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Evaluation of analytical methodology for the detection of hormones and their attenuation during aquifer recharge and recovery cycles

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Abstract

The hormones listed in the screening survey list 2 of the Unregulated Contaminant Monitoring Rule 3 (estrone, 17-β-estradiol, 17-α-ethynylestradiol, 16-α-hydroxyestradiol (estriol), equilin, testosterone and 4-androstene-3,17-dione) were analyzed by liquid chromatography electrospray ionization tandem mass spectrometry (LC-ESI-MS/MS). Two analytical methods were compared: EPA Method 539 and the Isotope Dilution Method. EPA Method 539 was successfully utilized in river and drinking water matrices with fortified recoveries of 98.9 to 108.5%. Samples from the Hillsborough River reflected levels below the method detection limit (MDL) for the majority of the analytes, except estrone (E1), which was detected at very low concentrations (<0.5 to 1 ng/L) in the majority of samples. No hormones were detected in drinking water samples. The Isotope Dilution Method was used to analyze reclaimed and aquifer storage and recovery (ASR) water samples as a result of strong matrix/solid phase extraction (SPE) losses observed in these more complex matrices. Most of the compounds were not detected or found at relatively low concentrations in the ASR samples. Attenuation of 50 to 99.1% was observed as a result of the ASR recharge/recovery cycles for most of the hormones, except for estriol (E3). Relatively stable concentrations of E3 were found, with only 10% attenuation at one of the sites and no measureable attenuation at another location. These results have substantiated that while EPA Method 539 works well for most environmental samples, the Isotope Dilution Method is more robust when dealing with complex matrices such as reclaimed and ASR samples.

Environmental impact

The presence of hormones in the environment and drinking water is a public concern. In order to adequately gauge the impact of these compounds on the environment, analytical methods with low detection limits are desired. Two methods were tested and optimized to achieve the highest sensitivity with simultaneous extraction of the target compounds. Matrix interferences were minimized and sensitive quantification of environmental samples was achieved using LC-ESI-MS/MS. Concentrations and attenuation of Unregulated Contaminant Monitoring Rule 3 (UCMR 3) listed hormones from aquifer storage and recovery (ASR) samples in Florida are reported here for the first time. The disclosure of these environmental data may aid

the scientific community and governmental agencies to define environmental standards and regulatory rules for these contaminants.

Introduction

Concerns over contamination of water resources by endocrine-disrupting chemicals (EDCs) have quickly prompted intensive research to better understand the removal of these compounds by drinking and wastewater treatment facilities.[1-5]

The seven hormones, estrone (E1), 17-β-estradiol (E2), 17-α-ethynylestradiol (EE2), 16-α-hydroxyestradiol (estriol, E3), equilin (EQ), testosterone (TT) and 4-androstene-3,17-dione (A4), investigated in this study are listed in the screening survey list 2 of the Unregulated Contaminant Monitoring Rule established in the Federal 1996 Safe Drinking Water Act (SDWA). Public Water Systems (PWS) and the United States Environmental Protection Agency (USEPA) have been performing pre-screen testing, screening surveys and assessment monitoring for a 12-month period between 2013 through 2015 as established in the UCMR 3.[6] As a result of the UCMR 3, the City of Tampa Water Department has been working on analytical methods for the determination of hormones in various water matrices.

EDCs, particularly natural and synthetic hormones, have been detected in environmental samples and are being continually introduced to the aquatic environment as complex mixtures via a number of routes.[1, 7] They may enter the environment via domestic or industrial wastewater discharge, application of biosolids from wastewater treatment processes, leaching from landfills and septic tanks, terrestrial runoff, and agribusiness among others pathways.[1, 7]Concentrations of hormones in aquatic environments usually vary from non-detectable to low nanograms per liter depending on local discharges and environmental conditions.[8-11] Some studies evaluating areas under heavy industrial and municipal wastewater influence reported hormones at higher levels, e.g., E2 concentration was up to 175 ng/L in Venice Lagoon, Adriatic Sea[12]; and E1 and E2concentrations up to 180 and 134 ng/L respectively in Licun River-Jiaozhou Bay, Qingdao, China.[13]

Some of the EDCs have been frequently detected in surface waters.[6] In a nationwide reconnaissance study, the U.S. Geological Survey detected TT in 2 of 70 (2.85%) samples at a median concentration of 116 ng/L and a maximum concentration of 214 ng/L.[14] An evaluation of hormones in aquatic environments found E1, E2, TT and A4 in a dairy waste lagoon at concentrations as high as 650 ng/L and sporadic presence of some hormones in nearby surface waters.[15] Hormones were detected in 86% of samples from rangeland creeks where cattle had direct access to the water. Concentrations as high as 44 ng/L were observed in surface waters shortly after rain events at the beginning of the winter wet season in California. Hormones were present at concentrations above the predicted no-effect concentrations in 10−20% of the fish samples. A4 was detected at concentrations higher than the response threshold for pheromonal communication in fish.[16] The detection of EDCs in the environment is concerning as some, such as TT and A4, have been shown to be relatively resistant to oxidation.[17]

EPA Method 539 for the Determination of Hormones in Drinking Water by Solid Phase Extraction (SPE) and Liquid Chromatography Electrospray Ionization Tandem Mass Spectrometry (LC-ESI-MS/MS) describes procedures for the extraction and quantification of seven hormones in drinking water.[18] Matrix suppression/enhancement, SPE losses and other analytical interferences may be present when working with matrices more complex than drinking water such as reclaimed or aquifer storage and recovery (ASR) water.[1, 18-20]Matrix effects may be caused by contaminants that are co-extracted from a sample. This can result in suppression or enhancement of the target analyte signal. Matrix components can directly interfere by producing a signal at or near the retention time of an analyte peak. The extent of matrix interferences will vary considerably from source to source, depending on the characteristics of the water. Humic and/or fulvic material in environmental samples may be co-extracted during SPE and can cause enhancement and/or suppression within the electrospray ionization component. Electrospray ionization (ESI) is known to be particularly predisposed to matrix suppression and isobaric interference when analyzing hormones. The highly efficient chromatographic separation as well as extensive purification steps are necessary to remove interferences arising from the matrix.[20-23]

The purpose of this study was to apply, compare and evaluate two analytical methods, EPA Method 539[18] and the Isotope Dilution Method[19], for the analysis of both synthetic and natural hormones. The analyses were performed with water samples from different sources including drinking, river, and reclaimed water before being recharged to ASR wells and water recovered from ASR wells after an extended period of storage in the aquifer. This information will provide a better understanding of the natural attenuation of hormones as a result of ASR programs.

