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Transformation of 17α-methyltestosterone in aquatic-sediment systems

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Abstract: Two sediment samples that differed with respect to total organic carbon and texture ("sand" and "clay") were exposed to radio inert 17α-methyltestosterone (MT) or [^{14}C]-radiolabeled 17α-methyltestosterone (^{14}C-MT), under both aerobic and anaerobic conditions, for up to 56 days, to characterize the fate of MT in the aquatic environment. Radio inert MT was quantified by a highly sensitive liquid chromatography/mass spectrometry (LC-MS) method and radioactive MT was quantified by HPLC using an in-line flow liquid scintillation counter (LSC). The data suggest that MT entering the aquatic environment is converted into metabolites that become tightly associated with the sediment. Half-lives for MT dissipation in the sediment systems ranged from 2-9 days, depending on the sediment type and the presence of oxygen. Sediment type had little effect on MT dissipation. The mineralization of MT under aerobic conditions was low (<9% conversion of MT to CO_2).

Keywords: 17α-Methyltestosterone (MT), Aquatic-Sediment, Radio-HPLC, LC-MS, Aerobic, Anaerobic

INTRODUCTION

Biodegradation of 17α-methyltestosterone, a 3-oxo Steroid (I, MT, Fig.1) by microorganisms from waste water treatment systems and sediments under aerobic condition has been studied to some extent (Sanchez, 2001, Wattanodorn *et al.*, 2007). Persistence of 17α-methyltestosterone (MT) in the environment after its use for masculinizing of Nile tilapia in nursery ponds has been reported (Sanchez, 2001). In a more recent study, activated sludge taken from an aeration tank of a municipal waste water treatment plant was used as bacterial seed and sediments were taken from masculinzing ponds of Nile tilapia. The results suggested that MT was biodegradable (Wattanodorn *et al.*, 2007).

Transformation of a series of 3-oxosteroids by *Trichoderma hamatum* KCh25 has been examined. The strain promoted 1-dehydrogenation, which has been rarely observed in fungi cultures. The other routes of biotransformations of these substrates included: hydroxylation at equatorial 11α, 6α, and 12β positions, ester bond hydrolysis, oxidation of 17α-hydroxyl group, C-17/C-20 bond scission, and the ring D-lactonization (Bartmañska and Gladysz, 2007). The fate of testosterone (T), a 3-oxo steroid closely related to MT is known in such systems and sheds some light on the possible fate of MT. The major product of testosterone metabolism was androstenedione. Lesser metabolites, tentatively identified as 16β-hydroxytestesterone and / or β-boldenone, were also produced by certain soils. After 24-31 hours of contact time, testosterone recoveries averaged between 23 and 90%, depending on the soil type. Low recoveries were probably due to the irreversible binding of T and its metabolites to the soil (Colucci *et al.*, 2001; Lee *et al.*, 2003 and Casey *et al.*, 2004). With all soil types, apparent sorption equilibrium was reached within a few hours. Measured sorption isotherms for the three parent chemicals and their principal transformation products were generally linear. Large log K_{oc} values (≈ 3-4) suggested that a significant fraction of these compounds would be associated with sediments. Half-lives for hormone dissipation in the aerobic soil and sediment slurries estimated assuming pseudo first-order processes ranged from a few hours to a few days with testosterone having the shortest half-life, ranging from 0.3 to 6.5 days, depending on the soil type. Biodegradation studies on 17 β-estradiol using aquatic/sediment samples collected from English rivers have been reported (Jurgens *et al.*, 2002).

The objective of the present investigation was to characterize the transformation of 17α-methyltestosterone (MT) in two water-sediment systems (sand and clay) under both aerobic and anaerobic conditions. This characterization included: 1) measuring the transformation rate of MT, including determination of half-lives ($t_{0.5}$); (2) the mineralization rate of MT to CO_2 under aerobic conditions; (3) the degradation of MT in the water and sediment phases; and (4) the measurement of the

Table 1. Physical characteristics of sediments used in MT transformation experiments.

Sediment	Sand %	Silt %	Clay %	Total nitrogen (mg/L)	Organic carbon (mg/L)	Inorganic carbon (mg/L)
Clay (loamy)	51	36	13	0.74	6.80	0.64
Sand (sandy)	85	6	9	0.06	1.75	1.60

distribution of MT between the two phases (water and sediment) during an incubation period in the dark at constant temperature (20±3°C).

MATERIALS AND METHODS

Radioinert 17α-methyltestosterone (I, MT, >99% pure) was purchased from Steraloids (Newport, RI). [4-^{14}C]-17α-methyltestosterone (^{14}C-MT) was obtained from Dr. William Hayton, College of Pharmacy, Division of Pharmaceutics, Ohio State University. Dr. Hayton purchased this material from Amersham International (Buckinghamshire, England). The ^{14}C-MT was purified by thin-layer chromatography (TLC) and had the following characteristics: purity, >97% (HPLC); specific activity, 45 mCi/mmol, or 148 microcuries per mg or 328,560 dpm per microgram, blank (carrier) solution = 100% ethanol. ^{14}C-MT labeled on the 4 position of the steroid backbone was required because the release of ^{14}C-CO_2 from [4-^{14}C]-17α-methyltestosterone denotes steroid ring cleavage and biological inactivation of the steroid molecule. 17β-Hydroxy-3β-methoxyandrost-5-en-7-one (II, I.S.) was synthesized in the laboratory (Marwah et al. 2001). Its structure was confirmed by NMR, (^{1}H and ^{13}C) and LC-MS, and purity (>99.5%) was established by UV at 245 nm and mass spectral data in electrospray ionization (ESI) mode. All solvents and reagents were HPLC grade and obtained from Sigma Chemical Company (St. Louis, MO) or Fischer Scientific (Hampton, NH).

