

# Challenges in Biodegradation of Trace Organic Contaminants—Gasoline Oxygenates and Sex Hormones

M. T. Suidan, M. Esperanza, M. Zein, P. McCauley, R. C. Brenner, A. D. Venosa

**ABSTRACT:** Advances in analytical methods have led to the identification of several classes of organic chemicals that are associated with adverse environmental effects. Two such classes of organic chemicals, gasoline oxygenates and sex hormones, are used to illustrate challenges associated with the biodegradation of trace organic contaminants. Gasoline oxygenates can be present in groundwater, alone, or commingled with xylene, at appreciable concentrations. However, target-treated water standards dictate that gasoline oxygenates be reduced to the microgram-per-liter concentration range before consumption. Sex hormones, on the other hand, are present in wastewater matrixes in the part-per-trillion concentration range, and the biggest challenge that must be met, before optimizing their removal, is facilitating their detection. *Water Environ. Res.*, 77, 4 (2005).

---

### Introduction

Challenges in biological water treatment evolved from control of gross organic matter and suspended solids, to the control of nutrients, and, more recently, to the biological transformation of specific organic chemicals. These chemicals are either removed by direct use as electron donors or via co-metabolic transformations, where other organic compounds present in the water provide the energy needed for microbial sustenance. Difficulties arise when the concentrations of organic chemicals are relatively low in the feedwater. In such cases, direct use of these compounds can only occur under prolonged solids residence times, necessitating the use of specialized reactors to harvest the low concentrations of synthesized biomass. Another challenge encountered in assessing the fate of trace concentrations of organic compounds is the availability of analytical procedures capable of quantifying these compounds in biological matrices. This manuscript illustrates the aforementioned challenges using biodegradation of methyl *tert*-butyl alcohol (MTBE) as an example of the difficulties encountered in treating organic compounds present at relatively low concentrations in contaminated groundwater plumes, while the analytical difficulties will be illustrated relative to the fate of sex hormones in wastewater treatment.

**Methyl *tert*-Butyl Alcohol.** The Clean Air Act Amendments of 1990 mandate seasonal or year-round use of oxygenated compounds in gasoline in certain areas of the country, which exceed the National Ambient Air Quality Standards (NAAQS) for carbon monoxide (CO) and ozone (O<sub>3</sub>). Methyl *tert*-butyl alcohol was first introduced to the United States in 1979, primarily as an octane enhancer to replace organo-lead compounds and to reduce air

pollution. Currently, MTBE is the most widely used gasoline oxygenate additive in the United States, where approximately one-third of all the gasoline sold contains MTBE in concentrations ranging between 11 and 15% by volume (U.S. EPA, 1998). However, the use of MTBE has created a significant and unacceptable risk to drinking water and groundwater resources, through its release, mainly from leaking underground gasoline storage tanks in states like California, New Jersey, Rhode Island, Illinois, Alaska, Texas, New York, Colorado, and others (Hartley et al., 1999; Squillace et al., 1996). A study conducted by the U.S. Geological Survey (USGS) found that MTBE was the second most common volatile organic contaminant that was detected in 5% of the wells monitored between 1993 and 1998 in urban areas nationwide (USGS, 2001).

Although there exist limited epidemiological and clinical studies assessing human health effects associated with MTBE exposure, many symptoms, including headaches, dizziness, ocular irritation, rashes, coughing, disorientation, and nausea were reported to result from MTBE inhalation (Borghoff et al., 1996; Mehlman, 2001; Nihlén et al., 1998). The U.S. Environmental Protection Agency (U.S. EPA) classified MTBE as a possible human carcinogen without establishing any drinking water standards. Instead, a drinking water advisory for MTBE of 20 to 40 µg/L (ppb), based on taste and odor thresholds, was issued (U.S. EPA, 1997a). Consequently, the remediation of aquifers contaminated with MTBE has become an active research area in the past few years.

