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# De-conjugation Behavior of Conjugated Estrogens in the Raw Sewage, Activated Sludge and River Water

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**Abstract**

The fate and behavior of estrone-3-sulphate (E1-3S), estradiol-3-sulphate (E2-3S), estrone-3-glucuronide (E1-3G) and estradiol-3-glucuronide (E2-3G) were studied in raw sewage, activated sludge and river water using microcosms. The glucuronide conjugates had a half-life of 0.4 h in raw sewage, yielding 40-60% of their free estrogens. Field observations at three activated sludge processes suggested complete transformation of the glucuronide conjugates in the sewer. In river water glucuronide conjugates half-lives extended to over two days yielding 60-100% of their free parent estrogens. Transformation of the sulphate conjugates in raw sewage and river water was slow with little formation of the parent estrogens. Sulphate conjugates could readily be detected in sewage influent in the field studies. In activated sludge the sulphate conjugates had half-lives of 0.2 h with the transient formation of 10-55% of the free parent estrogens. Field studies indicated transformation of sulphate conjugates across the sewage treatment, although a proportion escaped into the effluent. These results broadly support the view that glucuronide conjugates will be entirely transformed within the sewer largely to their parent estrogens. The sulphate conjugates may persist in raw sewage and river water but are transformable in activated sludge and, in the case of E2-3S, reform a high proportion of the parent estrogen.

**Keywords:**

De-conjugation; Glucuronide conjugates; Sulphate conjugates; Sewer; Activated Sludge; Natural estrogens.

## 1 Introduction

Where parent estrogens are excreted from human bodies as intact molecules, this is largely in the form of glucuronide and sulphate conjugates [1]. A wide range of conjugates can exist including for estrogen sulphate or glucuronide conjugation at C3- and C17- position of the basic parent estrogen structure. Also, some parent estrogens are conjugated with both glucuronide and sulphate groups together [2, 3]. The conjugated form makes them more water soluble and also relatively inactive as hormones [2]. However, the presence of free estrogens in the aquatic environment reveals some de-conjugation must have taken place in the sewage, or river environments. There is some evidence that the glucuronide forms are very susceptible to de-conjugation but much less certainty on the fate of the sulphate forms [4, 5, 6]. Unlike the glucuronides, residues of sulphate conjugates have been detected in the aquatic environment [6, 7, 8], indicating incomplete degradation at least of estrone-3-sulphate (E1-3S) in the sewage treatment plant (STP). In trying to assess risk, some have argued that both conjugate families are potentially available to conversion back to their parent forms [9], whilst others insist only the glucuronide form is relevant and the sulphate forms can be ignored [1]. As not just hormones, but many pharmaceuticals [10, 11] are excreted as different proportions of these two conjugates, this question has considerable relevance to aquatic risk assessments for pharmaceuticals as a whole. Their high water solubility, and in some cases high lability of conjugates makes them difficult to analyse and has left us with relatively few studies on these important compounds. To date our knowledge on the fate and behavior of the conjugates has been inferred from occasional observations on their presence in sewage or river water [8, 4, 6], and from laboratory studies with activated sludge [12, 13, 5] or with soil media [14]. Thus, in particular, no information on the extent, rates, or behavior of de-

63 conjugation is yet available for the sewer, river environments or in STPs. Using E1-3S,
64 estradiol-3-sulphate (E2-3S), estrone-3-glucuronide (E1-3G) and estradiol-3-
65 glucuronide (E2-3G) as model conjugates, the study tested the following hypotheses:

66 • Glucuronide de-conjugation is sufficiently rapid to permit complete
67 transformation to the free parent compounds within a sewer, or activated sludge
68 environment.

69 • Sulphate conjugate transformation does not yield the parent compound in the
70 sewer, sewage, or river environments. If sulphate transformation to the parent
71 compound occurs, then the rate and extent of de-conjugation in the sewer,
72 sewage and river environments is too small to be of environmental relevance.

73

74 **2 Materials and Methods**

75 **2.1 Chemicals**

76 Estrone (E1), 17β-estradiol (E2), sodium salt of E1-3S, sodium salt of E2-3S,
77 sodium salt of E1-3G and sodium salt of E2-3G were purchased from Sigma-Aldrich,
78 Japan. These conjugates were selected for the batch experiments on the basis of their
79 relative abundance in the urine [4, 1]. E1 and E2 were included in the experiments as a
80 form of positive control.

