

Biological and hydrogeological interactions affect the persistence of 17 β -estradiol in an agricultural watershed

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ABSTRACT

17 β -estradiol (E2), one of the natural estrogen compounds, is an endocrine disruptor, and low levels in natural waters can impair the reproductive health of aquatic organisms. Its presence has been reported in animal faecal wastes and some aquatic habitats, including surface waters impacted by intense animal agriculture or sewage contamination. Little is known about its transport in hydrological systems or its persistence in water supplies. We routinely sampled stream and soil water over the growing season in an instrumented 1.2-km² agricultural watershed in central Virginia. E2 concentrations in stream water ranged from 0.01 to 0.12 ng mL⁻¹; soil-water values ranged from 0.03 to 0.18 ng mL⁻¹. The highest concentrations were observed early in the growing season shortly after application of composted poultry litter to the cropped fields, and values decreased both with hydrological transport distance from the cropped field and over the course of the summer. Given the known application rate, E2 must be lost from the soil solution, and we explored biodegradation as a mechanism for this loss. A bacterial consortium cultured from the poultry compost biodegraded E2 in laboratory flasks amended with solutions of 1 : 1 acetate : glucose at concentrations ranging from 0.1 to 10 g L⁻¹ dissolved organic carbon (DOC), spiked with 1.8 ng mL⁻¹ E2, and incubated at different temperatures. A loss of 97–98% of the initial E2 occurred within 180 h in experiments at 22 °C and 28 °C with 1.0 or 0.1 g L⁻¹ DOC amendments. Higher DOC concentrations and lower temperatures slowed the rates of reaction, suggesting that more readily available carbon inhibits use of the E2 by degrading micro-organisms. The rapid rates of biodegradation in the laboratory incubations are inconsistent with the persistence of E2 in the watershed. This suggests that either the rates of biodegradation are reduced compared with the laboratory experiments or that E2 probably interacts with the components of the natural environment through complexation, sorption or abiotic transformation in the ageing process that leads to diminished bioavailability.

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INTRODUCTION

A number of chemical compounds have been identified recently as 'emerging contaminants' in the water supplies of the United States, including human and veterinary drugs, hormones, detergents, disinfectants, plasticizers, fire retardants, insecticides and antioxidants (Buxton & Kolpin, 2002). In a nationwide reconnaissance sampling of 139 streams, a stunningly high proportion of streams – 80% – was contaminated with one or more compounds ranging from acetaminophen to caffeine to antibiotics (Kolpin *et al.*, 2002). The introduction of many of these organic compounds that have household, pharmaceutical or personal-care use to rivers and streams may derive largely from discharge from sewage-treatment plants not designed for this type of contaminant removal (Harries *et al.*, 1996;

Routledge *et al.*, 1998; Ternes, 1999; Kolpin *et al.*, 2002). Yet, other important pathways to environmental occurrence are also clearly contributing. Runoff from livestock feedlots and manured fields has been implicated in the occurrence of a variety of exogenous (synthetic) and endogenous steroidal hormones as well as insecticides and antibiotics, especially in agriculturally intensive regions (Shore *et al.*, 1993a; Finlay-Moore *et al.*, 2000; Meyer *et al.*, 2000; Peterson *et al.*, 2000; Kolpin *et al.*, 2002; Ying *et al.*, 2002). Once in the environment, many of these compounds are detected only at low concentrations (Huang & Sedlak, 2001; Kolpin *et al.*, 2002). Nevertheless, the occurrence of one class of compounds in particular – hormones – presents significant cause for concern even when present at extremely low concentrations (National Research Council, 1999). The hormone-receptor systems in animals are

similar to those of humans, so the effects observed in wildlife species raises concerns of potential human health effects (Melnick, 1999; National Research Council, 1999).

Recent reports, not only of feminized wildlife, but also of the possibility of steep falls in sperm counts of men and the rise in hormone-related cancers in women, such as breast cancer, have brought popular attention to the issue of environmental occurrence of hormones (e.g. Colborn *et al.*, 1993; McLachlan & Arnold, 1996; Harrison *et al.*, 1997). The occurrence of hormones in the environment remains largely unquantified at present, because measurements of a number of synthetic (contraceptive) and natural male and female hormones have only recently been made (Huang & Sedlak, 2001; Ying *et al.*, 2002). The three natural estrogens – estrone (E1), 17 β -estradiol (E2) and estriol (E3) – are present in the faecal wastes of mammalian and avian species and are increasingly suspected of having relatively common occurrence in the environment.

Concern is now directed towards the environmental impacts of environmental estrogens and the implications of environmental loading of 17 β -estradiol (E2), the most potent of the natural estrogens (Nichols *et al.*, 1997; Ying *et al.*, 2002). Environmental loading of E2 can result from disposal of animal wastes (Knight, 1980), and observed concentrations in streams receiving runoff from farms have been higher than those in effluent from sewage treatment plants (Shore *et al.*, 1993a). Poultry litter and cattle manure are usually applied to agricultural fields for fertilization and for waste disposal. Poultry litter is clearly one source of E2 to surface waters (Shore *et al.*, 1988, 1993b). In a study by Nichols *et al.* (1997), E2 derived from poultry litter was persistent enough to produce significant concentrations in runoff from agricultural plots.

Relatively little is known about E2 occurrence in water supplies in agricultural regions. Reports in the United States Midwest suggest significant levels occur in surface waters impacted by runoff from pig and poultry farms (Barber *et al.*, 2000; Peterson *et al.*, 2000). Few other reports on this subject can be found. The literature suggests widespread occurrence, yet little is known of the behaviour of E2 in the environment. The ubiquity could result solely from the magnitude of the introduction into the environment. Alternatively, or perhaps additionally, E2 and similar compounds may persist in the environment as a result of either lack of biodegradability or because of interaction of the compounds with the physical matrix to render the compounds unavailable to attack by otherwise competent micro-organisms.