**Biodegradability of Methyl *tert*-Butyl Alcohol.** The physico-chemical characteristics of MTBE render most conventional remediation technologies like chemical oxidation, air stripping, and adsorption onto activated carbon inefficient or impractical in treating MTBE-contaminated aquifers (Braids, 2001; Rong, 2001). However, biological treatment of MTBE-contaminated groundwater appears to be the most economical, energy-efficient, and environmentally sound approach. Recent in-situ studies revealed the ability of several bacterial and fungal cultures to aerobically biodegrade MTBE, either as the sole carbon and energy source or co-metabolically, while growing on other organic substrates. Pure cultures of only two bacterial strains, *Rubrivivax gelatinosus* PM1 (Deeb et al., 2000; Hanson et al., 1999) and *Hydrogenophaga flava* ENV735 (Hatzinger et al., 2001; Steffan et al., 2000), were shown to be capable of

**Table 1—Summary of performance of biomass concentrator reactor at Pascoag, Rhode Island.**

Contaminant	Influent concentration (µg/L)	Effluent concentration (µg/L)	Removal efficiency (%)
MTBE	3526 ± 1182	5.39 ± 4.73	99.85
TBA	71 ± 79	0.61 ± 0.85	99.14
TBF	33 ± 12	0.02 ± 0	99.94
		(detection limit)	
Methanol	154 ± 288	1.69 ± 0.65	98.90
Acetone	493 ± 256	5.05 ± 2.29	98.98
TAME	563 ± 222	0.87 ± 0.84	99.84
DIPE	24 ± 13	0.04 ± 0.07	99.83
TAA	123 ± 42	0.05 ± 0.01	99.96
Benzene	161 ± 45	0.01 ± 0.03	99.99
Toluene	577 ± 158	0.15 ± 0.19	99.97
Ethyl benzene	289 ± 134	0.08 ± 0.16	99.97
o-xylene	437 ± 79	0.09 ± 0.11	99.98
m-xylene	624 ± 170	0.18 ± 0.15	99.97
p-xylene	728 ± 140	0.12 ± 0.12	99.98

complete use of MTBE as the sole carbon and energy source. In addition, a phylogenetically diverse group of other pure bacterial cultures, such as *Methylobacterium*, *Rhodococcus*, *Arthrobacter* (Mo et al., 1997) and *Mycobacterium* (François et al., 2002), were reported to partially degrade MTBE. Methyl *tert*-butyl alcohol has also been reported to be co-metabolized in the presence of pentane (Garnier et al., 1999), propane (Steffan et al., 1997), and ethanol (Hernandez-Perez et al., 2001). Hardison et al. (1997) isolated a filamentous fungus (*Graphium sp.*), capable of co-metabolically degrading MTBE in the presence of *n*-butane. Although MTBE was considered recalcitrant under anaerobic conditions because of its stable ether and tertiary alkyl moieties (Bradley et al., 1999; Sulfito and Mormile, 1993; USGS, 1999), a few studies have evaluated its biodegradability under methanogenic (Yeh and Novak, 1994), sulfate-reducing (Somsamak et al., 2001), and iron-reducing conditions (Finneran and Lovley, 2001; Pruden et al., 2004). In general, biodegradation rates that lend themselves to biotreatment were only observed under aerobic conditions.