Materials and Methods

Chemicals and standards

All neat materials, calibration standards and internal standards were of 98% purity or higher. All standards and reagents were obtained from Sigma Aldrich (St. Louis, MO) unless specifically provided in this section. Methanol, methyl-tert-butyl-ether, reagent water and ammonium hydroxide were purchased from Fisher Scientific (Pittsburgh); 4-androstene-3,17-dione (A4) from Cerilliant Corporation (Round Rock, Texas); labeled internal standards, 16-α-hydroxyestradiol-d2 (estriol-d_2), estrone-2,4,16,16-d_4, equilin-2,4,16,16-$d_4$from CDN Isotopes (Quebec, Canada); $^{13}C_6$-estradiol, $^{13}C_2$-ethynylestradiol and 4-androstene-3,17-dione-2,2,4,6,6,16,16-d_7 from Cambridge Isotope Laboratory (Andover, MA, USA). The analytes and surrogate stock standard solutions were prepared in methanol at 1000 µg/mL. Primary dilution standards (PDS) were prepared by diluting the stock standard solutions into 50% (v/v) methanol in HPLC-grade reagent water, followed by filtration with 0.22 µm polyvinyldene fluoride (PVDF) membrane from EMD Millipore (Billerica, MA, USA).

Sample collection, preservation, and storage

Water samples were collected in four west-central Florida counties: Hillsborough, Pinellas, Sarasota and Charlotte. Samples from the Hillsborough River were collected at several locations (State Park, Flatwoods, East Fowler Avenue, Temple Terrace, 56th Street, and 40th Street) and at the David L. Tippin Water Treatment Facility. Reclaimed water was collected at five locations (RW1, RW2, RW3, RW4 and RW5), with the last three used as the recharge water for the three ASR sites. Utilities with ASR wells that were able to provide both recharge and recovery samples were solicited to participate in this study. Due to the time constraints, only three utilities were able to provide samples for a full recharge/recovery cycle test. The recovered water from these sites, labeled as ASR1, ASR2 and ASR3 were collected on dates presented in Table 1. The corresponding storage periods were calculated based on their individual recharge and recovery cycles. The total dissolved solid (TDS) for all sites were well above the secondary drinking water standard of 500 mg/L, but still within the range typically observed for reclaimed water (Table 1). The total organic carbon (TOC) for both ASR1 and ASR2 were between 10 to 12 mg/L. No TOC data was collected by the participating utility for ASR3.

The three ASR sites are located within the wastewater treatment facilities, less than 1 mile away from the reclaimed pumping stations. All three locations provide tertiary treatment to the influent wastewater with filtration and disinfection as the final steps. The reclaimed water is used to recharge the ASR wells during low demand season and the water is subsequently recovered during high demand season. Schematic representation of the sampling locations and representative cross section of ASR1 are shown in Figure 1. The other ASR wells are structurally similar but with different depth as presented in Table 1.

Sample collection, preservation and storage were performed in accordance with EPA Method 539.At the time of collection, samples were dechlorinated using 80 mg/L of sodium thiosulfate and protected from biodegradation using 65 mg/L of 2 mercaptopyridine-1-oxide sodium salt (Table 2).The samples were kept on ice immediately following collection. Upon arrival, samples were kept at 4°C and analyzed within the 28-day maximum holding time as suggested by EPA Method 539. The samples were collected in triplicate, along with a travel blank (TB) and a field blank (FB). The laboratory fortified matrices (LFM) were processed and analyzed in triplicate for each location. Additional quality control measures included laboratory reagent blanks (LRB) and laboratory fortified blanks (LFB).

Solid-Phase Extraction

Extractions of 1000 mL samples were performed using end-capped 47 mm silica based C18 disks (Horizon Technology, Salem, NH, USA) with 5µm Atlantic fast flow pre-filters stacked on top to avoid clogging problems, controlled by an automated system, model SPE-DEX 4790 (Horizon Technology, Salem, NH, USA) equipped with the Envision Platform Controller. The samples were filtered prior to extraction with 0.22 µm polyvinyldene fluoride (PVDF) membrane from EMD Millipore (Billerica, MA, USA).The SPE disks were conditioned three times using 1 mL methanol followed by two additional rinses using 10 mL of water. The disk and container were rinsed with 15% (v/v) methanol for 30 seconds. The disk was then air-

dried for 10 minutes. An elution was performed using 5 mL of methanol with a one-minute soak time. This procedure was repeated three times. The combined extract was concentrated by a low flow of ultra-high purity nitrogen gas to a final volume of 0.5 mL. The volume was adjusted to 1 mL using a 50/50 (v/v) methanol/water solution. Internal standards were added to reach final concentrations of 5.0 to 20 ng/mL in the 1 mL extracts, as described in the EPA Method 539. The additional internal standards used in the Isotope Dilution Method not described in EPA Method 539 were added to a final concentration of 20 ng/mL. EPA Method 539 incorporates the internal standard solution after the SPE extraction while the Isotope Dilution Method adds the internal standard solution prior to the SPE extraction.

Liquid Chromatography

A Varian 212-LC High Pressure Liquid Chromatography System (Agilent Technologies, Palo Alto, CA) was used for the analyses. Hormones were separated using an Xterra MS C18, 2.1 x 150 mm, 3.5μm particle size column from Waters (Milford, MA, USA). A binary gradient consisting of HPLC-grade water (A) and methanol (B) was used at a flow rate of 200 μL/min. The pump gradient program started from 40% of B held for 5 minutes, stepped to 70% by 12 minutes, increased linearly to 90% at 17 minutes and held for 8 minutes, and finally decreased linearly to 40% from 25 to 29 minutes and held at 40% for 6 minutes. Representative chromatograms including the analytical retention times are shown in Figure 2. After the SPE each sample was analyzed twice, one to detect negative ions and the other to detect positive ions. For the negative ions, ammonium hydroxide 0.02% (v/v) was added into both mobile phase solutions A and B to a final pH of ~10.2. For the positive ions, acetic acid 0.1% (v/v) was incorporated into both solutions A and B to a final pH of ~3.2. In all cases, the analyses were divided into time segments to increase the dwell time for each analyte. E3 and estriol-d_2, grouped in the first segment, bisphenol A-d_{16} in the second segment and the rest of the negative ions in the third segment. Positive ions were analyzed separately in one segment.

Mass Spectrometry

The target compounds were identified using a Varian 320 Triple Quadrupole Mass Spectrometer (Agilent Technologies, Palo Alto, CA). Analysis was performed using electrospray ionization in both negative and positive modes. Nitrogen was used as the drying gas for both the negative and positive ions and also as the nebulizing gas for the positive ions. Air was used as nebulizing gas for negative ions. Argon was used as the collision gas for both the negative and positive ions. Selected reaction monitoring (SRM) in the negative and positive ionization modes was utilized to detect ion transitions. The ion transitions selected were the same as those presented in EPA Method 539. The precursor and product ions selected for the two internal standards not listed in EPA Method 539 were 273.3 and 144.7 for estrone-2,4,16,16-d_4 and 294.4 and 100.0 for 4-androstene-3,17-dione-2,2,4,6,6,16,16-d_7.

Method Detection Limit (MDL) Study

Method detection limits were determined for EPA Method 539 and the Isotope Dilution Method for the positive and negative ions. Seven replicates of laboratory fortified blanks spiked with 1 ng/L were extracted according to EPA Method 539[18] and Isotope Dilution Method[19]. The isotope labeled standards were added before or after SPE as specified in each method. The results of the MDL Study for both methods are shown in Table 3.