We undertook a study in a hydrologically well characterized watershed that is in mixed-use agriculture and has previously been the focus of our own studies on the fate and transport of herbicides, nutrients and colloids (Kauffman, 1998; Sprague *et al.*, 2000; Hyer *et al.*, 2001). The specific area we examined contains a 1000-bird turkey operation, approximately 15 young cattle that occasionally graze above the Stream 2 site (Fig. 1) and a 50-head dairy barn with summer grazing and manure disposal in the vicinity of Stream 4. Our objective was

to survey the occurrence of E2 in a typical agricultural region of Virginia. Furthermore, we sought to elucidate the processes limiting the apparent persistence of E2 in this environment by conducting biodegradation experiments in which we explored the effects of temperature and concentrations of co-occurring dissolved organic carbon on biodegradation rates and comparing those results with the apparent loss of E2 from the watershed.

METHODS

Site description

Our study site was the Muddy Creek subcatchment approximately 20 km north-west (38°32.93'N, 78°57.24'W) of Harrisonburg, VA, USA. The 1.2-km² subcatchment is drained by the Muddy Creek tributary (approximately 1.5 km in length), which eventually flows into the Shenandoah River and the Chesapeake Bay. Part of the watershed is forested, but most of the land is used for agricultural purposes, including corn, turkey and cattle production. Liquid cattle manure and dry poultry compost (pine bedding material and turkey faecal waste) are applied to the cornfields and pastures every year in mid- to late April as fertilizer and as a method of waste disposal (Hyer, 2001).

Five sites located on the Muddy Creek tributary were chosen for monitoring during the summer of 2001 (Fig. 1). Stream 1 is in a wooded area of a pasture, on which cattle were grazing during the first month of our sampling, and is located near an actively cultivated cornfield. Stream 2 is also in a pasture in which cattle were grazing during the latter portion of the sampling period. This stream at this site, however, was dry through most of July and August owing to the combined effects of the typical low rainfall and high evapotranspiration rates of late summer together with a long-term regional drought (http://va.water.usgs.gov/realtime/annual/2001/gw/gw_annualreport01.html). Spring 2 feeds a small tributary just upstream from the junction with the main stream. Stream 4 is in a pasture in which a very small number of cattle graze. The source of the main stream, a spring located in a forested area, was also monitored. Two additional sites downstream of the Muddy Creek subcatchment (downstream of the sites illustrated in Fig. 1) were also monitored. One site is located at the US Geological Survey (USGS) gauging station in Mt Clinton, VA, approximately 5 km west of the Muddy Creek site. Another site is approximately 1.5 km upstream from the Mt Clinton site along Muddy Creek Road in Harrisonburg, VA.

Pan lysimeters installed as part of a previous study (Kauffman, 1998) were also sampled in the present research. Lysimeters were installed at three depths – 15, 46 and 91 cm – at each installation site, and even at 91 cm the soils were aerobic (Kauffman, 1998). Pan 1 was upslope from Stream 1 at the edge of the cornfield. Pan 2 was installed between the junction of Spring 2 and Stream 2 in a field now used for grazing of a small number of young cattle. Pan 3 was installed within a