Sediment collection and preparation: Two different sediments were used in the study. Sediment 1 and associated water were collected from a marsh located at State Fish Hatchery, Lake Mills, WI, USA. The sediment had high organic carbon content (6.8%) and a fine texture (clay+silt content 49±3%). Sediment 2 and associated water were collected from Rock Creek, Lake Mills, WI. This sediment had a low organic carbon content (1.8%), and a course texture (clay+ silt content 15±3%). Sediments 1 and 2 are referred to as "clay" and "sand", respectively. Table 1 lists the physical properties of the sediments used in the investigation.

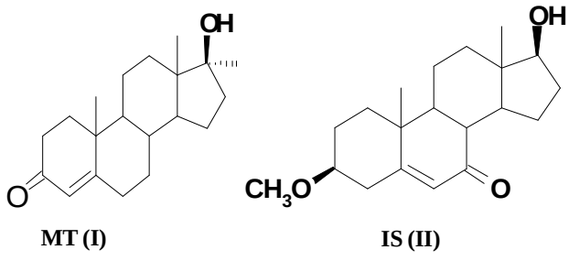

Fig. 1. *Chemical structures of 17α-Methyltestosterone (MT, I) and Internal Standard (17β-hydroxy-3β-methoxyandrost-5-en-7-one, II).*

For the aerobic experiment, the sediment and water samples were taken from the top layer (2 to 5 cm) of sediment. For the anaerobic study, sediment and water samples were collected deeper in the anaerobic zone, and handled and transported under exclusion of oxygen in tightly sealed glass bottles. Sediment texture and initial organic carbon content analyses were conducted at the University of Wisconsin Soil and Plant Analysis Laboratory (Verona, WI). A complete description of the analytical procedures used, including quality control measures, can be found at their website: http://uwlab.soils.wisc.edu/madison.

Water and sediment were separated using filter paper, and the sediment was wet-sieved with a 2 mm sieve using excess location water that was then discarded. For the anaerobic study, these steps were done under exclusion of oxygen in a glove box filled with nitrogen gas. Both the aerobic and anaerobic experiments were conducted using freshly sampled sediment and water with the exception of the "long-term" aerobic experiment that used water and sediment that had been stored for one week water logged (6-10 cm water layer), in the dark, at 4±2 °C, in an open containers with free access to air.

Aerobic experiments: The aerobic experiment was conducted using a gas flow-through system (OECD Guidelines, 2002). The incubation units were 250 ml polypropylene graduated cylinders (Fig. 2). The traps were 50 ml, polypropylene, conical-bottomed test tubes. Aerobic conditions were maintained throughout the experiment by passing a steady stream of air through the system. Alkaline traps containing 1.5N KOH were used to capture ^{14}C-CO_2 released by the mineralization of ^{14}C-MT during the experiment. Previous research has shown that the trapping efficiency for $^{14}CO_2$ in alkaline traps is greater than 95% (Nuck and Federle, 1996).

All experiments were conducted using 30 ml of settled sediment and 90 ml of water for a total incubation volume of 120 ml as per OECD guidelines (i.e., minimum 2.5 cm of sediment height within the incubation unit, and water to sediment ratio between 3:1 and 4:1). All incubations were performed in the dark by wrapping the incubation vessels in foil to completely exclude light, and at room temperature (20±3°C). The aquatic-sediment samples were preincubated for two weeks prior to adding MT to

Table 2. Half-life in days of total radioactivity and 17α-methyltestosterone in aquatic water.

	Curve fit total DPM in water	R^2	$T_{1/2}$	Curve fit MT in water	R^2	$T_{1/2}$
Aerobic						
Clay	$y= -0.0008x^3 – 0.023x^2 - 2.8x + 93.8$	0.97	**14.2**	$y=0.0005x^4–0.052x^3+1.8x^2–24.1x+105$	0.98	**2.8**
Sand	$y= 0.064x^2 – 5.3x + 99.7$	0.96	**10.8**	$y=0.0008x^4–0.081x^3+2.5x^2–29.1x+101$	0.96	**2.1**
Anaerobic						
Clay	$y= 0.05x^2 – 4.5x + 100.5$	0.99	**13.3**	$y= -0.0037x^3+0.37x^2-11.1x + 99.4$	0.99	**5.3**
Sand	$y= 0.05x^2 – 4.4x + 94.1$	0.96	**11.5**	$y= -0.001x^3+0.16x^2-7.3x+102.5$	0.98	**8.9**

