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ARTICLE *in* ENVIRONMENTAL SCIENCE AND TECHNOLOGY · SEPTEMBER 2000

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Mineralization of Steroidal Hormones by Biosolids in Wastewater Treatment Systems in Tennessee U.S.A.

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During the past several years, concern has risen over potential pollution of waterways with estrogenic compounds, including steroidal hormones from human and animal sources. One potential source of steroid hormone contamination is through the incomplete removal of these compounds in wastewater treatment systems (WTS). To address this issue, laboratory mineralization assays using ^{14}C-labeled estrogens and testosterone were performed with biosolids from four municipal treatment plants and one industrial system. The importance of adapted microbial populations in the removal of estrogen was shown by the dramatic differences in mineralization of ^{14}C-17β-estradiol by biosolids from a municipal plant compared to that from the industrial plant, 84% versus 4%, respectively. Indeed, biosolids from all of the municipal plants mineralized 70−80% of added ^{14}C-17β-estradiol to $^{14}CO_2$ in 24 h. Removal of ^{14}C-17β-estradiol from the aqueous phase by biodegradation and/or biosorption to cell matter was greater than 90%. A recombinant yeast estrogen assay (YES assay) also confirmed that biological estrogenic activity was removed from the biosolid samples to below the detection limit (1.56 nM). ^{14}C-Testosterone was mineralized to $^{14}CO_2$ in all four municipal biosolids in amounts ranging from 55% to 65%; moreover, total removal of ^{14}C-testosterone from the aqueous phase was 95%. First-order rate constants *k* were obtained for the mineralization and removal from the aqueous phase of natural and a synthetic steroid hormone in biosolids from one WTP. In these biosolids, ^{14}C-17β-estradiol and ^{14}C-testosterone were rapidly mineralized to ^{14}C-CO_2 ($k = 0.0042 \pm 0.0002$ min^{-1} and 0.0152 ± 0.0021 min^{-1}, respectively), whereas the mineralization of the synthetic estrogen ^{14}C-17α-ethinylestradiol was 25−75-fold less ($k = 0.0002 \pm 0.0000$ min^{-1}). In addition, mineralization of ^{14}C-ethinylestradiol did not reach completion in 24 h with only 40% mineralized to ^{14}C-CO_2. Approximately 20% of the ^{14}C-ethinylestradiol remained in the aqueous phase and was biologically active as determined by the YES assay. Changes in temperature of approximately 15 °C had a statistically significant effect on the rate of mineralization and removal of ^{14}C-17β-estradiol from the aqueous phase but not for ^{14}C-testosterone or ^{14}C-17α-ethinylestradiol. These results suggest that biosolids in municipal plants in this region have the capability to remove natural steroid hormones in their influents over a range of temperatures but may be less effective at removing the synthetic estrogen 17α-ethinylestradiol.

1. Introduction

Endocrine-disrupting chemicals in the environment have been linked to industrial chemicals, phytoestrogens, and steroid hormones (*1*). The most likely sources of estrogenic compounds in the aquatic environment are from discharge of municipal and/or industrial effluents, along with runoff from agricultural production. Concern about potential pollution by such steroid hormones dates back to the 1980s. However, there were conflicting reports as to whether estrogens were likely to be discharged into waterways after processing in wastewater treatment plants (*2*−*4*). Recently, vitellogenin (egg yolk precursor protein) production in different fish species has been shown to be higher downstream from wastewater treatment plants (WTPs) than upstream (*5*−*12*), suggesting that these waters contain estrogenic compounds. Additionally, studies have shown estrogenic activity in WTP effluents using fish or other biological assay systems (*13*−*15*). However, the type of estrogenic compounds reported in many of the studies was unknown or undetermined. Both steroidal estrogens and nonylphenols, derived from detergents, have been suggested as the possible sources of endocrine disrupters in fish (*7*, *9*, *11*, *16*, *17*). Other investigators reported detectable levels of natural and synthetic steroidal estrogens in WTP effluents using GC-MS methods (*18*−*20*) or radioimmunoassays (*21*). A few studies have directly linked steroidal estrogen concentrations in WTP effluents with vitellogenin production in fish downstream of the plants (*9*, *22*, *23*).

Upon examination of the literature concerning estrogen concentrations in effluents and surrounding waterways, several points may be inferred. First, detectable levels of estrogens (10−100 ng/L) are found in some, but not all, WTP effluents (*9*, *18*−*20*, *24*). Given that negative results are often not reported, many plants may be effective in removing estrogens from their influents. Estrogen concentrations in sanitary wastewater have been reported in only a few studies but consistently fall in the 40−100 ng per L range (*19*−*21*). Considering that some WTP effluents contain as high a concentration of estrogens as reported in their influents, these plants may not be effective in removing estrogens. In both humans and animals, estrogens are either excreted in urine as glucuronide or sulfated conjugates or simply eliminated in feces in the unconjugated form (*9*). Studies have shown that glucoronide conjugated compounds are readily cleaved in WTPs (*9*, *25*) and that the unconjugated forms of estrogens are more abundant in effluents and rivers than the conjugated forms (*18*, *24*).