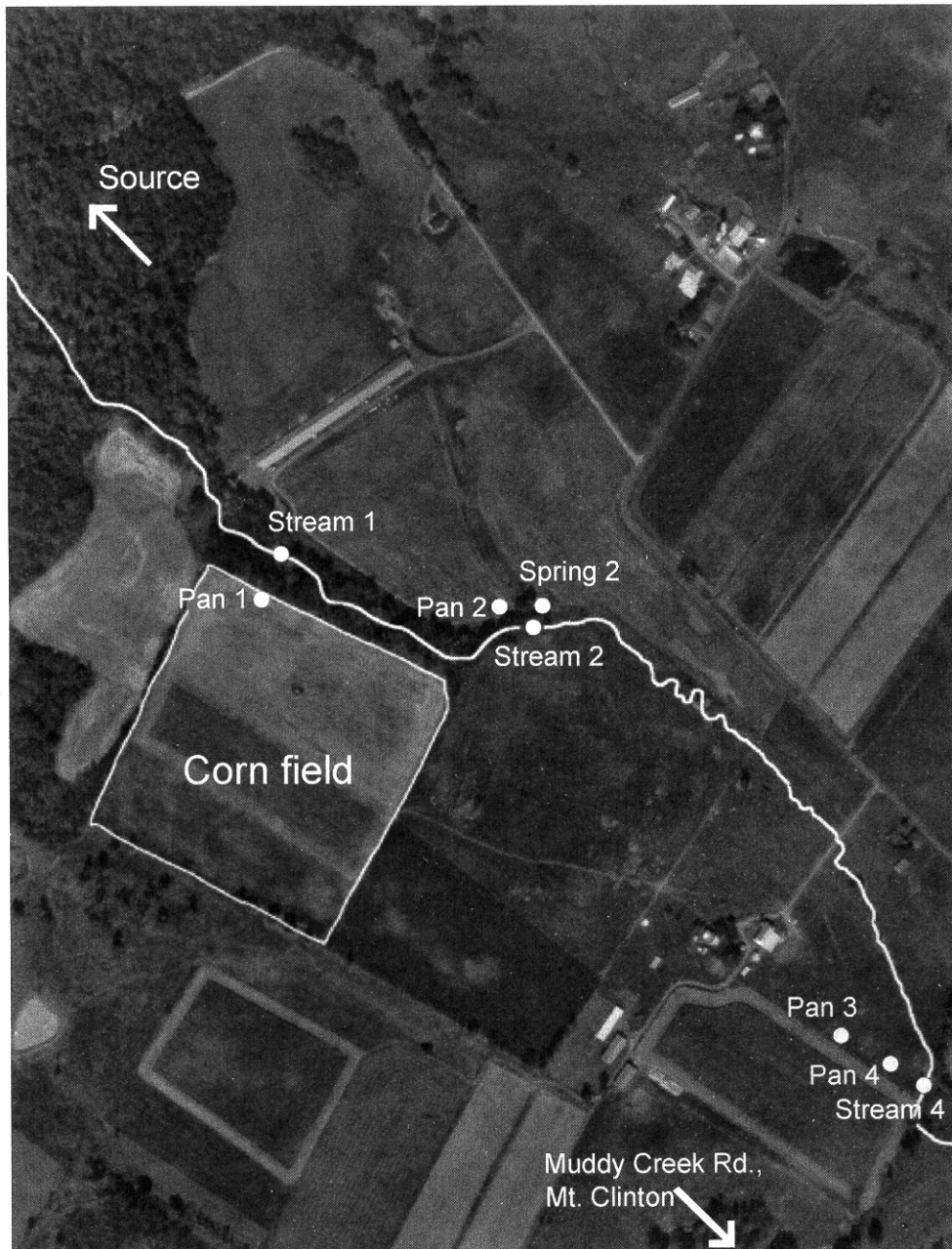


Fig. 1 Sampling sites in the Muddy Creek subcatchment. The site termed Source is about 500 m upstream from the edge of the figure, whereas the Muddy Creek Road and Mt Clinton sites are 3 and 8 km downstream, respectively. The cornfield that was the active area of poultry compost and cattle manure application during the study is marked. The ploughed area to the west was a fallow field for 3 years when the study began, and was not used during the period of the study.

pasture used for summer grazing of a larger herd as well as manure disposal and about 50 m from Stream 4. Pan 4 was located approximately 5 m from Stream 4 in the same pasture.

Field sampling

A series of preliminary experiments demonstrated the impact of filtration and of storage of samples on detected E2 concen-

trations (Franzosa, 2001). Prior to initiation of our routine sampling programme, replicate splits of samples were collected in the field and were either left unfiltered, filtered through a 0.45- μ m polysulphone membrane or filtered through a 0.20- μ m autoclaved syringe filter. The subsequent measured E2 concentrations varied minimally across treatments, so we adopted the standard recommended protocol of using a 0.45- μ m filter prior to sample storage (APHA, 1995). Sample splits

were also stored in both plastic and amber glass vials and stored both in the refrigerator and on the bench top. Measured E2 concentrations after 3 weeks of storage were lower than after 1 week of storage. These results are similar to those by Smith & Williams (1947), who observed that aqueous solutions of estrogen compounds stored in 5 °C in the dark experience less loss of activity than solutions exposed to light and room temperature. We subsequently stored samples in refrigerated amber glass vials in the dark for periods less than 10 days before analysis. Overall, sample handling and storage followed the practice of previous researchers (Peterson *et al.*, 2000). All collected samples were therefore handled accordingly.

Sampling at Muddy Creek began in early June 2001 and recurred on a regular 2-week basis into late August 2001. Samples were collected in acid-washed 250-mL polyethylene bottles that were rinsed thoroughly with sample water then filled. One sample was collected at each site. Sample collection from the lysimeter pans was limited owing to the limited infiltration during summer months; on numerous sampling dates the pans were empty. Each sample was filtered through a 0.45- μm polysulphone membrane into two 20-mL acid-washed amber glass vials that were rinsed three times with the filtered sample before collection of a portion for analysis. Latex gloves were worn during the entire sampling process. We changed gloves, rinsed the entire filter apparatus with deionized water and replaced the filter after sample collection at each site. The samples were stored on ice in a cooler until return to the laboratory, where all samples were refrigerated (4 °C) until analysis.

E2 biodegradation experiment

To obtain an idea of the highest concentrations of E2 likely to occur in our study watershed, the water-soluble E2 content of the cattle manure and poultry compost that is applied to the fields was determined. Cattle manure and poultry compost were collected at the site in plastic bags and stored in glass Mason jars until extraction on the following day. One litre of deionized water was combined with 20 g of moist cattle manure or 20 g of moist poultry compost in a flask. The slurries were placed on magnetic stir plates, and duplicate samples were collected at 120 min, the same duration of contact employed by Nichols *et al.* (1998). The solid phase was sedimented in a centrifuge at 12 000 g for 15 min. The supernatant fluid was filtered through 0.45- μm syringe filter into acid-washed amber glass vials. The samples were stored in the refrigerator (4 °C) until analyses for E2 were performed the following day.

A microbial culture was developed from the poultry compost in preparation for biodegradation experiments. The extrapolation of degradation potential from enrichment cultures based on poultry litter to the soil environment seemed reasonable, because the ultimate fate of the poultry litter in this agricultural operation is to be spread directly on the soil. Thus, the soil would be inoculated with the same organisms that were used in our enrichment experiments. Given the

practice of poultry litter application, the soil microbial community responsible for any E2 degradation should not differ substantively from that of the poultry compost. Furthermore, given that conditions in the soil and shallow groundwater are known to be aerobic (Kauffman, 1998; Hyer, 2001) the use of aerobic conditions for the enrichment was the best approximation of the *in situ* conditions. No effort was made to characterize the organisms within the enrichment culture, because the presence of activity was the essential characteristic of interest.