Results and Discussion

Mass spectra obtained by quantitative optimization showed a protonated molecular ion $[M+H]^+$ for two of the evaluated analytes, A4 and TT. All others were detected in negative mode in the form of their de-protonated $[M-H]^-$ ions. Fragmentation of the precursor ions yielded stable product ions, of which the strongest were chosen for quantification. The analytical conditions were optimized to achieve lower background noise from the matrix and favorable selectivity and sensitivity for all analytes and internal standards (IS), by dividing the analyses into negative and positive ions, modifying the pH of the mobile phase, and adding additional time segments to increase the dwell time of each compound.

EPA Method 539 was successfully applied to two types of matrices: river and drinking water. Sample results from the Hillsborough River (Table 4) demonstrated levels below the MDL for all of the analytes, except E1, which was detected at very low concentrations (<0.5 to 1 ng/L) in the majority of the river samples. Low concentrations of E3 (averaging 1.1 ng/L) were found in samples from the Flatwoods site. The hormone concentrations in the samples were comparable, and in some cases slightly lower, than those reported in previous studies.[9, 11, 24, 25] No hormones were detected in any drinking water samples.

The quality control samples, consisting of LRB, LFB, and LFM, have demonstrated that EPA Method 539 is robust for analyzing river and drinking water samples (Table 4). The surrogate compound, bisphenol A-d_{16}, had recoveries of 68.7 to 109.2% in the LFB, LRB and LFM in the river and drinking water samples. However, the same was not observed while analyzing reclaimed and ASR samples. The results of ASR samples and ASR LFM using EPA Method 539 has shown poor recovery for surrogate and target compounds ranging from negative values to 123.8% (Table 4). LFB and LRB analyzed in the same batch as the ASR samples presented acceptable recoveries of surrogate and target compounds, which demonstrated that low recoveries in ASR and ASR LFM were due to matrix effects.

Matrix interferences have been consistently reported in the analysis of hormones in water using SPE and LC-ESI-MS/MS techniques,[18-20] especially when considerable amounts of other organic contaminants, such as humic and fulvic acids, are present. To minimize matrix interferences and SPE losses, the Isotope Dilution Method as described by Vanderford and Snyder (2006)[19] was used for the analysis of reclaimed water and ASR samples. The results of reclaimed water before being recharged to ASR wells (ASR recharge) and water recovered from ASR wells (ASR recovery) after an extended period of storage in the aquifer using the Isotope Dilution Method is presented in the Table 5. The recoveries of the surrogate

compound in ASR samples were significantly improved using this method, increasing from an average of 56.2% using EPA Method 539 to an average of 86.7% using the Isotope Dilution Method for the ASR1 recharge sample and from an average of 56.1% to an average of 72.3% for the LFM-ASR1 recharge samples (Tables 4 and 5).LFM for ASR samples were more reliable and reproducible using the Isotope Dilution Method (Table 5).

The concentration of hormones in reclaimed water can vary widely depending on the source and amount of rainfall.[26] Overall, the wastewater treatment processes from municipal plants are considered highly efficient at reducing hormones, as seen in the analytical results from the reclaimed water sites, RW1 and RW2. A few hormones were found at very low concentrations, however, most were not detected (Figure 3).

The concentration of hormones in the ASR samples and the degree of attenuation after the recovery processes are shown in Figures 4, 5 and 6. Most of the compounds were not detected or found at very low concentrations. Only E1, E2, E3 and A4 were detected at levels significant enough to produce meaningful information about their fate as a result of ASR activities. The attenuation of E1, E2 and A4 varied from 50 to 99.1% and averaged at 82% concentration decrease during the recovery process. E3 appeared to be an exception. It was relatively stable with only 10% attenuation at ASR1 and its concentration increased at ARS2 during the recovery phase exhibiting no attenuation (Figures 4 and 5). No apparent correlation between the attenuation and water quality data, such as TDS and storage duration, was noticed.

The distribution of hormones presented in the pie diagrams (bottom portions of Figures 4 through 6) has changed dramatically between the recharge and recover phases as a result of the variability of the individual hormone's removal for each specific site and the variability between different sites. The concentrations initially present during the recharge phase can be reduced by dilution and biodegradation and hence may not be detected during the recovery process. The opposite trend, i.e., concentration increase during recharge/recovery cycles, is noted as well. Using ASR1 as an example, because E3 is recalcitrant to attenuation, it became dominant in the recovered water accounting for 95.6% of the total hormones present. The relative concentration of the remaining four hormones initially detected during the recharge phase from ASR1 (E2, E1, A4 and EQ) decreased from 44 to 4.4% in the recovery samples (Figure 4).

The persistence of E3 during ASR recharge and recovery cycles might be due to its relatively lower log K_{ow} of 2.7, making it less prone to adsorption. The log K_{ow} of E1 and E2 and A4 are 3.13, 4.01 and 3.32, respectively, suggesting that these three hormone are more polar and hydrophilic in nature and hence adsorption plays a significant role in their removal.[27] The increase of E3 at ASR2 from 0.22 ng/L to 0.69 ng/L during recharge and recovery cycles is confirmed with triplicate sample analysis (Figure 5). The reason for the increase remains to be further investigated with additional sites and data, but one likely explanation is the initial adsorption and subsequent release of the compound over time due to its low affinity to the adsorption sites.

The medium to high natural attenuation of E2 (62 to 99%) during ASR recharge and recovery cycles observed at all three ASR sites once again revealed the complexity of environmental degradation. Based on literature findings, under simulated bench-scale conditions, E2 exhibits a rapid biodegradation in native groundwater and reclaimed water under aerobic conditions with a half-life of approximately 2 days.[28, 29] However, under anaerobic conditions in the same matrix, no apparent degradation was observed within 70 days.[28, 29] The attenuation of E2 observed during this study suggests a combination of both aerobic and anaerobic conditions during the actual ASR operation. When the reclaimed water was recharged to ASR wells, E2 initially went through fast aerobic degradation. As the oxygen became depleted, anaerobic degradation took over and as a result, led to little or no further degradation of E2.

Another hormone, EE2, is reported to be very persistent under both aerobic and anaerobic conditions. Since it was not detected during ASR recharge and recovery cycles in this study, it cannot be confirmed. A fairly strong attenuation of A4 was observed with a 97.8% removal at ASR1 and with a 66% removal at the ASR3 site.E1 was removed by 97% at the ASR3 site and removed by over 50% at the ASR1 site. Direct comparison with literature for the attenuation of A4 and E1 and possible removal mechanisms can't be provided yet due to the lack of field studies available. The removal of E1 was demonstrated during a survey of surface water under the impact of dairy discharge.[15] E1 was detected close to the discharge point of the dairy waste and gradually decreased to eventually non-detectable in a group of groundwater monitoring wells downstream and away from the discharge point. It should be noted these wells were less than 82 feet and much shallower than the depth of ASR wells investigated in this study, which was up to 800 feet deep (Table 1).

