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Fate of Conjugated Natural and Synthetic Steroid Estrogens in Crude Sewage and Activated Sludge Batch Studies

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Steroids are excreted from the human body in the conjugated form but are present in sewage influent and effluent as the free steroid, the major source of estrogenic activity observed in water courses. The fate of sulfate and glucuronide conjugated steroid estrogens was investigated in batch studies using activated sludge grown on synthetic sewage in a laboratory-scale Husmann simulation and crude sewage from the field. A clear distinction between the fate of sulfate and glucuronide conjugates was observed in both matrices, with sulfated conjugates proving more recalcitrant and glucuronide deconjugation preferential in crude sewage. For each conjugate, the free steroid was observed in the biotic samples. The degree of free steroid formation was dependent on the conjugate moiety, favoring the glucuronide. Subsequent degradation of the free steroid (and sorption to the activated sludge solid phase) was evaluated. Deconjugation followed the first order reaction rate with rate constants for 17α-ethinylestradiol 3-glucuronide, estriol 16α-glucuronide, and estrone 3-glucuronide determined as 0.32, 0.24, and 0.35 h respectively. The activated sludge solid retention time over the range of 3−9 days had 74 to 94% of sulfate conjugates remaining after 8 h. In contrast, a correlation between increasing temperature and decreasing 17α-ethinylestradiol 3-glucuronide concentrations in the activated sludge observed no conjugate present in the AS following 8 h at 22 °C Based on these batch studies and literature excretion profiles, a hypothesis is presented on which steroids and what form (glucuronide, sulfate, or free) will likely enter the sewage treatment plant.

Introduction

The estrogenic activity in sewage effluent (*1*) and river systems (*2*) has been primarily attributed to the presence of free steroid estrogens due to incomplete removal during the sewage treatment plant (STP) (*1*). Though predominantly excreted from the body in the conjugated form, research has focused on the free steroids as they constitute the dominant form in influent and effluent, suggesting that deconjugation occurs between excretion and sewage effluent discharge (Figure 1). The sewerage system is an integral part of the STP and several studies have proposed that the long residence time in the sewerage system and the numerous observations of free steroids in crude sewage infer that deconjugation is likely to occur to a certain extent prior to entry to the STP (*3, 4*). Deconjugation is naturally mediated by β-glucuronidase and sulfatase enzymes, β-glucuronidase activity being exhibited by bacteria such as *Escherichia coli* which occur in the human intestine and are excreted in faeces (*5*). Such bacteria will be present in the sewerage system and STP and are therefore likely to be responsible for the deconjugation of glucuronides (*6*).

Analytical advances in the clinical field utilizing liquid chromatography mass spectrometry or tandem mass spectrometry (LC/MS/MS) have been recently extrapolated to environmental matrices. This has enabled direct determination of conjugated steroids, removing the hydrolysis step to the free steroid and derivatization which were required for GC determination (*7*). Using this approach, studies which directly assess steroid conjugate presence in wastewater matrices have identified sulfated steroids in the sewage influent and effluent (*8*−*11*).

Information about steroid deconjugation and subsequent free steroid behavior is essential for optimizing models which predict the influent and effluent concentrations of free steroids (*3*). Numerous factors need to be assessed, such as the extent of deconjugation, and the transformation of the free steroids produced upon deconjugation during transit in the sewerage system and the STP. However, there is a paucity of information regarding conjugate behavior and fate in sewage matrices, with current data limited to three studies. Two investigated 17β-estradiol glucuronides indirectly by measuring vitellogenin induction in fish (*12*) and by GC/MS (*6*). In both cases, diluted activated sludge (AS) (one laboratory, one field) was utilized. Chemical analysis was not carried out in the former study and for the latter only indirect analysis of the steroids was undertaken. A more recent study investigated the rate of deconjugation for eight conjugates, each individually spiking at 25 μg L^{-1} in condominium wastewater serving a population of 250 (*13*). Though this data was utilized for a revised prediction model of conjugate fate in the sewerage system (*14*), the microbial consortium of the condominium wastewater investigated will be more specialized and preferential to steroid breakdown compared to microorganisms (MO) entering STPs which have several inputs (drains, overflow, industrial contributions) to the sewerage system in addition to domestic waste. Additionally, the free steroids were not monitored and only natural conjugated steroids were investigated.

Contrary to other compounds that exhibit estrogenicity, the quantity of natural and synthetic steroids entering the STP is unlikely to decrease due to their origin and use respectively. As precursors to the estrogenic free steroids, knowledge of conjugate steroid occurrence and fate in the STP plays an important part in assessing the impact of operational parameters which may be utilized to improve STP removal efficiency, providing data for optimizing models for predicting steroid concentrations and evaluating environmental risk.