A sample of the poultry compost was obtained directly from the compost holding bins at the turkey barn. Two samples of approximately 50 g each, from different locations within the bin, were put in Mason jars and transported on ice to the laboratory for storage in the refrigerator. Aqueous stock solutions of a buffered mineral-salts (BMS) solution (Wodzinski & Johnson, 1968), E2 and dissolved organic carbon (DOC) solutions were used for culturing these samples. E2 (Sigma) was added to deionized water to create a stock solution of 0.9 $\mu\text{g mL}^{-1}$. The solution was shaken vigorously for 30 s, and then allowed to sit overnight in the dark. The stock DOC solution consisted of a 1 : 1 molar ratio of acetate and glucose in BMS to provide a DOC concentration of 100 g L^{-1} . Within a week of obtaining the poultry compost samples, two 125-mL Erlenmeyer flasks with 25 mL of solution containing BMS solution and 10 g L^{-1} DOC were autoclaved, and approximately 1 g of the poultry compost was added as an inoculum. The culture was immediately amended with 50 μL of E2 stock solution to yield a final concentration of 1.8 ng mL^{-1} E2. The two culture flasks were stored in a dark incubator at approximately 28 °C in aerobic conditions. After approximately 10 days, at which point substantial microbial growth was evident from the turbidity within the flasks, the culture suspensions were centrifuged at 8622 g for 15 min. The supernatant liquid was decanted from the centrifuge tubes and the remaining cells were washed three times by resuspending them in 25 mL of deionized water and centrifuging as before. The final pellet was resuspended in 25 mL of deionized water, and 1 mL of the microbial suspension was added to two new 125-mL Erlenmeyer flasks containing the BMS-E2-DOC growth medium prepared as above. This culture-enrichment process was repeated four times.

The treatments for the biodegradation experiment were combinations of temperatures of 4, 22 and 28 °C and DOC concentration of 10, 1 and 0.1 g L^{-1} . DOC (a glucose : acetate mixture) was added to allow microbial growth in the presence of the low concentrations of the target compound, E2. Each treatment was run in triplicate. A set of 66 125-Erlenmeyer flasks was prepared with 20 mL BMS solution and 5 mL of a DOC solution and autoclaved. Each treatment flask was spiked with 1.8 ng mL^{-1} E2 by adding 50 μL of the stock solution. The enriched culture was centrifuged and then resuspended in 25 mL of deionized water. Each treatment flask was inoculated with 250 μL of the cell suspension. Three flasks each of each treatment were spiked with 50 μL of deionized

water instead of E2 in order to control for the effects of background E2 or oestrogenic compounds that might interfere with subsequent E2 analyses. Three uninoculated controls (three flasks at each of three different DOC levels) were prepared with the E2 amendment. Three uninoculated control flasks without added E2 were also included. Sterile deionized water was used in place of the inoculum and the E2 stock solution. After all components were added and the cultures were thoroughly mixed, each flask was sampled by pipetting 1 mL of the contents into a 1.8-mL amber glass vial with a PTFE-lined screw-top cap.

Culture flasks of each DOC level were incubated in the dark at 4, 22 and 28 °C. In addition to the initial sampling ($t=0$), all flasks were sampled at 36 and 180 h. All sample vials were stored upright at -80 °C until analysis, as recommended by the immunoassay manufacturer. All samples were analysed within 1 week after sampling. Immediately before E2 analysis, the samples were allowed to come to room temperature.

Sample analysis

E2 concentrations were determined by analysis with an enzyme-linked immunosorbent assay (ELISA) kit (Oxford Biomedical Research, Oxford, MI, USA). The basis of the analysis is the competition between the enzyme conjugate and the E2 for a limited number of binding sites on an antibody-coated microtitre plate. First, dilutions were made from the E2 standard (1 $\mu\text{g mL}^{-1}$) and the provided ELISA buffer. For each well in the microassay plate, 1 μL of enzyme conjugate was added into 50 μL of ELISA buffer. The standards and samples were added into duplicate wells, as recommended by the kit manufacturer, in 50- μL amounts. E2-horseradish peroxidase, the enzyme conjugate, was added to each of the wells in 50- μL portions. The plate was then mixed gently and incubated at room temperature for 1 h. Diluted wash buffer was then prepared, and, after the incubation period, the wells were emptied and the plate was tapped on a clean, lint-free towel. Each of the wells was washed three times with 300 μL of wash buffer. Then, 150 μL of substrate was added to each well and the plate was shaken gently, and then left to sit at room temperature for 30 min. Absorbance (650 nm) in each well was read in a microplate reader. Generally, 2–8 wells were left blank with only substrate added to account for substrate background interference. The use of ELISA has allowed the detection of very low concentrations of E2 in water samples, and the extremely limited cross-reactivity with other compounds makes this method of analysis extremely successful for environmental samples (Huang & Sedlak, 2001; Isobe *et al.*, 2003). Oxford Biomedical Research literature reports the lower end of the assay range as 0.02 ng mL^{-1} E2, but personal communication with technical staff indicated a detection limit of 0.005 ng mL^{-1} according to a previous researcher (Peterson *et al.*, 2000). We read consistent values on a 0.01 mg mL^{-1} standard. The reported cross-reactivity with other estrogenic compounds was less than 0.5%.

Nitrate concentrations in water samples were measured using ion chromatography with a Dionex IonPac® AS4A, 4-mm column. A 1.44 mM Na_2CO_3 /1.36 mM NaHCO_3 eluent and a 0.028 N H_2SO_4 regenerant were used.

RESULTS

E2 in surface and soil water

Cattle manure and poultry compost were extracted with deionized water to determine the water-soluble E2 contents of the manure that is excreted by the cattle onto the grazing fields and the cattle manure and poultry compost that is spread as fertilizer over the agricultural and grazing fields. The E2 concentrations of these samples were 68.0 ± 4.9 and 316 ± 20.2 ng g^{-1} of wet material for cattle manure and poultry litter, respectively.

E2 occurred throughout the Muddy Creek subcatchment; concentrations ranged from 0.010 ng mL^{-1} to 0.120 ng mL^{-1} over the various sites during the Summer 2001 growing season (Fig. 2). Concentrations observed at Source, hydrologically upgradient from the farmed area with its active addition of manures, reveal a background concentration of 0.020–0.050 ng mL^{-1} that was present throughout the area examined. The stream water at Stream 1 had nearly steady background concentrations of E2 as well. Manure and poultry compost had been spread on the cornfield (Fig. 1) in mid- to late April. At the earliest sampling date on 5 June, E2 concentrations at the sites downgradient from that field, namely Spring 2 and Streams 2 and 4, were among the highest observed. Those concentrations declined rapidly in the downstream direction and at all sites over time, such that by the end of the growing season, E2 concentrations at all sites approximated the background value observed for the catchment.

