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Assessment of the efficacy of an advanced tertiary sewage treatment plant to remove biologically active chemicals using endocrine and genotoxicity bioassays

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A B S T R A C T

There has been much effort to assess the efficacy of sewage treatment plants to remove chemicals with estrogenic activity, but other modes of toxicity have received less attention. This study assessed the efficacy of advanced tertiary technologies to remove estrogen receptor (ER), aryl hydrocarbon receptor (AhR), retinoic receptor (RAR) agonists and genotoxicity using recombinant gene bioassays. Untreated sewage contained ER, AhR and RAR agonists and was genotoxic. Activated sludge treatment removed over 69% of the ER, AhR and RAR aqueous phase activities. Ozonation and biologically activated carbon processes removed genotoxicity and most receptor agonists to below detection limits. Estrogenic activity was associated with the semi-polar fraction of the aqueous phase and non-polar phase of the influent (maximum 30%). AhR and RAR activities were found in both the aqueous and particulate phases of influent. Only RAR activity was frequently found in the polar fraction of aqueous and particulate phases of the process effluent. In the influent, the greatest RAR activity was found in the polar fraction of the aqueous phase (>65%). Genotoxic activity was detected in all influent fractions but rapidly decreased. The results showed that ER, AhR and RAR agonists were greatly reduced by the advanced tertiary treatment processes.

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1. Introduction

Wastewater has been identified as a key source of micropollutants and their removal remains a key challenge for treatment processes [1]. Research on the efficacy of wastewater treatment technologies have mainly focused on endocrine disrupting chemicals (EDCs) in sewage effluent, particularly on estrogenic compounds due to global concern and their potential biological effects [2,3]. However, endocrine pathways can be disrupted through other receptor-mediated mechanisms. Many nuclear receptors have been characterised including the estrogen (ER), androgen (AR), *all-trans*-retinoic acid (RAR), and aryl hydrocarbon (AhR) and used to assess efficacy of sewage treatment plants [4,5]. The assessment of multiple receptors is important as there is a lot of 'cross talk' between receptors, and some EDCs are agonists to more than one type of receptor [6]. Some alkylphenols are not only estrogenic EDCs, but research shows that they are also RAR [7], and aromatase (CYP19) isoforms [8]. Parabens are both weakly estrogenic [9] and are RAR agonists [7]. Steroidal estrogens may be antagonists of the AhR receptor and have been shown to reduce the expression of cytochrome P450 (CYP1A1) as measured by ethoxyresorufin-O-deethylase (EROD) activity *in vitro* [10]. The mechanism is likely through direct interference with AhR agonism as it is not mediated by estrogen dependent protein transcription

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[10].

The AhR ligands have been widely studied including the halogenated aromatic hydrocarbons (HAHs) and non-halogenated polycyclic aromatic hydrocarbons (PAHs) groups of which 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) is the most potent [11]. Dietary plant compounds such as indoles and flavones, heterocyclic amines, drugs such as methylenedioxybenzenes and benzimidazole, and the pesticide carbaryl are AhR agonists [11]. The role of the AhR is not completely understood; however, the most well-defined role is in the metabolic pathway for detoxifying such chemicals and it is also involved in immune function, function of regulatory T-cells and as a transcriptional regulator in many tissue types [12]. The AhR-mediated effects include teratogenesis, immunosuppression and tumour promotion [13].

The RAR has also the potential to promote endocrine disruption. There are three forms of RARs, the α, β and γ and the natural ligands are retinoids (active forms of vitamin A) of which *all-trans* retinoic acid (*at*RA) and 9-*cis*-retinoic acid (9*c*RA) have the highest affinity for the RARs [14]. Retinoids and the RAR and the RXR are involved in a broad range of biological functions including cell differentiation, vision, immune response and embryonic development [15,16]. There is evidence to suggest that environmental exposure to RAR agonists is the cause of widespread deformities found in frogs in the USA [17]. Xenobiotic agonists of the RARγ include organochlorine pesticides, styrene dimmers, monoalkylphenols and parabens [7]. A toxicity identification evaluation (TIE) of RAR agonists in sewage effluent identified natural retinoic acid metabolites; all-*trans*-4-oxo-RA and 13-*cis*-4-oxo-RA as the causative compounds of RAR activity [18]. Of particular interest was 13-*cis*-4-oxo-RA, which had a potency 3.87 times that of *at*RA.

The aim of this study was to assess the efficacy of an operational state-of-the-art advanced tertiary sewage treatment plant (STP) to remove a range of biologically active chemicals. We used *in vitro* genetically modified yeast bioassays to measure the level of estrogen receptor (ER), aryl hydrocarbon receptor (AhR), retinoic receptor (RAR) agonists and genotoxicity activity was measured through the luminescent *umu* test in the non-polar to polar fractions of sewage effluent samples from various treatment stages within a modern multi-stage advanced STP.