Using field crude sewage and laboratory-scale Husmann simulated AS, the following research aims were undertaken to investigate the behavior and fate of glucuronide and sulfated conjugated steroids in the STP.

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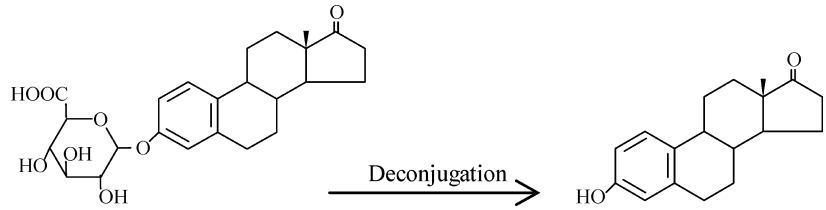

FIGURE 1. Deconjugation of the conjugate, estrone-3-glucuronide to form the free steroid, estrone.

(1) Provide direct evidence using LC/MS that free steroids are produced upon deconjugation of glucuronide and sulfate steroid conjugates.

(2) Investigate the deconjugation behavior of natural and synthetic steroid glucuronide and sulfate conjugates.

(3) Provide a mass balance of the conjugate and the fate of the evolved free steroid upon deconjugation.

(4) Effect of temperature on rate of deconjugation.

(5) Effect of AS solid retention time (SRT) on deconjugation.

Experimental Section

Reagents and Analysis. The free steroid estrogens, 17β-estradiol (E2), estrone (E1), estriol (E3), and ethinylestradiol (EE2) and their conjugates, estrone 3-sulfate (E1−3S), estrone 3-glucuronide (E1−3G), and estriol 16α-glucuronide (E3−16αG) were obtained from Sigma (Poole, UK). The EE2 conjugates, ethinylestradiol 3-sulfate (EE2−3S) and ethinylestradiol 3-glucuronide (EE2−3G) were from Steraloids (Newport, U.S.). Preparation and storage of free and conjugated steroid stock solutions have been previously described (*8*). Ten mL samples of Husmann AS or field crude sewage was centrifuged (Jouan S.A. C3*i*, Saint-Herblain, France) at 12 500 g for 10 min and the resultant aqueous phase was extracted (*8*). For the solid phase of the AS, excess water was removed by adding 2 g of oven-dried sodium sulfate before solvent extraction with 10 mL diethyl ether/hexane (10:1) for one hour (*15*). Analysis was by LC/MS according to established methods (*8*). Using the LC/MS method, both the conjugated and free steroids could be observed in the same analytical run. Therefore, the presence of a free steroid in the sample, irrespective of whether the spiked conjugate was a glucuronide or sulfate could only be present as a consequence of deconjugation of the spiked conjugate.

Behavior and Fate Studies. AS was taken from the aeration chamber of a laboratory-scale Husmann simulation of AS treatment (*16*). This was supplied with a synthetic settled sewage based on bacterial peptone and once sampled, was used immediately for the aerobic batch experiments. Base parameters were an operating temperature of 17 ± 1 °C, SRT of 5.0 days (containing AS suspended solids of 4000 mgL^{-1}) with dissolved oxygen content of 3.5 mg L^{-1} and pH of 7.1. Effluent suspended solids (SS) averaged 40 mg L^{-1} with a biological oxygen demand (BOD) removal of 85% (BOD in effluent averaging 25 ± 5 mgL^{-1}). Calculation of AS SS and AS volatile SS for organic carbon content followed the HMSO official gravimetric method (*17*). Using this, AS volatile SS was 70.2 ± 8.5% of AS SS in line with typical values (*18*). Experimental parameters were varied as follows for deconjugation experiments; temperature (4, 11, 17, and 22 °C) and SRT (3.2, 5.0, 6.7, and 8.8 days).

The sample of crude sewage was collected from a STP in South East England, which receives influent from both industrial and domestic sources. Crude sewage defined as the raw sewage obtained post the preliminary screening stage were obtained by grab sampling and kept at 4 °C under dark conditions until use in the batch tests. From sampling to use in the batch studies, the time duration was less than 18 h. To ensure that steroids (free or conjugated) in the crude sewage were present from the batch tests and not present prior to the experiment, a sample of crude sewage was tested for the presence of steroids and any steroids present subtracted from the spiked starting concentration. The solid content of the crude sewage in the 10 mL aliquots averaged 2 mg and was too limited to determine the partitioning of steroids.

