the observed genotoxicity. The large proportion of unknown toxicity calls for effect monitoring complementary to chemical monitoring.

Keywords

Effect-based monitoring, bioanalytical equivalent concentrations, mixture toxicity, reverse osmosis, recycled water

1 Introduction

Indirect potable reuse (IPR) of wastewater has become a necessity in many water-scarce regions of the world (National Research Council 1998, Rodriguez et al. 2009, Sedlak 2014). IPR schemes typically rely on advanced treatment of secondary wastewater effluents from wastewater treatment plant (WWTP). Such advanced treatment usually consists of a combination of membrane filtration (e.g. ultrafiltration and reverse osmosis) and oxidation processes (e.g. advanced oxidation, UV disinfection) to remove pathogens and chemicals, including inorganics and heavy metals, nutrients and organic micropollutants (Binnie and Kimber 2009). Recycled water is then introduced into aquifers or waters and can potentially be used as part of the drinking water supply. As reviewed recently (Rodriguez et al. 2009, van der Bruggen 2010), a large number of IPR schemes have been implemented in the US, as well as some in the UK, Namibia, and Singapore. To date, no adverse health impacts have been reported related to recycled water (Khan and Roser 2007).

In Australia, there are two major IPR projects. On the East Coast, the Western Corridor Recycled Water Project is Australia's largest water recycling scheme and the third-largest advanced water treatment project in the world. It was commissioned in 2008 but ultimately the scheme has not become operational up to 2014 because the 2003-2008 drought in Southeast Queensland ended with a period of heavy rainfalls and floods from mid-2010 onwards. On the West Coast, a pilot IPR scheme has been successfully implemented from 2010 to 2012 that treats secondary effluent from the Beenyup WWTP with ultrafiltration (UF) followed by reverse osmosis (RO) and final UV disinfection. The

recycled water is injected into the Leederville aquifer, which is a drinking water source for the city of Perth (Water Corporation 2013). This scheme has been approved to go to full scale, with stage one expected to be completed in 2016 (http://www.watercorporation.com.au). This managed aquifer recharge scheme is the focus of this paper.

Most IPR schemes extensively investigated potential environmental and human health impact of the replenishment of drinking water reservoirs with recycled water before implementation. Typically a large number of organic micropollutants known to occur in sewage or formed from natural precursors during treatment processes (e.g., disinfection by-products) are monitored through chemical analyses. These include pharmaceuticals and personal care products, pesticides, household and industrial chemicals. While micro- or ultra-filtration mainly remove bacteria, pathogens and high molecular-weight natural organic matter, most organic micropollutants are removed during RO treatment (Gupta and Ali 2013). However, low molecular weight and non-ionic (neutral) organic molecules (e.g. NDMA, dioxane, halogenated solvents) were less effectively rejected by RO membranes. As a result, these compounds are frequently detected in recycled water at low concentrations (Snyder et al. 2007, Drewes et al. 2008).

Taking a precautionary approach, frequently detected organic micropollutants in recycled water are tightly regulated in many countries. In the US, recycled water has to comply with drinking water guidelines. The Australian Guidelines for Water Recycling (AGWR) lists 348 organic chemicals with health-based guideline values (GVs) (NRMMC & EPHC & NHMRC 2008). The GVs generally match the Australian

Drinking Water Guidelines (ADWG) (NHMRC 2011) but almost twice as many chemicals are regulated in recycled water. Fifteen regulated organic micropollutants were occasionally detected in recycled water of the Western Corridor Recycled Water Project (Hawker et al. 2011) but concentrations never exceeded GVs. In addition the potential impact on the receiving dam was modeled, and concentrations of organic chemicals were expected to decrease further due to dilution and natural attenuation, mainly by biodegradation and sorption to sediments (Hawker et al. 2011).

In an initial investigation (2005 - 2008) of the IPR scheme in Perth, 396 parameters were monitored over three years (Van Buynder et al. 2009). While 23 organic chemicals and 6 metals/inorganics were detected in more than 25% of all RO waters investigated, all concentrations of chemicals in RO water were below GVs. The organics detected in RO permeate were mainly disinfection by-products (e.g., NDMA), small volatile organics (e.g., benzene, dioxane) and complexing agents (e.g., EDTA, NTA). Detected concentrations were below GVs and were not considered to pose any appreciable health risk, with one exception, the disinfection by-product NDMA (Linge et al. 2012). However, there remain unknowns because the detected chemicals could only explain a small fraction (~2-5 %) of the dissolved organic carbon in the RO permeate (Linge et al. 2012). While up to 95% of dissolved organic carbon in RO permeate could not be accounted for, chemicals below detection limit may have contributed to the residual DOC, along with low molecular-weight natural organic matter originally present in drinking water and wastewater, unknown anthropogenic micropollutants, chemicals

used during RO treatment or leached from RO membranes and soluble microbial by-products (Linge et al. 2012).

To bridge this knowledge gap, target and non-target screening were conducted recently in water post RO and post UV using an Orbitrap MS spectrometer (Busetti et al. 2013). Both target and non-target screening showed that (a) "suspect" or "unknown" chemicals did not make up the majority of the DOC in RO treated water, and (b) a large number anthropogenic chemicals targeted (i.e., pesticides, biocides, industrial chemicals, pharmaceuticals) were not detected in recycled water, further reducing the risk associated with human consumption of recycled water.

Furthermore, during Perth's Groundwater Replenishment Trial, which ended in 2012, 292 Recycled Water Quality Parameters were monitored over three years. The results of this extensive monitoring program confirmed 100% compliance of all water samples analysed with the required water quality guidelines (Water Corporation 2013).