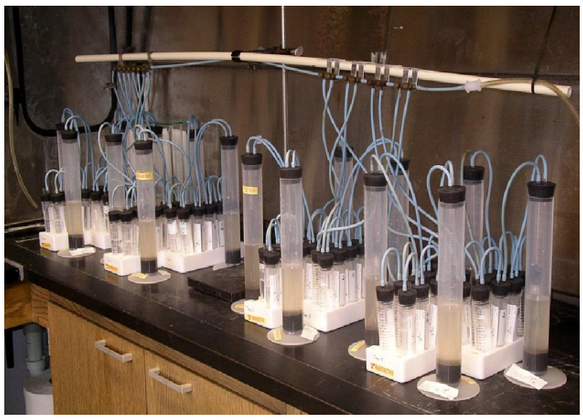

Fig. 2. *Photograph of the experimental setup used to evaluate the transformation of 17α-Methyltestosterone in Water-Sediment systems under Aerobic conditions. During the actual experiments the incubation vessels were covered with foil to completely exclude light. See text for complete description.*

the experimental units. The stability of the system during the acclimation period was evaluated by measuring pH and total organic carbon (TOC) in both the water and sediment at the beginning and end of the acclimation, as well as microbial biomass of the sediments and BOD of the water at the end of acclimation.

The experiment was started by adding MT to each of the experimental units. One initial concentration of MT (25 ng/ml) was tested in all experiments that was approximately 5-fold higher than the estimated concentration of unmetabolized MT that might be found in the discharge water from a large tilapia farm, and high enough to allow for observation of MT transformation by radio-HPLC or LCMS. This concentration was achieved by adding 2.25 mg of MT to each system in the form of either radioinert MT or ~740,000 dpm of ^{14}C-MT. The MT was applied directly to the water phase of each test system, and the water was gently mixed without disturbing the sediment to ensure uniform distribution of the MT.

There were a total of eight sampling times (in days): 0, 1, 3, 7, 14, 28, and 56. For logistical reasons, the aerobic experiment was split into a "short-term" (sampling times of 0, 1, 3 and 7 days) and "long-term" (sampling times of 0, 14, 28 and 56 days) study. There were duplicate incubation vessels at each sampling time. One of each duplicate received ^{14}C-MT and the other received radio-inert MT. During the "long-term" experiment four additional control incubation units (neither radio-inert MT nor ^{14}C-MT; 2 controls per sediment type) were prepared. One control incubation unit per sediment was used to estimate microbial biomass of the sediment and the total organic carbon of the water and sediment at the end of the acclimation period (start of incubation) and after 56 days of incubation (termination of the study). The other two control units (one per aquatic sediment) were used to monitor the required parameters in the sediment and water during the acclimation period.

The alkaline CO_2 traps were sampled on a weekly basis, and radioactivity was determined in duplicate by liquid scintillation counting (LSC). Ten ml of fresh 1.5 N KOH was added to replace the sampled volume. The replicate incubation vessels were sampled as scheduled. The surface water was carefully poured into a 100 ml polypropylene sampling bottle with minimum disturbance to the sediment. Duplicate 1 ml water samples were collected from the incubation vessels containing ^{14}C-MT and placed into 20 ml glass scintillation vials for determination of radioactivity by LSC. The remaining water and sediment (stored in the original incubation vessels) was stored at –40°C until analysis.

Anaerobic experiments: There were no traps used in the anaerobic experimental setup. This deviation from the approved protocol was made because very low mineralization rates were observed in the aerobic incubations (< 9% of the MT was converted into CO_2) and therefore little or no mineralization was expected to

Table 3. Fate of [^{14}C]-MT after 56 days in water/sediment under anaerobic and aerobic conditions.

Sediment	% MT in water	% MT in sediment	% MT mineralized to CO_2	% Non-extractable radioactivity
Clay (anaerobic)	8.2	0	---	91.8
Clay (aerobic)	8.0	0	7.4	84.6
Sand (anerobic)	4.3	0	---	95.7
Sand (aerobic)	3.0	0	8.7	88.3

