Fate of conjugated natural and synthetic steroid estrogens in crude sewage and activated sludge batch studies

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ABSTRACT

The transformation of free and conjugated steroid estrogens was investigated in batch studies using activated sludge grown on synthetic sewage in laboratory-scale Husmann simulation and crude sewage from the field. Deconjugation proved to be a biotic process, dependent on the conjugate moiety, positioning of the conjugate on the steroid body and the type of steroid (natural or synthetic). A clear distinction between sulfate and glucuronide conjugates was observed in both activated sludge and crude sewage, with sulfated conjugates proving far more recalcitrant. D-ring glucuronides were more resistant to deconjugation than A-ring as demonstrated by their half lives, inferring that conjugate positioning in the steroid plays a role in the rate of deconjugation. Deconjugation of the glucuronide conjugates was preferential in crude sewage than in Husmann activated sludge. Comparatively, sulfated conjugates proved far more recalcitrant in both matrices than the steroids conjugated with a glucuronide moiety Activated sludge solid retention time did not elicit any effect on sulfate deconjugation over the range of 3.2 – 8.8 days. In contrast, a strong correlation was observed between increasing temperature and decreasing 17α-ethinylestradiol 3-glucuronide concentrations in the aqueous phase after 8 hours incubation. Deconjugation followed the first order reaction rate with rate constants for 17α-ethinylestradiol 3-glucuronide, estriol 16α-glucuronide and estrone 3-glucuronide determined as 0.18, 0.24 and 0.35 hours respectively. A hypothesis is presented on the most likely steroids to enter sewage treatment, based on data from these batch studies and excretion profiles.

Keywords: steroid, conjugate, activated sludge, sewage, fate

Introduction

The estrogenic activity in sewage effluent (1) and river systems (2) has been primarily attributed to the presence of steroid estrogens due to incomplete removal during sewage treatment (3). Studies in Japan have shown that 17β-estradiol (E2) was responsible for 34 % of the overall estrogenicity identified in raw sewage influent (crude sewage), but accounted for 100 % in the final effluent after activated sludge (AS) treatment (4). Primary treatment is thought to contribute little to the observed overall removal of estrogenicity (5). Biological treatment via the AS process is an important unit treatment, with field evidence identifying it as the main site for steroid removal (6) and superior to trickling filter (7).

In order to reduce the estrogenic load entering receiving waters, reduction at source and/or improvements to sewage treatment are necessary. The origin of the steroid activity is excretion of naturally produced compounds within the human body and ingestion of the contraceptive pill hence reduction at source is not feasible. Therefore, knowledge of their behaviour and fate during sewage treatment and in particular secondary biological treatment is essential to assess the impact of operational parameters which may be utilised to improve removal efficiency.

Though predominantly excreted from the body in the conjugated form, research has focused on the free steroids as they constitute the dominant form in effluent, suggesting that deconjugation occurs between excretion and sewage effluent discharge. The sewerage system is an integral part of sewage treatment and several studies have proposed that the long residence time in the sewerage system and the fact that free steroids have been observed in crude sewage infer that deconjugation is likely to occur to a certain extent prior to entry to the sewage treatment plant (STP) (8,9). More recently, studies which directly assess steroid conjugate presence in wastewater matrices have identified sulphated steroids in the sewage influent and effluent (10-12).

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 (13). Such bacteria will be present in the sewerage system and STP and are therefore likely to be responsible for the deconjugation of glucuronides (14). There is a paucity of information regarding conjugate behaviour and fate in STP, with only two studies assessing E2 glucuronides via indirect methods (14,15) and one study directly measuring the rate of deconjugation for several glucuronide and sulphate conjugates individually spiked at 25 $\mu g\ l^{-1}$ in condominium wastewater serving a population of 250 (16). The studies reported here were undertaken on crude sewage and laboratory-scale Husmann simulated AS to investigate the behaviour and fate of glucuronide and sulphated conjugated steroids in the STP.

Experimental

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, USA). Preparation and storage of free and conjugated steroid stock solutions have been previously described (10). Ten ml samples of Husmann AS or crude sewage was centrifuged (Jouan S.A. C3*i*, Saint-Herblain, France) at 12,500 g for 10 minutes and the resultant aqueous phase was extracted (10). 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 (17). Analysis was by liquid chromatography mass spectrometry according to established methods (10).