In the present study, chemical analysis was complemented with bioanalytical tools. Cell-based bioassays are widely used for water quality assessment and monitoring (Escher and Leusch 2012) and have previously been applied to evaluate water quality from samples taken in the investigated IPR scheme (Leusch et al. 2014a, Leusch et al. 2014b).

Cell-based bioassays can provide a comprehensive profile of the biological activity of mixtures of organic chemicals and can also give information on the type of effect by choosing cells and assessment endpoints that are associated with defined modes of action (Escher and Leusch 2012). So far, investigated modes of action have

predominantly focused on estrogenic and other endocrine effects as well as genotoxicity (Escher and Leusch 2012). We previously applied 100 distinctly different bioassays to recycled water and demonstrated that a small number of indicator bioassays can be applied for monitoring of the treatment efficacy as well as for benchmarking the water quality of recycled water against other types of water (Escher et al. 2014a). According to these recommendations four bioassays were used in this study: non-specific toxicity (cytotoxicity) was evaluated with the bioluminescence inhibition test with *Vibrio fischeri* (Microtox) (Tang et al. 2013). Photosynthesis inhibition using the combined algae test (Escher et al. 2008) was a representative specific mode of action. We also determined estrogenicity with the ER-CALUX (Rogers and Denison 2000) and the activation of the aryl hydrocarbon receptor with the AhR-CALUX (Nagy et al. 2002) assay but these two bioassays did not show any responses and were therefore not suitable for the mixture modeling.

Reactive toxicity was assessed with the umuC assay for genotoxicity (Macova et al. 2011) and the AREc32 for oxidative stress response (Escher et al. 2012). In addition, we quantified 299 organic micropollutants during the same sampling campaign.

The aim of the study was to assess which of the detected chemicals drive the biological effect and which fraction of effect remains unexplained by detected chemicals. Therefore we mixed all chemicals that were (a) present at concentrations above the limit of detection (LOD) and (b) included in the AGWR. These chemicals were mixed in the concentration ratios that were detected by analytical chemistry in the various samples. These mixtures were termed “iceberg mixtures” as they constituted the visible “tip of the

144 iceberg" and allowed us to estimate the contribution of unknown chemicals and
145 chemicals below detection limits to the overall mixture effect. We have previously
146 performed such experiments with wastewater and recycled water and were able to show
147 that known chemicals can explain the majority of specific receptor-mediated effects
148 (Tang and Escher 2014) but for more general endpoints such as cytotoxicity (Tang et al.
149 2013) and oxidative stress response (Escher et al. 2013) less than 1% of effect could be
150 explained by known chemicals.

151 In addition to the four biological endpoints evaluated here, estrogenicity is a
152 highly relevant biological endpoint in wastewater and associated water types. However,
153 previous work has demonstrated that no estrogenic activity could be detected in recycled
154 water (Leusch et al. 2014a), and that the estrogenicity in typical source water can be fully
155 explained by known chemicals (Rutishauser et al. 2004). Therefore, and because no
156 estrogenic responses were detected in the investigated waters, this endpoint was omitted
157 in the present study.

158 The present study does not only apply this iceberg concept to a different and more
159 diverse set of samples in a recycling plant but goes a step further in that the iceberg
160 mixtures were subdivided into six chemical groups (pharmaceuticals (including personal
161 care products), endocrine disruptors compounds (EDCs), antibiotics, X-ray contrast
162 media (XRCs), pesticides (including transformation products) and others). By comparing
163 the effects of the individual groups and the effects of the combined iceberg mixtures, it
164 could be determined, which chemical group dominates or significantly contributes to the
165 biological effects at any stage of the treatment process.

2 Materials and Methods

2.1 Chemicals

The 65 chemicals used in the mixture experiments are listed in the **Electronic Supplementary Material** (ESM), Table S1. All chemicals were of analytical grade and purchased from Sigma-Aldrich or Novachem, Australia.

2.2 Sampling site

Grab samples were collected from a Wastewater Treatment Plant (WWTP) and an Advanced Water Recycling Plant (AWRP) located in Perth, Western Australia in July 2012 (Figure 1). The WWTP treats predominately urban residential sewage (Water Corporation 2013). Briefly, the raw wastewater is treated with grit removal and goes through sedimentation tanks (WWTP influent). This water then undergoes aeration, activated sludge treatment and clarification as a secondary treatment. The majority of the resulting secondary treated effluent (WWTP effluent) is discharged into the ocean and a small portion (~ 7 ML/day) is fed into the AWRP. The treatment train of the AWRP consists of chloramination for disinfection during treatment process, ultrafiltration, reverse osmosis and ultraviolet light (UV) disinfection. Samples were collected in the following points: after the sedimentation tanks in the WWTP (WWTP influent); after secondary treatment (WWTP effluent); after ultrafiltration (post UF); before reverse osmosis in the holding tank (mixing tank); after reverse osmosis (post RO); after UV disinfection (post UV). The reverse osmosis reject (RO reject) was also collected. The recycled water was injected into the groundwater system at a maximum of 4.5 ML/day. Routine water quality data was assessed by the plant operators at the time of sampling

and is given in the ESM, Table S2. In addition, a laboratory blank (LB) and a field blank (FB) were made up of ultrapure water.