The attenuation of EDCs in soil and groundwater has been reported to differ significantly among compounds under simulated conditions created in lab bench-scale studies. Some compounds may rapidly biodegrade or be adsorbed into rocks and organic matter, while others are much more resistant to biodegradation or adsorption.[26, 30-36] The field data collected in this study from ASR sites has demonstrated the variability of the seven hormones in regards to their detected levels and attenuation, and confirmed the environmental behavior of hormone at ASR sites is just as complex as the other environmental media, such as soil and groundwater. The field site attenuation data for hormones by ASR recharge and recycle operations is still very limited in the literature. This may be due to the challenge of finding appropriate field sites, requirement of time dedication, duration, and complexity of coordinated efforts between researchers and utilities in order to catch both the recharge and recycle stages. The closest previous study was conducted by Mansell and Drewes, who investigated the fate of E2, E3 and TT at two field sites where the treated wastewater was applied to surface spreading basins for groundwater recharge with monitoring wells no more than 121 feet deep.[37] In their study, all three hormones were attenuated to below detection limits, with E2 exhibiting similar attenuation and E3 presenting different attenuation when comparing to the results obtained in the current study. It is reasonable to assume that the difference of the E3's attenuation may be related to different geological structures, water characteristics and microbial activities between spreading basins and deep ASR wells, all of which could be part of the primary contributing factors.[38]

Conclusions

The evaluated methods were found to be sensitive and are suitable to simultaneously analyze trace levels of several structurally and chemically similar compounds such as the hormones listed in the screening survey list 2 of the UCMR 3 (E1, E2, EE2, E3, EQ, TT and A4). Two methods were evaluated using water matrices from different sources:

- EPA Method 539 was successfully applied to two types of matrices (river and drinking water) but strong matrix/SPE losses were seen in the more complex matrices evaluated during this study: ASR and reclaimed water.

- The Isotope Dilution Method was utilized to test reclaimed and ASR samples. The method dilutes the internal standards in the samples before the SPE, to minimize matrix suppression, SPE losses and instrumental variability.

Fortification of environmental samples resulted in recoveries ranging from 98.9 to 108.5%, using EPA Method 539. The detection limits for most compounds were lower than 0.66 ng/L and reporting limits ranged from 0.05 to 0.5 ng/L based on the extraction of a 1 L sample and a final concentrated volume of 1mL.

Hillsborough River samples demonstrated levels below the MDL for the majority of the analytes, except, E1, which was detected at very low concentrations (<0.5 to 1 ng/L) in most samples. Low concentrations of E3 (averaging 1.1 ng/L) were found in samples from the Flatwoods site. No hormones were detected in the drinking water.

The seven target hormones were found to be mostly below detection or at relatively low concentrations in the reclaimed water prior to being recharged into the ASR wells. The highest concentration detected was 250 ng/L of A4 which was found in the recharge water at ASR3. After 63 days of storage, A4 concentration dropped to 85 ng/L with 66% being removed by natural attenuation. High natural attenuation between ASR recharge and recovery cycles was also observed for E2 (99.1%, 62% and 98% for ASR1, ASR2 and ASR3) and E1 (50.9% and 97% for ASR1 and ASR3). E3 was found to be very resistant to natural attenuation, with only 10% attenuation observed at ASR1. At ASR2, despite the absence of E3 in the recharged water, a low concentration was detected in the recovered water after 82 days of storage, likely due to the release of the adsorbed hormone back into the water.

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LIST OF TABLES

Table 1. Reclaimed ASR site summary.

	Well depth (ft)	Reclaimed water		Recharge date	Recovery date	Storage Time (days)
		pH	TDS (mg/L)			
ASR1	400-600	7.05	576	February 3, 2014	May 27, 2014	113
ASR2	600	7.60	1100	March 27, 2014	June 17, 2014	82
ASR3	800	7.29	732	April 15, 2014	June 17, 2014	63

Table 2. Preservatives added to each sample and control blanks bottles prior to field collection.

	Type of Preservative	Amount (mg)	Purpose
Samples*	Sodium thiosulfate	80	Removes free chlorine
	2 mercaptopyridine-1-oxide, sodium salt	65	Microbial inhibitor
TB**	Sodium thiosulfate	80	Removes free chlorine
	2 mercaptopyridine-1-oxide, sodium salt	65	Microbial inhibitor
FB***	none	-	Quality control during collection

* Number of samples may vary on each sampling site, ** TB – Trip blank filled with reagent water and preservative, *** FB field blank is an empty bottle to be filled with reagent water in the field

Table 3. Method detection limit (MDL) and average recovery of UCMR3 listed hormones obtained using the EPA Method 539 and the Isotope Dilution Method.

MDL Study and Recovery of EPA Method 539 and Isotope Dilution Method								
		E1	**E2**	**EE2**	**E3**	**EQ**	**A4**	**TT**
EPA Method 539	**MDL (ng/L)**	0.49	0.66	0.53	0.47	0.4	0.37	0.2
	Fortification (ng/L)	1.00	1.00	1.00	1.00	1.00	1.00	1.00
	Recovery (%)	84.5	81.9	84.6	100.4	69.3	75.1	83.5
	Final concen. (ng/L)	0.84	0.81	0.84	1.04	0.69	0.75	0.83
Isotope Dilution Method	**MDL (ng/L)**	0.16	0.34	0.27	0.22	0.43	0.15	0.04
	Fortification (ng/L)	1.00	1.00	1.00	1.00	1.00	1.00	1.00
	Recovery (%)	87.9	113.7	108.7	104.9	86.1	92.2	102.1
	Final concen. (ng/L)	0.87	1.13	1.08	1.04	0.86	0.92	1.02

Table 4. Levels of hormones in samples from the Hillsbourough River, laboratory fortified blank (LFB), laboratory reagent blank (LRB) and laboratory fortified matrix (LFM) and ASR samples using the EPA Method 539.