Behaviour and Fate Studies. AS was taken from the aeration chamber of a laboratory-scale Husmann simulation of AS treatment (18). 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 ^{o}C, solid retention time (SRT) of 5.0 days (containing AS suspended solids of 4000 mg l^{-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 mg l^{-1}). Calculation of AS SS and AS volatile SS for organic carbon content followed the HMSO official gravimetric method (19). Using this, AS volatile SS was 70.2 ± 8.5 % of AS SS in line with typical values (20). Experimental parameters were varied as follows for transformation experiments; temperature (4, 11, 17 and 22 ^{o}C) and SRT (3.2, 5.5, 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 was grab sampled mid-morning and kept at 4^{o}C under dark conditions until use in the batch tests. From sampling to use in the batch studies, the time duration was less than 24 hours. 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. Ten ml samples of the crude sewage was analysed at each time point . 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.

Aerobic batch experiments were designed to investigate deconjugation under abiotic conditions (achieved by autoclaving) and biotic conditions. Conjugate presence in the aqueous phase of biotic AS and crude sewage was determined after 8 hours under continuous

aeration. From this, the percentage conjugate loss (C_{LOSS}) from the system was assessed using Equation 1 where 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.

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

A time course aerobic batch experiment was also conducted to investigate the rate of deconjugation in AS, using 200 ml of sample to allow for sample withdrawal during the course of the experiment. Each conjugate was individually spiked into a 200 ml sample at concentrations of 2348, 2450, 2517, 2125 and 2323 ng l^{-1} for E1-3S, EE2-3S, E1-3G, EE2-3G and E3-16αG respectively. Timing began upon addition of the spiked conjugate and 10 ml aliquots were withdrawn from each of the three flasks after 10 minutes, 30 minutes, 1 hour, 2 hours, 4 hours, 8 hours and 24 hours. Samples were immediately centrifuged at 12,500 g for 10 minutes and the supernatant separated for extraction and analysis.

For determining the degradation rate constants for the conjugates, the rate expression given in Equation 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.

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

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 ^{o}C and spiked

ultrapure water was used as a control. Experiments were carried out in triplicate to assess reproducibility.

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 ^{o}C, addition of 1 % sodium azide (NaN_3) or autoclaving at 121 ^{o}C for 20 minutes. After 8 hours continuous aeration in AS, only 8.0 ± 2.7 % of the original spiked EE2-3G remained in the biotic sample compared to 38.7 ± 3.1 % when chilled at 4 ^{o}C, 65.0 ± 3.6 % with the addition of 1 % NaN_3 and 100.3 ± 1.5 % after autoclaving. Only autoclaving achieved total biological inactivation of the samples, consequently it was utilized to achieve abiotic conditions.

Two hundred ml of biotic or abiotic AS were individually inoculated with approximately 2000 ng l^{-1} of each of the five conjugates and aerated for 8 hours. Under abiotic conditions, no deconjugation to the free steroids was observed for all 5 conjugates. In contrast, the concentration of each conjugate decreased in the biotic samples after 8 hours aeration and the free steroid was observed (Figure 1), demonstrating that deconjugation is a biotic process requiring the action of microorganisms (MO) to cleave the conjugate moiety. A clear distinction is demonstrated between the glucuronide and sulfate conjugates, inferring that the sulfate moiety imparts considerable resistance to the MO responsible for deconjugation. Thus, the primary removal mechanism for hydrophilic conjugates in the sewage environment is biodegradation to the free steroid. This observation is supported by clinical observations for glucuronide conjugated steroids, 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 (13). The observation of glucuronide deconjugation in AS concurs with results from previous investigations demonstrating the deconjugation of 17β-estradiol glucuronides in diluted simulated AS (15) and diluted AS from a field site (14).