Concentrations of E2 in soil water fluctuated throughout the growing season (Table 1). Pan 1 (see Fig. 1) was the

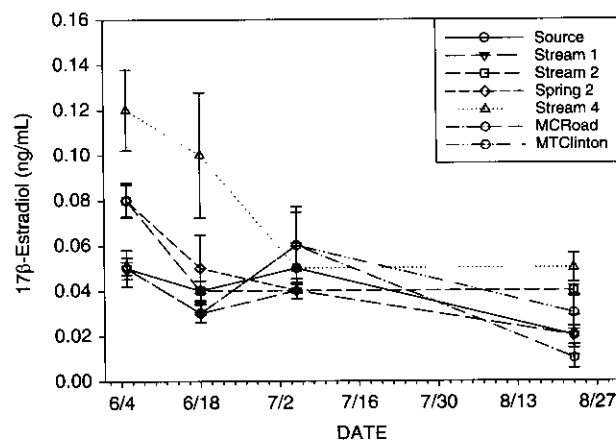


Fig. 2 E2 concentrations (ng mL^{-1}) on the Muddy Creek subcatchment during the Summer 2001 growing season. Standard deviations were calculated for two water samples at each site and each time.

Table 1 E2 concentrations in soil water collected from pan lysimeters in the Muddy Creek subcatchment. Values are the mean of three samples along with the standard deviation in units of ng E2 per mL. 'nw' indicates absence of water in the specific collector on that sampling date

Pan	Depth	Date			
		June 5	June 18	June 29	August 23
1	15	0.124 ± 0.018	0.039 ± 0.000	0.071 ± 0.012	0.040 ± 0.003
	46	0.160 ± 0.040	0.034 ± 0.001	0.069 ± 0.005	0.046 ± 0.006
	91	0.086 ± 0.022	0.043 ± 0.009	nw	0.023 ± 0.004
2	15	nw	nw	nw	0.035 ± 0.014
	46	nw	0.040 ± 0.008	nw	nw
	91	nw	nw	nw	0.040 ± 0.018
3	15	nw	nw	nw	0.046 ± 0.009
	46	nw	0.052 ± 0.005	nw	0.023 ± 0.006
	91	nw	nw	nw	nw
4	15	nw	nw	nw	nw
	46	nw	nw	nw	nw
	91	nw	nw	nw	0.035 ± 0.012

closest to the actively manure-fertilized and cultivated corn-field of all the stream and lysimeter sites sampled. The E2 concentrations observed on the first sampling date (5 June) at Pan 1, located directly adjacent to and downslope from the corn-field, were about four times higher than at any other site over the entire sampling period. The E2 concentrations in the soil water there (0.086–0.160 ng mL⁻¹) were significantly different than the stream water at Stream 1, the closest stream site to the manure-fertilized field ($P = 0.03$, Student's *t*-test). Concentrations of E2 at Pan 1 were comparable with those at Stream 2 at that time (0.120 ng mL⁻¹) suggesting that the soil water and groundwater move downstream some distance before entering the stream channel. Owing to the normally limited infiltration during the hot summer months, and perhaps exacerbated by the regional drought that began in 1998, water collection from the lysimeters was severely limited. As with the streams, the general trend in the soil water was that of decreasing E2 concentrations as time elapsed since the peak manure and poultry compost application on the fields.

The nitrate concentrations in the Muddy Creek subcatchment showed no correlation with the E2 concentrations collected on the same date (Fig. 3). Stream 4 had the highest E2 concentrations over the sampling period, yet relatively low nitrate concentrations. Furthermore, the high nitrate concentrations at the Muddy Creek Road and Mt Clinton sites were not reflective of high E2 concentrations.

E2 biodegradation

The average E2 concentration in all the unamended flasks remained nearly constant at a level carried over from the inoculum at 0.12 ± 0.11 ng mL⁻¹. The measured concentrations in the unamended flasks included for each treatment at each time were used as a background correction for the immunoassay results on the corresponding treatment flasks to which E2 had been added. E2 concentrations measured at each sampling

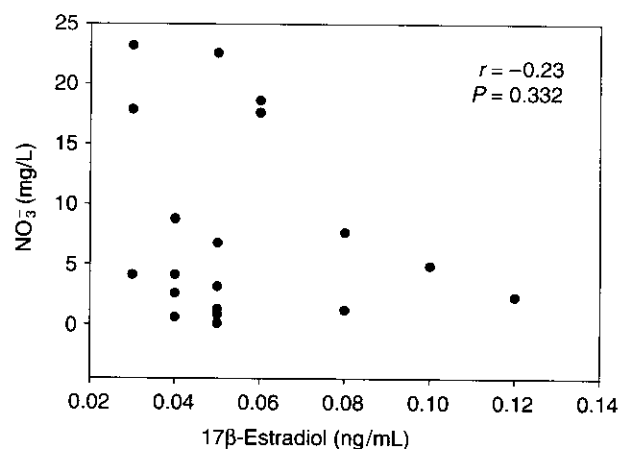


Fig. 3 Relationship of NO₃⁻ concentration to E2 concentration in samples from the Muddy Creek subcatchment. The slight negative correlation is due to the high NO₃⁻ concentrations at the downstream sites, Muddy Creek Road and Mt Clinton.

time were normalized by expressing them as the percentage of the initial measured concentration. Uninoculated controls showed no significant loss of E2 (Fig. 4). All inoculated treatments displayed a decrease in the concentration of added E2, with the greatest percentage lost in the first 36 h.