Concentration of UCMR 3 listed hormones and surrogate (n=3)									
Sampling Site		**E1**	**E2**	**EE2**	**E3**	**EQ**	**A4**	**TT**	**SUR**
Fowler Ave	**Conc. (ng/L)**	0.7	nd	nd	nd	nd	nd	nd	23.8
	Recovery (%)	-	-	-	-	-	-	-	95.2
State Park	**Conc. (ng/L)**	0.6	nd	nd	nd	nd	nd	nd	19.2
	Recovery (%)	-	-	-	-	-	-	-	76.8
Flatwoods	**Conc. (ng/L)**	0.6	nd	nd	1.1	nd	nd	nd	17.2
	Recovery (%)	-	-	-	-	-	-	-	68.7
Temple Terrace	**Conc. (ng/L)**	1	nd	nd	nd	nd	nd	nd	20.3
Terrace	**Recovery (%)**	-	-	-	-	-	-	-	81.2
56th Street	**Conc. (ng/L)**	0.9	nd	nd	nd	nd	nd	nd	21.6
	Recovery (%)	-	-	-	-	-	-	-	86.4
40th Street	**Conc. (ng/L)**	0.9	nd	nd	nd	nd	nd	nd	22.5
	Recovery (%)	-		-	-	-	-	-	90
30th Street	**Conc. (ng/L)**	nd	nd	nd	nd	nd	nd	nd	20.7
(river water)	**Recovery (%)**	-	-	-	-	-	-	-	82.8
30th Street	**Conc. (ng/L)**	nd	nd	nd	nd	nd	nd	nd	21.3
(drinking water)	**Recovery (%)**	-	-	-	-	-	-	-	85.2
LRB	**Conc. (ng/L)**	nd	nd	nd	nd	nd	nd	nd	26
	Recovery (%)	-	-	-	-	-	-	-	104.2
LFB	**Conc. (ng/L)**	47.7	51	49.4	51.8	52.6	49.9	49.5	25.1
(50ng/L)	**Recovery (%)**	95.4	102.1	98.8	103.6	105.3	99.9	99	100.3
LFB	**Conc. (ng/L)**	97.8	101.4	101.2	108.1	101.8	102.9	110.1	24.3
(100ng/L)	**Recovery (%)**	97.8	101.4	101.2	108.1	101.8	102.9	110.1	97
LFM 30th St.	**Conc. (ng/L)**	98.9	101.9	103.9	103.6	99.4	100.9	108.5	27.3
(100ng/L)	**Recovery (%)**	98.9	101.9	103.9	103.6	99.4	100.9	108.5	109.2
ASR 1	**Conc. (ng/L)**	4.3	nd	nd	132.2	nd	nd	1.5	14.0
Recharge	**Recovery (%)**	-	-	-	-	-	-	-	56.2
LFM - ASR 1	**Conc. (ng/L)**	17.0	12.38	nd	83.4	10.7	2.7	4.8	14.1
Recharge	**Recovery (%)**	127	123.8	< 0	< 0	107.6	27.6	32.7	56.4
LRB	**Conc. (ng/L)**	nd	nd	nd	nd	nd	nd	nd	26.4
SUR 25 ng/L	**Recovery (%)**	-	-	-	-	-	-	-	105.8
LFB 10 ng/L	**Conc. (ng/L)**	12.0	11.3	8.5	8.8	11.0	11.0	9.9	26.9
SUR 25 ng/L	**Recovery (%)**	120.0	113.0	85.0	88.1	110.4	110.5	99.2	107.9

*nd- non-detected, LRB - laboratory reagent blank, LFB - laboratory fortified blank, and LFM - laboratory fortified matrix, Surrogate spiked at 25 ng/L

Table 5. Levels of hormones in ASR samples, laboratory fortified blank (LFB), laboratory reagent blank (LRB) and laboratory fortified matrix (LFM) using the Isotope Dilution Method

Concentration of UCMR 3 listed hormones and surrogate* (n=3)									
Sampling Site		**E1**	**E2**	**EE2**	**E3**	**EQ**	**A4**	**TT**	**SUR**
ASR 1	**Conc. (ng/L)**	6.8	85.3	nd	124.3	0.3	6.7	nd	86.7
Recharge	**Recovery (%)**	-	-	-	-	-	-	-	86.7
LFM - ASR 1	**Conc. (ng/L)**	102.4	159.9	108.5	249.8	100.9	111.9	103.8	72.3
Recharge	**Recovery (%)**	95.6	74.6	108.5	125.5	100.6	105.2	103.8	72.3
ASR 1	**Conc. (ng/L)**	3.3	0.8	nd	111.5	0.4	nd	nd	77.3
Recovery	**Recovery (%)**	-	-	-	-	-	-	-	77.3
LFM - ASR 1	**Conc. (ng/L)**	100.3	109.3	102.5	211.0	105.0	104.5	109.3	80.5
Recovery	**Recovery (%)**	97.0	108.6	102.5	99.5	104.5	104.5	109.3	80.5
Field Blank	**Conc. (ng/L)**	nd	nd	nd	nd	nd	nd	nd	103.3
	Recovery (%)	-	-	-	-	-	-	-	103.3
LRB	**Conc. (ng/L)**	nd	nd	nd	nd	nd	nd	nd	96.1
	Recovery (%)	-	-	-	-	-	-	-	96.1
LFB 100 ppt	**Conc. (ng/L)**	102.9	96.8	111.8	110.1	113.5	106.7	101.6	91.4
	Recovery (%)	102.9	96.8	111.8	110.1	113.5	106.7	101.6	91.4
ASR 2	**Conc. (ng/L)**	nd	1.8	nd	nd	nd	nd	nd	78.7
Recharge	**Recovery (%)**	-	-	-	-	-	-	-	78.7
LFM - ASR 2	**Conc. (ng/L)**	94.2	101.6	96.9	101.9	100.6	103.8	104.6	100.2
Recharge	**Recovery (%)**	94.2	99.8	96.9	101.9	100.6	103.8	104.6	100.2
ASR 2	**Conc. (ng/L)**	nd	0.7	nd	1.9	nd	nd	nd	95.1
Recovery	**Recovery (%)**	-	-	-	-	-	-	-	95.1
LFM - ASR 2	**Conc. (ng/L)**	99.8	101.0	104.6	104.3	101.1	102.6	99.0	95.3
Recovery	**Recovery (%)**	99.8	100.3	104.6	102.3	101.1	102.6	99.0	95.3
Field Blank	**Conc. (ng/L)**	nd	nd	nd	nd	nd	nd	nd	95.3
	Recovery (%)	-	-	-	-	-	-	-	95.3
LFB 100 ppt	**Conc. (ng/L)**	95.3	101.4	106.0	100.4	99.5	102.2	99.0	81.4
	Recovery (%)	95.3	101.4	106.0	100.4	99.5	102.2	99.0	81.4
ASR 3	**Conc. (ng/L)**	5.0	14.4	nd	nd	nd	249.5	nd	91.0
Recharge	**Recovery (%)**	-	-	-	-	-	-	-	91.0
LFM - ASR 3	**Conc. (ng/L)**	99.6	103.5	98.7	102.4	101.7	353.6	101.9	92.1
Recharge	**Recovery (%)**	94.6	89.1	98.7	102.4	101.7	104.1	101.9	92.1
ASR 3	**Conc. (ng/L)**	0.2	0.3	nd	nd	nd	85.0	nd	90.3
Recovery	**Recovery (%)**	-	-	-	-	-	-	-	90.3
LFM - ASR 3	**Conc. (ng/L)**	96.2	102.0	103.7	104.7	100.0	179.9	97.5	100.6
Recovery	**Recovery (%)**	96.1	102.0	103.7	104.7	100.0	94.9	97.5	100.6
Field Blank	**Conc. (ng/L)**	nd	nd	nd	nd	nd	nd	nd	94.0
	Recovery (%)	-	-	-	-	-	-	-	94.0

*nd- non-detected, LRB - laboratory reagent blank, LFB - laboratory fortified blank, and LFM - laboratory fortified matrix, Surrogate spiked at 100 ng/L

FIGURE CAPTIONS

Figure 1. Study area showing the Hillsborough River sampling area (including State Park, Flatwoods, East Fowler Avenue, Temple Terrace, 56th Street, 40th Street and 30th Street); reclaimed water (RW1, RW2, RW3, RW4 and RW5) and recovery from ASR collection sites (ASR1, ASR2 and ASR3). Cross section of ASR1 illustrating construction standard of evaluated ASR sites on the left.