Second, bacterial populations in biosolids may vary in their ability to degrade estrogens. Ternes et al. (*20*) reported that 99.9% of the 17β-estradiol and 83% and 78% of the estrone and 17α-ethinylestradiol were removed from raw sewage in an aerator tank at a WTP in Brazil; however, 64% of the 17β-estradiol and essentially none of the estrone or 17α-ethinylestradiol were removed from raw sewage in a

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10.1021/es9914487 CCC: $19.00
Published on Web 08/09/2000

TABLE 1. Important Operation and Performance Features of the Wastewater Treatment Plants Examined in This Study

characteristic	industrial WTP	municipal WTP 1	municipal WTP 2	municipal WTP 3[a]	municipal WTP 4
flow capacity millions of gallons/day MGD (MLD)	30 (114)	15 (57)	4 (15)	40 (150)	10 (38)
mean cell retention time (MCRT) (days)[b]	16.7	NA	3.1	NA	NA
mixed liquor suspended solids (MLSS) (mg/L)	4981	3140	1950	2165	2126
av daily influent flow (MGD)	26.9	6.4 (max 12.1)	3.2 (max 7.6)	33.3 (max 100.3)	8.1 (max 14.7)
influent BOD5[c] load (mg/L)	803	97	140	273	146
% BOD removal	99.6%	94% (min 92%)	97.8% (min 96.4%)	94% (min 59%)	87% (min 66%)
influent suspended solids (mg/L)	NA[d]	96	163	339.5	129
% solids removal	NA	95% (min 93%)	98.3% (min 94.1%)	95.5% (min 58%)	89.8% (min 74%)
no. of bypass of treatment in 1 year[e]	NA	7	39	24	15
fecal coliform density (num/100 mL)	NA	29 (max 200)	25.8 (max 56)	10.5 (max 61)	2.9 (max 8.2)

[a] Data for plant is only for 1999 sampling. [b] Mean cell retention time. [c] BOD = biological oxygen demand. [d] NA = data not available. [e] Bypass of treatment = number of reported bypass of treatment for the year proceeding last sampling period.

plant in Germany. In diluted biosolid samples from a WTP in Germany spiked with 1 mg/L or 1 ng/L estrogens, 17β-estradiol was quantitatively converted to estrone and then removed at a slower rate (*25*). The synthetic estrogen, 17α-ethinylestradiol, was persistent in these spiked biosolid samples.

Third, several factors may affect estrogen removal by WTPs and subsequent accumulation in receiving waters; these include type and age of plant (*12*), geographic location (*20*), composition of feed stream, and estrogen substrate specificity. Currently, most reports of estrogens in effluents and receiving waters derive from areas with colder climates (e.g., Canada, Northern U.S.A., Sweden, and Germany) and in those with high population densities and voluminous effluent discharges (e.g., United Kingdom and Germany).

The goal of this investigation was to provide new quantitative information on the biodegradative fate of steroid hormones in biosolids from wastewater treatment plants in the southeastern region of the United States, which provides an extension of data to warmer treatment plants. The use of ^{14}C-labeled compounds allows demonstration of fate through partitioning and mineralization to CO_2, whereas other analytical methods primarily measure disappearance. Attempts were also made to relate analytical results with a biological assay to demonstrate reduction in activity. Because testosterone is structurally similar to 17β-estradiol and testosterone use is increasing in our society, demonstration of its removal is also important for the protection of the receiving waters.

In this study, four hypotheses were tested: 1. Waste stream influent affects the biosolids ability to mineralize steroid compounds. 2. Variations in operational parameters at municipal WTP affect the ability of the biosolids to mineralize steroid compounds. 3. The synthetic estrogen 17α-ethinylestradiol is mineralized more slowly than 17β-estradiol. 4. Temperature affects the ability of the biosolids to mineralize steroid compounds.

2. Methodology

Biosolids Samples from Wastewater Treatment Plants. Biosolids samples (0.5−1 L) were obtained from the aeration basin of four different WTPs on at least two separate dates for each plant between March, 1998 and April, 2000. All of these treatment plants processed primarily municipal sewage, employing aerobic biological treatment. Information on the efficiency of plant operations during the sampling period was obtained from water discharge permits maintained at EPA (www.epa.gov) through Surf your Watershed and Envirofacts Warehouse (Table 1). Effluent water temperatures were available for one plant for approximately 3 years. In this plant, effluent temperatures averaged 16.7 °C (± 4.2) and ranged from a low of 8.2 °C in January to 24 °C in July. The industrial WTP has an influent soluble total organic carbon (STOC) load of about 50 000 kg per day of strictly aqueous chemical waste (i.e., no sanitary wastewater). This load consists mainly of short-chained organic acids and alcohols (*26*).

^{14}C Mineralization Laboratory Assays. ^{14}C-Labeled compounds were obtained from NEN LifeScience Products (Boston, MA) and had a purity >97%. The 17 β-estradiol (specific activity 1.96−2.00 GBq/mmol), estrone (specific activity 2.10 GBq/mmol), 17α-ethinylestradiol (19-nor-17α-pregna-1,3,5(10)trien-2−4ne-3) (specific activity 2.15 GBq/mmol), and testosterone (specific activity of 2.05−2.10 GBq/mmol) were labeled on the C-4 carbon of the steroid backbone. Therefore, release of ^{14}C-CO_2 from these compounds would denote ring cleavage and concomitant inactivation of the steroid molecule. The glucose (specific activity 12.1−13.0 mCi/mmol; 0.4477−0.48 GBq/mmol) was uniformly labeled on all carbons and was used as a control for biosolids viability and metabolic activity.