2.3 Sampling and sample preparation

The water samples were collected in amber glass bottles and preserved with 0.1% sodium thiosulphate and concentrated hydrochloric acid to pH 2.5. The samples were split into two portions, for chemical analyses and bioassays. For bioassays, all samples were filtered with 0.45 μm microfiber glass Duo-Fine filter cartridges (PALL Life Sciences, NY, USA) before solid-phase extraction (SPE) in 20 mL custom-made cartridges from Supelco (Sigma-Aldrich, Sydney, Australia). The extraction material was comprised of 2 g SupelClean coconut charcoal and 1 g SupelSelect HLB with frits in between. The cartridges were conditioned with 20 mL acetone:hexane mixture (1:1, v:v), followed by 20 mL methanol and 20 mL ultrapure water at pH 3 at a flow rate of 5 mL/min. Samples were then loaded onto the custom-made cartridges using three 8-channel offline peristaltic pumps (Gilson, Middleton, USA) at a flow rate of 3 mL/min. The cartridges were dried under vacuum and wrapped in parafilm and aluminium foil and stored at -20 °C before shipping to the Entox laboratory for elution. The cartridges were eluted with 20 mL methanol and 20 mL acetone:hexane mixture (1:1, v:v) under gravity. The extracts were evaporated under gentle nitrogen flow and solvent-exchanged into 1 mL of methanol.

The SPE extracts were comprised of a mixture of known and unknown chemicals at unknown concentrations. The dose-metric is the relative enrichment factor (REF), which

is a measure of how much a sample is enriched (REF > 1) or diluted (REF < 1) in the bioassay as compared to the original sample (equation 1).

$$\text{REF} = \frac{\text{water volume equivalent in bioassay}}{\text{total volume of medium in bioassay}} \quad (1)$$

2.4 Chemical analysis

Water samples were analyzed using GC/MS-MS and LC/MS-MS at Queensland Health Forensic Scientific Services and at the Curtin Water Quality Research Centre (CWQRC). A total of 299 chemicals were analyzed between the two laboratories. More details on the sample preparation for chemical analysis and analytical methods are given in the ESM Data, Section S1.

2.5 Designed iceberg mixtures

Detected chemicals were mixed in the ratios of concentrations found (ESM, Table S3). The detected chemicals were clustered in six groups: endocrine disrupting compounds (EDCs), antibiotics, X-ray contrast media (XRCs), pesticides (including transformation products), pharmaceuticals (excluding antibiotics but including personal care products such as triclosan and consumer products such as coffeine) and "others" (ESM, Table S3). In addition, the individual chemical group mixtures were mixed according to the contributing fraction into one mixture comprising all detected chemicals termed as "iceberg mixture".

2.6 Bioanalytical assessment

All bioassays were previously applied and characteristics of the bioassays and literature references for the methods are given in Table 1. For each sample, the bioanalytical equivalent concentration BEQ was calculated from the effect concentration EC of the reference compound divided by the EC of the water sample.

$$BEQ_{water} = \frac{EC(\text{reference compound})}{EC(\text{water sample})} \quad (2)$$

In case of the water samples, the EC is in units of REF and the BEQ is termed BEQ_{water}. Analogously the BEQ of designed iceberg mixtures $BEQ_{iceberg}$ and the individual chemical groups $BEQ_{group\ i}$ can be derived with equation 3 by using the EC values experimentally obtained from the designed chemical mixtures (in units of mol/L) and converted to the EC in units of REF, EC(iceberg,REF), using the known chemical concentrations C in the mixture equivalent to the measured concentrations in the sample (sum of concentrations in units of mol/L).

$$BEQ_{iceberg} = \frac{EC(\text{reference compound})}{\frac{EC(\text{iceberg, M})}{C(\text{iceberg})}} = \frac{EC(\text{reference compound})}{EC(\text{iceberg, REF})} \quad (3)$$

The reference compounds and the associated BEQ for each bioassay are defined in Table 1. The limits of detection were derived by translating the effect of three times the standard deviation of the controls into the corresponding BEQ values (Table 1).

3 Results and Discussion

3.1 Chemical analysis

A total of 299 chemicals were analyzed in the water samples, of which 172 were included in the AGWR (ESM, Figure S1). In the paper, we focus the discussion on the regulated chemicals (ESM, Table S3), while results on additional non-regulated chemicals are compiled in the ESM, Table S4. The highest number of chemicals were detected in WWTP influent, WWTP effluent, post UF and mixing tank (50, 50, 49, 50, respectively, ESM, Figure S1). The concentrations of chemicals in the WWTP influent were typically higher than in WWTP effluent, although due to the higher LOD in the WWTP influent sample, some chemicals were not detected in the WWTP influent but found in the WWTP effluent. UF did not reduce concentrations of chemicals. Instead, RO was found to be a very effective removal process and only five chemicals were detected in the post RO sample however, no chemicals were detected post-UV disinfection. In the post RO sample, low levels of the anticorrosive chemical tolytriazole, the plasticizer bisphenol A, the pharmaceutical triclosan and the pesticides MCPA and the pesticide degradation product 3,4-dichloroaniline were detected. Tolytriazole (Busetti et al. 2013, Loi et al. 2013) and bisphenol A (Water Corporation 2013) were detected in previous monitoring programs but triclosan and the pesticides MCPA and 3,4-dichloraniline were detected in post RO water for the first time in this AWRP. No chemicals were detected in the post UV water sample.

The chemicals' concentrations in post RO and post UV samples were below the Australian GVs for recycled water (NRMMC & EPHC & NHMRC 2008). For

comparison, the GVs are indicated in Figure 2 by black bars. If at all, the concentrations exceeded the GVs for recycled water only in WWTP influent or RO reject. Exceptions were the pesticide MCPA, which exceeded the GV prior to the RO treatment but was two orders of magnitude below the GV in RO water, and resulted below detection in the post UV sample. Diatrizoic acid was also above GV up to the mixing tank but was below detection after RO.