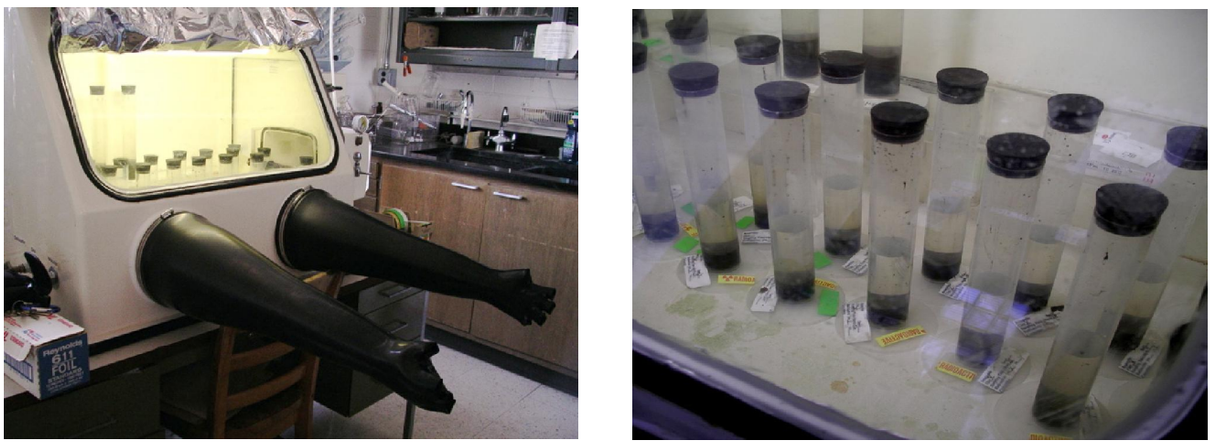

Fig. 3. *Photograph of the experimental setup used to conduct the anaerobic experiments. During the actual experiments the incubation vessels were covered with foil to completely exclude light. See text for complete description.*

occur in the absence of oxygen. All experimental procedures (e.g., adding sediment and water to the incubation units, adding MT to initiate the experiments, and sampling) were conducted under anaerobic condition within a nitrogen-fill glove box (Fig. 3). The anaerobic experiments were also conducted using 250 ml polypropylene graduated cylinders filled with 30 ml of settled sediment and 90 ml of water. Anaerobic conditions were maintained within the incubation vessels by sealing the incubation units with rubber stoppers, and placing the sealed units into a glove box filled with nitrogen gas. The nitrogen gas was bubbled though water to maintain a humidified atmosphere within the glove box. Anaerobic indicator strips (Anerogen brand, Oxoid, Hampshire, UK; Holthaus *et al.*, 2002) were placed into separate control incubation units, to verify that anaerobic conditions were maintained throughout the experiment.

The aquatic-sediment samples were preincubated for two weeks prior to adding MT to the experimental units. The stability of the system during the acclimation period was evaluated by measuring pH and total organic carbon (TOC) in both the water and sediment at the end of the acclimation period, as well as microbial biomass of the sediments and BOD of the water at the start of testing. The experiment was initiated by adding radio-inert MT or ^{14}C-MT to the experimental units as in the aerobic experiment. Duplicate incubation vessels were prepared for each of the five sampling times: 0, 7, 14, 28 and 56 days. No "short-term" experiment was conducted

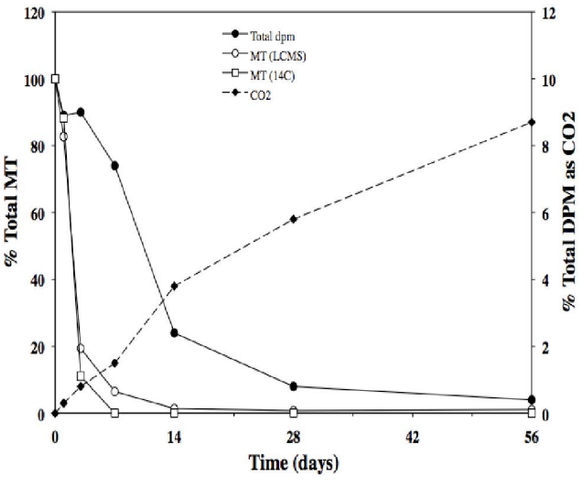

Fig. 4. *Fate of 17α-methyltestosterone (MT) in the water from an aquatic-sediment system under aerobic conditions. The sediment had a low organic carbon content (1.75%) and fine texture (clay + silt content 15%) and is referred to as "Sand". The total radioactivity in the water (solid dots), the radioactivity associated specifically with MT (open squares), and the radioactivity associated with CO_2 captured by alkaline traps (dashed line) are presented. MT was measured in a duplicate incubation by LCMS (open dot). See text for additional information.*

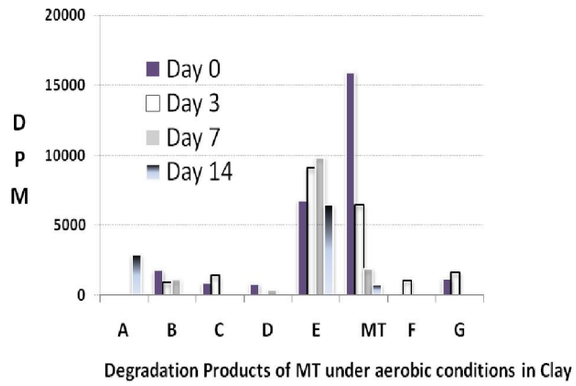

Fig. 5. *17α-methyltestosterone (MT) and MT degradation products present in the water from incubations of "Sand" under aerobic conditions. MT had a retention time of 8.95 minutes. Several of the degradation products were tentatively identified by LC-MS. The data shown on the y-axis are DPM from the incubations using radiolabeled MT and analyzed by radio-HPLC.*