2. Materials and methods

2.1. Sample collection and preparation

The Gerringong-Gerroa sewage treatment plant (GGSTP) includes BioDenipho biological treatment, sand filtration, ozonation and biological activated carbon filtration (ozone/BAC), microfiltration, and UV disinfection. All technical details about the GGSTP have been previously described [19,20]. On 24th January and 7th September 2007 samples representing high and low flow seasons were collected at GGSTP from each stages of the treatment train: raw influent, post-DN (denitrifying) tank (after Biodenipho biological treatment), clarifier, sandfilter, ozone contactor/biological activated carbon filtration, UV disinfection and from the final effluent storage dam (a diagram of the sampling sites at the GGSTP is provided in Fig. 1 of [20]. Samples were collected in solvent rinsed amber glass bottles and kept at 4 °C for less than 24 h until extracted using a previously described method [21]. To each 1.0 L sample from each treatment stage, 10 mL of acetic acid:H_2O:MeOH (1:9:90) was added before being filtered through 1.2 μm pore size Whatman glass fibre GF/C filters. The filtered samples were extracted using solid phase extraction (SPE) Empore SBD-XC disks for the January sampling and Empore C18-FF disks for the September samples. The full 1.0 L (+10 mL preservative) was extracted for all samples except the raw sewage influent sample, in which 100 mL was extracted. Extractions were performed on a multi-station glass filtration manifold. Extraction disks were preconditioned with 10 mL of MeOH and 20 mL of purified H_2O before drawing the sample through under vacuum at a flow rate of approximately 200 mL/min. The extraction disks were dried on a slide warming tray for at least an hour at 35 °C and individually wrapped in aluminium foil. The dried disks were sent to the National Institute for Environmental Studies (NIES) in Tsukuba, Japan for assaying using the hERα (human estrogen receptor), medERα (Japanese medaka fish; *Oryzias latipes* estrogen receptor) and RARγ (human retinoic acid receptor) two-hybrid yeast assays and the human aryl hydrocarbon receptor (AhR) recombinant yeast assay. Some of the glass fibre filters were also dried and sent to Japan for extraction and analysis by the yeast bioassays. The September samples were additionally analysed for genotoxicity using the luminescent *umu* test.

2.2. Sample fractionation process

The dry Empore disks were each extracted in a glass filtration manifold with 10 mL of MeOH. The disks were soaked and then the MeOH was drawn through under vacuum and into a screw cap glass centrifuge collection tube. The dry Whatman GF/C glass fibre filters were placed in a 50 mL screw cap glass tube with 20 mL of MeOH and ultrasonicated for 10 min. After ultrasonication, a 10 mL subsample of the extract was filtered through a Millex-HN filter (0.45 μL pore size Nylon) into a 10 mL glass screw cap centrifuge tube.

After the extraction procedure, the concentrated extracts from both the particulates and aqueous phases underwent solvent polarity-based fractionation. The sample (in MeOH) was evaporated to dryness under a gentle stream of N_2 gas and resuspended in 1 mL 3:1 hexane:DCM and quantitatively transferred onto a 500 mg 3 mL Bond-Elut FL florisil cartridge (Varian). Drawn through under vacuum, the eluate from the florisil column was collected in a glass centrifuge tube, and washed through with an additional 2.5 mL of hexane:DCM (non-polar fraction). A second fraction (semi-polar) was collected in a separate glass centrifuge tube by washing 5 mL of 1:9 mix acetone:DCM through the florisil column under vacuum and a final fraction (polar) was collected in a third glass centrifuge tube by washing through the florisil column with 5 mL of MeOH. The three fractions were then evaporated under a gentle stream of N_2 gas and redissolved in 100 μL of DMSO. The glass fibre filter samples were fractioned using the same method as that of the Empore disk extracts.

2.3. Yeast assays

The two-hybrid yeast assay system in *Saccharomyces cerevisiae* Y190 used was developed and modified to a high throughput method for analysing environmental samples where the yeast was transfected with either the human ERα, the medaka fish (*Oryzias latipes*) ERα or the human RARγ [22,23]. The rationale for using human and fish ERα is that there is evidence for species differences including affinity to EDCs [24]. The yeast contains an expression plasmid with the lacZ gene downstream of the receptor response element and the production of β-galactosidase is quantified as the measure of transcriptional activity.

The hER, medER and RARγ two-hybrid and AhR recombinant yeast bioassays were conducted on the three sample fractions using the protocol described [7,23,25] and used a miniaturised 96 well plate set up and chemiluminescent detection. The only variation was the standard; 17β-estradiol (E_2) was used for the hER and medER two-hybrid yeast assay, all-trans retinoic acid (*at*RA) for the RAR two-hybrid yeast assay and the standard for the AhR