The majority of detected chemicals fell into the group of pharmaceuticals with 34 out of 44 analyzed pharmaceuticals being detected in at least one sample (Figure 2). Five pharmaceuticals (citalopram, desmethylcitalopram, cyclophosphamide, fluoxetine and propranolol) were not detected in WWTP influent due to increased LODs in the complex sewage matrix but were present in the WWTP effluent. In general, concentrations were significantly reduced during secondary treatment (Figure 2) and nine pharmaceuticals (acetylsalicylic acid, acetaminophen, atorvastatin, cephalexin, ibuprofen, naproxen, ranitidine, salicylic acid and theophylline) were below detection limit after secondary treatment. Concentrations of carbamazepine, diclofenac, fluoxetine, gemfibrozil and indomethacin were very similar to previous studies (Busetti et al. 2009). Concentrations of pharmaceuticals remained fairly constant in the first steps of the AWRP because UF cannot efficiently remove organic micropollutants. RO reduced all chemicals to below detection except triclosan, which was detected for the first time at its LOD of 0.01 µg/L. In a previous study, clofibric acid, diazepam and naproxen had been occasionally detected but in less than 25% of the samples (Linge et al. 2012).

293 Of the EDCs, mainly xenoestrogens were quantified in this study as the previous
294 monitoring had shown that the estrogens ethinyl estradiol, 17β-estradiol and estrone were
295 always below detection (Van Buynder et al. 2009). In the present study, estrone levels of
296 5 ng/L in the WWTP effluent fell below detection limit thereafter. The surfactant 4-t-
297 octylphenol was only detected in the WWTP influent. The plasticizer bisphenol A was
298 also detected in the blanks. The concentrations of bisphenol A listed in the ESM, Table
299 S3 represent the measured values minus the blank value and are therefore of high
300 uncertainty but positive detections are consistent with previous work (Van Buynder et al.
301 2009).
302 Antibiotics were grouped separately from the pharmaceuticals because they are
303 relevant for the formation of resistant bacterial strains. Secondary treatment greatly
304 reduced the concentration of antibiotics with only erythromycin and sulfamethoxazole
305 detected in the WWTP effluent. Concentrations remained stable during the first steps of
306 the AWRP but RO efficiently rejected all antibiotics, which is again consistent with
307 previous work (Linge et al. 2012, Busetti et al. 2013).
308 XRCs are good indicator compounds as they are frequently detected in fairly
309 constant concentrations up to UF but are well removed by RO (Busetti et al. 2010), which
310 was confirmed in the present study (Figure 2).
311 Pesticides were generally well removed during treatment with only MCPA and
312 3,4-dichloraniline detected at very low levels. MCPA was detected at 50 times higher
313 concentration in the WWTP effluent than in previous work, therefore it is not astonishing
314 that it was detected post RO in the present study, and not previously (Rodriguez et al.

315 2012, Busetti et al. 2013). Concentrations in WWTP effluent were similar to previous
316 work for atrazine, 2,4-dichlorophenoxyacetic acid and simazine (Rodriguez et al. 2012).
317 The group of compounds called "others" was comprised of benzothiazoles,
318 fragrance chemicals and flame retardants. 5-Methyl-1H-benzotriazole (tolytriazole) was
319 the only chemical in this group detected at ng/L levels post RO, which is consistent with
320 previous findings (Busetti et al. 2013, Loi et al. 2013). The fragrance chemicals were
321 analyzed for the first time at this plant and while their concentrations were constant
322 during the WWTP and the initial AWRP steps, RO removed them below detection
323 (Figure 2). Previously, galaxolidon a biological transformation by-products of the musk
324 fragrance galaxolide, was detected in post RO and post UV samples at average
325 concentrations of 31 and 19 ng/L, respectively.
326 From comparison of the chemical analysis with previous works as discussed
327 above one can conclude that the grab samples taken for the present study are fairly
328 representative and are suitable for bioanalytical assessment and mixture effect studies.
329
330 ***3.2 Bioanalytical assessment***
331 The highest effect levels in all bioassays were observed in the WWTP influent and RO
332 reject samples, the effects decreased along treatment train (Table 2). Apart from
333 Microtox, effects were below detection limits post RO and post UV disinfection.
334 For the non-specific toxicity, the baseline-TEQ decreased from 26 mg/L in WWTP
335 influent to 9 mg/L after secondary treatment (WWTP effluent). The levels remained low
336 at 5 – 6 mg/L after ultrafiltration (post UF) and in the mixing tank between UF and RO.

337 The baseline-TEQ was further reduced to less than 1 mg/L post RO and post UV to levels
338 as low as the blanks (Table 2). These levels were similar to what was observed previously
339 in this plant (Leusch et al. 2014a) (ESM, Figure S2A) and in another Australian AWRP
340 (Macova et al. 2011, Escher et al. 2014a, Tang and Escher 2014), which uses the same
341 treatment processes (ESM, Figure S3A).

342 A consistent trend was observed in the PSII inhibition endpoint, the highest diuron
343 equivalent concentration (DEQ) was observed in RO reject (0.09 μg/L) and the DEQ
344 decreased along the treatment train from 0.07 μg/L in WWTP influent to 0.03 μg/L in
345 WWTP effluent and 0.02 μg/L post UF and mixing tank (Table 2). The DEQs in post RO
346 and post UV were below the detection limit of 0.004 μg/L. The EC were very similar to
347 previous work (Leusch et al. 2014a) (ESM, Figure S2B), although in the previous study
348 EC_{20} not EC_{50} were measured and the DEQ levels were much lower than in another
349 AWRP (Tang and Escher 2014) but the removal efficiency by reverse osmosis was again
350 similar (ESM, Figure S3B).