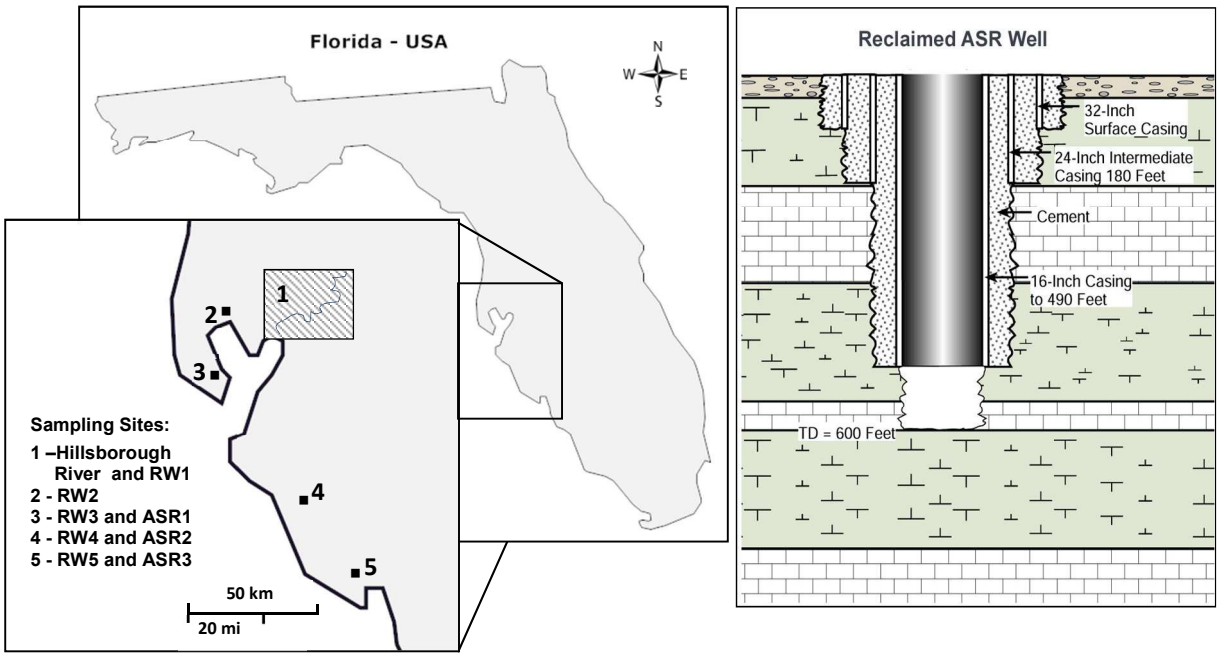
Figure2. Multiple Reaction Monitoring (MRM) Chromatogram of UCMR3 listed hormones: estrone (E1), estradiol (E2), ethynylestradiol (EE2), estriol (E3), equilin (EQ), androstenedione (A4), testosterone(TT),and surrogate compound, bisphenol-A-d16 (BA). Representative standard injection of 50 µg/L.

Figure 3. Concentrations of the UCMR 3 listed hormones in reclaimed water site 1 (RW1) and reclaimed water site 2 (RW2).

Figure 4. Concentrations of the UCMR3 listed hormones during ASR1 recharge and recovery phases illustrating attenuation after one cycle (top). Relative concentrations of the UMCR3 listed hormones, plotted as percent of total average concentration of the ASR1 samples during recharge (bottom left) and recovery (bottom right)

Figure 5. Concentrations of the UCMR3 listed hormones during ASR2 recharge and recovery phases illustrating attenuation after one cycle (top). Relative concentrations of the UMCR3 listed hormones, plotted as percent of total average concentration of the ASR2 samples during recharge (bottom left) and recovery (bottom right).

Figure 6. Concentration of UCMR3 listed hormones during ASR3 recharge and recovery phases illustrating attenuation after one cycle (top). Relative concentrations of the UMCR3 listed hormones, plotted as percent of total average concentration of the ASR3 samples during the recharge (bottom left) and recovery (bottom right).



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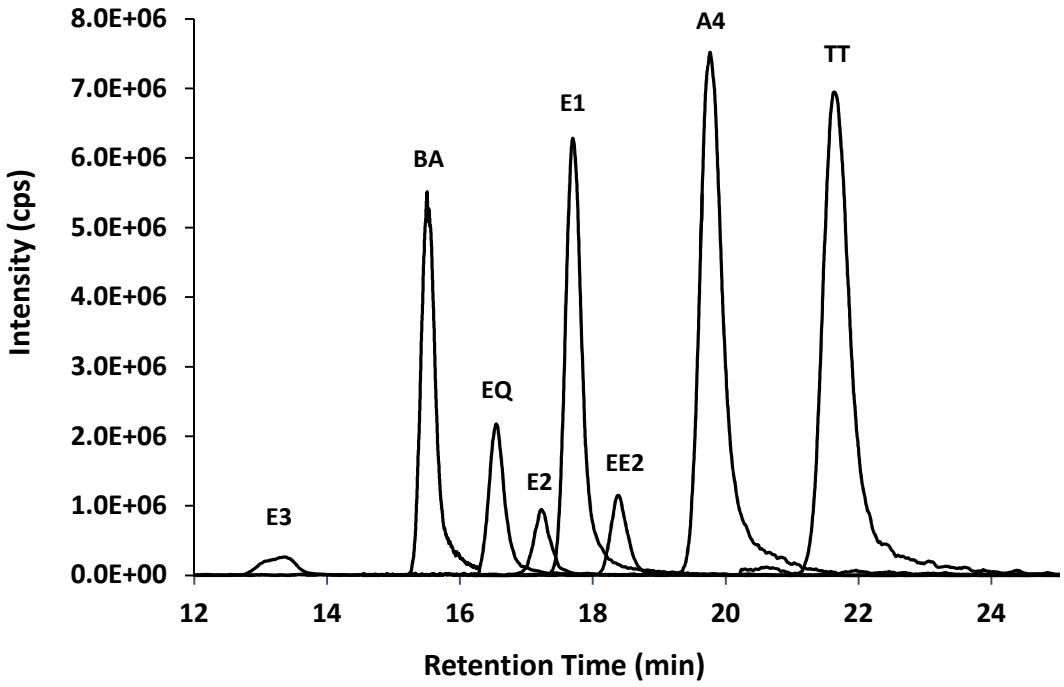