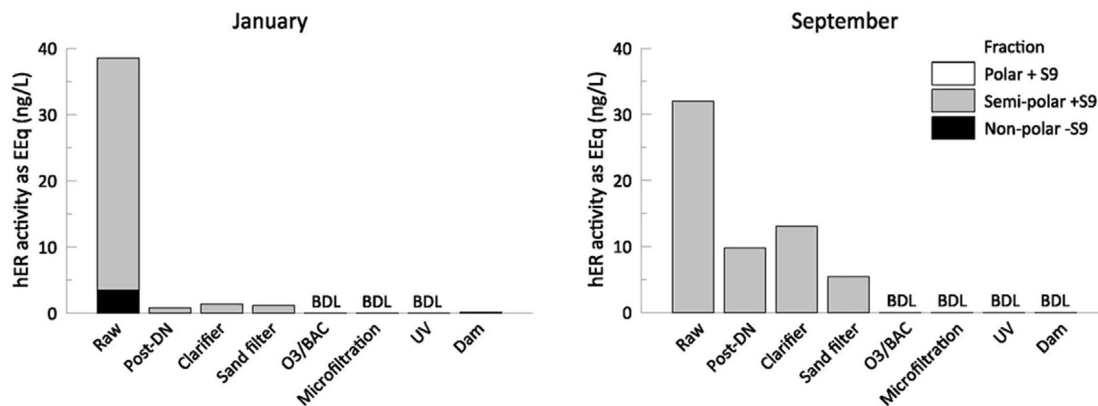

Fig. 1. Results of the hER two-hybrid yeast assay are the EC_{x10} of the sample fractions expressed as the equivalent concentration of estradiol (ng/L) for the 2 sampling events. White, grey, and black bars represent the responses in the polar, semi-polar, and non-polar fractions, respectively.

recombinant yeast was β-naphthoflavone.

2.4. Luminescent umu genotoxicity bioassay

A high throughput standardised luminescent umu test using genetically modified *Salmonella typhimurium* TL210 [21], was used to assess genotoxicity. The application of the luminescent *umu* test to environmental samples is based on the method previously described [26,27] with modifications to a more rapid, high-throughput assay, using a standard reference material [21]. The method used a stain of *S. typhimurium* TL210 that has been transfected with the plasmid construct pTL210, containing the luxA-E luminescent genes (from the luminescent bacteria *Vibrio fisheri*) downstream of *umu* D, C gene.

3. Results

3.1. hER and medER bioassays

Most of the estrogenicity measured by the hER two-hybrid assay was in the semi-polar fraction of the influent samples (Fig. 1). The non-polar fraction contributed 9% of the total 38.5 ng/L estrogen equivalent (EEq) in the January influent sample. The non-polar fraction of the influent sample contributed more frequently to the total estrogenicity as measured by the medER yeast bioassay. Both influent samples and the secondary stages of the September sampling had quantifiable activity in the non-polar fractions (Fig. 2). The medER yeast also measured low level activity (<0.91 ng/L EEq) in the polar fractions of the September samples from secondary treatment stages. The majority of the estrogenicity was measured in the semi-polar fraction regardless of treatment stage or ER type. There was a trend of increasing removal of estrogenicity with increasing number of stages of treatment as measured by the hER and medER yeast bioassays. No estrogenicity was detected in fractions following the O_3/BAC treatment stages (Figs. 1 and 2). Low responses were measured in the semi-polar fraction of the January dam sample at 0.17 and 1.1 ng/L in the hER and medER yeasts, respectively. Overall, the medER results show that it responded more strongly to the extracts than the hER.

3.2. AhR bioassay

AhR activity in the influent was different between the two sampling events. In January, the influent concentrations were only 11 and 71 ng/L β-naphthoflavone equivalent (β-NF Eq) in the non-polar and semi-polar fractions, respectively. In the September sample the response in the non-polar fraction (96 ng/L β-NF Eq) was higher than that of the combined total response of the January fractions while the semi-polar fraction had a response of 1396 ng/L β-NF Eq, which was 20 times higher than that in the January sample. The polar fraction of the January raw sewage influent sample was toxic to the yeast and the response of the assay could not be quantified. Interestingly, an Empore C18-FF replicate extraction of the raw influent had toxicity in both the semi-polar

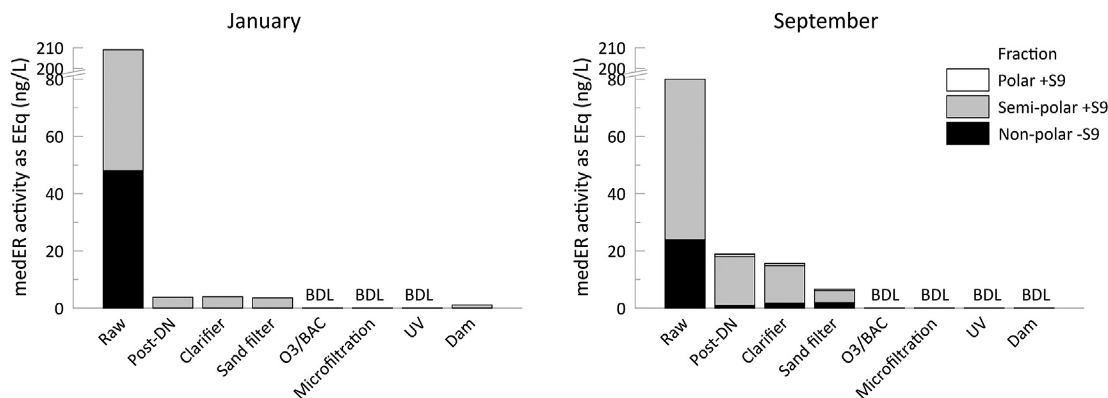

Fig. 2. Results of the medER two-hybrid yeast assay are the EC_{x10} of the sample fractions expressed as the equivalent concentration of estradiol (ng/L) for the 2 sampling events. White, grey, and black bars represent the responses in the polar, semi-polar, and non-polar fractions, respectively.