Biosolids obtained from WTPs were employed in mineralization experiments within 4 h of collection, following the protocols described by Sanseverino et al. (*27*). Accordingly, 5-mL aliquots were placed into sterile 40-mL Eagle Picher vials (Fisher Scientific, Pittsburgh, PA). Other 8-mL vials containing 0.5 N NaOH were added to each 40-mL vial to serve as ^{14}C-CO_2 traps. Individual vials were set up for 17β-estradiol, testosterone, and glucose mineralization assays. Each assay had at least three time points (ranging from 0- to 72-h incubation), and each time point included three experimental and two killed-control vials. Killed control samples were generated by acidification with 2 N H_2SO_4 before the addition of the radiolabeled compounds, and experimental samples were killed at the appropriate incubation time points, with 2 N H_2SO_4. A total of 2.5 μL (1×10^6−9×10^6 dpm) of radiolabeled compounds was added to the mineralization vials; the same quantity of radiolabeled compounds was also added to three separate scintillation vials, containing 1 mL of H_2O, to determine the initial radioactive counts. Mineralization vials were incubated on a gyratory shaker at ca. 200 rpm at appropriate temperatures. The starting concentrations of ^{14}C-17β-estradiol,^{14}C -testosterone, and 17α-ethinylestradiol in these assays were 198 nM (58 μg/L), 364 nM (99 μg/L), and 241 nM (72 μg/L), respectively.

The amount of ^{14}C from each compound was assayed in the CO_2 trap by suspending the 0.5 mL of 0.5 N NaOH in 1 mL of water, plus 10 mL of Ready-Safe scintillation fluid (Beckman Instruments, Inc., Fullerton, CA). The portion of ^{14}C in the aqueous phase was determined in 0.5 mL samples (plus 10 mL of Beckman Ready-Safe scintillation fluid) taken from the cleared liquid of a 1.5 mL aliquot of biosolids centrifuged for 10 min at 14 000 rpm. The amount of ^{14}C in the biomass was determined by air-drying 2 mL samples and oxidizing these samples in a Harvey Biological Oxidizer (Model OX-500, R.J. Harvey Instrument Co., Hillsdale, NJ). A Beckman liquid scintillation counter (Model LS3801) was used to determine radioactivity in all samples. The percentage of ^{14}C in each fraction (mineralized to CO_2, aqueous phase and biomass) was calculated from the measured radioactivity in each fraction divided by the radioactivity in 2.5 μL of untreated radiolabeled compound and multiplied by 100. The amount of ^{14}C in the biomass was derived from the oxidized value (normalized for the oxidation efficiency of each compound) minus the amount of ^{14}C in the aqueous phase.

First-order rate constants k were determined for the removal of steroid compounds from the aqueous phase and removal by mineralization. The rate expression

$$\frac{dC}{dt} = kC \quad (1)$$

where C is the concentration of the steroid (nM) and t is time (min) was assumed to model the removal of the steroid compounds. The rate expression was integrated and written in the linear form

$$\ln(C_0 - C(^{14}C\text{-}CO_2)) = kt \quad (2)$$

where C_0 is the initial concentration of the steroid and ^{14}C-CO_2 is the concentration of ^{14}C labeled CO_2. The value of the rate constant k for mineralization was estimated by plotting the data in the manner suggested by eq 2 and finding the value of k that gave the best fit of a straight line to the data.

The rate constant for the removal of steroid compounds from the aqueous phase, which can be both mineralized and incorporated into biomass, was estimated by finding the value of the rate constant k that gives the best fit to the integrated, linear form of the rate equation

$$\ln(C(^{14}C\text{-steroid})) = kt \quad (3)$$

where $C(^{14}C$-steroid$)$ is the concentration of ^{14}C labeled steroid (nM).

Yeast Estrogenic Assay. A recombinant *S. cerevisiae* strain, containing the human estrogen receptor integrated into the yeast genome and with the estrogen-responsive sequences carried on a *lac* Z reporter plasmid (*28*), was used as a bioassay for estrogenic activity (YES assay). In this assay, estrogen interacts with the receptor to activate the transcription of the *lac* Z genes, resulting in the production of the enzyme β-galactosidase. β-Galactosidase activity is measured colorimetrically at 540 nm using the chromogenic substrate, chlorophenol red-β-D-galactopyranoside. The absorbances in 96 well microtiter plates were measured using a Packard Spectra Count plate reader with I-Smart 1.0 software (Packard Instrument Co. Meridian CT). The YES assays were run as described previously using 2-fold serial dilutions of unlabeled 17β-estradiol starting at 2724 ng/L (well concentration) as a positive control and to construct standard curves (*29*). The estrogenic activity of biosolids samples amended with ^{14}C-labeled compounds was determined using 2-fold serial dilutions starting with 10 μL cleared aqueous phase subsamples from the ^{14}C-17β-estradiol killed-controls or 50 μL cleared aqueous phase subsamples from the live ^{14}C-17β-estradiol and ^{14}C-glucose assays. Relative estradiol concentrations in the test samples were calculated from regression values obtained from the 17β-estradiol standard curves.