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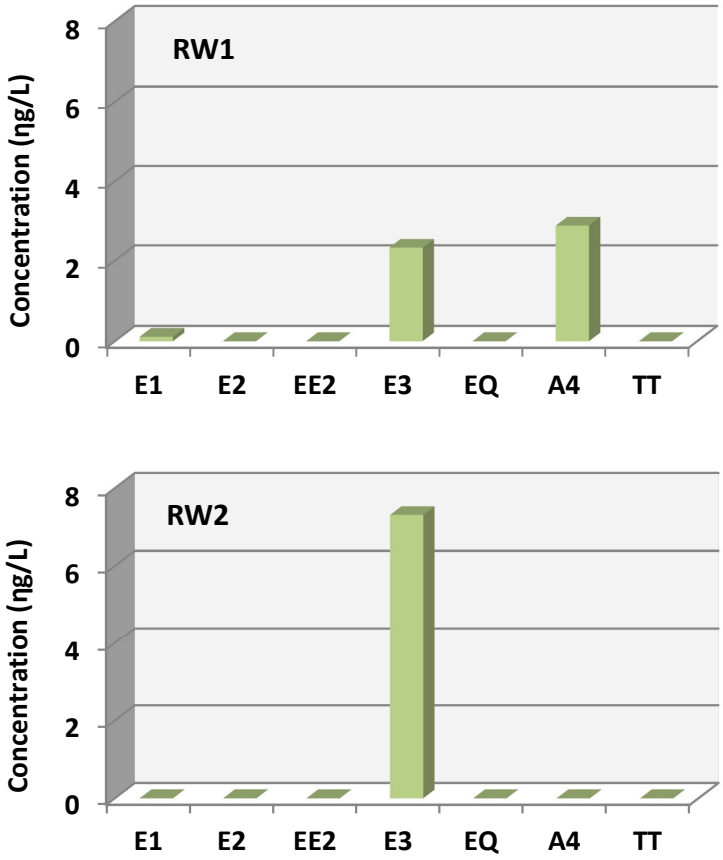
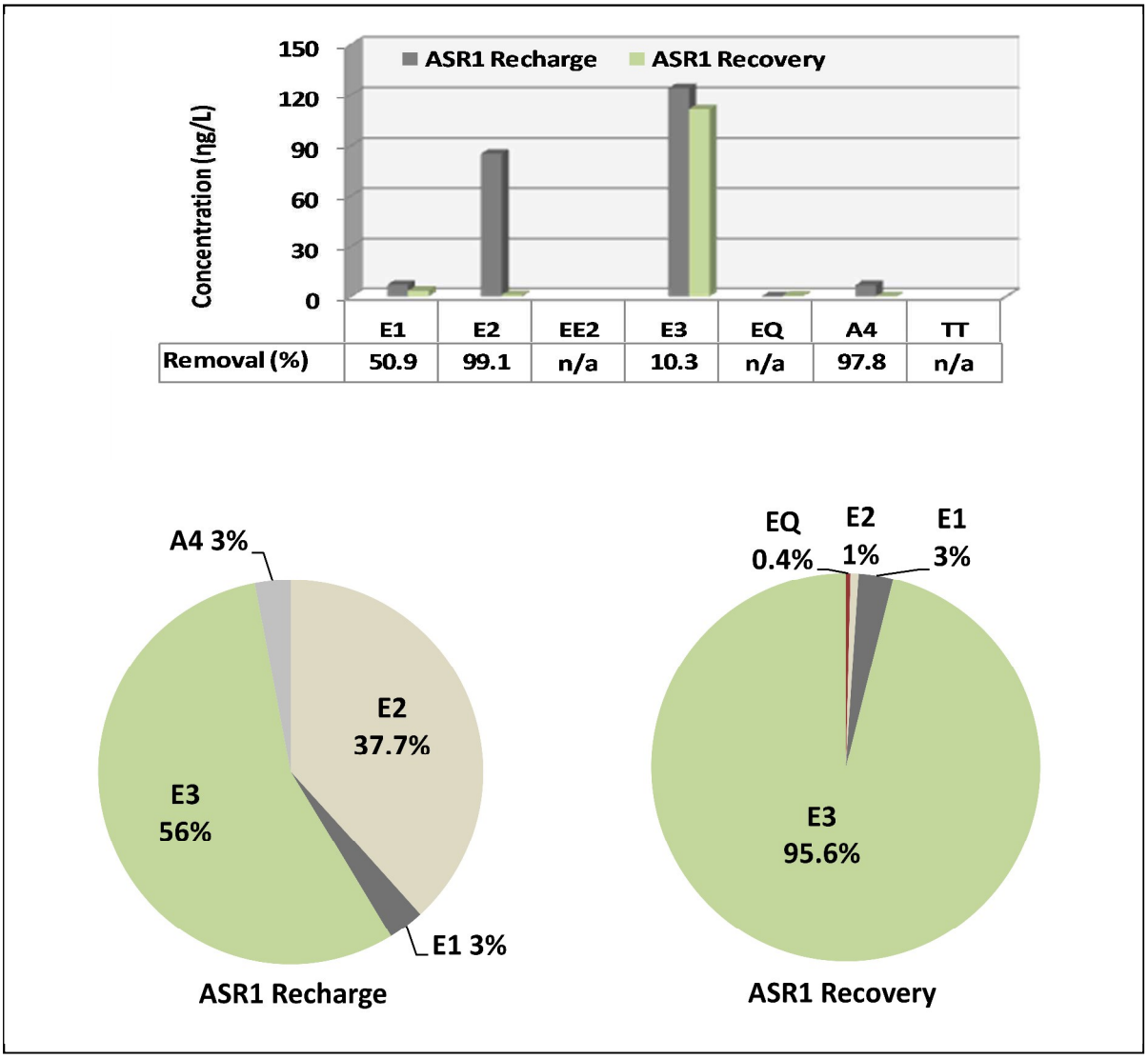


Figure 3. Concentrations of the UCMR 3 listed hormones in reclaimed water site 1 (RW1) and reclaimed water site 2 (RW2).