81 Stable isotope surrogate E1-$d_2$ (for E1), E2-$d_3$ (for E2), E1-3S-$d_4$ (for E1-3S),
82 E2-3S-$d_4$ (for E2-3S) and E2-17G-$^{13}C_4$ (For E1-3G and E2-3G) were obtained from
83 CDN Isotopes, Inc. (Pointe-Claire, PQ, Canada) and used as internal standard for
84 recovery analysis. Individual stock solutions of the standards were prepared in
85 methanol (MeOH), whilst for spiking the standards were prepared in Milli Q water.
86 Working standard mixtures of the compounds were prepared on a daily basis.

87

88 **2.2 Origin of sewage and river samples used in microcosm studies**

89 The sewage samples were collected from an activated sludge plant (ASP)
90 catering for 99,000 people (human PE) with a catchment area of 1,400 ha, and with a
91 mean flow of 57,000 $m^3$/day. The raw sewage, meant to represent the sewer, was
92 collected from the inlet of the plant after the screen. The activated sludge came from
93 the first third of one of the conventional plug flow aeration tanks. The samples were
94 collected in June 2008, when the water temperature at the plant was 21 °C. The time
95 from collection to use in the laboratory was 15 min., thanks to the proximity of the
96 ASP. The 2 L samples were vigorously shaken before decanting into the conical flasks.
97 The river water samples came from the Yodo River, 2 km south of Kyoto City
98 and were collected on 25$^{th}$ June 2008. A description of this river and local conditions
99 can be found in Kumar *et al*, [6]. River water temperature at the time was 18 °C.
100

101 **2.3 Sample preparation and extraction**

102 A pre-treatment method was developed for the extraction of the free and
103 conjugated estrogens from a 20 mL sub-sample. The samples were acidified (pH ~3.0)
104 with 20% acetic acid and then spiked with surrogates. Before loading the sample in
105 Oasis HLB cartridges (200mg/6cc, 30 μm particle size Waters Corp.) the sample was
106 first filtered by a glass fibre acrodisc syring filter (1 μm pore size) with the help of the
107 syringe [15]. Six mL of MeOH followed by 2 mL of 0.5% $NH_4OH$ in MeOH were
108 used for elution. The final elute was further evaporated to dryness under gentle
109 nitrogen stream at 37 °C. The residue was immediately dissolved in 1 mL of
110 acetonitrile (ACN) and Milli Q (1:9) solution. Finally, 10 μL was injected into the
111 UPLC/MS/MS system [15].
112

## 2.4 Chemical analysis

Chromatographic separations and analysis for the batch experiment samples were carried out on a ultra-performance liquid chromatography (ACQUITY UPLC$^{TM}$ system, Waters) coupled to tandem mass spectrometry system using an ACQUITY BEH C18 column (50 mm, 2.1 mm, 1.7μm particle size) for both free and conjugated estrogens. Separation was performed with a binary mobile phase of Milli Q (A) and ACN (B) at a flow rate of 0.2 mL/min. The gradient was as follows: Initial-2 min, 10% B; 2-4 min, 25% B; 4-6 min, 50% B; 6-8 min, 90% B; 9-10 min, 10% B. Mass spectrometry was performed on a Micromass Quattro Premier Tandem MS (Waters) fitted with an ESI interface. In negative ionization, multiple reaction monitoring (MRM) mode was used for the quantitative analysis. The parent/product ions pairs of *m/z* 446.5 to 271.3 for E2-3G, 444.5 to 268.8 for E1-3G, 351.1 to 270.8 for E2-3S, 349.1 to 268.7 for E1-3S, 271.0 to 144.8 for E2 and 268.9 to 144.8 for E1. Relative recoveries using stable isotope surrogate were between 70 (E2-$d_3$) to 100% (E2-17G-$^{13}C_4$).

## 2.5 Microcosm description

The raw sewage and activated sludge were taken from the ASP and immediately (within 15 min) utilized in the batch experiments. Initial measurements of temperature, dissolved oxygen, suspended sludge, and pH were taken (Table 1). Batch experiments were performed in triplicate for each individual estrogen and conjugate. Experiments were carried out in clean, wide necked 500 mL conical flasks. A series of laboratory batch experiments was conducted in different kinds of water as follows:

1. Raw Sewage: E1-3S, E2-3S, E1-3G, E2-3G, E1 and E2
2. Activated Sludge: E1-3S, E2-3S,

3. River Water: E1-3S, E2-3S, E1-3G, E2-3G, E1 and E2

In triplicate, 400 mL of the raw sewage, or activated sludge without filtration were decanted into the flasks, following stirring. Each flask was spiked with 2,500 ng/L MeOH free standard solution of studied estrogens and their conjugates, individually. That equates with initial concentration of 9.25, 9.19, 7.14, 7.10, 5.63, 5.60 nmol/L for E1, E2, E1-3S, E2-3S, E1-3G and E2-3G, respectively. The flasks were continuously stirred in an orbital shaker at 87 rpm and the temperature was maintained at 22±2°C. These values were set according to the trial experiments, where 87 rpm speed of the orbital shaker was found suitable for keeping the floc particles in suspension whilst 22±2 °C is a common sewage water and river temperature in Japan (Table 1). For river water, 2 L initial volumes were continually stirred in the 2.5 L. bottle reactor in an incubator. For river water, the initial concentration was 1.36 nmol/L for E1 and E2, 1.05 nmol/L for E1-3S and E2-3S, 0.83 nmol/L for E1-3G and 0.82 nmol/L for E2-3G (approximately 370 ng/L), respectively. Further, the transformations of the conjugated estrogens were assumed to follow first-order kinetics decay pattern and so half-lives were calculated on a first order basis. For sterile controls, conditions were the same as for the biotic treatments, but preceded by autoclaving at 121 °C and 15 psi for 30 min. Periodical temperature and dissolved oxygen (DO) were measured in the flasks (Table 1). At appropriate time intervals 20 mL sub-samples were taken from the sewage treatments, whilst 100 mL sub-samples were taken from the river water treatments. To preserve the samples before analysis 20 mg (for raw sewage and activated sludge sample) and 100 mg (for river water) of ascorbic acid were added to the sub-samples prior to storage at -80 °C.

**(Insert Table 1)**

### 2.6 STP survey and mass flux calculations

Three full-scale activated sludge process reactors were investigated in three STPs located in Japan. Twenty-four hour composite samples of influent, primary effluent, reactor exit, secondary effluent and final effluent water were collected in dry weather conditions (November, 2008; Figure 1).

**(Insert Figure 1)**

The entire sample pre-treatment process was carried out as described in a previous field study [16]. The limits of detection were 0.5, 0.2, 0.6 and 0.6 ng/L for E1-3S, E2-3S, E1-3G and E2-3G, respectively. Further, dissolved free and conjugated estrogen mass fluxes between the cumulative sampling points were determined as:

$$m_i = Q \times C_{Di} \quad \text{(Eq.1)}$$

where $m_i$ is the mass flux of the individual estrogen (i) (µg/L), Q is the flow (m3/d), $C_{Di}$ is the estrogen concentration in dissolved phase (µg/L). The following input data (Table 2) were used to calculate dissolved load in three activated sludge process reactors.

**(Insert Table 2)**

## 3 Results and Discussion

### 3.1 Experimental conditions

An inherent weakness of microcosm batch studies is their instability, this is particularly true where lots of bacteria and carbon are present since substrates are soon depleted, and toxic by-products formed. Thus, they are at their most realistic only in their first few hours. This is not as much as a handicap as it may at first seem for batch

188 studies as sewer travel times are typically in the order of only a couple of hours, and
189 activated sludge treatment typically 5-10 hours. The principal advantage of a batch
190 study is, at least in its initial stages, it is a good representation of the real environment.
191 In these studies the experimental temperature was similar to the real conditions, whilst
192 the DO rose in the sewage samples but not a great deal above that which might be
193 normal for those conditions (Table 1). The river water batch study is likely to be a
194 somewhat more stable system with lower carbon and bacteria than a STP and indeed
195 with less microbial activity incubations need to be longer. Fortunately, the
196 experimental conditions over the course of the 5 d period appeared to remain stable
197 (Table 1). Thus, at least at a superficial level, whether sewage, or river water, the batch
198 cultures resembled their original conditions throughout the incubation.
199
200 **3.2 Behavior of the conjugates in raw sewage representing the sewer**
201 The raw sewage incubation was intended to simulate the fate of the conjugates
202 in the sewer, i.e. prior to arriving at an STP. The sterile controls for the glucuronide
203 and sulphate conjugates showed little, or no, change in concentration over the course
204 of the experiments (Figures 2 and 3). The concentrations used in the experiments
205 (2,500 ng/L) were higher than would normally be found in the environment. It is
206 acknowledged that the concentration level can influence microbial behavior but in a
207 recent study with estrogens it has been demonstrated that between 30 to 10,000 ng/L
208 estrogens are degraded at similar rates in sewage [17]. After only 120 min incubation
209 both E2-3G and E1-3G were entirely transformed (Figure 3). This equated to a half life
210 of 0.4 h for both E2-3G and E1-3G in the raw sewage at 22 °C. However, perhaps
211 surprisingly, this did not yield a stoichiometric conversion to the parent estrogens as
212 the E2 and E1 formation was only 60 (3.36 nmol/L) and 40% (2.24 nmol/L)