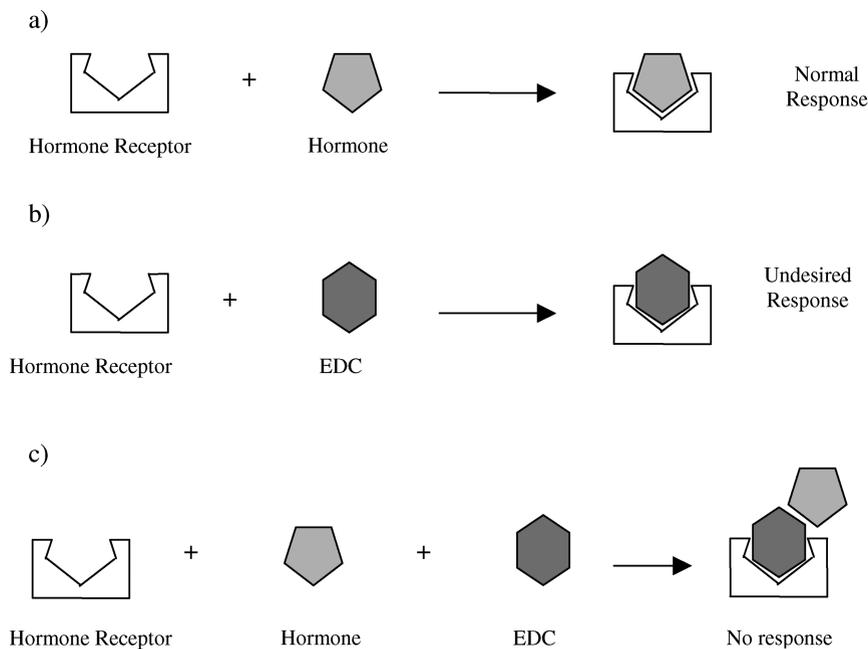
**Biological Treatment of Methyl *tert*-Butyl Alcohol.** The aforementioned research establishes that MTBE is biodegradable. However, what remains to be demonstrated is whether biological treatment can affect removal efficiencies that meet or exceed the U.S. EPA drinking water advisory of 20 to 40 µg/L. Wilson et al. (2001) used a porous pot to assess the biodegradation of MTBE in a continuous-flow reactor. This reactor consisted of a porous polyethylene membrane bucket placed in a nonporous container designed to collect the treated permeate and conduct it out of the reactor. The water to be treated is applied to the porous pot, where it comes to intimate contact with the retained biomass via aeration. The advantage of the porous pot reactor is its ability to afford the operator essentially complete biomass management control without the use of a troublesome settling tank. Using an influent MTBE concentration of 150 mg/L and a solids residence time (SRT) of 20 d, Wilson et al. (2001) reported that the performance of the bioreactor was very unstable, with effluent MTBE concentrations consistently higher than 88 µg/L. Subsequently, they limited biomass wastage to that associated with weekly sampling for volatile suspended solids (VSS) analysis, and the effluent concentration of

MTBE plummeted to a stable level below 6 µg/L. These data suggest that effective MTBE biodegradation to levels that meet the U.S. EPA drinking water advisory is achievable if a sufficiently high SRT is maintained in the bioreactor.

Wilson et al. (2001 and 2002) clearly demonstrated the need for a very long SRT to affect acceptable MTBE reduction in an activated-sludge-type bioreactor. Several researchers reported extremely low net microbial yield coefficients for biomass growing on MTBE alone (Morrison et al., 2002; Salanitro et al., 1994; Wilson et al., 2002). The reported values range between 0.10 and 0.15 g VSS/g MTBE mineralized, or 0.037 to 0.055 g VSS/g MTBE-chemical oxygen demand (COD) satisfied. This unusually low yield value poses problems when the treatment of contaminated groundwater is desired. The level of contamination in such a water resource is typically approximately 1 mg-MTBE/L, which will lead to the production of between 0.1 and 0.15 mg/L of VSS. Conventional biological treatment systems do not yield effluents with VSS concentrations below the 1 mg/L range and, consequently, will not be able to treat such waters. Membrane bioreactors (MBRs), on the other hand, can retain essentially all suspended matter and permit for the accumulation of elevated levels of biomass. Morrison et al. (2002) used a pilot-scale ceramic ultrafiltration MBR to treat simulated contaminated groundwater containing 5 mg/L MTBE. Effluent concentrations of MTBE were consistently below 1 µg/L, while VSS concentrations exceeded 3000 mg/L. This performance was achieved using a hydraulic retention time of 1 hour. Using the same reactor, Morrison (2003) demonstrated that similar results are achievable from the MBR when the influent concentration of MTBE was decreased to 1 mg/L. The presence of gasoline constituents (benzene, toluene, ethylbenzene, and xylene [BTEX]) did not adversely affect the performance of the system, either. Zein et al. (2004) reported similar results using a 1 m<sup>3</sup> pilot-scale novel gravity-operated biomass concentrator reactor (BCR). In their system, biomass separation and retention occurred across porous polyethylene barriers, similar to the ones used in the porous pot.