The greatest biodegradation of E2 was observed in the 22 °C and 28 °C flasks containing 1.0 and 0.1 mg L⁻¹ DOC (Fig. 4). After 180 h, only 2–3% of the initial E2 remained. Less extensive biodegradation was associated with greater concentrations of DOC at both 22 °C and 28 °C; significantly less E2 was biodegraded in the presence of 10 mg L⁻¹ DOC leaving 20% and 41% in solution, respectively. The pattern of greater biodegradation of E2 in treatments with higher DOC was obscured in the 4 °C cultures where the different DOC treatments resulted in virtually insignificant differences (overlapping error bars) in biodegradation rates. Regardless of DOC concentration, all the 22 °C and 28 °C treatments lost more E2 than any 4 °C treatment. At 4 °C, 51–72% of the initial E2 remained after 180 h. Consistent with the observed biodegradation of E2, very little microbial growth (turbidity) was observed in flasks incubated at 4 °C, whereas flasks held at 22 °C and 28 °C were turbid by 36 h.

DISCUSSION

Occurrence and distribution of E2 in natural waters

E2 occurred in stream water and soil water throughout the entire Muddy Creek subcatchment (Fig. 1), and the temporal relationship to the application of poultry compost to the fields was clear. Elevated concentrations of E2 were observed in all samples at the earliest sampling date, which was soon after the fertilization of the agricultural field, and concentrations generally decreased over the growing season (Fig. 2). Spatially,

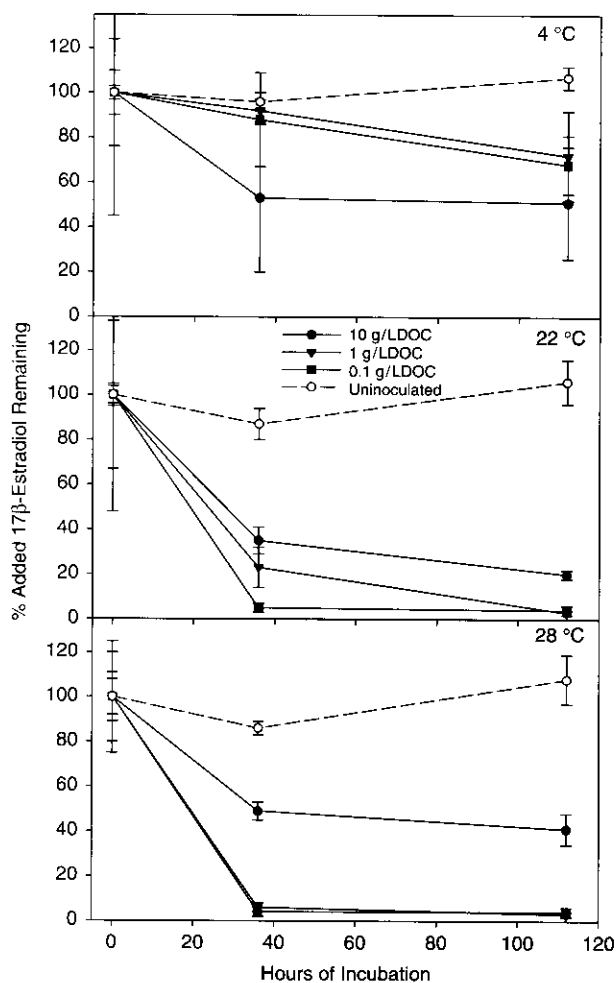


Fig. 4 Change in E2 concentration in flasks containing one of three concentrations of added DOC and incubated at one of three temperatures. Initial E2 concentration was 1.8 ng mL⁻¹. Data were corrected for background concentrations prior to normalizing.

the distribution of E2 reflects the complex relationship among hydrological pathways and E2 interactions with the geological and biological environment. The highest E2 concentration of stream water was not found at Stream 1, the site closest to the actively cultivated agricultural field, but at Stream 4. Earlier research in this watershed revealed the same pattern of occurrence for atrazine, a herbicide sprayed onto the soil surface for weed control in the cornfield (Hyer *et al.*, 2001). Stream 1 had very low levels of atrazine near background levels, whereas downstream locations consistently had more. Stream 4 (Fig. 1) is further downstream from the actively cultivated and fertilized cornfield yet is located just downstream from the fields that were used for cattle grazing and received some manure application as a waste-disposal practice. If E2 is moving through the surface-water environment by means of direct surface runoff from the fields to the stream channel, Site 1 ought

to have higher E2 concentrations. By contrast, if E2 infiltrates into the subsurface and migrates via soil and groundwater to the stream channel, the regional hydraulic gradient would direct water from the cornfield to a discharge point in the stream channel further downslope of Site 1. This reasoning is consistent with earlier observations of hydraulic connections at the site. Lingvai (2000) and Hyer (2001) performed dilution gauging on short sections of the stream channel and found groundwater discharge to the reach between Stream 1 and Stream 2 (i.e. a gaining stream), and therefore migration of chemicals from the cornfield to the stream is not primarily by overland flow but is the result of infiltration and subsurface flow.

The highest E2 concentrations found occurred in the soil water from Pan 1 on the first sampling date in early June (Table 1). This site was the closest of all of the sites sampled, stream and lysimeter, to the actively fertilized and cultivated field. The concentrations of E2 observed there ranged from 0.09 ng mL⁻¹ to 0.12 ng mL⁻¹. The E2 concentrations observed at this pan lysimeter site, located on the edge of and slightly downslope from the field, indicate the infiltration of E2 into the soil in runoff from the agricultural field. Plot studies following concentrations of E2 in surface runoff have shown that grass strips at the edges of fields promote infiltration (Nichols *et al.*, 1998; Finlay-Moore *et al.*, 2000). Sizable edge-of-field losses of E2 reduced its concentration in surface waters but increased the concentration in the bulk soil (Finlay-Moore *et al.*, 2000). Missing from these previous studies was any observation of soil water and consideration of its return to the surface drainage network.