213 respectively at their highest points. This suggests that glucuronide conjugates do not
214 necessarily entirely convert to their parent compounds in a sewage matrix. Earlier,
215 Gomes et al [5] have reported 83% formation of E1 from E1-3G after 8 h of incubation
216 in synthetic activated sewage. For the E2-3G it can be seen that the E2 formed is itself
217 being converted to E1 after 1.5 h (Figure 2). Thus, to some extent the sewer
218 environment has the potential to act as a preliminary sewage treatment stage. The rapid
219 formation of a large proportion of the estrogen parent from the glucuronide conjugates
220 in the raw sewage microcosms support the hypothesis that these forms will be
221 transformed prior to arrival at an STP [1] and are corroborated by field observations
222 where these forms are rarely seen in the influent [4, 18]. However, the apparent
223 incomplete formation of the parent compounds might indicate other metabolites were
224 formed. If this were the case it might imply that estrogen excretions models might be
225 overestimating the amount of E2 and E1 arriving at a STP [1]. E1 was slowly
226 transformed in raw sewage, with a 9 h half-life, whilst E2 had a half-life of only 2 h
227 being largely transformed to E1. This supports the view that transformation of E2 to
228 E1 occurs during sewer transit as proposed by Johnson and Williams [1].
229
230 **(Insert Figure 2)**
231
232 **(Insert Figure 3)**
233
234 The transformation of the sulphate conjugates in raw sewage was slow with no
235 more than 5% de-conjugated to the parent estrogens after 2 h (Figure 3). This equated
236 to a half life of 11.5 h for E2-3S and 13.9 h for E1-3S in the raw sewage at 22 °C.

Overall a much smaller proportion (total 12%) of the sulphate conjugates were transformed to their parent estrogens implying other metabolites are more important.

### 3.3 Behavior of the sulphate conjugates in activated sludge

With previous studies on glucuronide conjugates in activated sludge [5, 19] and the rapid transformation in raw sewage previously observed, the activated sludge studies here focused on the sulphate conjugates alone (Figure 4).

**(Insert Figure 4)**

E1-3S was rapidly transformed (half life of 0.19 h) in activated sludge with little formation of residual E1. E2-3S was similarly rapidly transformed but in this case a much higher proportion of a free estrogen, E1 was formed. Around 55% (3.94 nmol/L) E1 of original at 15 min. of incubation was detected. Intriguingly, E1-3S appeared to be one of the transient by-products of E2-3S transformation, thus E1-3S, E1 (and presumably E2) were amongst the products of E2-3S breakdown. Similar metabolites were reported by Scherr et al. [20], however, using a slightly different media (pasture soils) in a microcosm study.

### 3.4 Behavior of the conjugates in river water

Both glucuronide and sulphate conjugates concentrations were stable in the sterile controls (Figure 5). In the river water incubation E1-3G was transformed almost 1:1 to E1, with E2-3G forming a mixture of E2 and E1, representing 64% (1.87 nmol/L) of the original conjugate after 5 days incubation. The half lives were 15.4 and 12.4 h for E2-3G and E1-3G, respectively.

**(Insert Figure 5)**

Transformation of the sulphate conjugates was negligible, although a small fraction of the parent estrogen was detected (Figure 6). In the river water samples an E2 half-life of 1.4 d was recorded, whilst E1 degraded at a slower rate with a half-life of 4.1 d (data not shown). Half lives of 1.2 days for E2 was previously recorded in UK river water samples [21].

**(Insert Figure 6)**

### 3.5 Behavior of the conjugates in actual STPs

From examining the fate of the conjugates from three Japanese STPs, some clear observations can be made; firstly a complete absence of the glucuronide conjugates detected in the primary influent. Second, low concentrations of the sulphate conjugates could be found within the STPs (Table 2). E1-3S was detected at a maximum of 15.7 ng/L concentration, whilst E2-3S was 8.7 ng/L in the primary influent sample. Following their arrival, the concentration and load of the two measured sulphate conjugates declined throughout the sewage process (Figure 7). However, around 0.23 mg/day (16%) of E1-3S was detected in the secondary effluent in STP A reactor exit in contrast of STP B and C (>98%), indicating there incomplete de-conjugation in activated sludge processes. Glucuronide conjugates were never detected in the primary effluent sample and so it can infer conversion within the sewer. Hence, the role of the glucuronide conjugates can be neglected inside STP.