The University of Cincinnati (Ohio) and U.S. EPA have just completed a successful field demonstration of a 5-gpm BCR in Pascoag, Rhode Island, where a highly contaminated groundwater was treated. In addition to MTBE and BTEX, the groundwater contained several gasoline oxygenates and other contaminants including *tert*-butyl alcohol (TBA), *tert*-butyl formate (TBF), methanol, acetone, diisopropyl ether (DIPE), *tert*-amyl alcohol (TAA), and *tert*-amyl methyl ether (TAME). The chemical characteristics of the groundwater are presented in Table 1. A combination of well-field failures and problems with the well pumps control system prevented delivery of the full 5-gpm flowrate until four months into the project. Once these issues were resolved or minimized, the system was operated for two months at the design flowrate. No biomass was wasted from the BCR for the entire period of operation, except quantities withdrawn for sampling. The chemical characteristics of the treated effluent from the BCR during the last two months of operation are also summarized in Table 1.

In addition to the volatile organic compounds listed in Table 1, the BCR affected significant removal of nonpurgeable organic carbon. During the same operating period, the nonpurgeable organic carbon concentration decreased, from an influent value of 3.59 ± 0.36 mg/L to an effluent concentration of 1.87 ± 0.19 mg/L. It is very rare for biological treatment systems to affect mineralization of organic matter down to the effluent levels reported in Table 1. These levels were observed both when MTBE was the sole organic carbon



**Figure 1—Endocrine-disrupting mechanisms: (a) normal functioning, (b) agonist, and (c) antagonist.**

source (Wilson et al., 2001) and in the presence of various organic compounds (Pruden et al., 2001; Sedran et al., 2002; Wilson et al., 2002). Measurements of the concentration of MTBE in the mixed liquor revealed similar concentrations to those detected in the permeate, suggesting that biomass attached to the membrane did not contribute significantly to the observed high removal levels of MTBE. Because a very long SRT is maintained in the various membrane reactor systems discussed here, it is appropriate to use the concept of  $S_{min}$  to attempt to explain this behavior.  $S_{min}$  occurs when the rate of new growth is exactly equal to the rate of microbial decay, which defines the lowest concentration of substrate that can still maintain biomass (Rittmann and McCarty, 2001).

$$\frac{\mu_m SX}{K_s + S} = K_d X \quad (1)$$

Where

- $\mu_m$  = maximum growth rate,  $T^{-1}$ ;
- $S$  = substrate concentration,  $ML^{-3}$ ;
- $X$  = biomass concentration,  $ML^{-3}$ ;
- $K_s$  = monod half velocity constant,  $ML^{-3}$ ; and
- $k_d$  = microbial endogenous decay constant,  $T^{-1}$ .

This results in the following expression for  $S_{min}$ :

$$S_{min} = \frac{K_s}{\frac{\mu_m}{k_d} - 1} \quad (2)$$

For the effluent MTBE to reach the microgram-per-liter level,  $K_s$  must assume an exceedingly low value or the quantity  $\mu_m/k_d$  must be very large. Porous-pot, continuous-flow-reactor data collected by Wilson et al. (2002) appear to suggest that  $K_s$  for MTBE may be smaller than  $0.08 \mu\text{g/L}$ . Such a value for  $K_s$  supports the very small value of  $S_{min}$  needed to explain the results. Batch-spike-rate data by the same authors, however, suggest values for  $K_s$  exceeding  $50 \text{ mg/L}$ . The batch tests were performed at a higher MTBE concentration and may have led to a different mechanism of biodegradation than what was taking place in the porous-pot reactor. The dominant

microorganisms in the porous-pot reactor were identified as PM1-like organisms (these are microorganisms) (Pruden, 2002). These organisms are not filamentous and are not typically expected to act as scavengers. More research is needed to explain the observed data.