and polar fractions, indicating that there may be more toxic compounds co-extracted when using C18 disks than compared to SDB-XC disk. Concentrations in the secondary treatment stages of post-DN through to sand filter were more similar between seasons with the combined response in the September sample being only 58–96% higher than that of the January sample (Fig. 3). Secondary treatment samples had AhR responsive compounds in all three fractions, although the majority was eluted in the semi-polar fraction. In January, lower levels of response were measured in all fractions of the advanced tertiary treatment stages, which were lower after O_3/BAC treatment than in the final effluent. In the September, despite the much higher initial influent concentrations measured and higher responses of the secondary treatment samples than those of the January samples, advanced tertiary treatment samples had AhR responsive activity consistently below the detection limit (4.0 ng/L β-NP Eq). During both sampling events the dam sample displayed some activity in the AhR yeast, with a total of 43 and 22 ng/L β-NF Eq in January and September, respectively. It must be noted that the AhR yeast responses were the only bioassay that required blank correction as the non-polar and semi-polar fractions had quantifiably activity in the procedural blanks of 3.1 and 3.0 ng/L β-NF Eq, respectively.

3.3. RAR bioassay

RAR activity of the influent was found in all three polarity fractions during both sampling events (Fig. 4). The highest activity was measured in the polar fraction with 98 ng/L *at*RA Eq activity in the polar fraction of the September influent, and at least 116 *at*RA Eq in the polar fraction of the January influent. The polar fraction of the January influent was affected by toxicity and the true RAR activity is likely higher than the 116 *at*RA Eq measured. The secondary treatment stages of post-DN, clarification and sand filtration had low level responses in the polar fraction in the September sample and in the polar and semi-polar fractions in the January sample. Samples from the advanced tertiary treatment stages showed no detectable RAR yeast response, expect for the January UV sample that recorded 3.4 ng/L *at*RA Eq in the polar fraction. The January dam sample also had an elevated response in the polar fraction of 33 ng/L *at*RA Eq, whereas there was no detectable activity in the September dam sample.

3.4. Umu test

The September sample was analysed for genotoxic potential using the luminescent *umu* test. The results of the *umu* test have two components – 1) direct genotoxicity as measured by the response in the assay when run without S9 mammalian metabolic activation mixture, and 2) genotoxicity that may occur due to metabolic activation. Only the raw influent and sandfilter samples displayed direct genotoxicity with the non-polar fraction inducing responses equal to exposure of 0.31 ng/L and 0.01 ng/L of 4NQO, respectively. Metabolically activated genotoxicity was detected in samples of the raw influent, post DN, clarifier and sandfilter stages in the semi-polar fraction, and in the polar fraction of the influent and post-DN tank samples (Fig. 5). After O_3/BAC treatment and including the final storage dam, there was no detectable genotoxicity in any of the three fractions of these samples. The raw sewage influent had a much higher response than the other three stages where genotoxicity was measured, and O_3/BAC treatment removed the low levels of response remaining after secondary treatment.

3.5. Activity of particulate material

Particulate material was analysed for a limited number of samples. Samples containing particulate material were collected from the sandfilter, BAC, microfiltration treatment stages, and the dam in January and from the raw influent, post-DN tank and the dam in September. The highest response measured in the particulate fraction was that of the AhR yeast when exposed to the raw influent sample with a combined response of 860 ng/L β-NP Eq, however the majority (680 ng/L) of the response was from the semi-polar fraction. The high response in the particulate fraction in the AhR yeast, coincided with the high response in the aqueous phase. The particulate phase accounted for 38% of the total AhR response in the raw influent. Quantifiable responses in the particulate phase were measured in the AhR yeast for the post-DN, sand filter, BAC and both dam samples, although not for the microfiltration sample. This indicates decreasing particulate AhR activity with increasing treatment (Fig. S1). Particulate AhR activity was often a major contribution to the total AhR activity and was anywhere from 11 to 56% of the total activity (Table 1).

RAR activity of the particulate fraction was the only instance where the raw sewage influent response was not the highest response for all treatment stages. The sand filter sample had a higher response in the RAR yeast than that in the influent with a total of 20 ng/L compared to 7.3 ng/L *at*RA Eq, respectively (Fig. S2). Most of the response in the sandfilter sample was detected in the semi-polar phase, while the response in the raw influent sample was entirely in the non-polar fraction. This suggests that the activated sludge treatment removes most of the influent RAR activity as there was no detectable RAR activity in the post-DN stage and