Experiments were carried out in clean, silanised, wide-necked 250 mL Pyrex conical flasks. Aeration was achieved using two "Ghost 1" 1.5 L min^{-1} air pumps (Waterlife Research Industries Ltd., Middlesex, UK), each delivering air to flasks via a series of Pasteur pipettes. All experiments were conducted in a temperature controlled room at 17 ± 1 °C and carried out in triplicate to assess reproducibility. Controls were run alongside, the biotic control using spiked ultrapure water in place of the conjugate, and the abiotic control achieved by autoclaving the sample and spiked with the conjugate. Each sample was spiked with a single conjugate so any observation of a free steroid in the sample would be due to deconjugation of that spiked conjugate. Conjugates were spiked at 2348, 2450, 2517, 2125, and 2323 ng L^{-1} for E1−3S, EE2−3S, E1−3G, EE2−3G and E3−16αG, respectively. The following experiments were undertaken for each conjugate (unless otherwise specified):

(1) Assessment of obtaining true abiotic conditions.

(2) Assessment of site of biodegradation in AS (intracellular or extracellular, biomass related) on individual spiked representative free steroid (E2) and sulfate (E1−3S) and glucuronide conjugate (E1−3G).

(3) Deconjugation in crude sewage following 8 h incubation assessing for free steroid presence.

(4) Deconjugation in AS following 8 h incubation assessing for free steroid presence in the aqueous and solid phase.

(5). Time course 24 h study for EE2−3G in AS evaluating the presence of the free steroid evolved and subsequent fate.

(6) Time course 24 h study on deconjugation in AS with rate constants obtained for the glucuronide conjugates.

(7) Influence of AS SRT (3−9 days) on the sulfate conjugates, E1−3S and EE1−3S.

(8) Influence of AS temperature (4, 11, 17, 22 °C) for EE2−3G.

When obtaining a mass balance of the conjugate and resultant free steroid in a sample, the conversion between conjugate and free steroid must be assessed (e.g., 50 ng L^{-1} of E1−3S will deconjugate to form 38.57 ng L^{-1} of E1). Accounting for this, eq 1 gives the percentage loss of the spiked conjugate from the sample which represents the subsequent degradation of the evolved free steroid (C_{LOSS}), whereas C_O is the initial spiked conjugate concentration, C_f is the free steroid concentration, C_C is the conjugate concentration, and M_C and M_f are the molecular mass of the conjugate and free steroid, respectively.

$$C_{LOSS} = \left[1 - \frac{1}{C_O}\left(C_C + \frac{C_f M_C}{M_f}\right)\right]100 \qquad (1)$$

The time course experiments utilized 200 mL of sample to allow for sample withdrawal during the course of the experiment. Timing began upon addition of the spiked conjugate and 10 mL aliquots were withdrawn from each flask after 10 min, 30 min, 1, 2, 4, 8, and 24 h. For determining the degradation rate constants for the conjugates, the rate

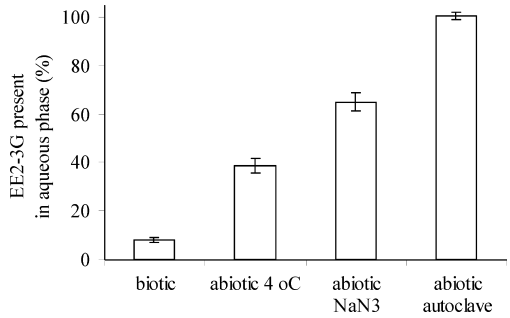

FIGURE 2. Degree of deconjugation of 17α-ethinylestradiol 3-glucuronide (EE2−3G) under biotic and abiotic conditions, the latter achieved by chilling to 4 °C, addition of sodium azide or autoclaving for EE2−3G.

expression given in eq 2 was used where C_O is the initial spiked conjugate concentration and C is the concentration of conjugate at the time sampled (t). Concentrations of the test substrate were low enough to ensure true first order kinetics.

$$-kt = \ln\left(\frac{C}{C_O}\right) \quad (2)$$

Results and Discussion

Deconjugation: A Biotic or Abiotic Process. To determine whether deconjugation is an abiotic or biotic process, several commonly used approaches for microbial inactivation were evaluated—chilling at 4 °C, addition of 1% NaN_3 or autoclaving at 121 °C for 20 min, using EE2−3G as a test conjugate. Following 8 h continuous aeration in AS, only autoclaving achieved total biological inactivation of the samples; consequently it was utilized to achieve abiotic conditions (Figure 2).