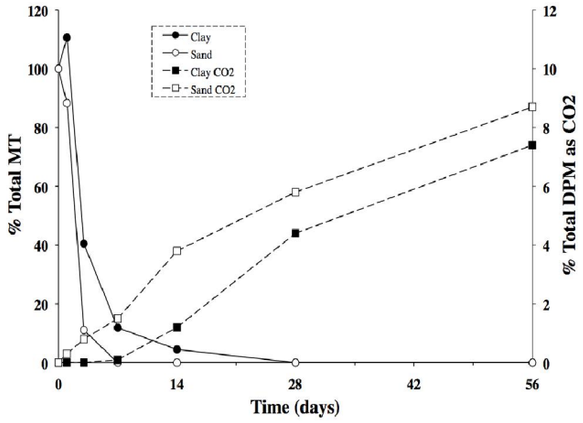

Fig. 6. *Comparison of the fate of ^{14}C-17α-methyltestosterone (MT) in the water from aquatic-sediment systems under aerobic conditions incubated with "Clay" or "Sand". The radioactivity in the water (solid lines) and the radioactivity associated with CO_2 captured by alkaline traps (dashed line) are shown.*

because MT metabolism was expected to be slow under anaerobic conditions. One control incubation unit per sediment was used to estimate microbial biomass of the sediment and the total organic carbon of the water and sediment at the start of incubation, and after 56 days of incubation (termination of the study). The replicate incubation vessels were sampled by carefully pouring the water into a 100 ml polypropylene sampling bottle. Duplicate 1 ml water samples were collected from incubation vessels dosed with ^{14}C-MT for determination of radioactivity by LSC. The remaining water was extracted and analyzed HPLC and LC-MS. The sediment remaining in the original incubation vessels was stored

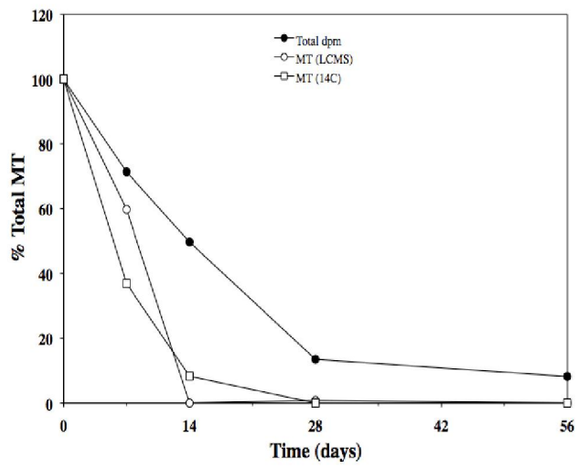

Fig. 7. *Fate of 17α-methyltestosterone (MT) in the water from an aquatic-sediment system under anaerobic conditions. The sediment had a high organic carbon content and fine particle size ("Clay"). The total radioactivity in the water (solid dots) and the radioactivity associated specifically with ^{14}C-MT (open squares). Radio-inert MT was measured in identical duplicate incubation vessels by LCMS (open dot).*

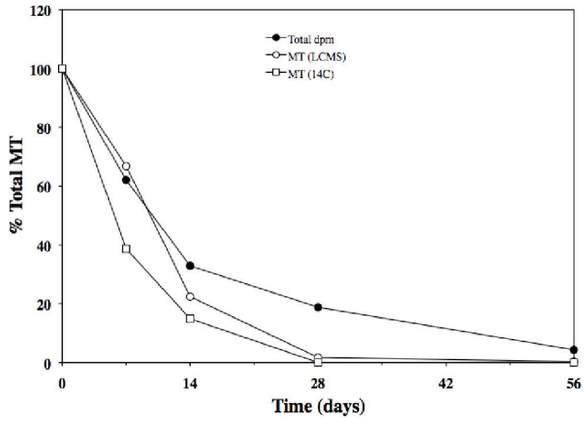

Fig. 8. *Fate of 17α-methyltestosterone (MT) in the water from an aquatic-sediment system under anaerobic conditions. The sediment had a low organic carbon content and large particle size ("Sand"). The total radioactivity in the water (solid dots), and the radioactivity associated specifically with MT (open squares) are presented. In addition, MT was measured in a duplicate incubation by LCMS (open dot). See text for additional information.*

frozen at -40°C until analysis.

Analytical methods

Water extraction: The quantity of MT in water samples was determined using LC-MS method reported earlier (Marwah *et al.*, 2010). In short, twenty-five ml of test water from each incubation in the aerobic and anaerobic trials was used for analysis. The water was centrifuged at 1000 g for 5 min at 4°C and applied to pre-conditioned solid phase extraction (SPE) cartridges (Oasis-HLB 6 c.c., Waters Corporation, Milford, USA). The loaded cartridges were washed and eluted with acetone into a graduated polypropylene tube (15 ml). The eluent was evaporated under nitrogen at 40ºC, and reconstituted in methanol-water (50:50) for radio-HPLC (^{14}C-MT samples) or LC-MS (radio-inert samples) analysis. The average extraction recovery of MT from water using this method was 95.1±1.8.

Analysis of radiation in alkaline traps and water: Radioactivity in the alkaline traps and water samples was measured using a Packard Tri-Carb Model 2900TR LSC. Duplicate 1 ml samples were added to 20 ml scintillation vials. Fifteen ml of scintillation fluid (Opti-Fluor, PerkinElmer, Waltham, MA, USA) was added to each vial, and the radioactivity was quantified by LSC.

