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Progesterone potentially degrades to potent androgens in surface waters

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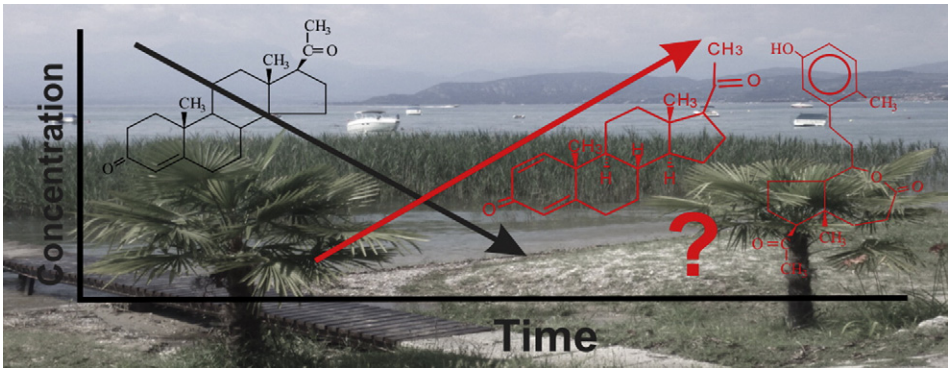
HIGHLIGHTS

- Progesterone degrades rapidly in the environment.
- Some of the main degradation products have been identified.
- Side chain modifications were observed.
- Dehydrogenation was identified as a pathway.
- Some of the products potentially possess biological activity.

GRAPHICAL ABSTRACT



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ABSTRACT

Progesterone is a natural hormone, excreted in higher concentrations than estrogens, and has been detected in the aqueous environment. As with other compounds, it is transformed during wastewater treatment processes and in the environment. However, minor modifications to the structure may result in transformation products which still exhibit biological activity, so understanding what transformation products are formed is of importance. The current study was undertaken to identify putative transformation products resulting from spiking river water with progesterone in a laboratory-based degradation study and hence to follow the metabolic breakdown pathways. On the basis of literature reports and predictions from the EAWAG Biocatalysis/biodegradation database, target putative transformation products were initially monitored under unit resolution mass spectrometry. The identity of these transformation products was confirmed by using accurate-mass quadrupole time-of-flight. The study results highlight that transformation of progesterone can potentially create other classes of steroids, some of which may still be potent, and possess other types of biological activity.

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1. Introduction

The regular detection and assessment of natural and synthetic steroidal hormones in effluent and surface water systems has raised significant environmental and public health concerns. This is due to their reported endocrine disrupting effects on biota at concentrations below 1 ng/L (Runnalls et al., 2010; Sumpter and Johnson, 2005; Ying et al., 2002). Progesterone (P4), a C-21 progestational steroidal ketone, is secreted naturally by females (Besse and Garric, 2009). The production rate and blood plasma levels of P4 are highest in females of reproductive age and during pregnancy (Quinkler et al., 2002). The

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production rate varies from 92 to 563 mg/day and blood plasma levels during pregnancy vary from 21 to 200 ng/mL in humans and 7 to 25 ng/mL in animals (EMEA, 2004), and P4 is rapidly conjugated and excreted by the body. In addition to the natural hormone P4, several synthetic forms of P4 (progestins) are now widely produced and regularly used in oral contraceptive formulations and as part of hormone replacement therapy (Fayad et al., 2013). Worldwide, progestins are probably the most widely used of all steroids (Zhang et al., 2014; Dinger et al., 2007), as contraception methods that use them have been reported to be the most desired by patients (Fayad et al., 2013). The increased use of progestogens may partly be due to their superior effectiveness in the inhibition of pituitary gonadotropins relative to the natural *P*4 compound (Dukes, 2003; Sitruk-Ware and Nath, 2010; Liu et al., 2011). Furthermore, their combination with certain estrogens in oral contraception formulations means consumers can have an all-in-one combination therapy.

Most studies on the prevalence of steroids in the water environment have focused on parent compounds and/or their human and animal metabolites and have ignored the possible interconversion of steroids caused by the myriad of transformation processes occurring in sewers, during water/wastewater treatment and in the natural environment (Liu et al., 2015a, 2015b; Liu et al., 2012; Tölgyesi et al., 2010; Ying et al., 2002). The evaluation of transformation products of steroids is highly relevant; especially as such TPs may exhibit distinct biological activities though differing only by slight structural modification to their side chains. All four classes steroids (progestogens, estrogens, androgens and glucocorticoids) making up this group of compounds have a common parent sterane backbone. Thus, slight modification in the arrangements of the peripheral atoms and/or functional groups of these compounds due to environmental transformation can modify their pharmacophore, thereby making their resultant TPs bind to different ligands (nuclear receptors) and eliciting unintended biological responses (e.g. activation or deactivation of certain genes) (Cwiertny et al., 2014; Runnalls et al., 2013; Jenkins et al., 2004). The pharmacophore refers to the electronic and spatial arrangement of a compound's atoms, which determines its optimal binding with a target biological site to trigger or block biological signalling (Overington et al., 2006).

1.1. Degradation of steroid hormones

Biological breakdown of progestogens starts on transit to wastewater treatment plants (WWTPs) in sewers, as these drains are known to be active bioreactors containing native biofilms with varying metabolic abilities (Jelic et al., 2015; Johnson and Williams, 2004). Generally, little is known about the possible TPs that result from biotic/abiotic degradation processes that steroids undergo and their associated toxicity (Evgenidou et al., 2015). This lack of knowledge is not unconnected with the underlying assumption in many engineered treatment approaches, which is that removal of previously identified parent compounds following treatment means the removal of earlier identified bioactivity (Cwiertny et al., 2014). This assumption, however, may be fundamentally flawed.