### Endocrine-Disrupting Chemicals

The publication of *Our Stolen Future* by Colborn et al. (1996) raised public concern over the harmful effects of endocrine-disrupting chemicals, or EDCs. The EDC problem is not recent, however. In 1938, the synthetic hormone diethylstilbestrol (DES) was administered to pregnant women to prevent miscarriages. Worldwide, this hormone was given to approximately 4.8 million women. In 1971, DES was linked to vaginal cancer in female offspring of women that used the drug during the first trimester of pregnancy. That same year, the U.S. Food and Drug Administration banned DES from use in both humans and animals. In 1972, the use of dichlorodiphenyltrichloroethane (DDT) was restricted in the United States, after a multi-year-long study demonstrated the estrogenic effects of DDT in mammals and birds (<http://www.ourstolenfuture.org>).

The U.S. EPA defines an EDC as “an exogenous agent that interferes with the synthesis, secretion, transport, binding action, or elimination of natural hormones in the body that are responsible for the maintenance of homeostasis, reproduction, development and/or behavior” (U.S. EPA, 1997b). In other words, the EDCs are compounds that interfere with the normal functioning of any of the tasks of the endocrine system.

Hormones induce an effect on cells when those cells possess a receptor specific for that particular hormone. Under normal conditions, hormones enter the nucleus of the cell and bind with the receptor. This hormone-receptor complex promotes the transcription and synthesis of new RNA, which induces the production of a new protein and the corresponding physiological response.

There are two main endocrine-disrupting mechanisms: agonist and antagonist (Figure 1) (Metzler, 2001). An agonist EDC is able

**Table 2—Average levels of estrogens in urine for women and men (Johnson et al, 2000).**

Sex hormone	Female (nonpregnant) ( $\mu\text{g}/24\text{h}$ )	Female (average during pregnancy) ( $\mu\text{g}/24\text{h}$ )	Female (post menopause) ( $\mu\text{g}/24\text{h}$ )	Male ( $\mu\text{g}/24\text{h}$ )
Estriol	4.8	6000	1.0	1.5
Estrone	8.0	600	4.0	3.9
Estradiol	3.5	259	1.0	1.6

to bind with a receptor and generate the same response expected from the specific hormone. On the other hand, there are molecules that occupy the hormone receptor and prevent RNA from being transcribed, resulting in no response, even if the hormone is present. These molecules are known as antagonists of the particular hormone. Some EDCs have a higher affinity for the receptor than the parent compound itself and, therefore, their disruption of the endocrine system is sustained.

The EDC problem is complex. A wide spectrum of compounds, such as pesticides, plasticizers, metals, pharmaceuticals, surfactants, phytoestrogens (plant estrogens), and natural and synthetic sex hormones, among others, are suspected of producing estrogen responses in the endocrine systems of fish, birds, and wildlife, in general. Regarding their chemical structures or properties, EDCs include a surprisingly wide variety of compounds, including halogenated compounds, phenols, phthalates, and polyaromatic hydrocarbons (Kolpin et al., 2002).

**Sex Hormones.** There are three main groups of sex hormones: progestins, androgens and estrogens. Estradiol, estrone and estriol, known as E2, E1, and E3, respectively, are the most important endogenous estrogens. Of the three, estradiol has the highest biological activity; estrone has one third that activity, while the activity of estriol is only 1% of that of estradiol. Estradiol and estrone are the main estrogens in nonpregnant women. They are also present in males, but at lower plasma concentrations. Estradiol is readily converted to estrone. Although this enzymatic reaction is reversible, the formation of estrone is favored. Estriol is a metabolic product of both estradiol and estrone. Estriol concentration, which is generally low, increases during pregnancy, becoming by far the predominant sex hormone in both mother and fetus (Table 2) (Johnson et al., 2000).