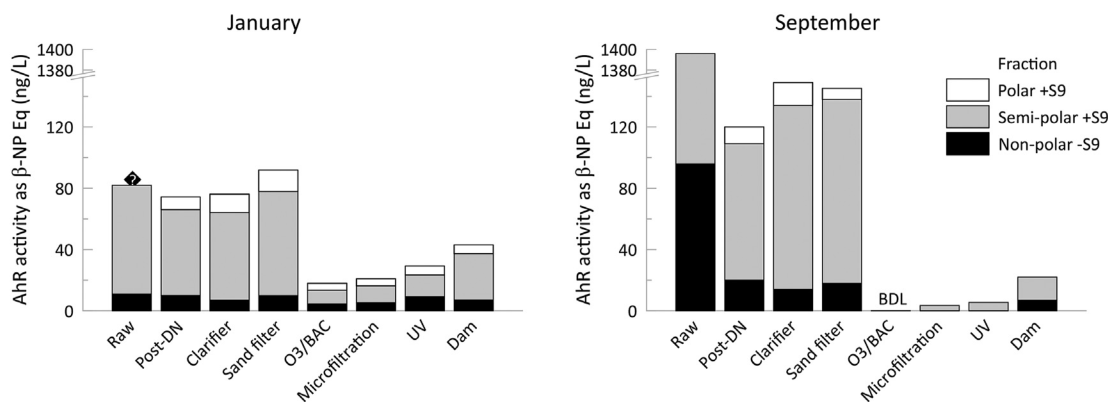

Fig. 3. Results of the AhR two-hybrid yeast assay are the EC_{x10} of the sample fractions expressed as the concentration equivalent to β-naphtoflavone. White, grey, and black bars represent the responses in the polar, semi-polar, and non-polar fractions, respectively.

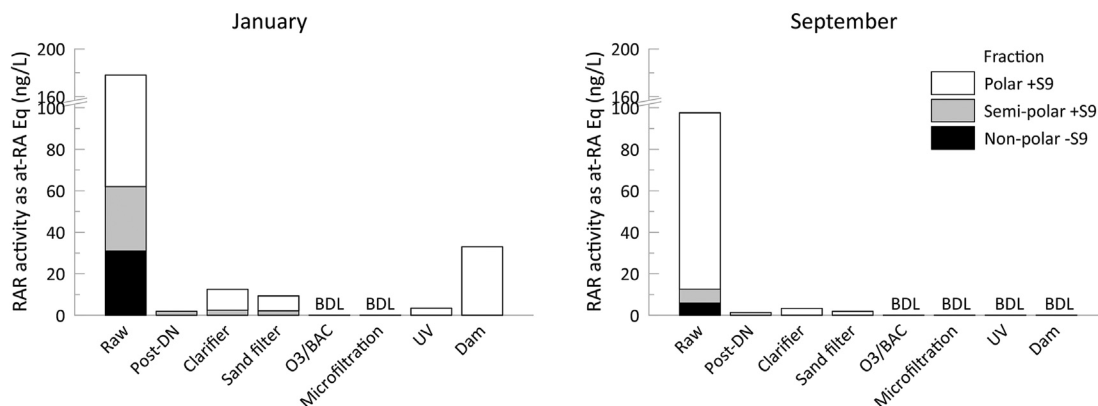

Fig. 4. Results of the RAR two-hybrid yeast assay are the EC_{x10} of the sample fractions expressed as the equivalent concentration of all-trans retinoic acid (ng/L). White, grey, and black bars represent the responses in the polar, semi-polar, and non-polar fractions, respectively.

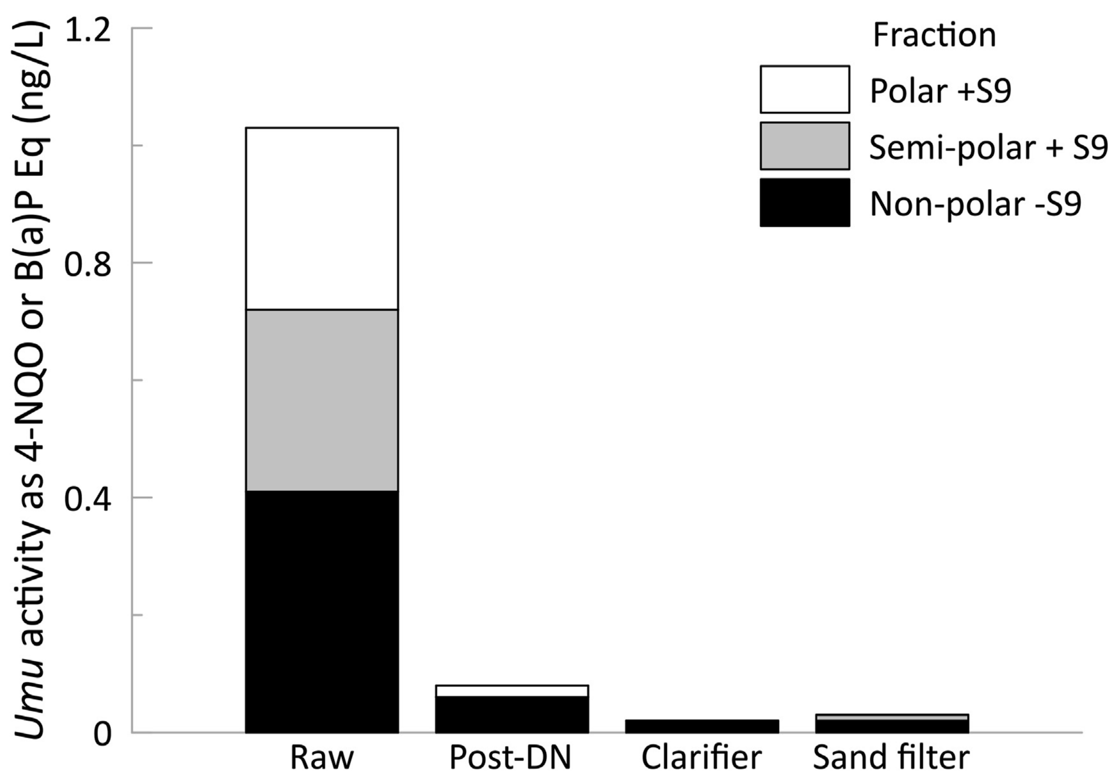

Fig. 5. Genotoxicity as measured by expression of the umu gene in *S. typhimurium* TL210. Sample responses are expressed as the equivalent concentration in either 4-nitroquinoline-1-oxide (4NQO) in ng/L for the samples not incubated with metabolic activator (−S9) and as benzo(a)pyrene (BaP) in ng/L for samples incubated with metabolic activator (+S9) for the September 2007 samples.