Sediment extraction and analysis: Sediment (~30 ml in volume) was diluted with acetone to 100 ml and shaken in a metabolic shaker for 4 h at room temperature (~22° C), and then allowed to stand for 1 h. The acetone layer (~70 ml) was decanted and centrifuged at 1000 g for 5 min at 4°C. A 25 ml volume was evaporated under nitrogen and the residue reconstituted in 10 ml water and subjected to solid phase extraction as described earlier. Cartridges were extracted with methanol (3 ml) and acetone (3 ml) to

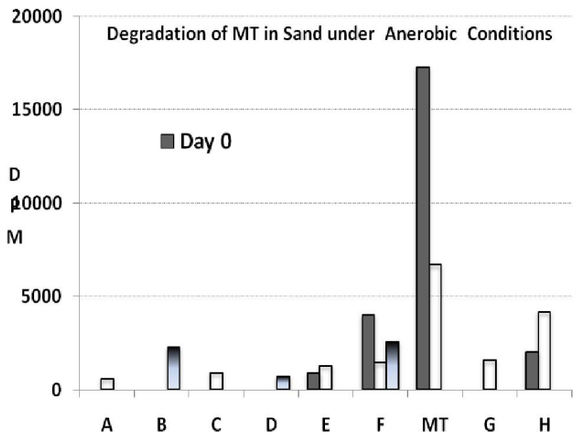

Fig. 9. *MT and MT degradation products present in the water from incubations of "Sand" under anaerobic conditions. Several of the degradation products (A to H) were tentatively identified. The data shown are DPM from the incubations using radiolabeled MT and analyzed by Radio-HPLC.*

elute all possible products of MT. MT was then measured by LC-MS. The concentration of MT was measured at every sampling time in water and sediment. Mineralization rates, and the amount of non extractable (bound) residues in sediment were calculated at each sampling point, as was the distribution of' radioactivity between water and sediment. The half life of MT was calculated from the untransformed data using the curve-fitting program GraphPad Prism (La Jolla, CA).

RESULTS AND DISCUSSION

Aerobic experiment: Total radioactivity in the Clay-water, as measured by liquid scintillation counting, steadily declined with time (Fig. 4, solid dots). The half-life of total radioactivity in the water was calculated to be 14.2 days (3rd level polynomial curve fit, $R^2 = 0.97$, Table 2). The fraction of the total radioactivity accounted for specifically by MT declined faster than the total radioactivity (Fig. 4). On day 7, for example, approximately 70% of the added radioactivity was still present in the water, but only about 10% of this radioactivity was MT (Fig. 4). The results indicate that MT is rapidly converted into other water-soluble MT metabolites that then slowly disappear from the water, a conclusion supported by the appearance of various unidentified MT degradation compounds with time (Fig. 5). The results from both the radio-HPLC analysis of "hot" samples, and LCMS analysis of "cold" samples were similar. The half-life of total MT in the water was calculated to be 2.8 days (4th level polynomial curve fit, $R^2 = 0.98$, Table 2). MT was mineralized into CO_2 over the course of the experiment. The mineralization rate of MT was slow, however, and CO_2 production did not account for the dissipation of MT from the water column (Fig. 4, dotted line).

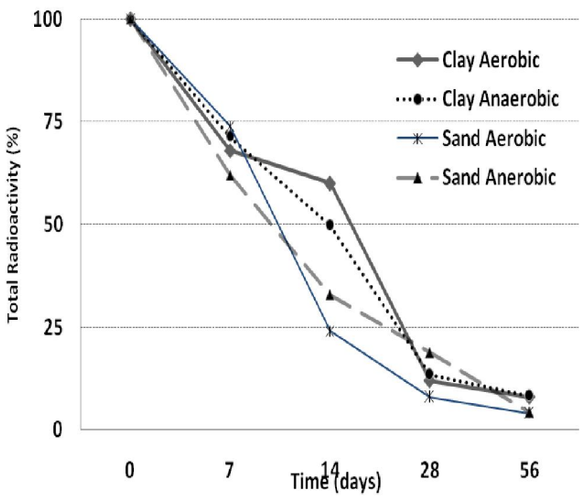

Fig. 10. *Comparison of the fate of radioactive 17α-methyltestosterone (MT) in the water from aquatic-sediment systems with "clay" and "sand" under aerobic and anaerobic conditions.*

No MT was detected in the extracted sediment at any time point. This observation, together with the low mineralization rate, indicates that a non-extractable MT transformation product(s) become strongly associated with clay sediment. Mass balance analysis indicated that almost 85% of the radioactivity was associated with this non-extractable fraction by day 56 of the incubation.