Structural modification to key locations in the P4 molecule (Fig. 1) has been shown to not only modify the progestagenic activity, but resultant TPs may also demonstrate activity in other steroid pathways (e.g. androgenicity) (Besse and Garric, 2009). Target nuclear receptors for each steroid (progesterone, estrogen, androgen or glucocorticoid) are relatively specific. For example, a hydroxyl group on carbon 3 confers estrogenic activity. However, small modifications to the respective structures of these steroids can switch the activity of one to that of another. To illustrate, a subtle change in progesterone molecular structure due to biological degradation of the compound has been shown to yield the androgen, testosterone (Fig. 2) (Peng et al., 2014). The biological activity of progesterone TPs may therefore be different from the activity of the parent compound.

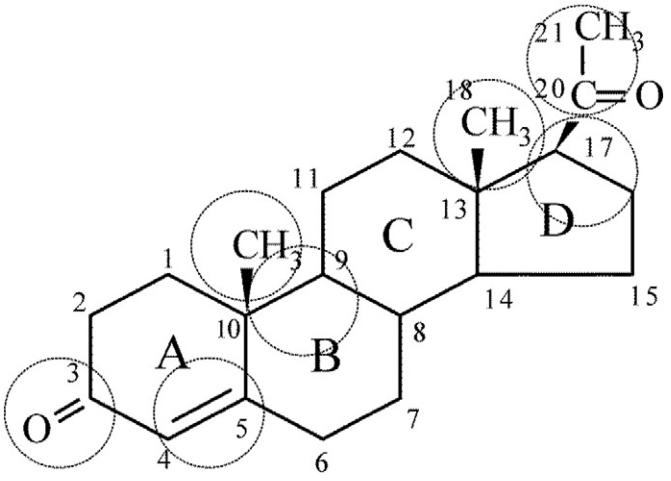

Fig. 1. Key points (circled) in the progesterone (P4) chemical structure where changes resulting from substitution or addition of a functional group can alter the affinity for receptors and consequently biological effects.

Accordingly, the biotic and abiotic degradation processes that occur in water/wastewater treatment infrastructures and in the environment are relevant in understanding the occurrence of steroidal hormones in surface water systems. Until new research facts proving otherwise are available, it is plausible that certain steroids regularly detected in WWTP influent/effluents and in surface waters are products of transformation processes. Thus, the current study was undertaken to identify putative TPs resulting from spiking river water with P4 in a laboratory-based degradation study and to elucidate P4 metabolic breakdown pathways. This has not been previously reported.

2. Materials and methods

2.1. Reagents

Progesterone (P4), its isotopically labelled compound (P4-d_9) used as internal standard, and other reagents used in this study, were >98% in purity. P4 was purchased from QMx (Essex, UK) and P4-d_9, from Sigma Aldrich (Gillingham, UK).

Reference standards were prepared in methanol (Rathburn Chemicals, Walkerburn, UK) and reagent grade MilliQ water (18.2 MΩ) (Millipore, Watford, UK) was used throughout the study. Ammonium hydroxide (NH_4OH) and formic acid used for aqueous phase preparation were also purchased from Sigma Aldrich (Gillingham, UK). Acetonitrile was purchased from Fisher Scientific (Loughborough, UK).

Progesterone and progesterone-d_9 1000 mg/L stock solutions were prepared in methanol by dissolving 10 mg of the standard in 10 mL methanol. A 10 mg/L (10,000 ng/mL) calibration standard of progesterone was prepared by a 1 in 100 dilution of the 1000 mg/L stock. Four other calibration standards, namely 1000 ng/mL; 100 ng/mL; 10 ng/mL and 1 ng/mL, were prepared from the 10 mg/L standard by 1–10 serial dilution with each new standard serving as the stock for the next. Similarly, 800 ng/mL standard of P4-d_9 was prepared by appropriate dilution (10 μL in 12.5 mL MeOH) of the stock solution (1000 mg/L) in methanol.

Five-point calibration series from 0.25 to 2500 ng/mL, each containing 200 ng/mL of the internal standard, were prepared for chromatographic analysis. Working standards were freshly prepared for each run by the mixture of appropriate amount of the intermediate sub-standards. Internal standard was added to samples prior to analysis.

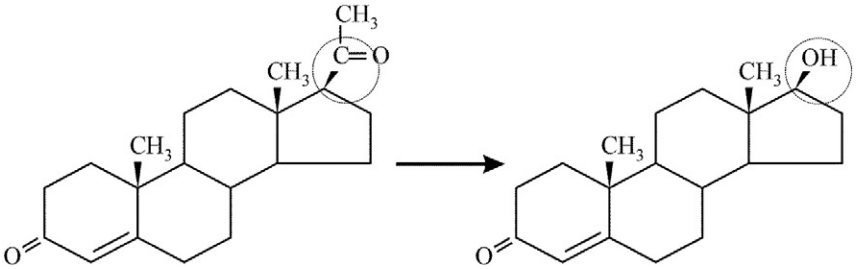

Fig. 2. Transformation of progesterone to testosterone formed by side chain transformation of the parent molecule at C17.

2.2. Instrumental analysis

2.2.1. Unit resolution LC-MS/MS

The unit resolution analytical system consisted of a Perkin Elmer Series 200 pump equipped with autosampler and mobile phase mixer connected in series to a PE SCIEX API365 triple quadrupole mass spectrometer with turbo ion spray source in positive ionisation mode (LC/ESI$^+$/MS/MS). System parameters were optimized in order to obtain higher signal strength (sensitivity). Instrument calibration for progesterone mass and the optimization of source-dependent parameters was achieved by manual tuning. Optimized parameters were; nebulizer gas: 9 psi; curtain gas: 8 L/min; ionspray voltage: 4500 V: collision gas: 10 L/min; gas temperature: 350 °C; declustering potential: 35 V; focussing potential: 92 V; entrance potential: 4.5 V; collision energy: 36 J and collision cell exit potential: 8 V.