Table 1
Relative percentages of the total AhR or RAR yeast response between the particulate and aqueous phases for the total response of the three solvent fractions. n.a. = not applicable as both phases were below the detection limits.

	AhR		RAR	
	Particulate	Aqueous	Particulate	Aqueous
Raw	38	62	7	93
Post-DN	40	60	0	100
Dam 1	56	44	0	100
Sand filter	11	89	69	31
O3/BAC	23	77	100	0
Microfiltration	11	89	n.a.	n.a.
Dam 2	12	88	12	88

only a small proportion of the sandfilter response was in the non-polar fraction (0.44 ng/L *at*RA Eq, 6%). The elevated response in the sandfilter stage also equated to a much higher percentage of the total RAR activity in the sandfilter sample being present in the particulate phase (69%). RAR activity was detected in the BAC sample and the January dam sample, but not in the microfiltration sample, indicating that microfiltration removed the low level particulate response that remained after O_3/BAC treatment. The BAC sample had 100% of the RAR activity in the particulate phase, with no detectable activity in the aqueous phase.

The only estrogenic response detected in the particulate fraction was 77 ng/L EEq measured in the non-polar fraction of the raw sewage influent when tested with the medER two-hybrid yeast.

This was almost equal to the combined response of the aqueous phase for this sample. The same sample did not have a corresponding particulate phase response in the hER two-hybrid yeast and 100% of the estrogenicity measured by the hER yeast was in the aqueous phase.

4. Discussion

Assessing the efficacy of sewage treatment is important to minimise impacts on receiving environments using bioassays based on multiple mechanisms of toxicity [5,28]. The presence and removal of estrogenic activity from the sewage influent was as expected from the previous assessment of the GGSTP [20]. Both hER and medER results reflected the fact that estrogenic EDCs are amenable to biodegradation through activated sludge treatment, but not completely removed. Advanced tertiary treatment was able to remove estrogenic activity in the wastewater to below detection limits. Only the influent had appreciable estrogenic activity found in the non-polar phase. Of the low levels of estrogenicity measured in the influent of a Beijing reclamation pilot plant, non-polar and polar fractions had similar levels of estrogenicity [29]. During treatment, varying levels of estrogenic activity was found in the different polarity fractions, with the semi-polar fraction only dominating after secondary treatment and ozonation. It is likely that this reflects the complexity of influents between the two STPs, as GGSTP is of a purely domestic origin and contains high concentrations of steroid estrogens in the influent which generate 10's-low 100's of ng/L EEqs. The Chinese study reported estrogenicity in the pg/L range, which is extremely low for STP influent [29].

The only unexpected result was the activity (77 ng/L) detected in one influent sample in the non-polar fraction of the particulate component by only the medER two-hybrid yeast. The non-polar fraction is supposed to contain a greater portion of synthetic phenols which are more potent in the medER assay. The magnitude of the response was particularly high considering the fact that no hER activity was detected and the potencies of synthetic phenols in the medER are still at least 1000 times less than E_2 (see Table 4.2)." to " [20]. Low levels of activity have been found in the particulate phase of other wastewater influents at levels less than 15% of the total estrogenicity of the influent [30]. Another point worth noting is that the response of the medER was much more pronounced than the hER which suggests the presence of ligands of higher affinity for the fish receptor [24].

Due to the hydrophobicity of AhR agonists, most studies have either focused on sediments, landfill leachates, incineration waste or on wastewater sludge, particularly from industrial waste [31]. Secondary treated STP effluent in Tunisia used for irrigation had AhR activity in the HeLa cell line equal to 319.5 pM (102.9 ng/L) TCDD Eq/L [32], while in an advanced wastewater treatment plant in south-east Queensland, Australia influent EC_{20} was 0.83 ng/L TCDD Eq in the AhR CAFLUX assay and was reduced to 0.33 ng/L TCDD Eq after coagulation, flocculation, DAFF (dissolved air floatation filtration), sand filtration, O_3 and BAC treatments [33]. At that stage, the final effluent response was not significantly different to that of the blank. Similarly to the AhR response of samples collected after clarification and sandfiltration in the current study, Macova et al. [33] also noted that sandfiltration did not reduce AhR activity after secondary treatment.

In our study, advanced tertiary treatment had variable results in reducing AhR activity to below detection limits. The January sample had low levels of activity remaining in all three fractions after O_3/BAC treatment while for the September sample, AhR activity was reduced to less than 4.0 ng/L β-NP Eq. This may be due to poor performance of the BAC treatment at this sampling period as other studies found that ozonation alone appeared to be unable to completely remove AhR activity. Macova et al. [33] found that the activity after ozonation was not significantly different to that after secondary treatment but a spike in activity was reported after ozonation in another study [29]. Both studies found that biological treatment applied after ozonation was able to significantly reduce AhR activity. This is comparable to the results of the current study that showed that secondary treated effluent had a higher AhR activity that was then reduced by the combination of O_3/BAC treatment.