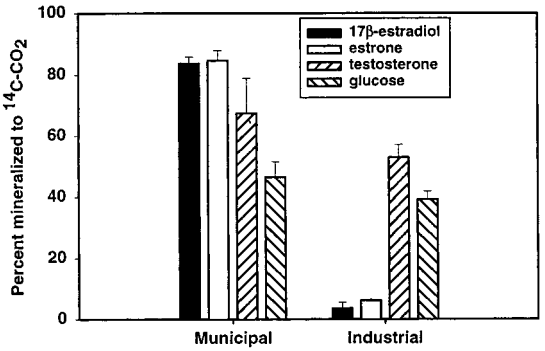

FIGURE 1. Comparison of mineralization of ^{14}C-17β-estradiol, ^{14}C-estrone, ^{14}C-testosterone, and ^{14}C-glucose after 24 h in municipal versus industrial biosolids.

3. Results

Hypothesis 1. Waste Stream Influent Affects the Biosolids Ability To Mineralize Steroid Compounds: Laboratory Comparison of ^{14}C-Steroid Mineralization between Industrial and Municipal WTP Biosolids. Mineralization of steroid hormones in 24 h was initially examined with biosolids from municipal WTP3 and biosolids from an industrial plant that receives no sanitary sewage. In the municipal biosolids, equivalent amounts of ^{14}C-17β-estradiol and ^{14}C-estrone were mineralized to ^{14}C-CO_2 (84% and 85%, respectively) (Figure 1). Slightly less ^{14}C -testosterone was mineralized to ^{14}C-CO_2 (68%). Approximately, 47% of the uniformly labeled ^{14}C-glucose was mineralized to ^{14}C-CO_2, consistent with about half of the carbon in the sugar being assimilated into cell biomass.

Mineralization of ^{14}C-17β-estradiol and ^{14}C-estrone in the industrial biosolids was considerably less than in the municipal biosolids (4%). The estrogens added to the industrial biosolids remained biologically active, as measured by the YES assay (data not shown). Mineralization of ^{14}C-testosterone and ^{14}C-glucose to ^{14}C-CO_2 in the industrial biosolids were almost equivalent to the mineralization of these compounds in municipal biosolids (Figure 1), indicating that the industrial biosolids were metabolically active.

Hypothesis 2. Variations in Operational Parameters at Municipal WTP Affect the Ability of the Biosolids To Mineralize Steroid Compounds: Mineralization and Partitioning of ^{14}C-17β-Estradiol, Testosterone, and Glucose in Municipal WTP Biosolids. The ability to mineralize ^{14}C-labeled 17β-estradiol, testosterone, and glucose in biosolids from four different municipal WTPs was examined. The mineralization of ^{14}C-estrone was not examined because the amount of 17β-estradiol and estrone mineralized by WTP3 in a 24 h period was almost identical (previous section). All four of the municipal WTPs, from which biosolids were examined, efficiently removed wastes and met EPA limits for percent BOD removal and percent suspended solids removal (average = 85%, minimum = 40% for each) and for fecal coliform numbers in effluent (average = 200/100 mL and maximum = 1000/100 mL) (Table 1). For all four plants, dissolved oxygen in the effluent was greater than 5.0 mg/L and the pH was greater than 6.0. Effluents from three of the WTPs discharged into a river, whereas effluent from one WTP discharged into a creek.

The amount of ^{14}C-labeled products in the gas phase (as CO_2), aqueous phase, and solid phase was measured in each vial. The mineralization data for the 24- and 72-h timepoints

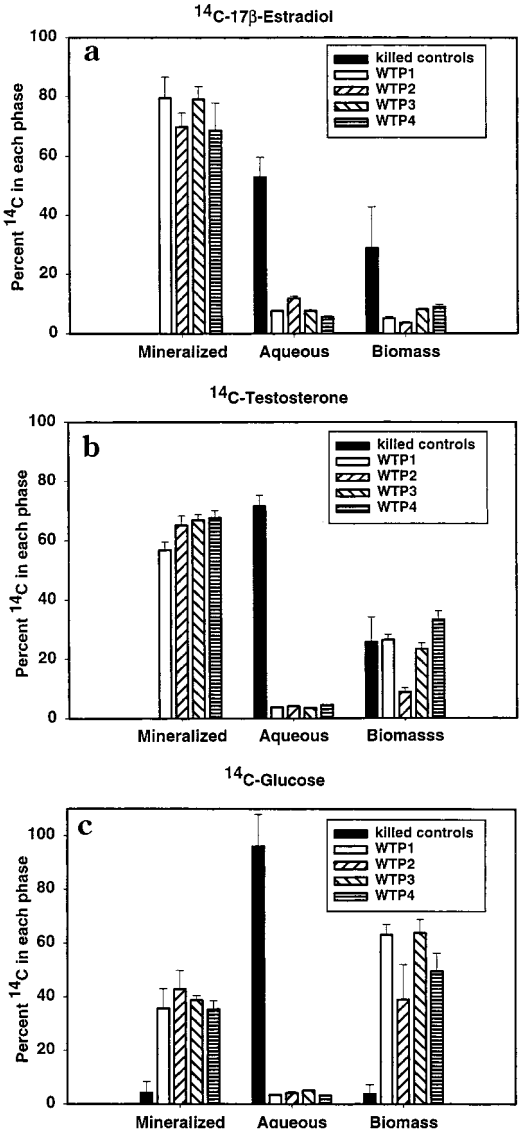

FIGURE 2. Partitioning and mineralization of ^{14}C-labeled compound in biosolids after 24 h from four municipal wastewater treatment plants (WTPs): A. ^{14}C-17β-estradiol, B. ^{14}C-testosterone, and C. ^{14}C-glucose.