Liquid chromatographic separations were achieved by using an ACE C18 (100 × 2.1 mm) 3 μm particle size column (Hichrom, UK) protected by a C18 guard column. Separation was achieved by pumping two eluents (Mobile phase A and B) at a flow rate of 210 μL/min. The aqueous phase was a mixture of MilliQ water and formic acid (999:1 v/v) (pH: 2.6). Phase B was acetonitrile. Total run time was 43 min with data acquisition over a gradient program of 5% acetonitrile for 11 min, then a linear gradient to 75% acetonitrile over 19 min and held at 70% for 1 min. The sample injection volume was 50 μL. Data were acquired with Analyst 1.4 software.

2.2.2. Accurate mass measurements and collision induced dissociation

Separation was conducted on a 1290 Series LC from Agilent (Stockport, UK) equipped with the same column as before. The injection volume was 20 μL. The mobile phase consisted of water (A) and acetonitrile (B), with both eluents containing 0.1% formic acid. An elution gradient (0.3 mL/min) was applied with the initial concentration (5% B) held for 1 min, after which it was linearly increased to 75% B for 9 min and then held for 5 min. A 3-min post run was required after analysis. The LC was connected to an Agilent 6540 accurate-mass quadrupole time-of-flight mass spectrometer. Sample ionisation was achieved with jet stream electrospray operated in positive ion mode under the following conditions: sheath gas temperature 250 °C; nebulizer 45 psi, gas flow 8 L/min; gas temperature 250 °C; skimmer 65 V, fragmentor 175 V, nozzle 0 V; octopole RF 750 V; and capillary 3500 V. Accurate mass spectra were acquired in scan mode (50–1200 *m/z*). Reference masses were 121.0509 and 922.0098 *m/z* with a resolution of 19,546 at 922.0106 *m/z*. Data were acquired with Agilent MassHunter software. Considering *P4* as the parent compound, a mass defect filter (0.2246 ± 0.05 Da) was applied to identify TPs.

Ion fragmentation was performed by collision-induced dissociation (CID) mass spectrometry in the Agilent 6540 time-of-flight instrument equipped with the column used previously. Acquisition parameters remained the same, with fragmentation carried out at a collision energy of 15 V. Ion fragmentation was monitored over an *m/z* range of 40–400.

2.3. Progesterone degradation study

2.3.1. River water sampling

River water (20 L) was collected at approximately 1 km downstream of the effluent discharge point of a WWTP that serves a population of about 500,000 people. The sample was collected with the aid of a sampling bucket attached to an adjustable 3 m handle. Prior to sample collection, the sampling bucket and sample container were rinsed with river water. A representative sample was taken from a point approximately 2 m from the bank of the river. The water sample in a 20 L plastic container was transported immediately to the laboratory. River water was not filtered to remove sediment/biota.

2.3.2. Laboratory degradation study

?twb=0.27w?>Two 9 L degradation tanks were used for the degradation study. Prior to the study set-up, the degradation tanks were rinsed twice with river water, after which 6 L of river water was measured into each tank. The tanks were left to stabilise for 24 h. Prior to the installation of air pumps and stirrer, the initial dissolved oxygen (DO) levels, pH and temperature of water in the tanks were measured. Subsequently, these parameters were monitored during each sampling time in the spiked and control tanks, so that any observation from chemical analysis can be placed in proper context, taking into account any observed fluctuations in these parameters. Two aerators with fitted air stones connected to air pumps, as well as two agitators (magnetic stirrers), were installed in each tank to ensure even aeration and good mixing of tank content.

Following the 24-hour equilibration period, one of the tanks was spiked with 6 mL of a 1 mg/mL progesterone stock solution and the other left as control. Thus, a nominal concentration of 1000 μg/L was expected in the tank for samples collected at the start time (0 h). Sampling regimes in hours were 0 (20 min following spiking), 4, 8, 12, 24, 48 and 72. For each sampling period, 0.6 mL of water was sampled respectively from the spiked and non-spiked tanks into a chromatographic vial, followed by the addition of 0.3 mL each of methanol and the internal standard. Three replicate samples were taken at each sampling time so that LC/MS analyses could be repeated.

2.4. Identification of target transformation products (TPs) to be monitored

The choice of target progesterone TPs to be monitored under low resolution was guided by the outputs from the EAWAG BBD predictions alongside literature reports of products resulting from the biodegradation of P4 (Peng et al., 2014; Ellis and Wackett, 2012). Putative products detected under low resolution and those confirmed by high resolution mass spectrometry (HMRS) are presented in the Results section.

3. Results and discussion

3.1. Target transformation products monitored

A summary of target TPs predicted by the EAWAG BBD is shown in Fig. 3 and compounds selected from the literature and that were