In another study, the polar fraction had the most AhR activity, at around 8 pg/L TCDD Eq (as measured by EROD in H4IIE rat hepatoma cells), the non-polar fraction had about one third of the activity of the polar fraction and the semi-polar fraction had less than one tenth the activity of the polar fraction [29]. This is different to the results of the current study where the majority of AhR activity in the influent was measured in the semi-polar fraction and no activity was detected in the polar fraction. The different fraction results are likely due to the different polarity of the solvent mixture used to partition the semi-polar fraction.

In Croatia, untreated sewer effluent of domestic and industrial origins as well as street run-off had an average EROD response in rainbow trout hepatocytes of 6.4 ng/L TCDD Eq [34]. When applying a fractionation procedure using HPLC, AhR activity was detected in 12 out of the 30 different fractions of which the nine most polar contained no activity [34]. This is more comparable to the results of the current study and identified likely causative PAHs. Despite identifying many contributing compounds, only 12% of the activity could be attributed to the concentrations of the measured agonists.

Converting the β-NP Eq response data generated in our study to a TCDD Eq is not practical due to adsorption problems associated with plastic containers. The potency of TCDD is dependent on the material used in the bioassay as TCDD has a higher potency than β-NP in glass than plastic in the AhR yeast assay [35]. It was found that the human AhR receptor yeast had an EC_{50} of 10 nM for TCDD and 5 nM for β-NP [36]. Although theoretically the molar responses of the effluent samples in the current study could be doubled to convert to TCDD Eq for comparison with other studies, it will probably produce unreliable outcomes. This is one of the disadvantages of using the yeast-based assay, as TCDD is the accepted standard for reporting equivalent AhR activity.

Removal of RAR activity was effectively achieved by activated sludge treatment with O_3/BAC treatment on both sampling occasions. The influent had a high proportion of the RAR activity associated with the polar fraction of the aqueous phase, which contrasted with influent where ER and AhR activities were primarily measured. Most of the RAR activity was in the aqueous phase except in the sandfilter sample, which had a higher semi-polar particulate response than the total response of the aqueous phase. Few studies have been conducted on RAR activity in wastewater. An environmental study in Japan showed that two-hybrid yeast RARα activity in rivers was not elevated downstream of STPs and that RAR activity was more likely to be caused by general surface run-off [37]. One of the STP studies found that across seven STPs in China, influent RAR activity is mostly found in the semi-polar fraction with no significant activity found in the polar fraction [18]. This is different to the results of this study where variable activity between fractions was found. Zhen et al. [18] also detected antagonistic effects in influent samples. Spiking experiments showed that whole (unfractionated) samples had 86–103% inhibition. The concentration of RAR activity measured in the influent ranged from 6.6 to 13.4 ng/L *at*RA Eq [18], which is approximately 10 times lower than what was measured in our study. The higher RAR activity measured coupled with the presence of activity in more fractions probably indicates a wider range of RAR agonists in our study compared to the retinoid metabolites all-

Table 2
Summary of the data from all the bioassays from the 2 sampling events. The shading level represents the relative activity for the endpoint measured from high activity (black) to below limit of detection of the assay (light grey). For the *Umu* test, only 4 fractions were tested from the September sample.

Assay	Fraction	January (summer)								September (autumn)							
		Raw	Post-DN	Clarifier	Sandfilter	O_3/BAC	μFilter	UV	Dam	Raw	Post-DN	Clarifier	Sandfilter	O_3/BAC	μFilter	UV	Dam
hER	Polar	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
	Semi-polar	+++++	++	++	++	−	−	−	−	++++	++	++	++	−	−	−	−
	Non-polar	+++	−	−	−	−	−	−	+	−	−	−	−	−	−	−	−
medER	Polar	−	−	−	−	−	−	−	−	−	+	+	−	−	−	−	−
	Semi-polar	++++	+	+	+	−	−	−	+	++++	++	++	−	−	−	−	−
	Non-polar	+++	−	−	−	−	−	−	−	+++	+	+	+	−	−	−	−
AhR	Polar	+	+	+	+	+	+	+	+	−	+	+	+	−	−	−	−
	Semi-polar	++++	++++	++++	++++	++	++	++	++	++++	+++	+++	+++	−	++	++	++
	Non-polar	++	++	++	++	++	++	++	++	+++	++	++	++	−	−	−	++
RAR	Polar	++++	−	++	++	−	−	++	+++	++++	+	+	+	−	−	−	−
	Semi-polar	+++	−	−	−	−	−	−	−	+	−	−	−	−	−	−	−
	Non-polar	+++	+	+	+	−	−	−	−	+	−	−	−	−	−	−	−
Umu	Polar																
	Semi-polar																
	Non-polar																

trans-4-oxo-RA and 13-cis-4-oxo-RA identified by Zhen et al. [18]. Although the influent concentrations were higher, the advanced tertiary treatment processes reduced effluent RAR activity to levels similar or lower than those measured in the secondary treated effluents [18]. A laboratory study compared the efficacy of different advanced treatment to reduce RAR activity after secondary treatment [37]. The authors found that the most effective treatments for reducing RAR agonist activity was RO or NF and removal by ozonation > MF > UV > coagulation with ferric sulphate ≫ coagulation with PAC. RAR activity was completely removed from secondary effluents by ozone treatment even at a low dose of 2 mg/L [38]. UV and ultrafiltration provided little removal, while chlorination and RO were only able to remove about 75% of the activity.