Testosterone is the most potent androgen. Testosterone is present in females, but plasma concentrations are significantly higher in males. The presence of testosterone during male fetus gestation is responsible for the sexual differentiation of the genitals. Later, during the stage of puberty, it is indirectly responsible for the sustenance of secondary sexual characteristics. It also promotes muscle and skeletal growth, by acting on skeletal muscle (Fraser et al., 1998).

Androstenedione is another major androgen in both males and females. The main function of androstenedione is as a prohormone, which gets converted in the target tissues to testosterone, estrone, and estradiol (Fraser et al., 1998).

Progesterone is the most important progestin in circulation. Its function is associated with the menstrual cycle and the maintenance of pregnancy. Progesterone is mainly metabolized in the liver, producing pregnandiol, a metabolite eliminated via urination as the glucuronate conjugate (Fraser et al., 1998).

In addition to levels naturally present in urine, all the above and several other natural and synthetic sex hormones are used for thera-

peutic and nontherapeutic purposes. Administered quantities of hormones are generally high, and, in most cases, a significant percentage is not assimilated, but rather excreted (Johnson et al., 2000).

Replacement hormones are prescribed to women that have undergone ovary removal surgery or after menopause. More often, the prescribed pills contain estradiol or estrone and a progestin (typically progesterone). Approximately 65% of the administered estrogens are found in urine and approximately 15% in feces (Johnson et al., 2000).

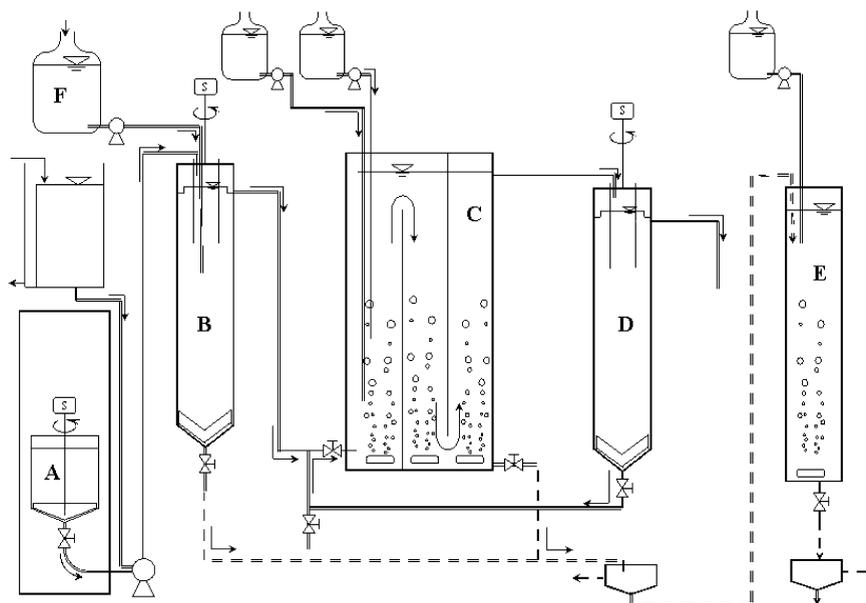
Birth control pills contain synthetic hormones, such as ethinylestradiol, which sometimes is combined with a progestin. The typical dosage of ethinylestradiol is 20 to 50  $\mu\text{g}/\text{day}$ , over a period of 21 days. After administration, approximately 16.5% of unmetabolized but conjugated ethinylestradiol is found in urine and 9% in feces (Johnson et al., 2000). Both testosterone and androstenedione are used for therapeutic purposes on both men and women. Additionally, other substances, like dehydroepiandrosterone (DHEA) or androstenedione, are sold over the counter as hormone supplements used to balance decreases in testosterone production.