**(Insert Figure 7)**

## 4 Conclusions

As predicted, the selected glucuronide conjugates were quickly transformed in raw sewage representing a sewer environment although they were not entirely de-conjugated to their parent forms. The field observations also indicate the complete de-conjugation of glucuronide conjugates in the sewer. In contrast, the sulphate conjugates were only slowly transformed. The presence of sulphate conjugates in all three STPs influent samples confirmed the limited transformation suggested for sewer transport. E2 also was transformed in the raw sewage study suggesting that a proportion of the E2 would be converted to E1 in the sewer. The sulphate conjugates demonstrated their greater persistence to the glucuronides in river water studies. Contrary to expectations, with one of the sulphate conjugates, E2-3S over 50% was transformed to the estrogen parent molecules in the activated sludge study. The STP studies indicated substantial but incomplete transformation of sulphate conjugates across the different stages of the STPs. Returning to the original hypotheses:

- Glucuronide de-conjugation would be sufficiently rapid to permit complete transformation to the free parent compounds within a sewer, or activated sludge environment.

  Strictly speaking this hypothesis has been falsified as transformation was not quite complete after 2 h in raw sewage and complete conversion to the parent compounds did not occur. However, the studies demonstrated the potential for substantial conversion of the glucuronide conjugates in a sewer environment to their parent estrogens and were not found in the Japanese STP influent.
- Sulphate transformation does not yield the parent compound in the sewer, sewage and river environments.

311 This hypothesis was also falsified as a proportion of the parent compounds could
312 be released.
313 Overall these data suggest that neither the model of Johnson and Williams [1], or
314 Cunningham et al. [5] has an entirely correct understanding of the behavior of the
315 different conjugates. Nevertheless a broad interpretation, that glucuronide conjugates
316 are important (being readily transformable to their parent compounds) whilst sulphate
317 versions are less so, remains a good starting place for a risk assessment for human
318 excreted hormones or pharmaceuticals.
319

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391 **Figure Captions**

392 Figure 1 Schematic description of surveyed STPs with sampling location (Solid arrow:
393 Composite samples; PST=Primary Settling Tank; SST= Secondary Settling tank).

394 Figure 2 Time course study for the single spiked glucuronide conjugates in raw sewage
395 (mean and SD, S.C.: Sterile control).

396 Figure 3 Time course study for the single spiked sulphate conjugate in raw sewage
397 (mean and SD, S.C.: Sterile control).

398 Figure 4 Time course study for the single spiked sulphate conjugate in activated sludge
399 (mean and SD, S.C.: Sterile control).

400 Figure 5 Time course study for the single spiked glucuronide conjugate in river water
401 (mean and SD, S.C.: Sterile control).

402 Figure 6 Time course study for the single spiked sulphate conjugate in river water
403 (mean and SD, S.C.: Sterile control).

404 Figure 7 Dissolved mass fluxes of free and conjugated estrogens (in box) in three STPs
405 (error bar shows range of the detection).

Table 1 Water quality parameters during microcosm experiments

| **Water Quality Parameters** | **Raw Sewage** | **Activated Sludge** | **River Water** |
|---|---|---|---|
| Initial Temperature (°C) | 16.8 | 21.0 | 18.4 |
| pH | 7.4 | 7.4 | 7.1 |
| SS (mg/L) | 128 | 2830* | 4.1 |
| DO (mg/L) | 1.5 | 2.0 | 9.2 |
| **During Experiment** | | | |
| Incubation time | 24 h | 24 h | 5 days |
| DO (mg/L) | 3.8~4.2 | 2.5~3.5 | 7.2~9.2 |
| Temperature (°C) | 22±2 | 22±2 | 22±2 |