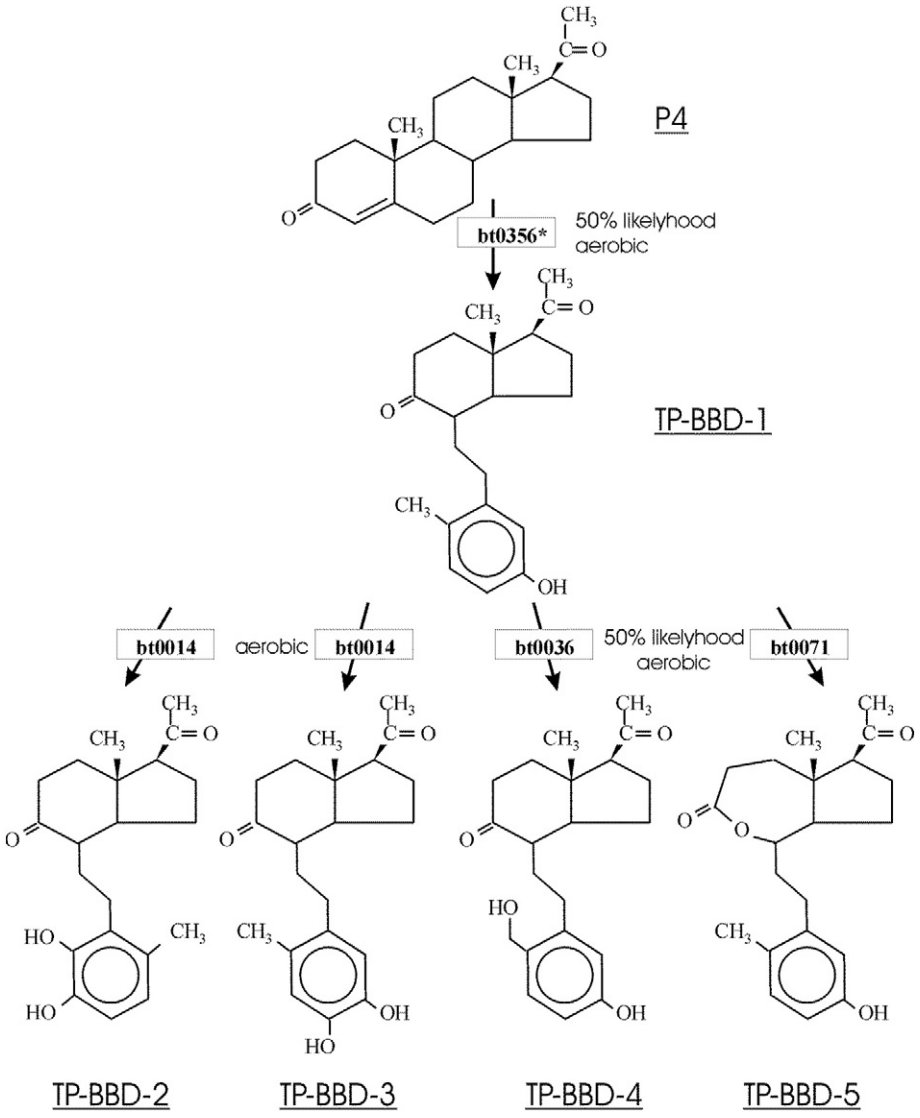

Fig. 3. The EAWAG BBD prediction for the environmental transformation of progesterone.

produced through algal degradation of P4 are shown in Fig. 4. The details of compounds monitored in the target analysis are summarised in Table 1. Progesterone details are also included in this table for easy reference, especially when comparing the properties of individual TP with the parent molecule. Table 1 also details the chromatographic retention times (RT) of the target compounds and their relative retention times (R/RT) to that of the P4-d_9 internal standard. The biodegradation database predicted a number of products (TP-BBD) resulting from the cleavage of the 'B' ring in the P4 C21 pregnane skeletal backbone (Fig. 3). The literature search, however, identified algal products (TP-ALG) (Fig. 4) that do not show ring cleavage.

3.2. Degradation study

Conditions within the spiked and control tanks were relatively stable for the entire study period. Of the parameters measured, DO was stable at 8.1 to 8.9 mg/L, pH started at 8.7 in each tank, and decreased to 8.2 in the spike and 8.5 in the control. Temperature in both tanks ranged from 20 to 22 °C. There were no significant differences ($p > 0.05$) between all these parameters in the spike and control tanks.

Overall, the outcome of the degradation study was that progesterone degraded rapidly from an initial 1000 μg/L nominal concentration (Fig. 5), with the concentration falling to <0.7 μg/L after 72 h. Unit resolution results showed the formation of six putative transformation products of the nine target products monitored, as indicated by the relative retention times provided in Table 1. High resolution time-of-flight results confirmed the presence of two of the products detected in unit resolution with their chemical formulae and accurate masses determined to four decimal places (Table 2). A third product seen under unit resolution was also detected in HRMS (TP-ALG-2; mass: 316.2402), however, this was with a lower degree of confidence, and lacked a molecular formula, as the intensity was very low.

By applying a mass defect filter (0.2246 ± 0.05 Da) in the identification of TPs in HRMS, we found two features at *m/z* 313.2170 (TP-ALG-1) and *m/z* 287.1997 (TP-ALG-4). The former corresponded to a mass of 312.2097 (−2.48 ppm error), a double bond equivalent of 8 and proposed molecular formula $C_{21}H_{28}O_2$. The latter resulted in a mass of 287.1997 (0.28 ppm error), double bond equivalent of 7 with $C_{19}H_{26}O_2$ being its molecular formula (see Table 2). CID fragmentation of the aforementioned features showed similar fragment ions at *m/z* 121.0648 and 121.0643 respectively (Table 3 and Fig. 6). Fragmentation of both TP-ALG-1 and TP-ALG-4 is believed to have occurred in the A-ring, most probably due to protonation of the ketone group attached to C_3. Subsequent relocation of the positive charge either on carbon 1 or 5 due to resonance results in the dissociation of the bond between C_9 and C_{10} (Guan et al., 2006). CID experiments for *m/z* 287.1997 helped