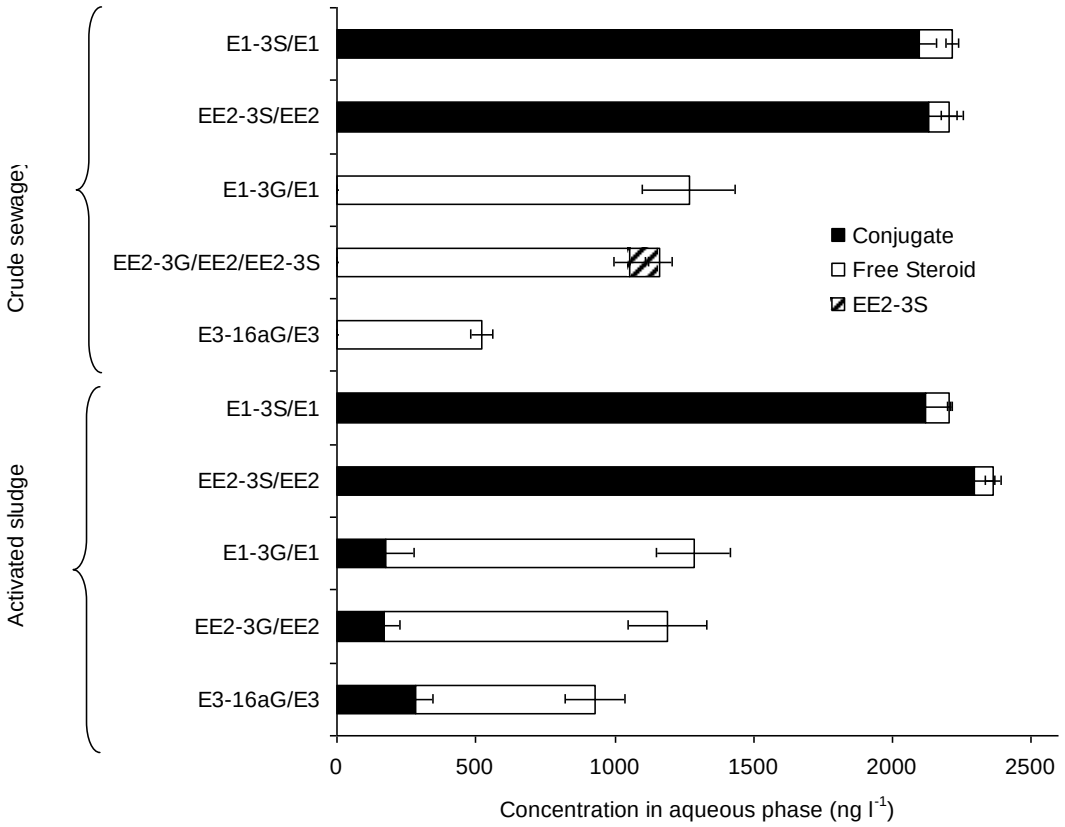


Figure 1. Deconjugation of individually spiked steroid conjugates and formation of the free steroid in the aqueous phases of biotic crude sewage and Husmann simulation activated sludge samples after 8 hours of continuous aeration (each carried out in triplicate).

To further determine where biodegradation may be occurring, samples of AS were filtered with 0.45 μm and 0.22 μm cellulose acetate membrane filters to remove solids. The filtered samples were individually spiked with approximately 2000 ng l^{-1} concentrations of representative free, glucuronide and sulfate conjugate steroids (E2, E1-3S and E1-3G) and

left for 8 hours under continuous aeration. Concentrations remained stable in the filtrate with no deconjugation observed. Enzymes would have been present in the filtrate due to lysis and/or secretion however 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.

Influence of Temperature on Deconjugation. Using EE2-3G, the influence of temperature on the rate of deconjugation was assessed. Following 8 hours continuous aeration under biotic conditions with Husmann AS at temperatures between 4 and 22 ^{o}C, a strong correlation between increasing temperature and decreasing conjugate concentrations in the aqueous phase was obtained (R^2 of 0.994). At 4^{o}C, 38.7 ± 3.1 % of the spiked EE2-3G remained in the aqueous phase of the AS. At 22 ^{o}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 free estradiol (21) and could be expected since the majority of MO in the sewerage system originate from excretion and so are adapted to higher temperatures.