To further determine where deconjugation of the conjugate or degradation of the free steroid may be occurring, samples of AS were filtered with either 0.45 or 0.22 μm cellulose acetate membrane filters to remove solids and viable cells. Each filtered sample was spiked with either a free (E2), glucuronide (E1−3G) or sulfate (E1−3S) conjugate steroid and left for 8 h under continuous aeration. For each filtrate, the spiked steroid concentration remained stable with no deconjugation of the sulfate or glucuronide steroid, or degradation of the free steroid E2 being observed. Though intracellular enzymes would have been present in the filtrate due to lysis and/or secretion from the cell, the lack of degradation after removal of viable cells infers that the activity is associated with the biomass and is responsible for deconjugation and degradation.

Deconjugation in Crude Sewage and Activated Sludge. Following 8 h in contact with either crude sewage or AS, the presence of each conjugate, and any free steroid formed upon deconjugation was evaluated under biotic and abiotic conditions. Under abiotic conditions, no free steroid was observed in any of the samples confirming that deconjugation of the spiked conjugate had not occurred. In contrast, the concentration of each conjugate decreased in the biotic samples and the free steroid was observed (Figure 3), demonstrating that deconjugation is a biotic process requiring the action of MO to cleave the conjugate moiety.

Comparing conjugate behavior between AS and crude sewage, glucuronide conjugates completely deconjugated in the crude sewage after 8 h. Depending on the residence time in the sewerage system, it infers that glucuronides may deconjugate on-route to the STP and be negligible in the influent. In contrast, both sulfate conjugates are present in the crude sewage and AS at significant levels after 8 h. This clear distinction between the glucuronide and sulfate conjugates is due to the type of conjugate moiety which are cleaved by different, specific enzymes (*5, 19, 20*) and infers that the sulfate moiety imparts considerable resistance to the MO responsible for deconjugation. These batch studies and the condominium fate study (*13*) provide evidence that deconjugation of sulfate conjugates is difficult to achieve and limited.

Contrasting hypotheses have been proposed relating to the presence of conjugates in the STP influent, with steroids present mainly in their free form (*3, 4*) or conjugated form (*1, 21, 22*). These findings illustrate that the conjugate moiety (sulfate or glucuronide) is influential in deciding which hypothesis will prove true. A further consideration is that the sewerage system can be aerobic or anaerobic depending on the design. As these batch studies were under aerated conditions, future studies evaluating anaerobic deconjugation is necessary.

Observing the free steroid in biotic samples spiked with a conjugate confirm that the primary removal mechanism for both glucuronide and sulfate steroid conjugates in the sewage environment is deconjugation to the free steroid. For glucuronide conjugated steroids this observation is supported by clinical observations where faecal excretion contains a higher ratio of free to conjugated steroids compared to excretion in urine, as a consequence of action of the microflora in the gut (*5*). The observation of glucuronide deconjugation in AS concurs with results from previous investigations demonstrating the deconjugation of 17β-estradiol glucuronides in diluted simulated AS (*12*) and diluted AS from a field site (*6*).

Formation of Sulfate Conjugate in Crude Sewage. For crude sewage samples spiked with EE2−3G, the presence of EE2−3S was observed after 8 h. No EE2−3S was observed in the abiotic sample spiked with EE2−3G and neither had this been detected in the biotic AS sample spiked with EE2−3G. As the experiment was undertaken in triplicate and controls were carried out alongside, the EE2−3S was not due to contamination at the spiking stage, inferring that its presence is due to interaction with the crude sewage.

Sulfate conjugation of steroid estrogens in the human body occurs by sulfotranferases (SULT1 E1) which utilize 3′-phosphoadenosine- 5′-phosphosulfate (PAPs) as the sulfur donor. The observation of sulfate conjugate production that sulfotransferase and a sulfur donor are present, thereby allowing for sulfate conjugation to occur following initial cleavage of the glucuronide moiety. This is supported by a recent study of a STP with AS which observed an increase in E1−3S and E2−3S across the STP of 74% and 360% respectively (*11*). If sulfate conjugation of free steroids can occur in sewage then this could have profound implications, with the sulfate group effectively "protecting" the free steroid from the biodegradation and partitioning processes in the STP. The question then arises about the deconjugation ability of the microbial community in the watercourse, which coupled with the long duration, may have sufficient deconjugation ability to cleave the conjugate and produce the free steroid.

Deconjugation and Stability of Free Steroid Estrogens. To understand the fate of conjugated steroids, a mass balance is required that includes observing the free steroid released following deconjugation, and the subsequent degradation and to a lesser extent sorption of the free steroid. A mass balance for each conjugate after 8 h in either AS or crude sewage was determined. The percentage distribution for the spiked conjugate remaining in the sample and the presence of the free steroid in the aqueous and solid phase (for AS) is given in Table 1. Equation 1 was used to determine the percentage loss of the spiked conjugate from the sample which represents degradation of the free steroid in the sample (following its formation upon deconjugation).