351 The *umuC* genotoxicity assay only gave responses when metabolism was not
352 activated with metabolic enzymes. The only sample that was active after metabolic
353 activation by rat liver S9 was the WWTP influent with a 2AAEQ of 2 μg/L. The results
354 for 2AAEQs were therefore omitted from Table 2 as they were mainly non-detects.
355 Without metabolic activation, the highest response in the *umuC* assay was found in
356 WWTP influent and reject with a 4NQOEQ of 0.6 μg/L (Table 2). The 4NQOEQ levels
357 decreased along the treatment train and were below the detection limit of 0.1 μg/L in post
358 RO and post UV samples. A comparison of the $EC_{IR1.5}$ with previous work on the same

AWRP (Leusch et al. 2014a) showed again consistent results (ESM, Figure S2C), although the secondary effluent still showed an effect after metabolic activation in the previous work while it was below detection limit in the present study.

For the oxidative stress response, the highest tBHQ equivalent concentration (tBHQEQ) was observed in the RO reject sample at 73 μg/L (Table 2). The tBHQEQ levels decreased along the treatment train from WWTP influent (32 μg/L) to post RO and post UV samples (<9 μg/L). Again a comparison with the other AWRP (Escher et al. 2013) revealed a consistent pattern of reduction, although the levels in the WWTP effluent were five times lower in the present study and the levels in the post-UV sample were slightly higher but in the same range as the blanks (ESM, Figure S3C).

3.4 Contribution of known chemicals to the observed biological effects

The iceberg mixtures explained less than 3% of the observed cytotoxicity (Figure 3A and Table 3). A smaller fraction of effect could be explained for WWTP influent as compared to the samples along the AWRP treatment train (Figure 3A) and the fraction explained was not related to the number of chemicals detected (ESM, Figure S1). The fraction explained in WWTP effluent was similar to previous work (Tang et al. 2013), but larger fractions than in previous work were explained in the other samples (Figure 3A).

In contrast, the photosynthesis inhibition was higher in the iceberg mixtures than in the samples (Figure 3B and Table 3), which indicates that PSII-herbicides dominate the mixture effects toward algae, which had previously been confirmed for similar types of samples (Tang and Escher 2014). The lower effects in the samples as compared to the

381 iceberg mixtures can be rationalized by the fact that the chemical analysis was corrected
382 for SPE recovery while for the bioassays the composition of the samples is unknown and
383 one cannot correct for SPE recovery. While SPE recovery of pesticides is typically close
384 to 100% (Escher et al. 2014b), any recovery lower than 100% will cause the effect of the
385 icebergs appear to be higher than of the extracted samples.

386 The detected chemicals explained only 0.04% to 0.7% of the observed oxidative
387 stress response (Figure 3C and Table 3), which was in the same order of magnitude as
388 previous work (Escher et al. 2013). Interestingly the WWTP influent was an outlier with
389 an unusual high fraction explained (0.7%), while for the cytotoxicity assay there was a
390 remarkably low fraction explained (0.2%). This observation is presumably an artifact as
391 the WWTP influent also had a high organic matter content and the detection limits of
392 individual chemicals were higher, so that in some cases chemicals were below the LOD
393 even though they were present in the WWTP effluent (ESM, Figure S1 and Table S3).

394 Overall, the fraction of BEQ explained by known chemicals was generally higher
395 in this study that in the previous study (empty diamonds in Figure 3). This can be
396 explained by the fact that a higher number of chemicals were quantified in the present
397 study than in the previous studies (Escher et al. 2013, Tang et al. 2013) and is likely not
398 related to a different composition of the water samples.

399

400 ***3.5 Contribution of individual chemical groups to the overall iceberg mixtures***

All individual chemical groups were tested in all bioassays. Positive responses were found only in the assays for cytotoxicity, photosynthesis inhibition and oxidative stress response and there was no response in the genotoxicity assay (Table 2).

Figure 4 shows the cumulative BEQs of the six chemical groups in comparison with the experimental BEQ of the entire iceberg mixture. With one exception, the individual group BEQs summed up to the experimental BEQ of the entire iceberg mixture, which confirms the suitability of the experimental design and concentration addition of individual groups.

For the cytotoxicity endpoint, pesticides and pharmaceuticals had an equal share to the BEQ in the WWTP influent sample, while pesticides dominated in all other samples (Table 2, Figure 4A). This is consistent with the general notion that many pesticides are more recalcitrant towards secondary treatment than many pharmaceuticals. Of the other four chemical groups only the EDCs had a minor contribution of 3% in the WWTP influent and 12% in the RO reject (Table 2). Post RO the BEQ levels were very low with pharmaceuticals and others dominating the BEQ.

As expected, the group of pesticides dominated the overall DEQ quantified in the photosynthesis inhibition assay. Antibiotics contributed only 1% to the DEQ in the WWTP influent but were below detection limit thereafter. Pharmaceuticals contributed between 0.3% and 1.8% to the DEQ. Post RO, no photosynthesis inhibition was detected. In the RO reject the pharmaceuticals had a nominal contribution, which must be an artifact of the mixture calculations, which are extrapolations, as the iceberg mixture itself was not active.