Deconjugation in Crude Sewage. Deconjugation under abiotic and biotic conditions was also investigated using crude sewage obtained from a STP in South East England. The results observed are very similar to those obtained from the AS of the laboratory-scale Husmann simulation (Figure 1) substantiating the earlier observation in AS that sulfate conjugates are considerably more recalcitrant than glucuronide conjugates. However, when crude sewage was spiked with EE2-3G, after 8 hours EE2-3S was also observed at 106.3 ± 42.5 ng l^{-1}. No EE2-3S was observed in the abiotic sample spiked with EE2-3G and neither had this been detected in the AS sample. 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.

Sulphate conjugation of steroid estrogens in the human body occurs by sulphotranferases (SULT1 E1) which utilise 3'-phosphoadenosine- 5'-phosphosulfate (PAPs) as the sulphur donor. The observation of EE2-3S after 8 hours following the spiking of EE2-3G in crude sewage infers that sulphotransferase and a sulphur donor are present, thereby allowing for sulphate conjugation to occur following initial cleavage of the glucuronide moiety. Conjugation of cholesterol in a laboratory based aerobic sewage treatment simulation has been tentatively reported (22). Additionally, several kinds of arylsulphate sulphotransferases have been identified from intestinal bacteria present in human and animal faeces. The sulphating capability was assessed for several parabens and was found to be preferential when using the human bacterium, *Eubacterium* A-44 (23). This illustrates that sulphate conjugation of a phenolic compound may be possible in a matrix which has faecal sources.

From Figure 1 comparing conjugate behaviour in AS and crude sewage, glucuronide conjugates completely deconjugated in the crude sewage after 8 hours. Depending on the residence time in the sewerage system, it infers that glucuronides may deconjugate on-route to the STP. In contrast, both sulfate conjugates are present in the crude sewage and AS at levels between 87 - 93 % after 8 hours.

Deconjugation and Stability of Free Steroid Estrogens. When assessing the percentage conjugate loss from the aqueous phase of the Husmann AS and crude sewage after 8 hours incubation, the resulting free steroid produced on deconjugation must also be accounted for. However, formation of the free steroid as a result of deconjugation is not equivalent to the precursor conjugate, as the ratio between the two must take account for the loss of the conjugate moiety. To evaluate transformation of conjugated forms to the free steroids, mass volume concentrations were converted to molar values. Once the free and conjugate steroids

in the crude sewage and aqueous phase of the AS have been accounted for, any loss can then be attributed to a combination of two factors:

1. Sorption of the free steroid produced from deconjugation to the sludge solids
2. Degradation of the free steroid (produced from deconjugation)

Using Equation 1, the loss of the conjugate from the aqueous phase as a percentage of the original conjugate spike was evaluated in the AS. Loss of the original spiked conjugate from the aqueous phase was most evident for E3-16αG > E1-3G > EE2-3G > E1-3S > EE2-3S. In order to determine how much of the loss was due to the free steroid sorbing to the AS solids, the solid phase was analysed and any free steroid identified was adjusted to the equivalent conjugate amount (Table 1). Therefore, any of the original conjugate still unaccounted for was due to degradation of the free steroid (as the standard deviation attributed to method recoveries was 2.1-3.7 %). Degradation was preferential to E3 (40 %) > E1 formed from E1-3G (12 %) > EE2 from EE2-3G = E1 from E1-3S (3 %) > EE2-3S (1 %). The limited sulfate deconjugation meant that relatively little free steroid was formed and was thus not available for further degradation. Therefore, degradation of E1 produced from deconjugation of E1-3G was greater than E1 degradation in the E1-3S spiked sample (12 % versus 3 %). Due to the ethinyl group at carbon 17 of the D-ring, EE2 is more persistent and this was reflected with degradation at 3% and 1% in the glucuronide and sulfate spiked samples respectively.

For the crude sewage samples, the conjugate loss from the aqueous phase after accounting for transformation processes to the free steroid was between 4.19 - 63.91 % with the greatest loss for E3-16aG mirroring that observed in AS samples. No free steroids were identified from analysis of the solids in the crude sewage possibly a consequence of the limited solids concentration in the 10 ml sample size (<3.56 mg). Degradation occurred at a faster rate in the crude sewage compared to the AS. Though the EE2 and E1 conjugate concentrations in

1 the aqueous phases of both AS and crude sewage was similar, the contribution of free to
2 conjugate presence within the aqueous phases varied.