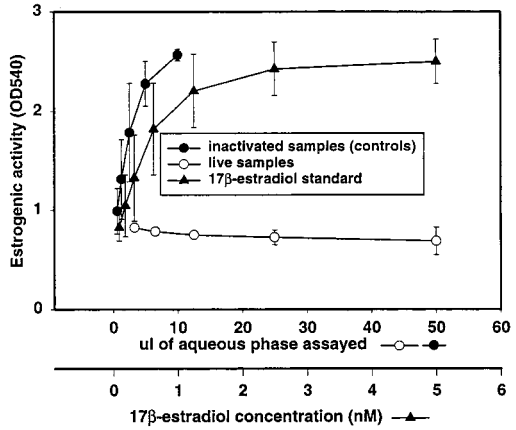

FIGURE 3. Response of the yeast estrogenic assay with aqueous samples from biosolids amended with ^{14}C-17β-estradiol. Killed biosolids = biosolids killed by acidification before addition of ^{14}C-17β-estradiol. Live biosolids = biosolids incubated with ^{14}C-17β-estradiol for 24 h.

were essentially identical and, therefore, are only presented for the 24-h timepoint (Figure 2 A–C). Killed-controls were sampled at 0-, 24-, and 72-h and averaged across all samples for each compound. Mass balance recoveries for all the ^{14}C-17β-estradiol amended samples were 80.4% (±11.3) and 94.7% (±11.0) for the ^{14}C-testosterone amended samples, and 95.3% (±17.4) for the ^{14}C-glucose amended samples.

The mineralization of ^{14}C-17β-estradiol was similar in all biosolids samples with an average of 74.2% (±5.8) being completely oxidized to CO_2 after 24 h for all treatment plant sources (Figure 2A). After 24 h, 8.1% (±2.7) of the ^{14}C count was dissolved in the aqueous portion of the sample, and 6.4% (±2.4) of the ^{14}C count was associated with the biomass. In the killed-controls, 0.1% (±0.1) of the ^{14}C activity was in the gas phase (nondiscernible in Figure 2A), 52.9% (±6.7) of the ^{14}C-17β-estradiol was in the aqueous phase, and 28.8% (±14) was attached to the solids (biomass). With one municipal WTP biosolids, mineralization of ^{14}C-17β-estradiol remained consistent in samples taken 1 year apart, i.e., 84% (±2.2) mineralized in March, 1998 (Figure 1) and 79% (±4.4%) mineralized in April, 1999 (Figure 2A, WTP 3).

The mineralization of ^{14}C-testosterone in the biosolids samples was similar to the mineralization of ^{14}C-17β-estradiol (Figure 2B). In these samples, 64.1% (±5.5) of ^{14}C-testosterone was mineralized to CO_2. The amount ^{14}C-activity remaining in the aqueous phase was 3.9% (±0.8); 23.0%(±10.1) of the ^{14}C-activity was retained in the biomass. In killed controls, 0.1% (±0.1) of the ^{14}C-testosterone was in the gas phase, 71.5% (±3.7) was in the aqueous phase, and 25.8% (±8.4) was associated with the biomass.

In biosolids samples amended with ^{14}C-glucose, 39.2% (±7.2) was mineralized to CO_2. The amount of ^{14}C-activity remaining in the aqueous phase was 4.2% (±1.7), and 55.4% (±17.4) of the ^{14}C-activity resided in the biomass (Figure 2C). The high amount of ^{14}C in the biomass is consistent with the incorporation of carbon from glucose into cell material. In the ^{14}C-glucose killed-controls, 4.2% (± 4.1) was mineralized to CO_2, 96% (±11.9) was found in the aqueous phase, and 3.8% (±3.4) was associated with the biomass.

Attempts were made to correlate plant operating conditions with the data obtained from ^{14}C studies. The rate of ^{14}C-compound mineralized to $^{14}CO_2$ was not correlated to the percent BOD removal during the test period for each plant ($r^2 < 0.1$ for each regression analysis). There was also no correlation between the percent of ^{14}C remaining in aqueous phase and the percent suspended solids removed by each plant ($r^2 < 0.6$ for each regression analysis).

Biological Activity of ^{14}C-17β-Estradiol before and after Degradation in Biosolids. The amount of metabolically active estrogen was measured in aqueous samples from vials amended with ^{14}C-17β-estradiol and ^{14}C-glucose using the YES assay. Estrogenic activity in the aqueous phase, as determined by the YES assay, was detectable in the inactivated biosolid controls [145 nM (±61)] but not in any of the live biosolids samples after 24 or 72 h (Figure 3). The measured estrogenic activity in the inactivated biosolid controls from all of the plants was slightly higher than the expected activity of 98 nM based on the percentage of ^{14}C counts in aqueous phase (52.8% × 189 nM added ^{14}C-17β-estradiol). Also, no estrogenic activity was seen in any of the aqueous phase samples from the glucose mineralization experiments, indicating that the starting estrogen concentration in the biosolids samples was below the experimental detection limit of 1.56 nM. The expected concentration of 17β-estradiol in the live samples was 15 nM, based on the initial amount of ^{14}C-17β-estradiol added and the percentage of ^{14}C-label remaining in the aqueous phase after mineralization (8.1% × 189 μM ^{14}C-17β-estradiol). Therefore, the estrogenic activity was at least 10-fold less than expected by the ^{14}C-assay data, indicating that the residual ^{14}C counts are not due to bioactive estrogen.