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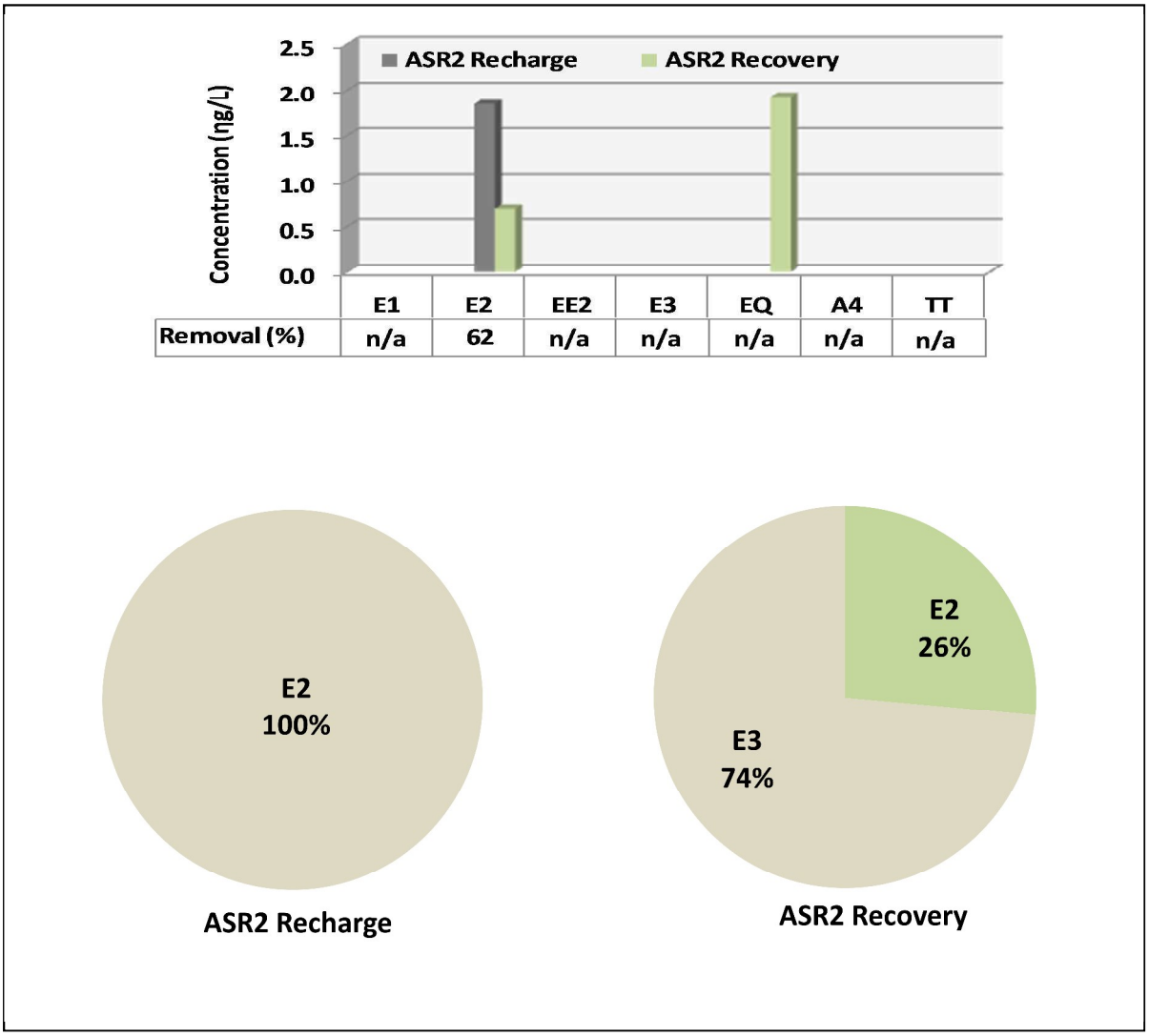

Figure 5. Concentrations of the UCMR3 listed hormones during ASR2 recharge and recovery phases illustrating attenuation after one cycle (top).Relative concentrations of the UMCR3 listed hormones, plotted as percent of total average concentration of the ASR2 samples during recharge (bottom left) and recovery (bottom right).

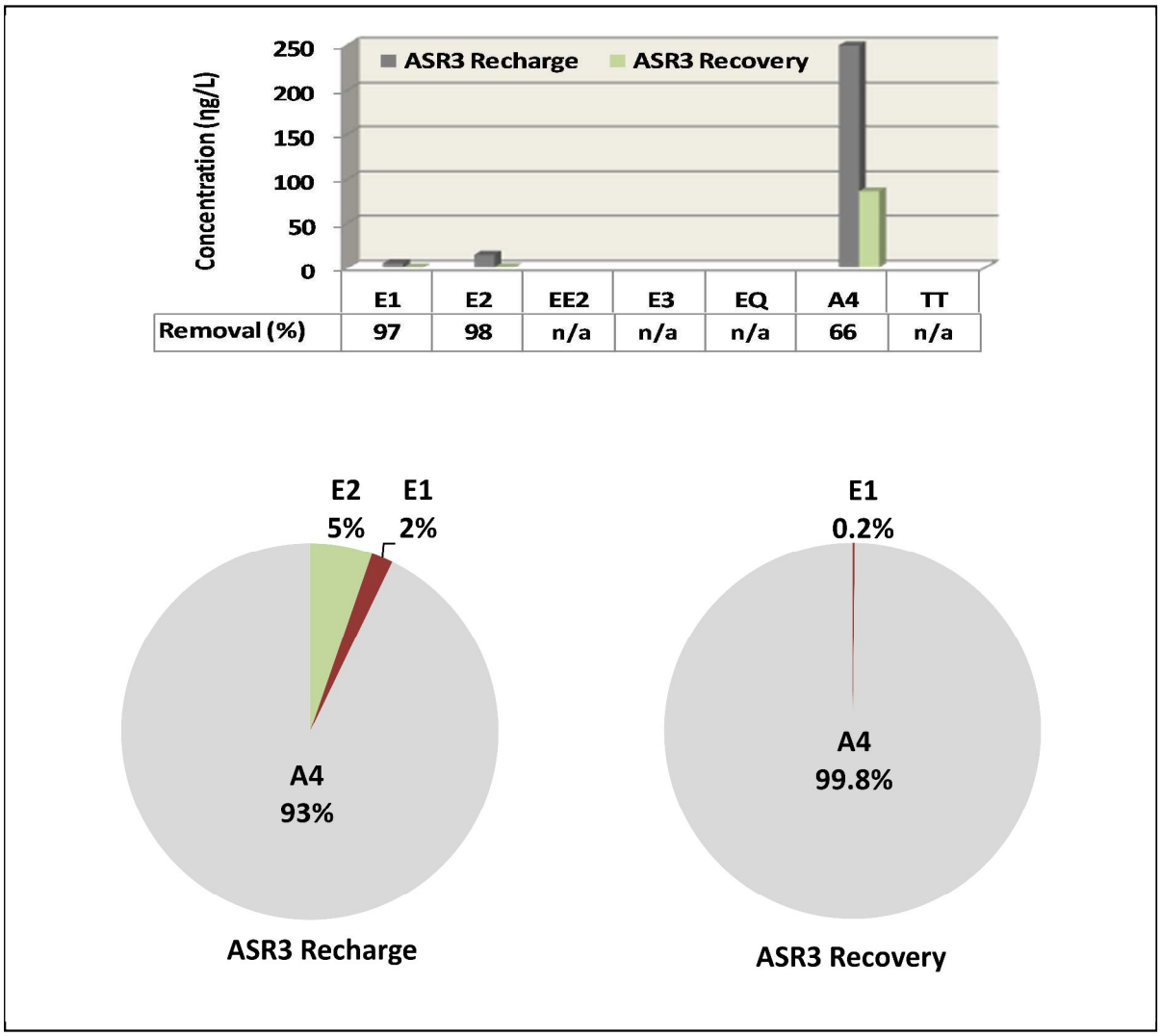

Figure 6. Concentration of UCMR3 listed hormones during ASR3 recharge and recovery phases illustrating attenuation after one cycle (top). Relative concentrations of the UMCR3 listed hormones, plotted as percent of total average concentration of the ASR3 samples during the recharge (bottom left) and recovery (bottom right).