Before their excretion, sex hormones are inactivated via sulfation or glucuronidation. Elimination occurs mainly through urination, with smaller quantities of sex hormones also excreted with feces. However, several studies agree that bacterial activity in sewers and wastewater treatment plants cleave the conjugated sex hormones, reverting them to their endocrine active forms (Baronti et al., 2000; Desbrow et al., 1998; Ternes et al., 1999a and 1999b).

**Fate and Detection.** Several analytical methods have been reported for the quantitation of sex hormones in environmental samples (Lopez de Alda and Barceló, 2001; Snyder et al., 1999; Ternes et al., 2002). Furthermore, a sizable body of information has been compiled on the occurrence and concentration of sex hormones in municipal wastewater treatment plant effluents, rivers, groundwater, sediments, and sludge (Andersen et al., 2003; Belfroid et al., 1999; Baronti et al., 2000; Kröner et al., 2000; Solé et al., 2000; Kolpin et al., 2002; Ternes et al., 1999a).

Some information is also available on biodegradation rates and removal of sex hormones from wastewater. These data were collected either from laboratory-scale batch tests, in which mixed liquor from a wastewater treatment plant was spiked with the compounds of interest, or from influent and effluent samples collected at full-scale wastewater treatment facilities (Kröner et al., 2000; Layton et al., 2000; Ternes et al. 1999b). Limitations of the batch studies center around the spike concentrations and effect of absorption of these compounds on the biomass on the calculation of rate. Furthermore, questions arise as to the applicability of these data to understanding the fate of these compounds in full-scale plants. Limitations of data collected at full-scale facilities include seasonal changes (temperature and rainfall), variability in influent levels (unexpected discharges), and speciation (conjugated or unconjugated steroids). Other important issues pertain to analytical difficulties and level of effort needed for the determination of sex hormones in wastewater and sludge matrices.

Data elucidating fate, occurrence, and levels of sex hormones in soils, sediments, or sludges are very limited. Ying et al. (2003) performed batch experiments to study the sorption and degradation of five EDCs, including E2 and EE2, in aquifer material, under both aerobic and anaerobic conditions. Lee et al. (2003) conducted sorption and degradation studies for E2, EE2, and testosterone using five soils of varying characteristics. Although interesting information can be obtained from the studies mentioned above, direct extrapolation is difficult. The experiments were conducted as batch



**Figure 2—Pilot plant with aerobic digestion scheme: (A) synthetic-water-feeding system; (B) primary clarifier; (C) aeration tank; (D) secondary clarifier; (E) aerobic-sludge digester; (F) return-flow reservoir.**

experiments and, more importantly, the concentration of sex hormones used was in the microgram-per-liter range, a much higher concentration than what is expected in real samples. Ternes et al. (2002) published an analytical procedure for analysis of the sex hormones E2, EE2, and E1 in sediments and sludge. Their procedure included three cleanup steps for the sediment extracts and two for the sludge extracts before derivatization and injection to a gas chromatograph–tandem mass spectrometer (GC–MS–MS) system. Even with the implementation of extensive cleanup of the samples and the enhanced selectivity of the MS–MS analytical instrument, the authors were not able to obtain acceptable results for primary sludge. The same research group published a complete study, in which the authors report the fate of three sex hormones, E2, EE2, and E1, in a real wastewater treatment plant (Andersen et al., 2003). In this study, the levels of these three hormones are reported in liquid and solid phases (except for primary sludge), for every operational unit. The authors reported excellent performance of the plant regarding the elimination of hormones. They suggest a link between the existence of denitrification in the process and the observed improvement in the elimination of the sex hormones. They also suggest that the sorption–desorption processes of the sex hormones are slow, and that there is no equilibrium between the levels of sex hormones in the liquid and sludge phases. Definitive assessment of the fate of these compounds in wastewater treatment plants is limited by the fact that the concentrations of the sex hormones in the influent are constantly varying, and sorption of these compounds onto the solids surfaces confounds interpretation because of large differences in time scales between liquid and SRTs.