Total radioactivity in the Sand-water, as measured by LSC, steadily declined with time and less than 10% of the initial MT levels were present in the water column by day 28. The half-life of total radioactivity in the water was calculated to be 10.8 days (2nd level polynomial curve fit, $R^2 = 0.96$, Table 2). The fraction of the total radioactivity accounted for by MT declined faster than the total radioactivity. The results indicate that MT is rapidly converted into other water-soluble MT metabolites that then slowly disappear from the water, a conclusion supported by the appearance of various unidentified MT degradation compounds with time. The MT dissipation results from both the radio-HPLC analysis of "hot" samples, and LCMS analysis of "cold" samples were essentially identical. The half-life of total MT in the water incubated with "sand" was calculated to be 2.1 days (4th level polynomial curve fit, $R^2 = 0.96$, Table 2). MT was mineralized into CO_2 over the course of the experiment. The mineralization rate of MT was slow, and CO_2 production did not account for the disappearance of MT from the water column. MT was not detected in the extracted sediment at any time point. This observation, together with the low mineralization rate, indicates that a non-extractable MT transformation product (or products) that becomes strongly associated with the sand sediment. Mass balance analysis indicated that over 84% of the radioactivity was associated with this non-extractable

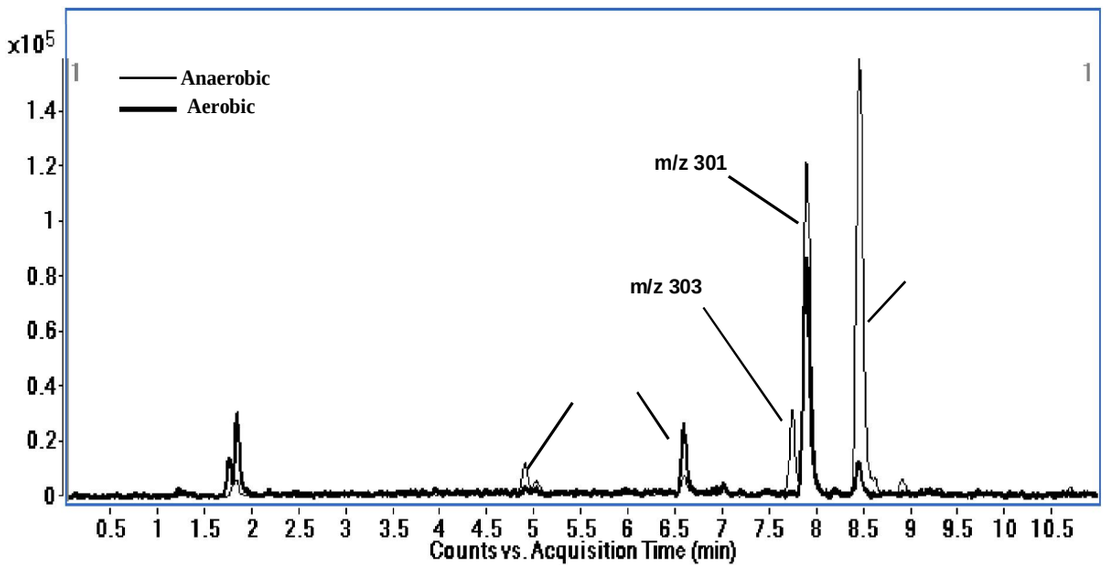

Fig. 11. *Transformation of methyltestosterone in clay under anaerobic and aerobic conditions on day seven of incubation. The samples were extracted with methanol and acetone and after solid phase extraction analyzed by LC-MS in ESI (+) mode; data subjected to extracted ion analysis for the probable degradation products.*

fraction by day 56 of the incubation (Table 3).

The changes in total radioactivity in the water from the "clay" and "sand" incubations are shown together in Fig. 6. Total radioactivity initially declined more rapidly in sand compared to clay, but by days 28 and 56 the levels of MT in the water were almost identical in both sediment types. The radio-labeled MT concentrations fell more rapidly in the sand compared to the clay $^{14}CO_2$ levels were higher in the sand incubations at every time point compared to the clay incubations This faster mineralization rate in the sand may at least partially explain the more rapid dissipation of MT from the water in sand compared to clay.

Anaerobic experiment: Total radioactivity in the Clay water, as measured by liquid scintillation counting, steadily declined over the total 56-day experimental period (Fig. 7, solid dots). The fraction of the total radioactivity accounted for by MT declined faster than total radioactivity, and MT was almost absent from the water column by day 14 (Fig. 7, open squares). The results from both the radio-HPLC analysis of "hot" samples, and LCMS analysis of "cold" samples were similar. As in the aerobic experiments, MT was below the method detection limit in the extracted clay sediment at every time point. Total radioactivity in the sand water, as measured by LSC, steadily declined over the total 56-day experimental period (Fig. 8, solid dots). The fraction of the total radioactivity accounted for by MT declined faster than total radioactivity, but this difference between the total radioactivity, and total MT was less than in other treatment groups suggesting that the formation of MT metabolites occurs at a slower rate under anaerobic conditions. MT was almost absent from the water column by day 28 (Fig. 8, open squares). The results from both the radio-HPLC analysis of "hot" samples, and LCMS analysis of "cold" samples were similar. As in the aerobic experiments, not even a trace of MT was detected in the extracted sand sediment at any time point. Several of the degradation products (Fig. 9) of MT present in the Water from incubations of "Sand" under anaerobic conditions were tentatively identified using LCMS, though further work is needed to establish their structures conclusively. The rate of dissipation of radio-labelled MT from the water was very similar in both the clay and sand incubations.