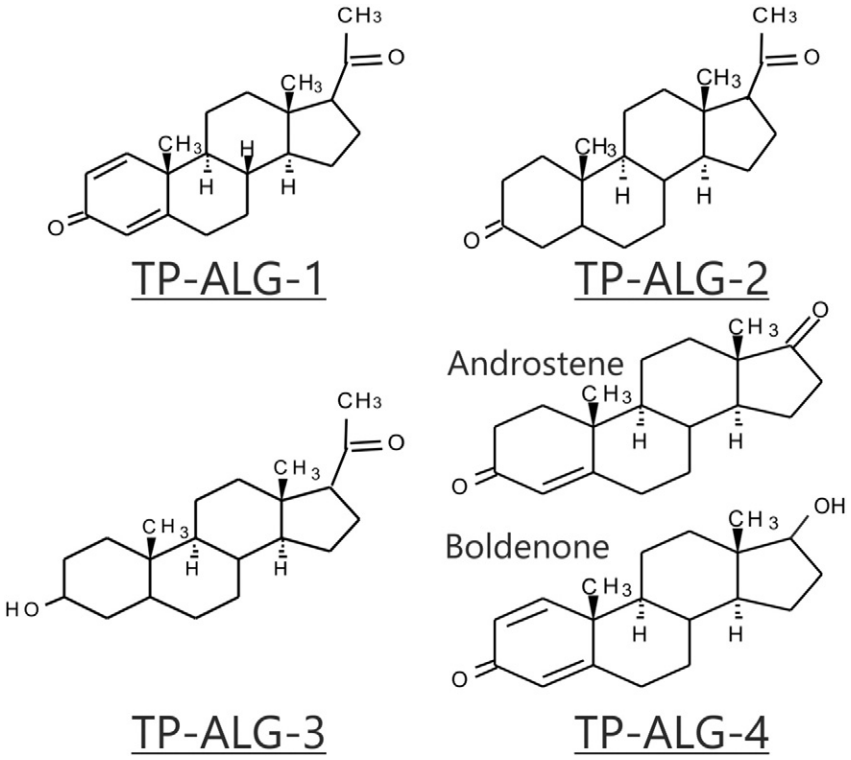

Fig. 4. The predicted transformation products from the literature. Peng et al., 2014 identified these products from their algal studies (TP-ALG). There are two possible compounds identified for TP-ALG-4, see also Table 1.

to identify this feature as boldenone since its two main product ions *m/z* 121.0643 and 135.1161 (Fig. 6) have been previously reported elsewhere (Guan et al., 2006).

Under both unit and high resolution monitoring, a number of target masses corresponding to TPs were detected as the progesterone concentration declined. TPs were typically observed as peaks in chromatograms in unit resolution, as shown for *m/z* 287.3, which represents TP-ALG-4 (Fig. 7). For this example, two peaks were observed for the *m/z* monitored. However, the blank also had a response at RT 12.56 min, so this peak was discounted as being linked to spiking with P4. The response at RT 18.46 min was recorded as no similar response was seen in the blank. In all instances where *m/z* responses linked to possible TPs were identified, target ions eluted before the parent (Table 1). In reversed-phase chromatography this is indicative of increasing polarity, which is reflected in the structures shown in Figs. 3 and 4. The greater number of compounds detected in unit resolution was probably a result of operating the triple quadrupole in selected ion monitoring mode, increasing sensitivity for the specific ions. The high-resolution work was undertaken in scanning mode, only targeting specific ions for the collision induced fragmentation. However, other, instrument specific factors, such as the ionisation source, may also play a role in determining sensitivity.

The algal metabolic reactions were the dominant pathways observed in the degradation experiment, with fewer occurrences of the bacterial degradation products predicted by the BBD. Of these latter potential compounds, only TP-BBD-1, predicted as a first level product of progesterone degradation, was detected in the study. The *m/z* associated with this TP was detected only in the 4 h spiked sample (Fig. 8). The

Table 1
Characteristics of the BBD and algal metabolites monitored in this study.

Name	Abbr.	Mol. wt.	CAS	*m/z*	RT (%RSD)[a]	R/RT[a]
Progesterone	P4	314.5	57-83-0	315.3	25.4 (0.6)	1
1-Acetyl-4-[2-(5-hydroxy-2-methylphenyl)ethyl]-7a-methyl-octahydro-1H-inden-5-one	TP-BBD-1	328.5		329.3	12.4 (<0.1)	0.49
1-Acetyl-4-[2-(2,3-dihydroxy-6-methylphenyl)ethyl]-7a-methyl-octahydro-1H-inden-5-one	TP-BBD-2	344.5		345.3	14.8	0.58
1-Acetyl-4-[2-(4,5-dihydroxy-2-methylphenyl)ethyl]-7a-methyl-octahydro-1H-inden-5-one	TP-BBD-3	344.5				
1-Acetyl-4-{2-[5-hydroxy-2-(hydroxymethyl)phenyl]ethyl}-7a-methyl-octahydro-1H-inden-5-one	TP-BBD-4	344.5				
6-Acetyl-1-[2-(5-hydroxy-2-methylphenyl)ethyl]-5a-methyl-octahydro-1H-cyclopenta[*c*]oxepin-3-one	TP-BBD-5	344.5				
1,4-Pregnadiene-3,20-dione	TP-ALG-1	312.5	1162-54-5	313.3	23.3 (0.8)	0.92
3,20-Allopregnanedione	TP-ALG-2	316.5	566-65-4	317.3	21.5 (0.8)	0.85
3b-Hydroxy-5a-pregnan-20-one	TP-ALG-3	318.5	516-55-2	319.3	21.5 (0.6)	0.85
Androstene or 17b-boldenone	TP-ALG-4	286.5	846-48-0	287.3	18.5 (0.1)	0.73

[a] Information for degradation products is only available if they were detected.