Indvidually spiked steroid conjugate[1]	Husmann activated sludge				Crude sewage	
	Percentage remaining in aqueous phase (%)	Average concentration in solid phase (%)	Loss from the system (degradation of the free steroid following deconjugation) (%)	Average concentration in aqueous phase (%)	Average concentration in solid phase (%)	Unaccounted (%)
	Conjugate Free					
E3-16αG	56.91 ± 9.85	2.42 ± 0.76	40.67 ± 15.01	36.09 ± 2.64	NA	63.91 ± 3.74
EE2-3G	84.55 ± 13.14	11.98 ± 1.89	3.47 ± 21.24	85.49 ± 6.79	NA	14.51 ± 9.60
E1-3G	79.66 ± 12.74	8.19 ± 0.45	12.15 ± 18.65	83.11 ± 10.99	NA	16.49 ± 15.55
EE2-3S	97.05 ± 4.52	1.62 ± 0.19	1.33 ± 6.66	90.69 ± 6.46	NA	9.31 ± 9.13
E1-3S	94.98 ± 4.29	1.59 ± 0.37	3.42 ± 6.59	95.81 ± 3.81	NA	4.19 ± 5.39

Conjugate spiked	Activated sludge			Crude sewage		
	Average[1] concentration in aqueous phase (%)	Average concentration in solid phase (%)	Unaccounted* (%)	Average concentration in aqueous phase (%)	Average concentration in solid phase (%)	Unaccounted (%)
E3-16αG	56.91 ± 9.85	2.42 ± 0.76	40.67 ± 15.01	36.09 ± 2.64	NA	63.91 ± 3.74
EE2-3G	84.55 ± 13.14	11.98 ± 1.89	3.47 ± 21.24	85.49 ± 6.79	NA	14.51 ± 9.60
E1-3G	79.66 ± 12.74	8.19 ± 0.45	12.15 ± 18.65	83.11 ± 10.99	NA	16.49 ± 15.55
EE2-3S	97.05 ± 4.52	1.62 ± 0.19	1.33 ± 6.66	90.69 ± 6.46	NA	9.31 ± 9.13
E1-3S	94.98 ± 4.29	1.59 ± 0.37	3.42 ± 6.59	95.81 ± 3.81	NA	4.19 ± 5.39

[[1] = carried out in triplicate; * = loss due to degradation and/or experimental error; NA = not analysed due to insufficient material]

Table 1. Mass balance of individually spiked conjugates in Husmann activated sludge or crude sewage following 8 hours incubation. The presence of the free steroid following deconjugation in both the aqueous and solid phase is provided.

Deconjugation results in formation of the free steroid which is then subject to degradation and sorption processes. Mass balances in AS were calculated, accounting for the percentage loss of the conjugate moiety (e.g. 50 ng l^{-1} of E1-3S will deconjugate to form only 38.57 ng l^{-1} of E1). Due to the recalcitrant nature of the sulfate conjugate, degradation/sorption of the free steroid was limited inferring that free steroids are more likely to survive the STP. Indeed sulfated conjugates have been recently been identified in sewage effluent (16,24-26). Relatively little E3 is likely to survive sewage treatment if excreted in the glucuronide form, due to rapid degradation of the free steroid after cleavage of the conjugate. As E3 is rarely analysed, this conclusion is more difficult to substantiate. However, in one study at a Spanish STP, E3 was detected at 262 ng l^{-1} in crude sewage but was not detected in the resulting final effluent. All other steroids were below detection limits in both matrices (27).

Timecourse Study. The consequence of deconjugation resulting in the free steroid is illustrated for EE2-3G over a 24 hour period in the AS (Figure 2). A lag phase was observed for the first hour, followed by rapid deconjugation between 1 and 8 hours with a corresponding increase in EE2, demonstrating deconjugation to the free steroid. At the 8 hour sampling point, the EE2 concentration peaked followed by a gradual decline of 14.7 % between 8 and 24 hours.