Aerobic vs. anaerobic: The rate at which total radioactivity disappeared from the water was faster under aerobic conditions compared to anaerobic conditions in both the clay and sand water/sediment systems (Figs. 10). Under both oxygen environments, there was no radioactivity associated with the water by 28 days of incubation. Table 3 shows the mass balance of radioactivity between the water and sediment on day 56 of the anaerobic experiment.

Overall fate of MT in water/sediment systems: The data suggest that MT entering the aquatic environment is converted into metabolite(s) that then become tightly associated with the sediment. Half-lives for MT dissipation in the sediment systems were estimated using best-fit quadratic curve fitting equations, and ranged from 2 to 9 days, depending on the sediment type and the presence or absence of oxygen.

Fate of testosterone in the soil has been studied and documented (Lee *et al.*, 2003; Colucci *et al.*, 2001; Casey

et al., 2004). We have earlier studied degradation of MT under accelerated stress situations at high and low pH (Marwah et al. 2005). Selected samples from aerobic and anaerobic experiments were subjected to LC-MS analysis under scan mode and data subjected to the extracted ion analysis for the expected degradation products. Transformation of MT in clay under anaerobic and aerobic conditions on day seven of incubation is shown in Figure 11. The samples were extracted with methanol and acetone and after solid phase extraction analyzed by LC-MS in ESI (+) mode; data subjected to extracted ion analysis for the probable degradation products. Degradation products at m/z 301, 303 and 319 were observed, which are supposed to be formed by oxidation and/ or dehydration of MT. Although similar products are formed under aerobic and anaerobic conditions, but there relative quantities differ as expected. For example only a little MT remained unreacted under aerobic conditions in one week as compared to anaerobic conditions. Similarly oxygenated product (m/z 319) is found in much more abundance under aerobic conditions than under anaerobic conditions. The degradation product (m/z 301) is most likely formed by the dehydration of oxygenated product (m/z 319). However further studies are required to completely characterize these degradation products.

Total radioactivity vs. MT : Under aerobic conditions, the half-life of ^{14}C-MT based on total radioactivity in water (> 10 d) was ~5 times longer than the half-life specifically associated with intact ^{14}C-MT (~2 days). Similarly, under anaerobic conditions, the half-life of ^{14}C-MT based on total radioactivity in the water (~11-13 days) was longer than the half-life specifically associated with intact ^{14}C-MT (~5-9 days). The best interpretation of this data is that MT is rapidly converted into water-soluble metabolite(s) that remain associated with the water column longer that MT itself.

Effect of oxygen: The dissipation of MT occurred in both the presence and absence of oxygen. MT dissipated 2-4 times faster under aerobic conditions (half-lives of 2.1-2.8 days) compared to anaerobic conditions (half-lives of 5.3-8.9 days). This likely reflects the higher microbial biomass associated with the aerobic sediments. The rapid degradation of steroids under anaerobic conditions has been reported (Jurgens *et al.*, 2002).

Mineralization under aerobic conditions: The faster dissipation of MT under aerobic conditions does not appear to be due to mineralization. Cleavage of the MT steroid ring was demonstrated by the release of radiolabeled CO_2, but overall, the mineralization rate was low in both sediment systems (<9%). In at least one other experiment, the mineralization rate of testosterone was shown to be much greater than found in the present investigation. Layton *et al.* (2000) investigated the mineralization of ^{14}C-testosterone (^{14}C-T) using biosolids from four municipal sewage treatment plants and one industrial system. In 24 hrs, from 55% to 65% of added ^{14}C-T was mineralized to $^{14}CO_2$ by all five biosolids tested; total removal of ^{14}C-T from the aqueous phase was 95%. Temperature (5-10°C vs. 22-25°C) had no statistically significant effect on the rate of mineralization and removal of ^{14}C-T. This difference in mineralization rate is probably explained by differences in microbial biomass, which would be much greater in the sewage system compared to the sediments used in the present investigation.

Effect of sediment type: There was little difference between the two sediment types on the rate at which MT left the water column indicating that particle size, organic matter content, and specific surface area may not play key roles in determining the sorption of MT or MT metabolites to sediments. Given the Log P (octanol-water) partition coefficient of MT (3.36), hydrophobic partitioning of MT or an MT metabolite was expected to be the dominant sorption mechanism. Non-extractable MT metabolites were present in both the clay and sand sediments, suggesting that the organic content of the soil is not the primary factor determining the degree of sorption to the sediment. In a similar manner, it has been reported that sediment organic content and particle size had little effect on the sorption of testosterone to different soil types (Casey *et al.*, 2004). Very high non-extractable binding of steroid metabolites (i.e., low recoveries of steroid and steroid metabolites from sediments), on the order found in the present investigation, have been previously reported. For example, testosterone and its metabolites were shown to irreversibly bind to soil (Lee *et al.*, 2003; Colucci *et al.*, 2001; Casey *et al.*, 2004).

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