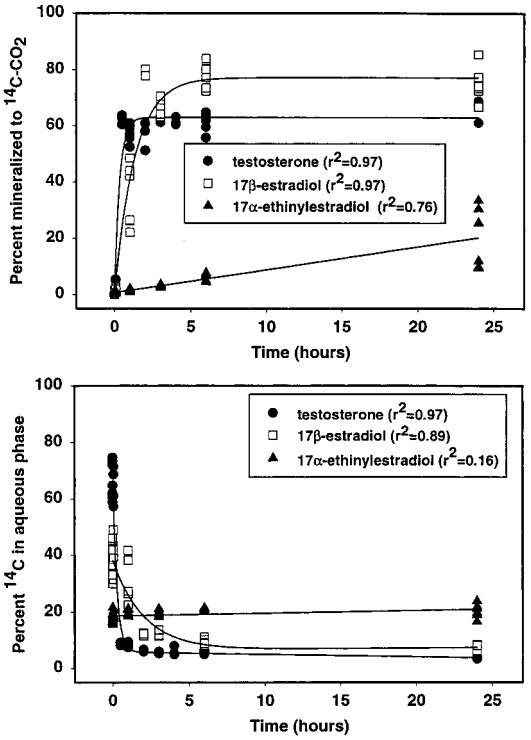

FIGURE 4. (a) Mineralization of ^{14}C-labeled compounds in municipal WTP biosolids between 0 and 24 h. Data for ^{14}C-CO_2 from ^{14}C-17β-estradiol and ^{14}C-testosterone were fit to first-order rate equations $C(^{14}C\text{-}CO_2) = a(1 - \exp^{-b(t)})$, and data for ^{14}C-CO_2 for ^{14}C-glucose were fit to linear equation. (b) Partitioning of ^{14}C-labeled compounds in municipal WTP biosolids between 0 and 24 h. Data for ^{14}C-compound in the aqueous phase for ^{14}C-17β-estradiol, ^{14}C-testosterone, and ^{14}C-glucose were fit to the first-order rate equations $C(^{14}C\ aq) = a(1 - \exp^{-b(t)})$.

Hypothesis 3. The Synthetic Estrogen, 17α-Ethinylestradiol, Is Mineralized More Slowly Than 17β-Estradiol: Mineralization and Sorption Rates of 17β-Estradiol, Testosterone, and 17α-Ethinylestradiol. Mineralization of ^{14}C-17α-ethinylestradiol was compared to mineralization of ^{14}C-17β-estradiol and ^{14}C-testosterone in biosolids from WTP3 (Figure 4A). The percent mineralization of ^{14}C-17α-ethinylestradiol (20.2% ± 11) was considerably less than the mineralization of ^{14}C-17β-estradiol (75.2% ± 5.0) and ^{14}C-testosterone (65.7% ± 4.0)

The removal of these ^{14}C-labeled steroid compounds from the aqueous phase also differed between ^{14}C-testosterone, ^{14}C-17β-estradiol, and ^{14}C-17α-ethinylestradiol. Both ^{14}C-testosterone and ^{14}C-17β-estradiol were rapidly removed from the aqueous phase, whereas 20% ± 2.5 of the ^{14}C counts from ^{14}C-17α-ethinyl estradiol remained in the aqueous phase 24 h after incubation (Figure 4B). In addition, estrogenic activity of 17α-ethinylestradiol in biosolids as measured by the YES assay was present in the aqueous phase even 24 h after incubation (T0 = 202 ± 131 nM vs T24 = 50 ± 42 nM). The measured 17β-estradiol activity at T0 and in the inactivated controls was 167 ± 67.5 nM and was below the detection limit in the aqueous phase after 24 h.

Hypothesis 4. Temperature Affects the Ability of the Biosolids To Mineralize Steroid Compounds. Mineralization assays were performed at low temperatures (5−10 °C) using one to two separate assay dates for each compound and at higher temperatures (22−25 °C) using two to three separate assay dates for each compound to determine the effect of temperature on mineralization. The temperatures of the biosolids at the time of sampling at WTP3 ranged from 11 to 16 °C. In these experiments, ^{14}C-testosterone had the fastest rates of mineralization and the fastest rates of removal from the aqueous phase (Table 2). ^{14}C-17β-Estradiol mineralization was also relatively fast, whereas ^{14}C-17α-ethinylestradiol mineralization was slow. Temperature did not have an effect on the first-order rate constants (k) for mineralization of ^{14}C-testosterone or ^{14}C-17α-ethinylestradiol but did have a statistically significant effect on the first-order rate constant (k) for the mineralization of ^{14}C-17β-estradiol (Table 2). The k values for the removal of each ^{14}C-labeled compound from the aqueous phase and by mineralization were similar indicating that the two processes are linked (Table 2).