AhR and RAR activity in the particulate phase appeared to have no relationship to each other. AhR activity in the recombinant yeast test had a high response in the influent and appeared to be progressively reduced by the treatment process. Conversely, RAR activity was lower in the influent than the sandfilter despite having been undetectable in the post-DN tank. Low levels were still present after O_3/BAC treatment, but no activity was detected in any fraction after microfiltration. Both the AhR and RAR activities were preferentially associated with the aqueous phase during treatment of the effluent. For the AhR activity the particulate phase still contained a reasonably high proportion of the activity (up to 40%) and RAR activity in the sandfilter and O_3/BAC treatment stages were preferentially associated with the particulate phase. The RAR activity in the sandfilter and O_3/BAC particulate phase may indicate the generation of hydrophobic RAR agonists during the treatment process. The results show that the advanced tertiary treatment process reduces the presence of AhR and RAR active compounds in particulate phase; however, as there is no replication of the particulate sample analysis, these results should be interpreted with caution.

No studies were found in the literature that analysed sewage effluent particulate material for RAR activity. Only one study analysed the partitioning of AhR agonism in sewage effluent between the aqueous and particulate phases using the AhR transfected cell line HAhLP [30]. For the three STPs studied, the particulate fraction of the influent had a higher AhR activity by mass (μg/g) than the sludge, and a higher activity per volume (μg/L) than the aqueous phase when taking into account the volume of influent used to obtain the particulate sample. This is different to the current study where the aqueous phase of the influent had higher activity than the particulate fraction. In this study, a 1.2 μm pore size filter was used, which would allow colloidal material to be associated with the aqueous phase, whereas Mnif et al. [30] used centrifugation to separate particulates from the aqueous phase.

The *umu* luminescent genotoxicity assay measures the potential of genotoxic, mutagenic and carcinogenic effects as upregulation of the *umu* gene is in response to lesions in the DNA. The method used in this study uses a standard to correct for inter-assay variability. The study of a sewage treatment plant in Beijing, China using 4-NQO to standardise measurements of luminescent *umu* tests reported higher activities than our study at the equivalent level of treatment [39]. The final effluent after AS treatment, coagulation and sandfiltration had a genotoxic response of 7.0 ng/L 4-NQO, which is 700 times higher than the activity measured in samples collected after sandfiltration and seven times higher than the total genotoxic activity in the influent of the current study.

Although the magnitude of response in the *umu* assay cannot be directly compared to studies not using a standard, the performance of treatment for removing genotoxic activity can be compared. At the GGSTP the influent genotoxicity was much higher than that after activated sludge treatment, clarification and sandfiltration. After the O_3/BAC treatment no genotoxicity was detectable regardless of whether metabolic activation was used. Some studies have found that the influents of some STPs are not genotoxic under the assessment criteria of the 1.5 sample: negative control ratio [40]. However, this is clearly dependent on influent characteristics. In a German study, only one out of five STP influents tested was not genotoxic and the differences between samples assayed with and without metabolic activation also varied between STPs [41]. Although all the STPs studied [41] had secondary effluents that were not above the 1.5 ratio, a study [40] found one STP that had no genotoxic activity in the influent, had genotoxic secondary treated effluent (measured in the -S9 assay). Daily measurements of samples from secondary STP effluent consistently showed no genotoxicity in the −S9 assay but was genotoxic when metabolic activation was applied (+S9) [42]. In an advanced STP genotoxicity was not significantly different in samples between the influent and after denitrification and ‘pre-ozonation’ treatments [33]. After coagulation/flocculation/DAFF and ozonation, genotoxicity was still detectable in the samples, but was significantly lower than that in the influent. However, it was not until after BAC treatment that genotoxicity of the sample was undetectable, which concurs with the genotoxicity findings of the effluent from the GGSTP. Removal of genotoxicity by ozone treatment improves linearly with

increasing ozone dose and removal to a level not different to the negative control can be achieved when applying ozone to secondarily treated effluent [39]. However, a higher ozone dose is needed to remove genotoxicity compared with that needed to remove RAR activity.

5. Conclusions

The bioassays results are summarised in Table 2 and show that the efficacy of the treatment was not influenced by season as reported previously [20]. The sewage influent at the GGSTP contained AhR, RAR, ER active and genotoxic compounds. Out of all the analyses, it was only the AhR activity on one sampling day that was not fully removed by activated sludge treatment and even persisted after O_3/BAC treatment in both the aqueous and particulate phases of the effluent. No activity was detected for hER, medER, AhR and RAR in particulate phase after microfiltration but further investigations are required to fully assess the risk. The advanced tertiary treatment processes removed the receptor agonists and genotoxicity activities in the effluent. Some activity was detected in the holding dam suggesting other sources of contamination that could likely originate from storm water, leaching from lining material or natural compounds.

Declaration of competing interest

The authors declare no conflict of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.emcon.2021.03.003.

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