* MLSS

Table 2 Input parameter and estrogen concentration (ng/L) in three STPs

| | | Primary Influent | Primary Effluent | Reactor Exit | Secondary Effluent | Final Effluent |
|---|---|---|---|---|---|---|
| STP A | PE:775,500 | | | | | |
| | HRT: 12.1 hr | | | | | |
| | SRT: 19 days | | | | | |
| | Q ($m^3$/d) | 221,130 | 197,316 | 256,511 | 197,316 | 194,560 |
| | SS (mg/L) | 126 | 41 | 1350 | 2 | 0 |
| | Estrogen Concentration in dissolved phase (ng/L) | | | | | |
| | E1 | 14.5 | 35.3 | 24.3 | 16.5 | 8.3 |
| | E2 | 19.8 | 42.6 | 5.1 | 2.2 | 1.6 |
| | E1-3S | 6.8 | 5.4 | 2.2 | ND | ND |
| | E2-3S | 5.6 | 2.2 | 0.3 | 0.2 | 0.2 |
| | E1-3G | ND | ND | ND | ND | ND |
| | E2-3G | ND | ND | ND | ND | ND |
| STP B | PE:84,000 | | | | | |
| | HRT: 9.9 hr | | | | | |
| | SRT: 22 days | | | | | |
| | Q ($m^3$/d) | 29,060 | 29,060 | 43,590 | 29,060 | 28,860 |
| | SS (mg/L) | 81 | 46 | 1600 | 2 | 0 |
| | Estrogen Concentration in dissolved phase (ng/L) | | | | | |
| | E1 | 19.5 | 22.0 | 3.5 | 2.2 | 0.4 |
| | E2 | 38.9 | 42.0 | 1.8 | 0.5 | ND |
| | E1-3S | 11.2 | 9.4 | 1.0 | ND | ND |
| | E2-3S | 6.6 | 1.8 | 0.6 | 0.2 | ND |

| | | | | | | |
|---|---|---|---|---|---|---|
| | E1-3G | ND | ND | ND | ND | ND |
| | E2-3G | ND | ND | ND | ND | ND |
| STP C | PE:604,000 | | | | | |
| | HRT: 13.2 hr | | | | | |
| | SRT: 10 days | | | | | |
| | Q ($m^3$/d) | 42,221 | 42,221 | 61,468 | 42,221 | 53,880 |
| | SS (mg/L) | 212 | 71 | 2830 | 2 | 0 |
| | | Estrogen Concentration in dissolved phase (ng/L) | | | | |
| | E1 | 26.9 | 31.1 | 3.9 | 2.8 | ND |
| | E2 | 38.4 | 40.0 | 2.0 | 1.0 | ND |
| | E1-3S | 15.7 | 9.1 | ND | ND | ND |
| | E2-3S | 8.7 | 3.1 | 0.2 | 0.2 | ND |
| | E1-3G | ND | ND | ND | ND | ND |
| | E2-3G | ND | ND | ND | ND | ND |

Q=flow
SS=Suspended Solids
PE=Population Equivalent
ND=Non-detect (or below detection level)

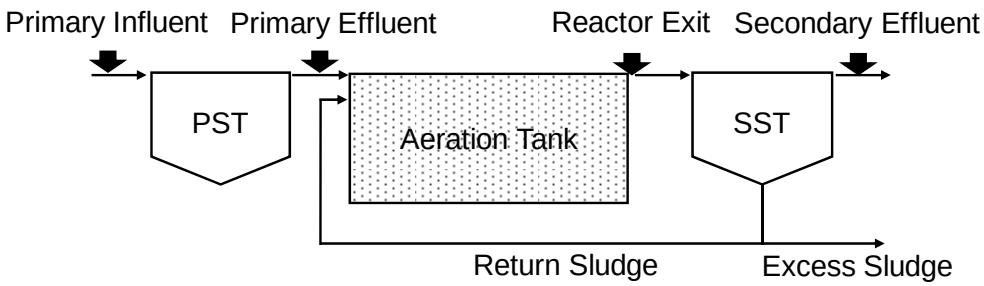


Figure 1 Schematic description of surveyed STPs with sampling location (Solid arrow: Composite samples; PST=Primary Settling Tank; SST= Secondary Settling Tank).

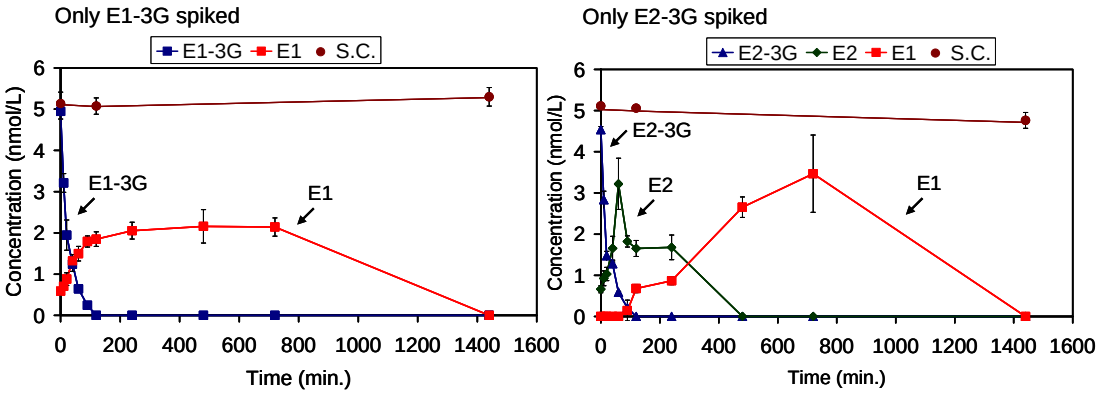


Figure 2 Time course study for the single spiked glucuronide conjugates in raw sewage (mean and SD, S.C.: Sterile control).