For the oxidative stress response, there was generally a good agreement between the BEQ of the iceberg mixtures and the sum of the BEQ of the individual groups (Figure 4C), with the exception of the WWTP influent sample where the pharmaceuticals were below detection limit, which is probably an extrapolation artifact and not real. In the remaining samples, the pesticides caused approximately 60% of tBHQEQ, the pharmaceuticals 30% and the others 10%, and these proportions did not vary much during treatment despite the overall tBHQEQ varying by more than ten-fold, indicating that there was no preferential removal for any group.

4. Conclusions

A previous study had compared, qualitatively, chemical analysis with *in-vitro* and *in-vivo* bioassays and found that treatment of wastewater in the investigated plant reduced chemicals as well as effects below the detection limit (Leusch et al. 2014a). The present study confirmed previous findings of Leusch et al. (2014a) and went a step further: for the first time chemical monitoring was linked with effect-based assessment in a quantitative manner and related to the individual groups of chemicals.

Mixture toxicity modeling applying the mixture model of concentration addition, which is valid for chemicals acting according to the same mode of action, confirmed previous findings that chemicals typically present in wastewater act concentration-additive in the applied bioassays (Escher et al. 2013, Tang et al. 2013, Tang and Escher 2014). After this was confirmed, it was possible to quantify (a) which fraction of effect

could be explained by the detected chemicals and (b) which groups of chemicals influenced or even dominated the mixture effects.

Although a total of 299 chemicals were screened and a higher fraction of biological effect could be explained than in previous studies (Escher et al. 2013, Tang et al. 2013), the detected chemicals explained less than 3% of cytotoxicity and less than 1% of oxidative stress response. As in earlier work (Tang and Escher 2014), all responsible chemicals for photosynthesis inhibition were included in the analytical target list. This finding can be rationalized by the fact that pesticides explained the majority of this effect, which does not come unexpected because the pesticide group contained several highly potent photosynthesis inhibitors such as diuron, hexazinone and simazine (ESM, Table S3). What was even more interesting is the novel finding that pesticides were also responsible for around two third of the effects of the iceberg mixtures in the cytotoxicity and oxidative stress response assays. Thus it appears that in addition to a focus on endocrine disruptors (Leusch et al. 2014a), pesticide monitoring is of high relevance despite the source water is of domestic origin and Australia has a separate sewerage systems. This observation has implications for risk assessment and management. Given that even the most thorough chemical analysis could account for only a small fraction of the non-specific toxicity and adaptive stress response, we propose to always complement chemical monitoring with cell-based bioassays, which constitute efficient and high-throughput monitoring tools.

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476

477 **Appendix A. Supplementary Data**

478 Supplementary data related to this article can be found at
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480

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Figure Captions

Figure 1. Overview of the treatment processes at the Wastewater Treatment Plant (WWTP) and Advanced Water Recycling Plant (AWRP). The blue boxes denote the points where the samples were collected (text in *italics*).

Figure 2. Concentration of 65 chemicals detected in at least one water sample and used for the iceberg mixture experiments (Table S3) (from 299 analyzed chemicals and a total of 95 detected chemicals); (n) refers to the number of samples with concentrations above the limit of detection. The detected chemicals were clustered in six groups: pharmaceuticals, endocrine disrupting compounds (EDCs), pesticides, antibiotics, x-ray contrast media (XRC), and others. N refers to the number of samples that were above the detection limit. The different symbols denote the different water samples (circle: WWTP influent, diamond: WWTP effluent, square: post UV, down-facing triangle: mixing tank, up-facing triangle: RO reject, star: post RO). The black bars denote the guideline values (GV) of the AGWR (NRMMC & EPHC & NHMRC 2008). The only chemical that was included and does not have an AGWR GV is fipronil (but included in ADWG).

Figure 3. Contribution of detected chemicals for (A) non-specific toxicity as baseline-TEQ (Microtox), (B) DEQ (IPAM) and (C) oxidative stress response as tBHQEQ (AREc32). Filled diamonds represent experimental data from the present study, open diamonds represent reported data from other recycled water plants and surface water (Tang et al. 2013, Escher et al. 2014b).

Figure 4. Cumulative bioanalytical equivalent concentrations of the iceberg mixtures in comparison with the cumulative BEQs of the six chemical groups: (A) non-specific toxicity (Microtox), (B) photosynthesis inhibition (IPAM), (C) oxidative stress response (AREc32).

Tables

Table 1. Bioassays used in this study, reference chemicals for the derivation of BEQ and their effect concentrations EC.

Mode of action	Bioassay	Literature reference (assay principle) / (method applied)	Reference compound	Effect concentration EC	Bioanalytical equivalent concentration BEQ	Limit of detection[a]
Non-specific: cytotoxicity	Bioluminescence inhibition test with *Vibrio fischeri* (Microtox)	(ISO11348-3 1998)/ (Tang et al. 2013)	Virtual baseline toxicant (a model chemical with $\log K_{ow} = 3$ and a molecular weight of 300 g mol^{-1})	EC_{50} = 66 ± 6.7 mg/L	Baseline toxicity equivalent concentration (Baseline-TEQ)	0.13 mg/L
Specific: photosynthesis inhibition	Combined algae test with *Pseudokirchneriella subcapitata*	(Muller et al. 2008)/ (Escher et al. 2008)	Diuron	EC_{50} = 1.81 ± 0.45 µg/L	Diuron equivalent concentration (DEQ)	0.004 µg/L
Reactive: genotoxicity	umuC assay –S9	(ISO13828 1999)/ (Macova et al. 2011)	4-Nitroquinoline-N-oxide (4NQO)	$EC_{IR1.5}$ = 9.1 ± 3.8 µg/L	4NQO equivalent concentration (4NQOEQ)	0.10 µg/L
Reactive: genotoxicity after metabolic activation	umuC assay +S9	(ISO13828 1999)/ (Macova et al. 2011)	2-Aminoathracene (2AA)	$EC_{IR1.5}$ = 46.7 ± 27.6 µg/L	2AA equivalent concentration (2AAEQ)	0.05 µg/L