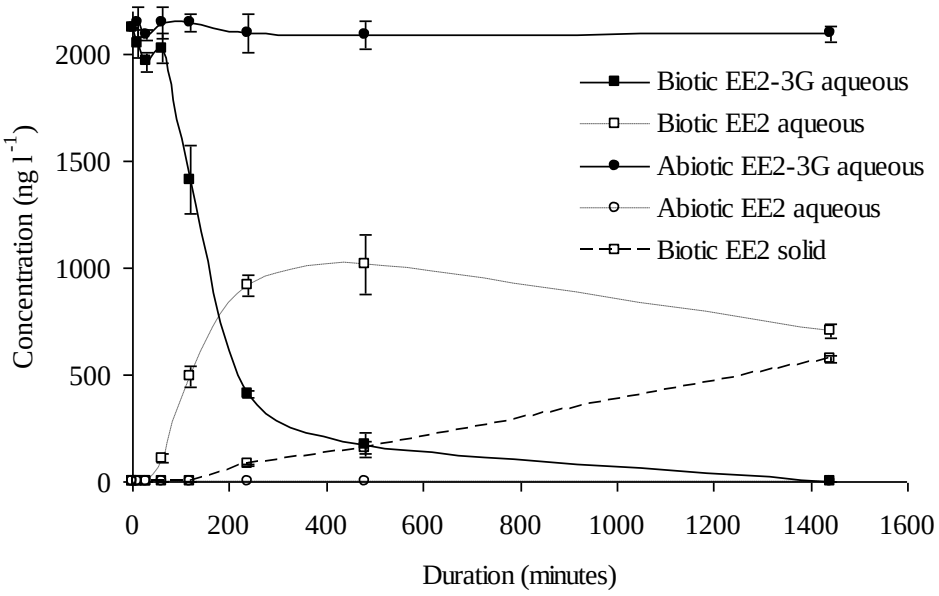


Figure 2. Time course study for individually spiked conjugate EE2-3G in Husmann activated sludge (AS) over 24 hours. The presence of the free steroid, EE2 released as a result of deconjugation has been plotted in both the aqueous and solid phase of the AS

To obtain a mass balance, the solid phase was also analysed. For the 4, 8 and 24 hour time points, the total conjugate mass recovered was 94.0 - 96.5 % indicating near complete recovery of the original conjugate spike. During this period, 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 hours is primarily between EE2 in the aqueous phase and EE2 in the solid phase.

The Impact of Conjugate Moiety on Deconjugation. It is apparent from data already presented that the form of conjugate (glucuronide or sulfate) has a significant impact on degradation rates. To quantify this, a time course study in AS was carried out and the behaviour of the five conjugates throughout the 24 hour period is illustrated in Figure 3. After 2 hours, a clear distinction in behaviour was observed between the glucuronide and sulfate

conjugates. At 24 hours, none of the three glucuronides could be detected above the detection limits (29.4 ng l^{-1}). In contrast, the two sulfate conjugates had only undergone minimal deconjugation with the synthetic steroid sulfate proving slightly more recalcitrant than the natural sulfated steroid conjugate.

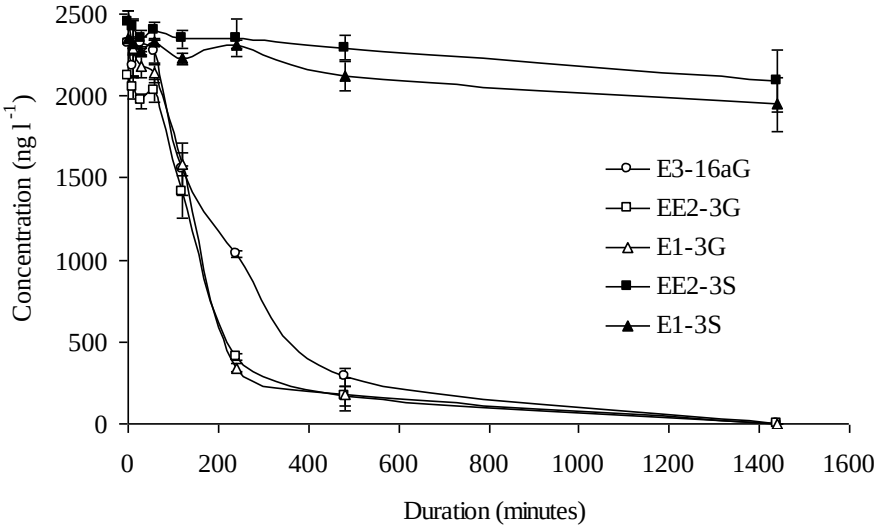


Figure 3. Concentration against experiment duration for the five steroid conjugates in activated sludge