TABLE 2. First-Order Rate Constants k (min^{-1}) for the Removal of ^{14}C-Labeled Compounds by Mineralization to ^{14}C-CO_2 and First-Order Rate Constants k (min^{-1}) for the Removal of ^{14}C-Labeled Compounds from the Aqueous Phase in Biosolids from Municipal WTP3

	rate constant k for mineralization to ^{14}C-CO_2 (min^{-1})		
temp	testosterone[a]	17β-estradiol	17α-ethinylestradiol[b]
5−10 °C	0.0161(±0.0016) (r^2 = 0.94)[b]	0.0029 (±0.0002) (r^2 = 0.89)	0.0001 (±0.0000) (r^2 = 0.98)
22−25 °C	0.0152(±0.0021) (r^2 = 0.77)	0.0042 (±0.0002) (r^2 = 0.92)	0.0002 (±0.0000) (r^2 = 0.96)
	rate constant k for removal of ^{14}C from aqueous phase (min^{-1})		
temp	testosterone[c]	17β-estradiol[c]	17α-ethinylestradiol
5−10 °C	0.0216 (±0.0024) (r^2 = 0.88)	0.0024 (±0.0002) (r^2 = 0.80)	NC[d]
22−25 °C	0.0235 (±0.0022) (r^2 = 0.86)	0.0036 (±0.0016) (r^2 = 0.92)	NC[d]

[a] k(min^{-1}) was calculated from the linear regression using the formula $\ln[C_0(^{14}C\text{-compound}) - C(^{14}C\text{-}CO_2)] = kt$. [b] r^2 of linear regression used to calculate k value. [c] k (min^{-1}) was calculated from the linear regression using the formula $\ln[C(^{14}C$ − compound in aqueous phase)] = kt. [d] Not calculated. The amount of ^{14}C-17α-ethinylestradiol in the aqueous phase remained constant over time.

Discussion

The ^{14}C assay used in this study is a powerful tool for analyzing the removal of steroid hormones from wastewater by the biosolids treatment process. In this assay, two types of removal can be detected. First, removal of the steroid hormones may occur through bacterial metabolism and use of the compounds as a carbon-energy source; evidence of this process is the mineralization of the ^{14}C-steroid to ^{14}C-CO_2. In this assay, both the estrogens and testosterone contain ^{14}C at the fourth carbon of the A-ring. Oxidation of ^{14}C to ^{14}C-CO_2 requires ring cleavage and thus complete inactivation of the steroid compound. Second, the removal of steroid compounds by biosolids may occur through the physical binding of the compound to the bacterial biomass without biological degradation. The process of physical binding (i.e., biosorption) can be detected in the oxidized fraction (termed biomass) of the killed controls of the ^{14}C assay because the biological activity is removed. However, in the experimental treatments, the oxidized fraction may contain ^{14}C biologically incorporated into the biomass via bacterial assimilation as well as undegraded compound sorbed to the bacterial biomass. In this assay, ^{14}C remaining in the cleared, aqueous phase after the bacterial solids were settled by centrifugation contains the soluble portion of the ^{14}C-labeled compound.

In this study, only the unconjugated ^{14}C estrogens were used for the mineralization assays. The use of the unconjugated ^{14}C steroid hormones is valid because other studies have shown that glucoronide conjugated compounds are readily cleaved in WTPs (*25*) and that the unconjugated form of estrogens is more abundant in effluents and rivers than the conjugated form (*18*, *24*). Other studies indicate that

17β-estradiol is rapidly converted to estrone and then estrone is removed at a slower rate (*23*, *25*). In this study 55% of ^{14}C-17β-estradiol was mineralized to ^{14}C-CO_2 in 1 h, and maximum mineralization (approximately 75%) was achieved in 2−3 h suggesting that small amounts of estrone accumulated. In addition the high percentage of ^{14}C-17β-estradiol and ^{14}C-testosterone converted to ^{14}C-CO_2 (75% and 65%, respectively) suggests that ring cleavage of these compounds primarily serves as an energy source. The approximately 15−25% difference between the amount of ^{14}C removed from the aqueous phase and the amount of ^{14}C-CO_2 accumulated may be attributable to carbon incorporated into biomass or cometabolism.

Hypothesis 1. Waste Stream Influent Affects the Biosolids Ability To Mineralize Steroid Compounds. In this study, considerable differences were seen in the mineralization of ^{14}C-17β-estradiol in the municipal and industrial WTP biosolids (84% vs 4%) confirming the importance of an adapted microbial population in the biological removal of estrogens from wastewater. However, equivalent amounts of ^{14}C-testosterone and ^{14}C-glucose were mineralized in the municipal and industrial biosolids. It is unclear why larger amounts of ^{14}C-testosterone, rather than ^{14}C-17β-estradiol and ^{14}C-estrone, were mineralized to ^{14}C-CO_2 in the industrial biosolids. The waste influent does not include sanitary waste, and testosterone and estrogens have similar chemical structures. However, it is likely that the industrial WTP biosolids includes bacteria, such as *Comamonas testosteroni*, capable of using testosterone as a carbon-energy source (*30*, *31*).