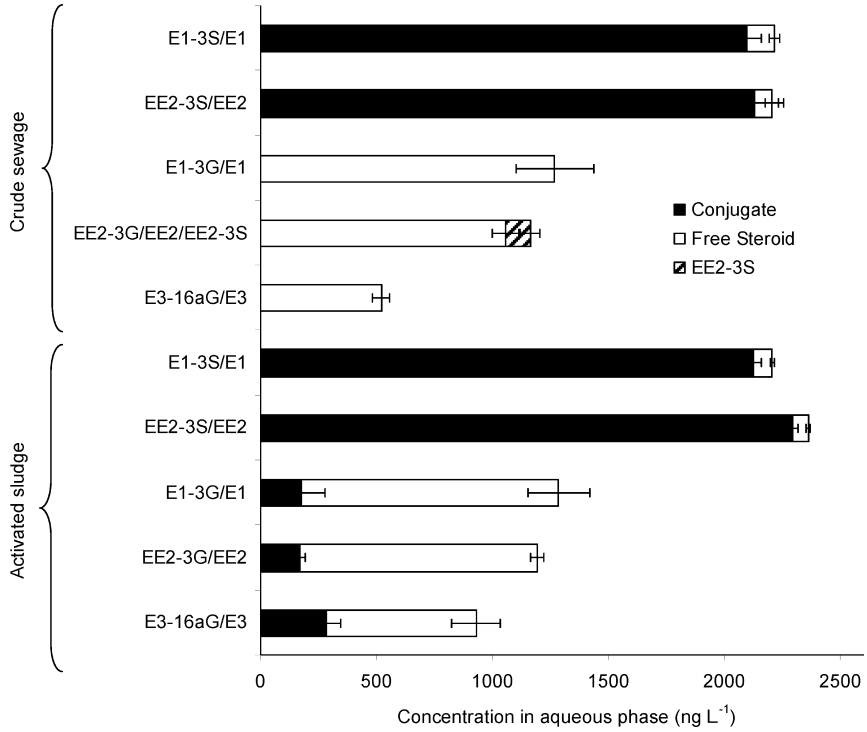

FIGURE 3. Deconjugation of steroid conjugate each spiked in separate samples and formation of the free steroid in the aqueous phases of crude sewage and laboratory-scale Husmann activated sludge samples after 8 h of continuous aeration (each carried out in triplicate).

TABLE 1. Mass Balance of Individually Spiked Conjugates in Laboratory-Scale Husmann Activated Sludge or Crude Sewage from the Field Following 8 h Incubation[a]

	laboratory-scale Husmann activated sludge				crude sewage from the field			
individually spiked steroid conjugate[1]	percentage in aqueous phase (%)		percentage in solid phase (%)	percentage loss of conjugate from the sample [2] (%)	percentage in aqueous phase (%)		average concentration in solid phase (%)	percentage loss of conjugate from the sample [2] (%)
	conjugate	free			conjugate	free		
E3−16αG	12.3 ± 2.6	44.6 ± 7.3	2.4 ± 0.8	40.7 ± 4.5	0.0	36.1 ± 2.6	NA	63.9 ± 2.6
EE2−3G	8.0 ± 1.2	76.6 ± 2.1	12.0 ± 1.9	3.5 ± 1.5	EE2−3G = 0.0 EE2−3S = 6.3 ± 2.5	79.2 ± 4.3	NA	14.5 ± 5.9
E1−3G	7.0 ± 4.0	72.7 ± 8.7	8.2 ± 0.4	12.2 ± 10.7	0.0	83.1 ± 11.0	NA	16.9 ± 11.0
EE2−3S	93.7 ± 0.9	3.4 ± 0.4	1.6 ± 0.1	1.3 ± 0.6	87.0 ± 5.0	3.7 ± 1.5	NA	9.3 ± 5.9
E1−3S	90.3 ± 1.6	4.7 ± 0.5	1.6 ± 0.4	3.4 ± 1.6	89.3 ± 2.5	6.5 ± 1.3	NA	4.2 ± 3.4

[a] The percentage distribution between the spiked conjugate remaining in the sample, and the free steroid in the aqueous and solid phase (for AS) is provided. The percentage loss of the spiked conjugate from the sample represents degradation of the free steroid which is present in the sample due to deconjugation of the conjugate. ([1] = carried out in triplicate; [2] = due to degradation of the free steroid following deconjugation; NA = not analyzed due to insufficient material).