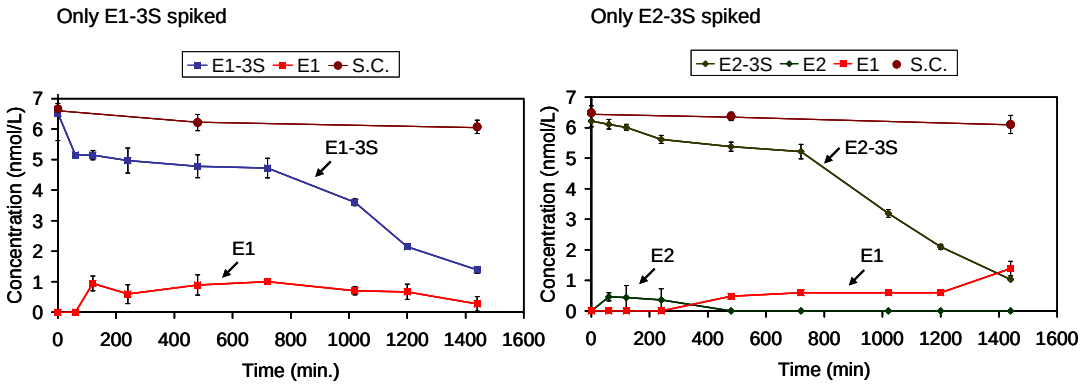


Figure 3 Time course study for the single spiked sulphate conjugate in raw sewage (mean and SD, S.C.: Sterile control).

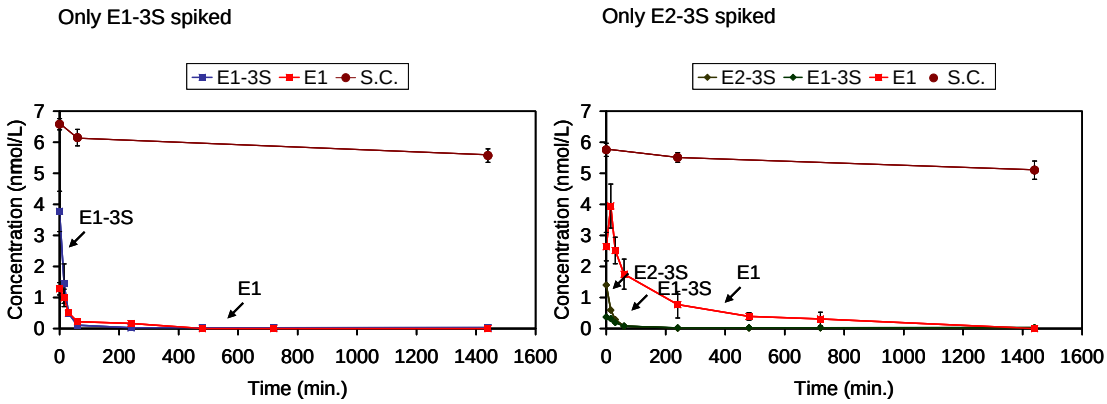


Figure 4 Time course study for the single spiked sulphate conjugate in activated sludge (mean and SD, S.C.: Sterile control).

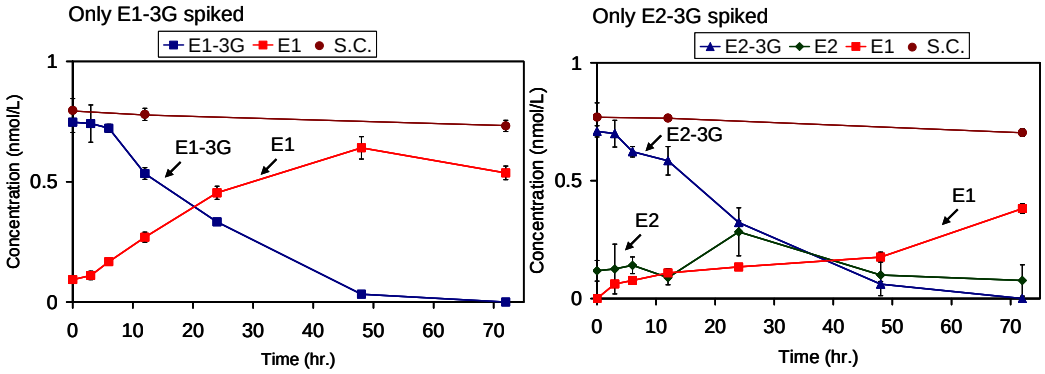


Figure 5 Time course study for the single spiked glucuronide conjugate in river water (mean and SD, S.C.: Sterile control).