After 4 hours, there was a difference observed between the three glucuronides with the percentage of original spike concentrations for the two A-ring glucuronides, EE2-3G (19.2 ± 0.9 %) and E1-3G (13.6 ± 1.1 %) being less than half the amount present for the D-ring glucuronide E3-16aG (44.6 ± 2.8 %). The half life ($t^1/_2$) for E3-16αG, EE2-3G and E1-3G were 212.0, 162.5 and 152.0 minutes respectively. After 8 hours, deconjugation favoured E1-3G > EE2-3G > E3-16αG > E1-3S > EE2-3S with the three glucuronides nearing complete deconjugation. The clear distinction between sulfate and glucuronide conjugates observed in both AS and crude sewage is due to the conjugated moieties of these steroids which are cleaved by different, specific enzymes (13,28,29).

At 8 hours incubation in crude sewage completely deconjugated glucuronide conjugates. Hence, depending on the residence time in the sewerage system, it is probable that concentrations of glucuronide conjugates entering the STP will be negligible. With a large percentage of the sulfate conjugates still present in the crude sewage after 8 hours incubation, levels of these conjugates can be expected to enter the STP. Contrasting hypotheses have been proposed relating to the presence of conjugates in the influent to STPs, with steroids present mainly in their free form (8,9) or conjugated form (1,30,31). These findings illustrate that the moiety of the conjugate (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, an evaluation of anaerobic deconjugation is required to determine the effect on deconjugation.

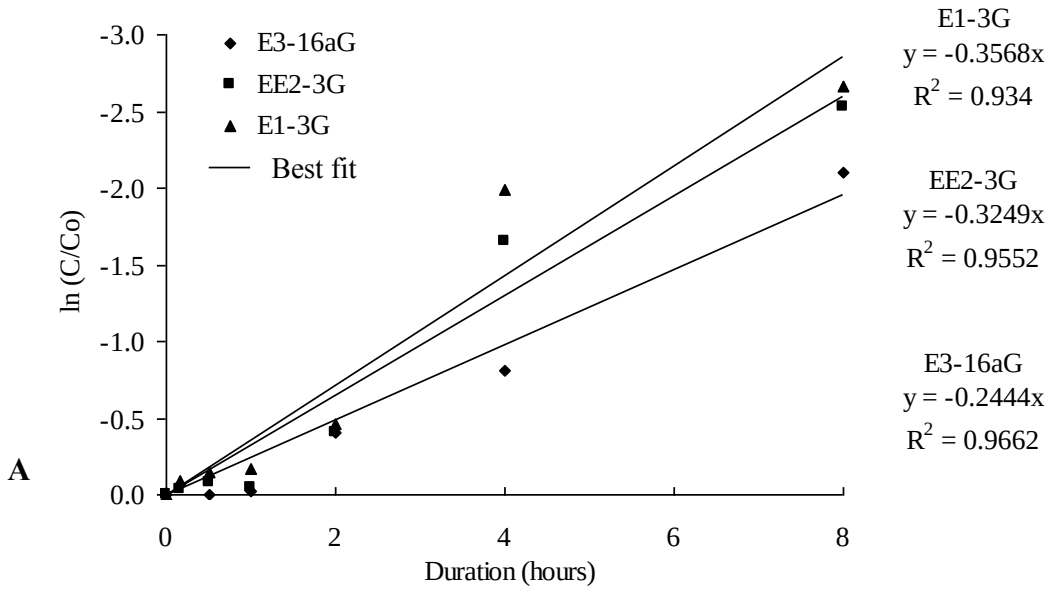
In addition to conjugate moiety, deconjugation is also influenced by positioning of the conjugate on the steroid with D-ring conjugates proving more resistant than A-ring conjugates. This is evidenced by the half lives obtained in the batch studies inferring that conjugate positioning in the steroid plays a role in the rate of deconjugation. The effect of conjugate positioning has recently been identified and substantiates the above findings (16).