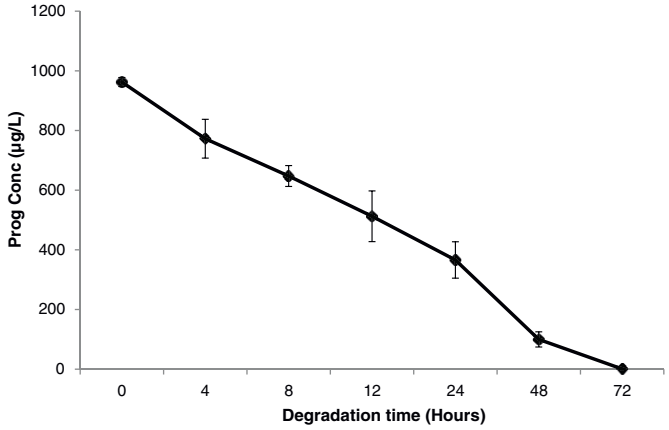

Fig. 5. Progesterone degradation with time (Instrument LOD 0.35 µg/L).

products predicted by the BBD have all undergone ring cleavage, and TP-BBD-1 eluted earlier than other TPs observed, with a R/RT of 0.58 (Table 1).

The reactions that resulted in detection of the *m/z* linked to the TPs observed in the degradation study were hydroxylation, hydrogenation, dehydrogenation and side-chain breakdown (Peng et al., 2014). Of these, dehydrogenation was dominant, as it yielded the major transformation product of the study (TP-ALG-1), as determined empirically by its peak area (Fig. 9). Although peak area is not a quantitative measure, and is related to ionisation efficiency, it provides an empirical assessment of the possible significance of the products.

The metabolic reactions identified for P4 degradation have earlier been reported as the main transformation pathways for progesterone degradation by some strains of fungi, namely the Cyanobacterium *Microchaeta tenera* and two freshwater microalgae, *Scenedesmus obliquus* and *Chlorella pyrenoidosa* (Pollio et al., 1996; Safiarian et al., 2012; Peng et al., 2014). Accordingly, it is not impossible that P4 transformation could be carried out by a consortium of microorganisms in the environment, using P4 as a carbon source and utilizing diverse reaction pathways.

The ring cleavage of P4 predicted by the BBD may hardly have occurred. A transitory peak corresponding to the predicted mass of TP-BBD-1 was seen at low resolution. This peak appeared only at 4 h after spiking. Possible factors that may have accounted for this observation are:

a. The degradation tank was an aerobic environment, whereas pathways that yield BBD-predicted transformation products may not be aerobic.
b. BBD-predicted transformation products are rapidly formed and then degraded at a rate that the sampling regime of the present study was unable to detect.
c. The microbial community available in the degradation tank may be less diverse/active and thus not included bacterial strains predicted by the BBD database to carry out reactions that yield its predicted TPs.
d. Cleaving of the P4 ring may require relatively high energy compared to the energy required to access the alternative carbon source available at the compound's side chain, such that degrading microorganisms may have favoured the latter over the former.

Table 3
Fragmentation pattern of the putative transformation products.

Precursor ion			Base peak			
Targets	*m/z*	Putative formula	*m/z*	Putative formula	Loss mass	Loss formula
TP-ALG-1	313.2173	$C_{21}H_{28}O_2$	121.0648	C_8H_9O	192.1514	$C_{13}H_{20}O$
TP-ALG-4	287.2001	$C_{19}H_{26}O_2$	121.0643	C_8H_9O	166.1358	$C_{11}H_{18}O$

Biotransformation rule 0356 that facilitated the formation of TP-BBD-1 has been reported to facilitate several steps in steroid metabolism including aromatization, spontaneous ring cleavage and oxidation (Philipp, 2011; Horinouchi et al., 2005). However, it has only 50% likelihood of occurrence in an aerobic system.

The metabolic pathways identified in this study as the dominant reaction pathways for P4 transformation all acted on the side-chains of the molecule, leaving the core tetracyclic parent backbone unaffected. The conservation of this parental backbone in TPs is of interest as P4 bioactive potency, like other steroidal hormones, is dependent not only on its localised functional groups but also on its tetracyclic structure (Cwiertny et al., 2014). TP-ALG-4 had earlier been identified by Peng et al., 2014 as 4-androstene-3, 17-dione (AED), an androgen. 17β-boldenone, used as a veterinary anabolic agent (Cwiertny et al., 2014; Oda and El-Ashmawy, 2012; O'Connor et al., 1973), also has similar mass (Table 1) as TP-ALG-4, but differs in the conversion to a hydroxyl group of the ketone in C17. Previously, Thomas et al. (2002) reported AED as one of the steroids responsible for the high androgenic activity observed in UK rivers. Two years later, Jenkins et al. (2004) reported the transformation of progesterone by *Mycobacterium smegmatis* to androgens. Progesterone, 17β-boldenone and AED were among the steroids reported by Liu et al. (2015c)to be present in water, sediment, and mollusc and fish samples from South China. It is not unlikely, therefore, that at some point in the degradation process, certain TPs formed were biologically active steroids, a condition that can only be confirmed with biological assay data, which the scope of the present study did not cover, but will be investigated in subsequent studies.

The high-resolution data provided accurate masses for TP-ALG-1 and TP-ALG-4 as 312.2097 and 286.1933 respectively. For TP-ALG-1 this matches the theoretical monoisotopic mass/formula for 1, 4-pregnadiene-3, 20-dione, a reported derivative of P4 degradation. The accurate mass of 286.1933 for TP-ALG-4, fits the theoretical monoisotopic mass/formula for androstenedione and boldenone, both reported derivatives of P4 (Peng et al., 2014). The CID fragmentation of these peaks shows that both TPs had the base peak *m/z* 121.064 in common. This fragment has been previously reported as the major fragment resulting from collision-induced dissociation of a number of microbially synthesized monohydroxylated progesterones (2α-hydroxyP4, 7β-ydroxyP4 and 9α-hydroxyP4), compounds recognized for their pharmacologically active properties (Kang et al., 2004). The significance for the water industry of bioconversion of one class of steroid into another is that there is a chance that there are diverse sources of androgen in surface waters as there are multiple transformation pathways in the water environment. A range of synthetic progestogens are used clinically, primarily in hormonal contraceptives. These synthetic progestogens differ appreciatively in structure from each other, and from P4. For example, quite a few, such as the widely used levonorgestrel, is a C19

Table 2
High resolution MS data (Accurate mass measurements).