The limited sulfate deconjugation meant that relatively little free steroid was formed which provides less opportunity for degradation/sorption processes to act on the released free steroid, e.g., degradation of E1 produced from deconjugation of E1−3G was greater than E1 degradation in the E1−3S spiked sample (12% versus 3%). This is likely to have implications on optimizing STPs for steroid removal. As steroids conjugated with a sulfate group are more resistant to cleavage releasing the free steroid, they will be more likely to survive the STP compared to glucuronide conjugates. This has been substantiated in the field utilizing the more recent application of LC/MS/MS to environmental analyses (*13, 23−26*). Degradation of the free steroid formed upon deconjugation was most evident for E3 suggesting that relatively little E3 is likely to survive STP if excreted as a glucuronide conjugate. This is due to rapid degradation of the free steroid following cleavage of the conjugate. As E3 is rarely analyzed, this conclusion is more difficult to substantiate. However, 100% removal for E3 has been observed for a Spanish STP, where E3 was detected at 262 ng L^{-1} in crude sewage (*27*), while elimination of E3 in three German STPs was 58−69% for AS and only 34% for trickling filter. Due to the ethinyl group at carbon 17 of the D-ring, EE2 is more persistent and this was reflected with degradation at 3.5% and 1% in the glucuronide and sulfate spiked samples, respectively. Efforts should be directed toward improving deconjugation during STP so that the released free steroid has more time in contact with sewage thereby allowing removal (degradation/sorption) processes to take place. This would result in decreased concentrations of free and conjugated steroids in sewage effluent and receiving watercourses.

Timecourse Study. A mass balance showing deconjugation of spiked EE2−3G resulting in the presence of the steroid

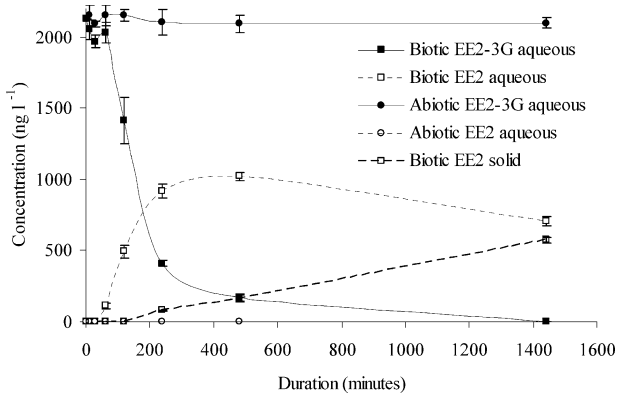

FIGURE 4. Time course study for the single spiked conjugate EE2−3G in laboratory-scale Husmann activated sludge over 24 h (in triplicate). The presence of the free steroid, EE2 released as a result of deconjugation has been plotted in both the aqueous and solid phase of the activated sludge.

and the subsequent fate of the free steroid over a 24 h period in the AS is illustrated in Figure 4. A lag phase was observed for the first hour, followed by rapid deconjugation between 1 and 8 h with a corresponding increase in EE2, demonstrating deconjugation of the spiked EE2−3G to the free steroid. At the 8 h sampling point, the EE2 concentration peaked in the aqueous phase followed by a gradual decline between 8 and 24 h.

To obtain a mass balance, the solid phase was also analyzed. For the 4, 8, and 24 h time points, sorption processes occurred resulting in increased removal of EE2 from the aqueous to the solid phase. Thus for the synthetic glucuronide, the distribution of original spiked conjugate at 8 and 24 h is primarily between EE2 in the aqueous phase and EE2 in the solid phase. Due to the high level of deconjugation for glucuronide conjugates and the resistant nature of EE2 to degradation, a mass balance of the EE2−3G over the 24 h period provides an excellent example of the emergence of the free steroid, EE2 over time and then subsequent partitioning to the solid phase of the AS. Comparatively, as shown in Table 1, there is minimal deconjugation of the sulfate conjugate to the free steroid, which subsequently limits the degradation and partitioning activity on the free steroid.

The Impact of Conjugate Moiety on Deconjugation. It is apparent from data already presented that the conjugate moiety (glucuronide or sulfate) has a significant impact on deconjugation rates. To quantify this, a time course study in AS for each conjugate was carried out to determine the behavior of the five conjugates throughout the 24 h period (Figure 5). After 2 h, a clear distinction was observed between the glucuronide and sulfate conjugates. At 24 h, none of the three glucuronides could be detected above the detection limits (20 ng L^{-1}). In contrast, the two sulfate conjugates underwent minimal deconjugation, with the synthetic steroid sulfate proving slightly more resistant to deconjugation than the natural sulfated steroid conjugate.