As the difference in deconjugation of the synthetic and natural sulfate is also mirrored with the EE2-3G and E1-3G, it infers that the synthetic steroid also imparts 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.

Influence of SRT on Degradation. Due to the recalcitrant nature of the sulfate conjugates, the influence of SRT on sulfate deconjugation was assessed in the AS. At an SRT of 5 days, the concentrations of E1-3S and EE2-3S remaining after 8 hours were 82.9 ± 6.9 % and 85.4

± 7.8 % respectively. When repeated with the SRTs of 3.2, 5.5, 6.7 and 8.8 days, degradation at all SRTs were between 74-93 % for the two sulfate conjugates and no correlation between degradation and SRT was observed. 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 (32). These findings suggest that E1-3S and EE2-3S could pass through the sewerage system predominantly in their conjugated form and may even survive AS treatment. Therefore, sulfate conjugates may be identified in STP effluent and this has been validated recently by direct determination (25,26).

Determination of Kinetic Rate Constants for Deconjugation. Conjugate degradation rate parameters were determined in AS using Equation 2. Figure 4 illustrates the deconjugation behaviour of the five conjugates. For the glucuronides, a first order reaction rate is followed with the degradation rate constants (k) for EE2-3G, E3-16αG and E1-3G being 0.18, 0.24 and 0.36 $hours^{-1}$ respectively (R^2 = 0.923 - 0.966). Reaction rates for the two sulfated conjugated steroids could not be determined within the 24 hour incubation period. This finding is in line with E1-3S deconjugation spiked at 25 ug/l in condominium wastewater (16) where a lag phase of approximately 24 hours was observed and a half life of 2.5 days.



A

Figure 4. Deconjugation behaviour of the conjugated glucuronides by the first order reaction rate

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

B

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 favour 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 favour:

1. E3 > E1 > E2 at 64 > 3-20 > 0.5-5 μg day^{-1} (14), which are predominately excreted as E3-16αG, E2-3G (14,33) and E1-3S (33-35).
2. EE2 favours conjugation as a glucuronide on the C3 (50-90 %) with the remainder as EE2-3S (36,37).
3. The ratio of synthetic to natural steroid excretion is 1:50 (38).

Using this information, the steroids most likely to enter the STP can be hypothesized (Figure 5). 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.

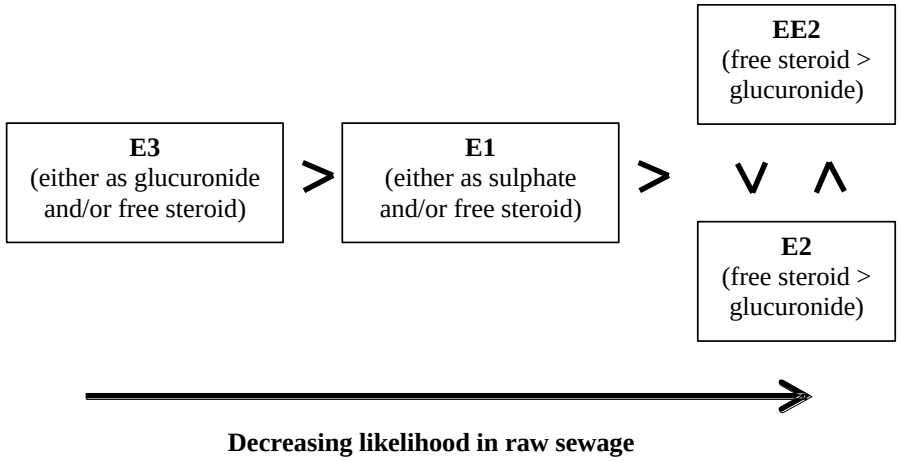


Figure 5. Hypothesis from batch studies and excretion profiles on the most likely steroids to enter the sewage treatment plant.

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), competing

compounds for sorption sites and seasonal variation. However, assessing the behaviour of the precursor conjugates under wastewater conditions; in particular the sulfates will allow a greater understanding of their behaviour and ultimately facilitate optimization of STPs for their removal.

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