Sources of E2 in an agricultural watershed

Both poultry compost and cattle manure are sources of E2 in the Muddy Creek subcatchment. The cattle manure E2 concentrations averaged 68.0 ng g⁻¹ (i.e. 68 000 ng kg⁻¹, expressed on a weight equivalent with the water samples). The poultry compost E2 concentration average 316 ng g⁻¹ was well within the range of previous reports of 14–904 ng g⁻¹ dry waste (Shore *et al.*, 1995; Nichols *et al.*, 1998). Although the cattle manure contained significant concentrations of E2, poultry compost was far richer in estrogen. Previous studies showed that grazing cattle had no effect on E2 runoff from a field, whereas application of poultry litter did (Finlay-Moore *et al.*, 2000). In our study, there was a clear association between the time that the poultry compost was spread on the field and when elevated E2 concentrations were observed. In the Muddy Creek subcatchment, the determining source for E2 was probably not the cattle manure that is introduced rather consistently all year long, but rather the E2-rich poultry compost that is stored with little loss of estrogenic activity (Shore *et al.*, 1993a) and then spread intensively over the fields in May.

Water samples were analysed to determine if there was a correlation of E2 concentrations with nitrate, a good indicator of contamination by manure (e.g. Nolan & Stoner, 2000). The

lack of a strong correlation (Fig. 3) does not necessarily indicate distinct sources for the two chemicals, but it does suggest that both the biological and the physical behaviour of E2 and nitrate differ. Furthermore, manure is not the only source of nitrate; ammonium nitrate fertilizer was applied to the cornfield (Hyer, 2001), and applied fertilizers also contribute substantially to high surface-water and soil-water concentrations of nitrate (Nolan *et al.*, 1997).

Biodegradation and environmental persistence of E2

Given the high concentration of E2 in the poultry compost that is used to fertilize the fields, the question remains as to what happens to the E2 following leaching by rainfall. Because the first sample date was a little over a month after the field was fertilized, the E2 concentrations possibly could have decreased significantly as a result of the high temperature and sunlight (Smith & Williams, 1947). Yet, the highest concentrations observed were in soil water near the cornfield on 5 June, suggesting persistence since the time of infiltration. Additionally, all downgradient samples and all later samples had lower, but non-zero, E2 concentrations. It would be reasonable to conclude that if biodegradation is occurring at all, rates are slow.

Certainly biodegradation observed in the batch cultures developed from the microbial consortium in the poultry litter pile was adequate to consume all the E2 introduced to soil and stream water in a very short period of time. In just 180 h, all but 2–3% of the initial $\sim 2 \text{ ng mL}^{-1}$ E2 was biodegraded at 22–28 °C and 0.1–1.0 mg L^{-1} DOC (Fig. 4). We initially hypothesized that enhanced DOC concentrations would yield enhanced microbial growth in the cultures leading to enhanced E2 removal. An excess of soluble organic matter, however, actually slowed the biodegradation reaction. The trend of decreasing E2 degradation with increasing DOC suggests that in the presence of an abundant energy source, E2 is ignored. Most degradation experiments by other researchers have not examined the ability of E2 to support microbial growth as the sole source of carbon and energy (Ternes *et al.*, 1999; Raman *et al.*, 2001), although Layton *et al.* (2000) did show production of $^{14}\text{CO}_2$ was 70–80% of the added E2 in municipal biosolids, but only 4% was mineralized in sludge from an industrial wastewater plant (note the inclusion of substantial organic matter in the experiments of Layton *et al.*).

Degradation studies most often use disappearance of the parent molecule to indicate biodegradation. The typical concentrations of E2 found in the environment ($<0.01 \text{ ng L}^{-1}$ in most surface water and groundwater; Ying *et al.*, 2002) seem too low to support extensive microbial growth. Therefore, we added DOC to support the inoculum that had been enriched on DOC in the presence of 1.8 ng mL^{-1} E2. It must be pointed out, however, that the natural occurrence of DOC in soil and stream water is far below the large concentrations selected for our co-metabolism experiments. Typical surface water might have 0.01 g L^{-1} DOC and soil water may be more

in the range of 0.05 g L^{-1} (Drever, 1997). The total organic carbon (TOC) in a soil would be even higher, but much of that carbon would not be labile (Tate, 1987). The soil in the Muddy Creek catchment is a clay-rich residuum sitting on top of deeply weathered limestone, and there is very little organic matter in this soil compared with many locations in the humid, temperate mid-Atlantic region. The soil at Pan 1 contains only 0.4–2.6% organic carbon (Sprague *et al.*, 2000). DOC concentrations in soil water at this site ranged widely depending on the depth of the pan and the amount of antecedent rainfall, but monthly samples of lysimeters in the watershed yielded DOC values of $0.0032\text{--}0.146 \text{ g L}^{-1}$ with a typical growing season value of 0.015 g L^{-1} (Kauffman, 1998). Our laboratory experiments suggest that the glucose–acetate mix used in the flask experiments successfully competed with the degradation of E2, but it is impossible to infer the resultant biodegradation rates that would occur at any DOC concentrations characteristic of the field environment. Regardless, E2 persists in the watershed at concentrations considerably diminished from the levels in leached poultry compost.