In addition to conjugate moiety, deconjugation may also be influenced by positioning of the conjugate on the steroid with the D-ring conjugate (E3−16αG) proving more resistant than A-ring conjugates (EE2−3G and E1−3G) after 4 h. This is evidenced by the half-lives for E3−16αG, EE2−3G and E1−3G being approximately 200, 150, and 140 min, respectively inferring that conjugate positioning in the steroid plays a role in the rate of deconjugation (Figure 5). It should be noted that as the steroid type (EE2 or E1) was different, it cannot be ruled out that this variable may be part responsible for this observation. The effect of conjugate positioning has recently been identified for natural steroids, substantiating that conjugate positioning on the steroid plays a role in the rate of deconjugation. Approximate half-lives of 7 h were

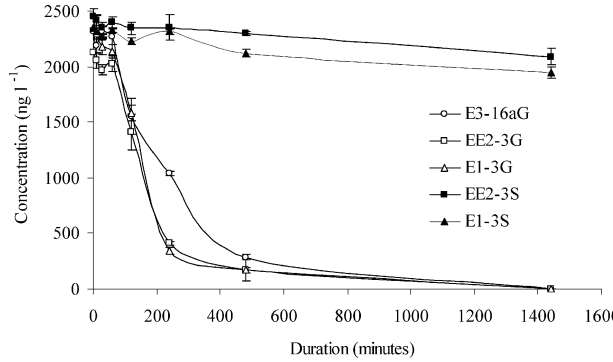

FIGURE 5. Concentration against experiment duration for the five steroid conjugates in activated sludge.

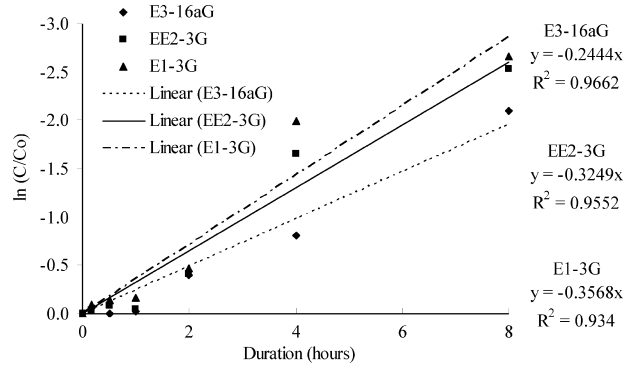

FIGURE 6. Deconjugation behavior of the conjugated glucuronides by the first order reaction rate in laboratory-scale Husmann activated sludge.

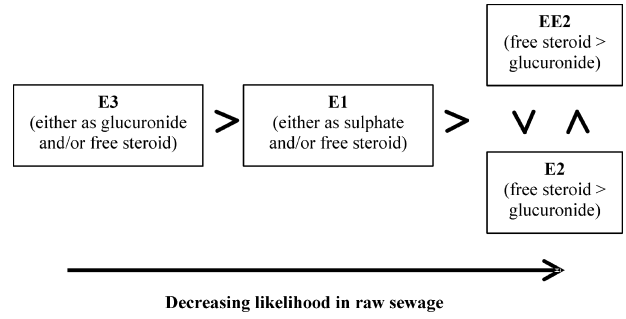

FIGURE 7. Hypothesis from batch studies and excretion profiles on the most likely steroids to enter the sewage treatment plant.

observed for E1−3G and E3−16αG when individually spiked at 25 μg L^{-1} in condominium wastewater, with the D-ring conjugated steroid experiencing a 5 h lag phase (*13*).

Following 8 h, deconjugation was preferential to E1−3G > EE2−3G > E3−16αG > E1−3S > EE2−3S with the three glucuronides nearing complete deconjugation. From this data, there is the possibility that the synthetic steroid may be responsible for imparting some resistance to deconjugation, albeit to a far more limited effect than conjugate moiety and positioning. Thus, factors influencing the rate and extent of deconjugation is conjugate moiety > conjugate positioning on steroid > type of steroid.

Determination of Kinetic Rate Constants for Deconjugation. Conjugate degradation rate parameters were determined in AS using eq 2. Figure 6 illustrates the deconjugation behavior of the glucuronide steroid conjugates and followed a first order reaction rate. The degradation rate constants (k) for EE2−3G, E3−16αG and E1−3G were 0.32, 0.24, and 0.36 h^{-1}, respectively ($R^2 = 0.934 - 0.966$). Reaction rates for the two sulfated conjugated steroids could not be determined within the 24 h incubation period. This finding is in line with

E1−3S deconjugation spiked at 25 μg L^{-1} in condominium wastewater (*13*) where from the graph, a lag phase of approximately 18 h with a half-life of 2.5 days was observed.