Targets	Monoisotopic mass	Measured mass	*m/z*	Putative formula	Score (MFG)	DBE	Error
TP-ALG-1	312.2089	312.2097	313.2170	$C_{21}H_{28}O_2$	90.42	8	−2.48
TP-ALG-4	286.1932	286.1932	287.1997	$C_{19}H_{26}O_2$	99.8	7	0.28

m/z: mass-to-charge ratio; MFG: molecular formula generator; DBE: double bond equivalents.

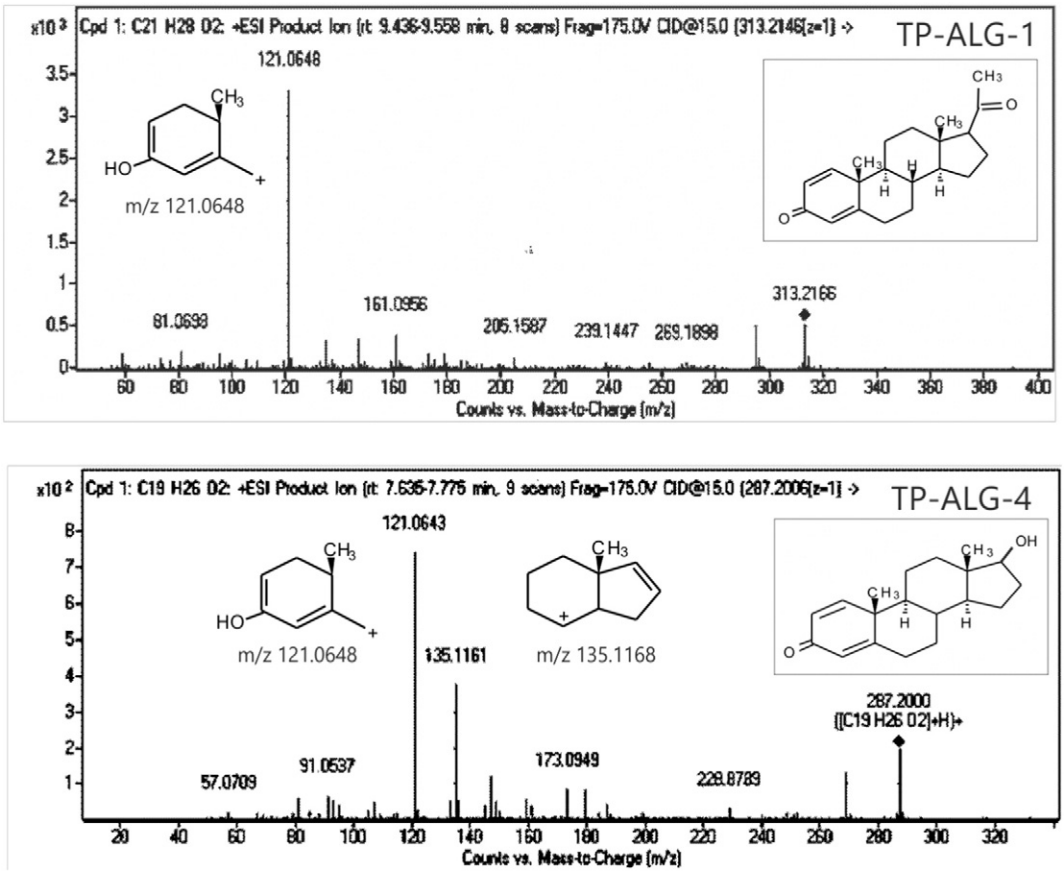

Fig. 6. ESI-CID mass spectra for putative transformation products TP-ALG-1 (top) and TP-ALG-4 (bottom). The major fragments of 121.0643 and 135.1161 are likely to be produced via the fragmentation pathway for boldenone proposed by Guan et al. (2006).