Reactive: oxidative stress	AREc32 assay	(Wang et al. 2006)/ (Escher et al. 2012)	t-butyl-hydroquinone (tBHQ)	$EC_{IR1.5}$ = 0.15 ±0.03 mg/L	tBHQ equivalent concentration (tBHQEQ)	8.64 µg/L

[a]Limit of detection calculation from the equivalent concentration caused by a effect of 3 times the standard deviation of the controls.

663 Table 2. Summary of all bioassay results expressed as bioanalytical equivalent concentrations

Sampling site / treatment	WWTP Influent	WWTP Effluent	Post UF	Mixing Tank	Post RO	Post UV	RO Reject	Lab Blank	Trip Blank
***V. fischeri* bioluminescence inhibition assay**									
Baseline-TEQ_{water} (mg L^{-1})	25.9 ± 0.72	9.15 ± 0.07	5.12 ± 0.78	5.83 ± 0.65	0.43 ± 0.09	0.74 ± 0.10	29.9 ± 1.0	0.40 ± 0.04	0.29 ± 0.0
Baseline-$TEQ_{iceberg}$ (mg L^{-1})	0.04 ± 0.02	0.11 ± 0.03	0.16 ± 0.03	0.13 ± 0.02	0.003 ± 0.004	n.t.	0.37 ± 0.07	n.t.	n.t.
Baseline-TEQ_{EDC} (µg L^{-1})	1.81 ± 1.67	0.30 ± 0.07	0.44 ± 0.10	0.04 ± 0.01	0.30 ± 0.09	n.t.	2.22 ± 0.49	n.t.	n.t.
Baseline-TEQ_{XRC} (µg L^{-1})	<0.07	<0.05	<0.05	<0.05	n.t.	n.t.	<0.2	n.t.	n.t.
Baseline-$TEQ_{antibiotics}$ (µg L^{-1})	0.15 ± 0.04	0.09 ± 0.03	0.10 ± 0.05	0.06 ± 0.04	n.t.	n.t.	0.16 ± 0.18	n.t.	n.t.
Baseline-$TEQ_{pesticides}$ (µg L^{-1})	25.4 ± 10.7	96.0 ± 34.1	132 ± 31.0	69.8 ± 35.8	0.14 ± 0.03	n.t.	252 ± 109	n.t.	n.t.
Baseline-$TEQ_{pharmaceuticals}$ (µg L^{-1})	25.6 ± 35.7	9.1 ± 2.7	7.3 ± 2.7	11.7 ± 2.7	0.94 ± 0.67	n.t.	39.0 ± 13.5	n.t.	n.t.
Baseline-TEQ_{others} (µg L^{-1})	0.05 ± 0.01	2.8 ± 0.6	4.0 ± 1.0	2.8 ± 0.9	1.1 ± 0.2	n.t.	15.8 ± 5.6	n.t.	n.t.
IPAM photosynthesis inhibition assay									
DEQ_{water} (µg L^{-1})	0.073 ± 0.023	0.033 ± 0.012	0.025 ± 0.006	0.017 ± 0.004	< 0.004	< 0.004	0.11 ± 0.02	< 0.004	< 0.004
$DEQ_{iceberg}$ (µg L^{-1})	0.10 ± 0.04	0.11 ± 0.03	0.10 ± 0.03	0.10 ± 0.03	$2.5 \pm 1.2 \times 10^{-5}$	n.t.	$< 5.5 \times 10^{-4}$	n.t.	