Influence of SRT on Degradation. Due to the recalcitrant nature of the sulfate conjugates, the influence of AS with SRTs of 3.2, 5.0, 6.7, and 8.8 days were assessed. Following 8 h incubation with AS, the percentage of the EE2−3S and E1−3S remaining in the samples was between 74 and 94% across the SRT range. In the field, AS processes are typically operated at SRTs between 8 and 12 days, so the limited deconjugation for the sulfates observed in this study may in part be due to the lower SRTs employed. High SRT retains slower growing MO in the AS process which would otherwise be washed out at lower SRTs, the increased number and diversity of the MO consortium at higher SRTs promoting biodegradation (*28*). In addition, the laboratory synthetic AS used in this study will be unlikely to have the diverse microbial consortium that is present in a field AS process. These findings suggest that E1−3S and EE2−3S will survive AS treatment and be present in sewage effluent. This is substantiated by field observations of natural steroid sulfate conjugates in sewage effluent (*24, 25*) and the 24 h lag phase that was experienced by natural sulfate conjugates in condominium water (*13*).

Influence of Temperature on Deconjugation. Using EE2−3G, the influence of temperature on deconjugation was assessed. Following 8 h continuous aeration under biotic conditions with AS at temperatures between 4 and 22 °C, a strong correlation between increasing temperature and decreasing conjugate concentrations in the aqueous phase was obtained (R^2 of 0.994). At 4 °C, 38% of the spiked EE2−3G remained in the aqueous phase of the AS. At 22 °C, no EE2−3G was observed in the aqueous phase having undergone complete deconjugation to the free steroid, EE2.

Thus in the field environment, warmer weather or sites in hotter climates will result in more rapid deconjugation on glucuronides. The influence of increased temperature on steroid removal has been suggested in a previous study for the free steroid, E2 (*29*) and would be expected since the majority of MO in the sewerage system originate from excretion and so are adapted to higher temperatures.

Factors Controlling the Entrance of Steroid Estrogens into Plants. From the batch studies, the factors affecting the rate and extent of deconjugation are.

(1) Glucuronide deconjugation is preferential to cleavage of the sulfate conjugate from the steroid.. Therefore, it would be expected that conjugate concentrations in the crude sewage and through the STP would favor these compounds, e.g., E1−3S.

(2) Conjugates on the D-ring of the steroid are more resistant to deconjugation than A-ring conjugates. Thus, concentrations of E3−16αG or E3 will be higher rather than E1−3G which primarily conjugates with a glucuronide on the A-ring.

(3) The synthetic steroid is slightly more resistant to deconjugation than natural steroids.

However, these assumptions are dependent on the residence time in the sewerage system. If prolonged, there is the possibility that deconjugation followed by complete degradation of the resultant free steroid may occur. Therefore, in point two above, although E3−16αG is more resistant to deconjugation than E1−3G, as degradation of the free steroid is preferential for E3 it is possible that E1 may be present in higher concentrations.

Clinical studies on excretion rates for steroids favor.

(1) E3 (64 μg day^{-1}) > E1 (3−20 μg day^{-1}) > E2 (0.5−5 μg day^{-1}) (*6*), which are predominately excreted as E3−16αG, E2−3G (*6, 30*) and E1−3S, respectively (*30−32*).

(2) EE2 favors conjugation as a glucuronide on the C3 (50−90%) with the remainder as EE2−3S (*33, 34*).

(3) The ratio of synthetic to natural steroid excretion is 1:50 (*35*).

Using this information, the steroids most likely to enter the STP can be hypothesized (Figure 7). It is difficult to assess whether EE2 or E2 will be dominant, though EE2 is far more resistant than E2, the concentration of synthetic steroid on excretion are fifty times less. Thus, 100 ng L^{-1} of E2−3G (corresponding to 60.65 ng L^{-1} E2) will equate to 2 ng L^{-1} EE2−3G or 1.25 ng L^{-1} of EE2.

Determining steroid presence in final effluent is far more difficult dependent on numerous factors such as treatment type (e.g., AS, trickling filter) and optimization (SRT, hydraulic retention time), competing compounds for sorption sites and seasonal variation. However, assessing the behavior of the precursor conjugates under wastewater conditions; in particular the sulfates will allow a greater understanding of their behavior and ultimately facilitate optimization of STPs for their removal.

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