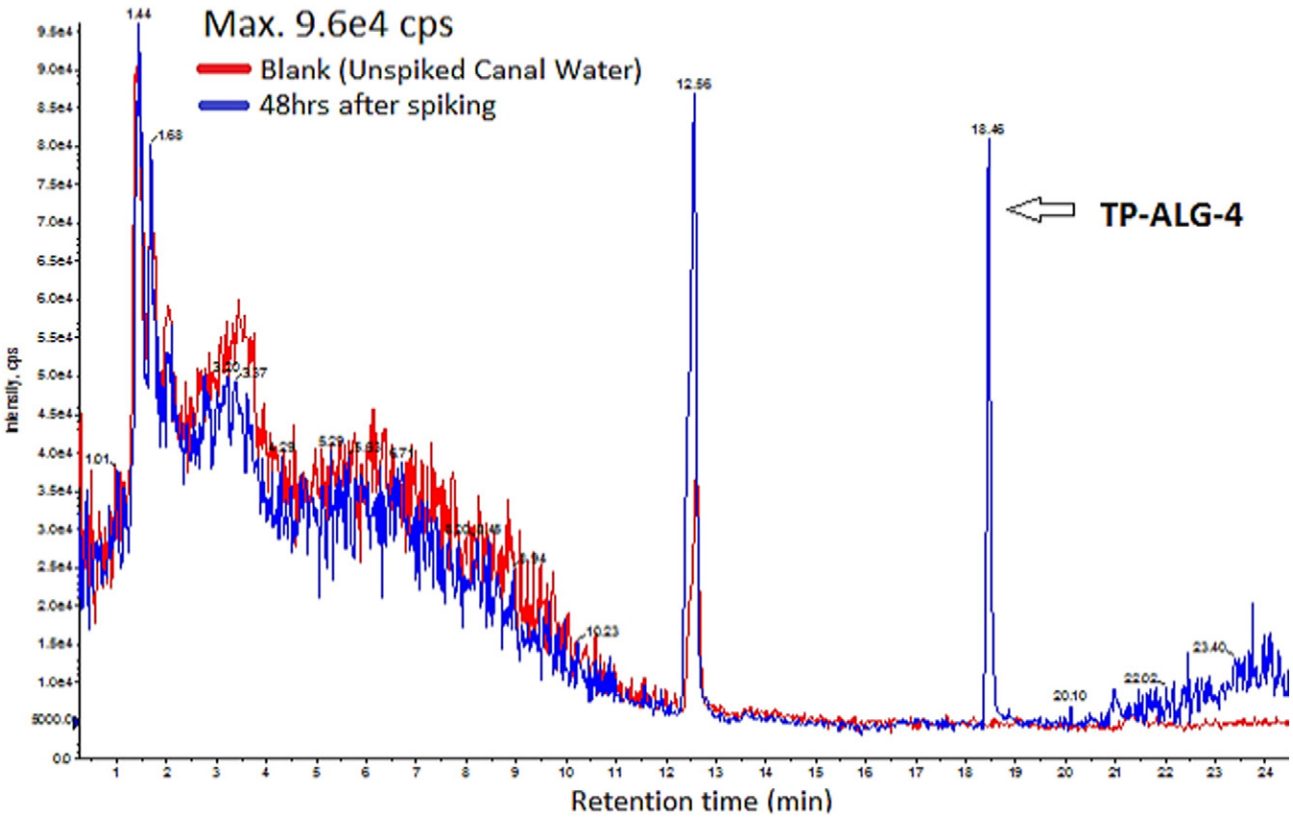

Fig. 7. Overlaid chromatograms showing the formation of TP-ALG-4 by side chain breakdown (-2C and -4H) of the parent progesterone molecule.

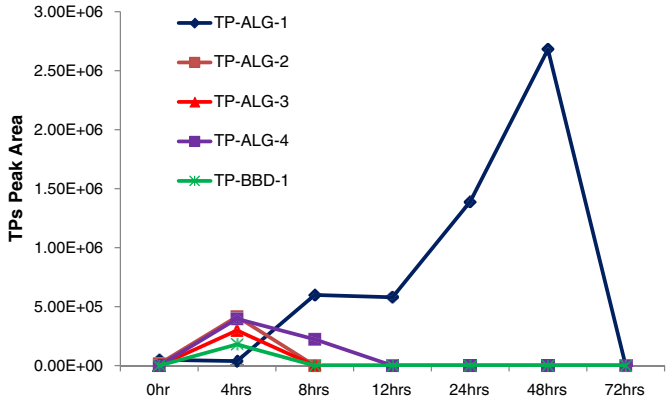

Fig. 8. Formation of target progesterone TPs with time, using peak area as a measure of occurrence.

steroid, not a C21 steroid like P4. These significant differences in structure mean that it is impossible to know whether or not our results presented here are applicable to the biodegradation of synthetic progestogens. Nevertheless, we consider it quite likely that our main finding – that as a steroid hormone degrades, it can lead to the formation of other steroids with different biological activities – will be applicable to the degradation of other steroid hormones, both natural and synthetic.

4. Conclusions

Generally, the present study highlights that minor transformation of progestogens can create other classes of steroids, some of which may be potent, possessing other types of biological activity, an outcome supported by findings by other workers (Sangster et al., 2016), who observed that progesterone degraded into potent androgens. Thus, even without prior ecotoxicological investigations or determination of physical-chemical properties that drive the fate of identified TPs, the significance of the transformation processes that progesterone undergoes in rivers cannot be underestimated. Other published work on P4 degradation has been conducted with pure algal culture (Peng et al., 2014) and sediments (Sangster et al., 2016). The present study, which supports those findings, highlights the relevance of possible degradation products by identifying them in surface waters at environmentally relevant conditions. Furthermore, there is a need for improved treatment technology that ensures complete removal of steroids and their stable metabolites. Toxicity testing of treated effluent for potent TPs prior to discharge is also necessary. As identification of TPs alone without parallel toxicity testing does not provide a comprehensive risk assessment tool for understanding their ecotoxicity, further studies aimed at assessing the relative toxicities of progesterone TPs with respect to the parent molecule will be undertaken.

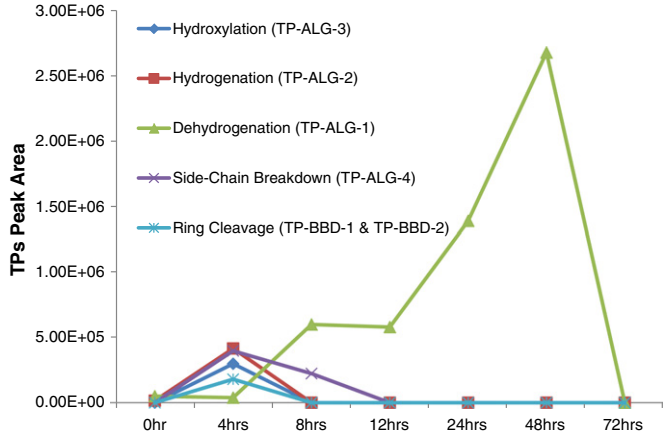

Fig. 9. Chemical reactions that yielded the transformation products identified in this study. Based on peak areas, dehydrogenation was the most dominant reaction.

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