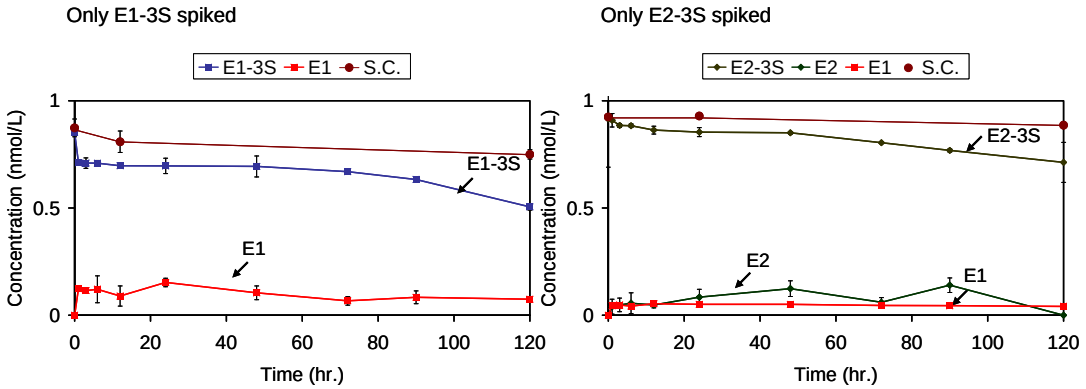


Figure 6 Time course study for the single spiked sulphate conjugate in river water (mean and SD, S.C.: Sterile control).

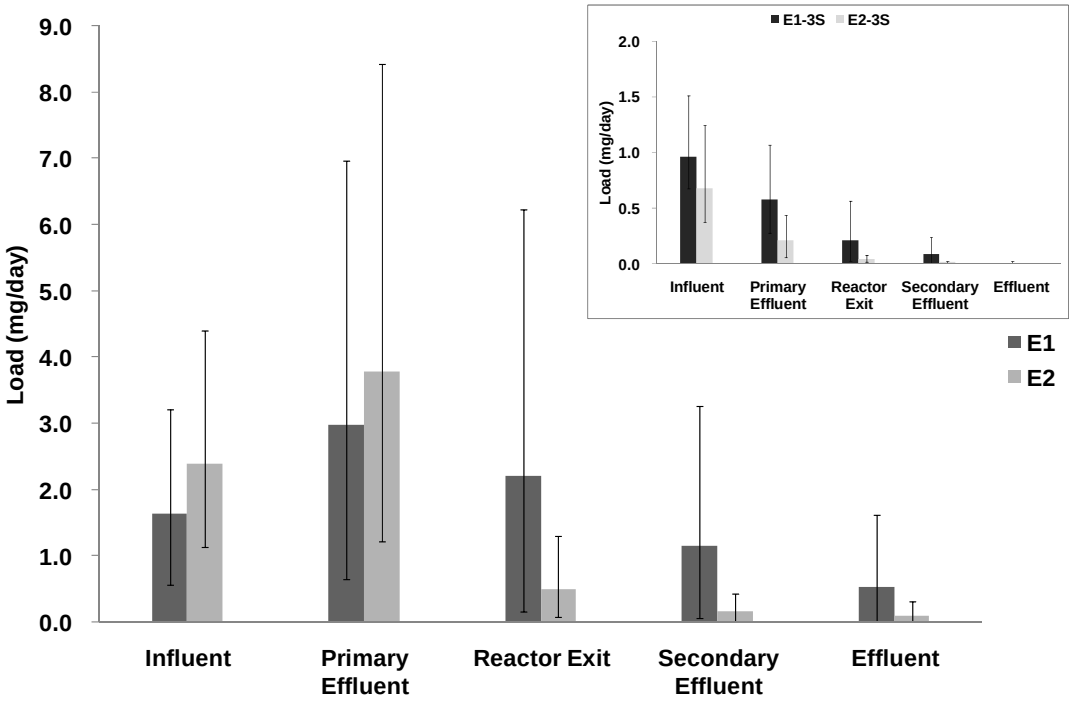


Figure 7 Dissolved mass fluxes of free and conjugated estrogens (in box) in three STPs (error bar shows range of the detection).