Poultry compost is applied at the rate of 4500 kg ha^{-1} for fertilization of the cornfield (Hyer, 2001). Using the application rate together with the E2 concentration determined for the wet compost in the leaching experiments, we calculate a loading rate of $1422 \text{ mg E2 ha}^{-1}$ of agricultural field. The rainfall record for the nearest federally maintained climatological station at Dale Enterprise, Virginia, revealed that over the 4-month period of May–August 2001, a total of 37.3 cm of rain fell (http://www.dnr.state.sc.us/climate/sercc/products/historical/historical_va.html). This value was near the 53-year average for those 4 months of 36.3 cm even though the mid-Atlantic region was midway through a protracted drought (http://waterdata.usgs.gov/va/nwis/discharge/?site_no=01621050). Distributing the summer 2001 rainfall amount over a hectare, a volume of rainfall per hectare is obtained. Diluting the mass of E2 loaded on the agricultural field into that volume of water yields a concentration of E2 of 0.392 ng mL^{-1} . Using a regionally appropriate estimate of the ratio of evapotranspiration to infiltration plus runoff of 33%, the concentration of E2 in the water subject to further transport in the watershed becomes 0.585 ng mL^{-1} E2. This concentration is in the range observed by others. Runoff immediately after poultry litter application was 1.28 ng mL^{-1} but a week later was 0.44 ng mL^{-1} from a plot planted in tall fescue that received a significantly higher litter application of $898\,000 \text{ kg ha}^{-1}$ (Nichols *et al.*, 1997). In another study by Nichols *et al.* (1998), a lower application rate of 5000 kg ha^{-1} was employed, but because their poultry litter has a higher E2 content of 903.9 ng g^{-1} relative to the present study, their loading rate of E2 was 4520 mg ha^{-1} . As expected, their runoff concentration was higher than previously observed values of 3.5 ng mL^{-1} . In a study employing lower poultry-litter application rates of $2550\text{--}4750 \text{ kg ha}^{-1}$ that resulted in E2 loading rates of $49\text{--}179 \text{ mg ha}^{-1}$, the flow-weighted runoff concentration

was appropriately lower at 0.095–0.45 ng mL⁻¹ (Finlay-Moore *et al.*, 2000). Although satisfyingly within the context of other studies, our estimates indicate that more E2 is probably released to the runoff by leaching than we observed in any soil solution or stream water. In contrast to our estimated release of 0.585 ng mL⁻¹ of E2, our entire range of field observations fell within 0.01–0.18 ng mL⁻¹.

Loss of E2 from solution could be attributed to biodegradation or partitioning into the soil. Estrogens would certainly be expected to sorb to soil particles, especially organics, as the log K_{OW} of E2 is 3.94 (Lai *et al.*, 2002) and the reported sorption constant K_{OC} is 3300 (Lai *et al.*, 2000). Consistent with anticipated preferential partitioning into the organic phase, 49–81% of the added E2 in batch slurries was adsorbed to granular activated carbon in less than 12 min (Fuerhacker *et al.*, 2001). Indeed, Finlay-Moore *et al.* (2000) quantified the amount of E2 found in the bulk soil receiving runoff from a controlled poultry-litter application experiment and found that concentrations of E2 increased from a background level of 55–65 ng kg⁻¹ to 145–190 ng kg⁻¹ 7 days after litter application. By the time another 7 days had passed, however, the soil content of E2 had returned to background, suggesting either loss or redistribution within the system.

In contrast to the rapid loss due to biodegradation by the micro-organisms present in the poultry compost that is applied to the field, the remarkably slower rates we infer for the field setting could stem from lack of nutrients to support adequate biochemical activity, inhibition of the reaction as a result of the presence of some other constituent (e.g. DOC), or an inadequate number of E2-degrading organisms in the soil consortia. The nitrate measurements, concentrations in the range 0.1–44 mg L⁻¹, contradict speculation about lack of nutrients. The levels of available DOC in the soil water are not high enough to inhibit E2 biodegradation rates compared with the carbon-rich laboratory cultures. Because the soil is essentially inoculated with micro-organisms demonstrated to degrade E2 every time poultry compost is spread, it seems likely that an adequate number of degraders is present in the soil.

Alternatively, the reduced rate of biodegradation in the field may derive from diminished bioavailability of E2 as a result of interaction with inorganic or organic components of the soil or natural water (Knezovich *et al.*, 1987; National Research Council, 2002). The bioavailability of E2 subsequent to complexation, sorption and reconfiguration upon ageing in the soil environment remains an open question. The details of the processes associated with ageing are poorly known but include slow diffusion through intraparticle micropores, bonding with soil constituents, sorption at high-energy surface sites and entrapment within the molecular structure of organic matter (Hatzinger & Alexander, 1995). The outcome of ageing is reduced bioavailability of organic compounds for microbial degradation. We have no direct determination of ageing of E2 in the soils of Muddy Creek subcatchment, but the persistence of E2 in the watershed may indicate an effect of decreased

bioavailability. The details of the physicochemical phenomena involved as E2 interacts with the geological environment may have direct impact on biologically mediated processes that limit the occurrence and persistence of E2 in natural waters.

The fact that the mass of E2 applied to these fields would yield substantially higher concentrations in the stream water if the behaviour of E2 was conservative means that E2 is being removed from solution. In part, the compound may be biodegraded. Our work and that of others has shown that the chemical structure of the estrogens is amenable to attack by enzymes in a wide range of organisms. Given the rapid rate of degradation obtained in the laboratory, however, the concentrations observed at the field site are higher than might be expected by as much as an order of magnitude (97–98% removal in the laboratory under ideal conditions in 1 week vs. 30–95% apparently removed in the field over a period of several months). Certainly factors such as retention by sorption onto clay minerals and particulate organic matter in soils, abiotic chemical transformations or photochemical transformations could contribute to limiting the migration of E2 in natural waters in much the same way a multitude of other organic compounds are influenced (Schwarzenbach *et al.*, 1993). Although the most plausible process decreasing the abundance of E2 subsequent to introduction to aquatic systems remains biodegradation, the persistence of E2 must be an effect of the interaction of biotic and abiotic constraints on the degradation resulting in ageing (and therefore reduced bioavailability) of the compound in the natural environment.

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