Hypothesis 2. Variations in Operational Parameters at Municipal WTP Affect the Ability of the Biosolids To Mineralize Steroid Compounds. The four selected municipal WTPs, utilizing biosolids processes, effectively removed both natural estrogen and androgen steroid hormones. There were no statistical differences in the mineralization of the ^{14}C-labeled compounds between WTPs. The ^{14}C-labeled compounds mineralized or removed from the aqueous phases were not correlated to percent BOD removal or percent suspended solids removal. The stable pattern of steroid mineralization in biosolids from the four municipal plants examined in this study may be due to moderate temperatures found in the geographic region and continuous exposure of these plants to estrogenic substances in the influent sanitary waste.

One of the drawbacks to experiments utilizing spiked compounds is that higher concentrations than found in the environment are needed for accurate quantification. In this study, 58 μg/L ^{14}C-17β-estradiol was used, which is approximately 1000 times higher than the concentrations reported in sewage influents (*19*−*21*). However, the ability of the biosolids from WTP3 to mineralize 24 μg/L in 1 h suggests that the load of steroid hormones supplied in normal influents (<100 ng/L) is well below their biodegradative capacity.

In addition to biodegradation of steroids (as measured by the ^{14}C assays), all of the municipal biosolids samples removed the estrogenic activity from samples with ^{14}C-17β-estradiol as measured by the YES assay. Based upon the YES assay, the ^{14}C counts remaining in the aqueous phase (approximately 8%) were less estrogenic than expected i.e., < 1.5 nM compared to 15 nM. Unfortunately, the sample volumes used in the ^{14}C assay were too small to allow analytical fractionation and detection of estrogen metabolites in the expected 100 ng/L range. Further chemical analysis would be useful in determining whether the residual ^{14}C counts in the aqueous phase were 17β-estradiol or other metabolites, such as estrone.

Estrogenic activity was not seen in biosolids samples without added ^{14}C-17β-estradiol. However, estrogenic activity would probably not be detectable in the YES assay in biosolids samples with <100 ng/L (0.4 nM) of 17β-estradiol. 17β-Estradiol concentrations as low as 15 ng/L in raw sewage samples have been observed, well below the detection limit of this YES assay (*20*).

Hypothesis 3. The Synthetic Estrogen, α-Ethinylestradiol Is Mineralized More Slowly Than 17β-Estradiol. Previous studies indicated that both 17β-estradiol and 17α-ethinylestradiol were effectively eliminated from the influent by the biosolids in a WTP in Brazil but not from a WTP in Germany (*20*, *25*). In this study, removal of ^{14}C-17α-ethinylestradiol by mineralization in WTP3 was considerably less than the removal of ^{14}C-17β-estradiol, 75% vs 20%, indicating that the ethinyl group inhibits degradation. The slow mineralization rate of ^{14}C-17α-ethinylestradiol may also contribute to lack of removal of ^{14}C-17α-ethinylestradiol from the aqueous phase. The importance of mineralization in the biosolids for effective removal of steroid hormones is also demonstrated by the fact that 50% of ^{14}C-17β-estradiol and 70% of ^{14}C-testosterone remained constant in the aqueous phase in the inactivated biosolids samples over the 24−72-h test period.

Hypothesis 4. Temperature Affects the Ability of the Biosolids To Mineralize Steroid Compounds. Steroid removal by biosolids in WTPs has been reported to differ between geographic localities. These differences may be attributable to differences in temperature (*20*). In these studies no significant differences were seen in the first-order rate constants k for removal by mineralization or removal from the aqueous phase for ^{14}C-testosterone or ^{14}C-17α-ethinylestradiol at temperatures differing by 10−15 °C. Differences in the rate constants for the removal of ^{14}C-17β-estradiol by mineralization and from the aqueous phase at different temperatures were statistically significant. However, it should be noted that the initial mineralization rates of ^{14}C-17β-estradiol at 5−10 °C were 200 ng of 17β-estradiol/L biosolids in a minute, which is higher than reported influent concentrations. This suggests that even at cold temperatures 17β-estradiol is rapidly removed by the biosolids at this WTP.

The mineralization and removal of ^{14}C-testosterone from aqueous phase was approximately 2× faster than the mineralization and removal of ^{14}C-17β-estradiol suggesting that testosterone is easier to metabolize than 17β-estradiol. This is also supported by the fact that ^{14}C-testosterone, but not ^{14}C-17β-estradiol, was mineralized in the industrial biosolids.

In this study, mineralization rates for ^{14}C-17β-estradiol were approximately the same as the rates of removal from the aqueous phase indicating sorption to the biomass was not the rate-limiting step at these concentrations. Additional studies at very low concentrations (10 ng/L) would be useful in determining whether sorption to the biomass becomes a rate-limiting step and thus contributes to the presence of estrogens in the effluent.

Acknowledgments

This study was supported in part by the United States Department of the Interior, U.S. Geological Survey Grant No. 1434-HQ-96-GR-0279, Project 17 through Mississippi Water Resources Research Institute at Mississippi State University and in part by the Waste Management Research and Education Institute at the University of Tennessee. We would like to thank Dr. John Sanseverino for advice on ^{14}C mineralization assays, Dr. Paul Frymier for discussions on rate analysis, and Dr. Arthur Meyers, Eastman Chemical Company, for his thorough review of the manuscript.

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Received for review December 30, 1999. Revised manuscript received June 16, 2000. Accepted June 26, 2000.

ES9914487