DEQ_{EDC} (ng L^{-1})	2.9×10^{-2}	$8.8 \pm 3.3 \times10^{-4}$	$1.2\times10^{-3} \pm 3.0\times10^{-4}$	$1.0 \pm 0.3\times10^{-4}$	$7.7 \pm 5.0\times10^{-4}$	n.t.	n.t.	n.t.	n.t.
DEQ_{XRC} (ng L^{-1})	$< 3.4\times10^{-3}$	$< 2.4\times10^{-3}$	$< 2.5\times10^{-3}$	$< 2.3\times10^{-3}$	n.t.	n.t.	$< 9.4\times10^{-3}$	n.t.	n.t.
$DEQ_{antibiotics}$ (ng L^{-1})	0.35 ± 0.15	$< 2.6\times10^{-3}$	$< 2.4\times10^{-3}$	$< 2.4\times10^{-3}$	n.t.	n.t.	$< 2.4\times10^{-3}$	n.t.	n.t.
$DEQ_{pesticides}$ (ng L^{-1})	30 ± 16	93 ± 24	60 ± 30	61 ± 35	$< 1.2\times10^{-4}$	n.t.	< 0.42	n.t.	n.t.
$DEQ_{pharmaceuticals}$ (ng L^{-1})	0.55 ± 0.36	0.25 ± 0.14	0.19 ± 0.15	0.37 ± 0.12	$< 7.8\times10^{-5}$	n.t.	1.34 ± 0.72	n.t.	n.t.
DEQ_{others} (ng L^{-1})	$< 5.2\times10^{-4}$	$< 6.0\times10^{-3}$	$< 6.6\times10^{-3}$	$< 6.2\times10^{-3}$	$< 8.2\times10^{-4}$	n.t.	$< 2.6\times10^{-2}$	n.t.	n.t.
umuC genotoxicity assay without metabolic activation									
$4NQOEQ_{water}$ (µg L^{-1})	0.56 ± 0.17	0.24 ± 0.10	0.09 ± 0.02	0.13 ± 0.07	< 0.10	< 0.10	0.62 ± 0.18	< 0.10	< 0.10
$4NQOEQ_{iceberg}$ (µg L^{-1})	$< 4.2\times10^{-3}$	$< 6.4\times10^{-3}$	$< 7.2\times10^{-4}$	$< 6.1\times10^{-4}$	$< 3.7\times10^{-6}$	n.t.	$< 2.1\times10^{-3}$	n.t.	n.t.
$4NQOEQ_{EDC}$ (µg L^{-1})	$< 3.1\times10^{-6}$	$< 3.9\times10^{-7}$	$< 4.6\times10^{-7}$	$< 4.0\times10^{-8}$	$< 2.9\times10^{-7}$	n.t.	$< 2.7\times10^{-6}$	n.t.	n.t.
$4NQOEQ_{XRC}$ (µg L^{-1})	$< 3.8\times10^{-5}$	$< 1.4\times10^{-5}$	$< 1.4\times10^{-5}$	$< 1.3\times10^{-5}$	n.t.	n.t.	$< 5.3\times10^{-5}$	n.t.	n.t.
$4NQOEQ_{antibiotics}$ (µg L^{-1})	$< 9.3\times10^{-6}$	$< 1.9\times10^{-6}$	$< 1.7\times10^{-6}$	$< 1.7\times10^{-6}$	n.t.	n.t.	$< 8.9\times10^{-6}$	n.t.	n.t.

4NQOEQ$_{pesticides}$ (µg L^{-1})	< 4.3×10^{-4}	< 1.6×10^{-1}	< 1.9×10^{-1}	< 1.5×10^{-1}	< 8.1×10^{-1}	n.t.	< 4.9×10^{-1}	n.t.	n.t.
4NQOEQ$_{pharmaceuticals}$ (µg L^{-1})	< 3.9×10^{-3}	< 5.6×10^{-5}	< 5.4×10^{-5}	< 5.4×10^{-5}	< 5.6×10^{-8}	n.t.	< 2.5×10^{-4}	n.t.	n.t.
4NQOEQ$_{others}$ (µg L^{-1})	< 2.0×10^{-6}	< 2.2×10^{-5}	< 2.5×10^{-5}	< 2.3×10^{-5}	< 3.1×10^{-6}		< 9.6×10^{-5}	n.t.	n.t.
AREc32 oxidative stress response assay									
tBHQEQ$_{water}$ (µg L^{-1})	32.4 ± 0.4	19.5 ± 7.0	< 8.64	< 8.64	< 8.64	< 8.64	73.3 ± 18.8	< 8.64	< 8.64
tBHQEQ$_{iceberg}$ (ng L^{-1})	219 ± 47	5.6 ± 1.5	5.6 ± 3.3	6.3 ± 1.4	0.15 ± 0.07	n.t.	25.7 ± 5.02	n.t.	n.t.
tBHQEQ$_{EDC}$ (ng L^{-1})	0.01 ± 0.01	0.03 ± 0.01	0.02 ± 0.01	0.0007 ± 0.0004	0.005 ± 0.001	n.t.	0.05 ± 0.06	n.t.	n.t.
tBHQEQ$_{XRC}$ (ng L^{-1})	<0.08	<0.06	<29	<27	n.t.	n.t.	<110	n.t.	n.t.
tBHQEQ$_{antibiotics}$ (ng L^{-1})	<0.05	<0.01	<0.01	<0.01	n.t.	n.t.	<0.06	n.t.	n.t.
tBHQEQ$_{pesticides}$ (ng L^{-1})	1.75 ± 0.64	2.79 ± 0.75	4.07 ± 1.65	2.85 ± 0.56	0.003 ± 0.001	n.t.	10.0 ± 3.0	n.t.	n.t.
tBHQEQ$_{pharmaceuticals}$ (ng L^{-1})	<51	1.11 ± 0.76	2.75 ± 0.56	1.44 ± 0.47	0.002 ± 0.001	n.t.	14.6 ± 6.3	n.t.	n.t.
tBHQEQ$_{others}$ (ng L^{-1})	<0.01	0.43 ± 0.18	0.48 ± 0.22	0.46 ± 64.7	0.09 ± 0.03	n.t.	1.84 ± 0.71	n.t.	n.t.

n.t. = not tested.

666 Table 3. Fraction of BEQ explained by detected chemicals ($BEQ_{iceberg}/BEQ_{water}$).

Sampling site / treatment	WWTP Influent	WWTP Effluent	Post UF	Mixing Tank	Post RO	RO Reject
***V. fischeri* bioluminescence inhibition assay**						
Baseline-$TEQ_{iceberg}$/ Baseline-TEQ_{water}	0.2%	1.2%	3.1%	2.2%	0.8%	1.3%
IPAM photosynthesis inhibition assay						
$DEQ_{iceberg}$/ DEQ_{water}	141%	323%	405%	581%	-	0.1%
AREc32 oxidative stress response assay						
$tBHQEQ_{iceberg}$/ $tBHQEQ_{water}$	0.68%	0.03%	0.07%	0.09%	-	0.04%

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