## Methodology

**The Challenge.** A study is underway at the University of Cincinnati to elucidate the fate of seven sex hormones in municipal wastewater treatment plants. These compounds are as follows: testosterone and androstenedione as representative androgens; progesterone as a representative progestin; and estrone (E1), estradiol (E2), estriol (E3), and ethinylestradiol (EE2) as representative estrogens. This work is challenging because of the fact that these

compounds are present in wastewater at very low concentrations, and their detection in wastewater matrices is very difficult. It is further desired to perform the work using instrumentation that is readily available in most laboratories. To accomplish this objective, it was decided to use a complex synthetic wastewater as the feed to a pilot plant, because fluctuations in the concentration of these compounds in real wastewater can render the task of determining their fate very difficult (Esperanza et al., 2004). Furthermore, because sex hormones are present in very low concentrations in wastewater, they tend to partition favorably to solid surfaces. To minimize this potential problem, all surfaces in contact with the wastewater in the pilot-plant system used in this study were constructed of stainless steel. The pilot-plant system is a 20 L/h plant, consisting of primary sedimentation, a three-stage aeration tank, a final settling tank, and aerobic sludge digestion. Primary and waste-activated sludge are dewatered and fed to the digestion system. The dewatering supernatant is combined with the digested sludge supernatant and fed to the primary settling tank. The complex synthetic wastewater is prepared 24 hours before use to allow for the sex hormones to reach equilibrium in partitioning to the solids in the feed. A schematic diagram of the pilot plant is given in Figure 2, while a detailed description of the individual unit processes is given in Esperanza et al. (2004).

The pilot plant was operated at steady-state conditions and intensively sampled for several months for COD, total suspended solids and VSS, and the various forms of nitrogen. A summary of these data is given in Table 3.

Sex hormones were extracted from 1 L of the aqueous matrix of interest by solid-phase extraction (SPE). Triplicate samples were taken for each sampling point. The cartridges used were superclean Envi-18 (Supelco, Sigma-Aldrich, St. Louis, Missouri) prepacked with 500 mg of solid-phase material. The cartridges were conditioned using 10 mL of methanol and 20 mL of distilled water. Methanol (1%) was added to all samples. Surrogates were spiked just before extraction. These were 13C2-E2, D4-testosterone. The samples were loaded in the cartridges at a flowrate between 5 and 10 mL/min. The cartridges were then washed with 20 mL of distilled water and dried for 15 minutes by applying vacuum. The

**Table 3—Average steady-state values for chemical oxygen demand, total Kjeldahl nitrogen (TKN), nitrate, ammonia, and suspended solids during the sampling period.**

Parameter	Influent	Primary effluent	Final effluent	Digester supernatant
Total COD (mg/L)	308 ± 17	262 ± 8	22 ± 4	506 ± 73
Soluble COD (mg/L)	270 ± 16	230 ± 10	19 ± 4	426 ± 53
TKN as nitrogen (mg/L)	44 ± 1	Not applicable	1 ± 1	Not applicable
Ammonia as nitrogen (mg/L)	36.0 ± 1.5	36.1 ± 1.5	0.1 ± 0.1	13.1 ± 1.5
Nitrate as nitrogen (mg/L)	Not applicable	Not applicable	22 ± 2	709 ± 38
	Aeration tank		Final effluent	
Suspended solids (g/L)	TSS	VSS	TSS	VSS
	2.12 ± 0.17	1.50 ± 0.15	0.022 ± 0.010	0.016 ± 0.007

sex hormones were eluted with 10 mL of methanol and collected in a silanized conical bottom culture tube.

To remove interferences during the GC analysis, the extract was cleaned using SPE neutral alumina (Supelco) cartridges containing 1 g of the adsorbent. Precleaned anhydrous sodium sulfate (1 g) was placed in the alumina tubes before conditioning. The alumina cartridges were conditioned with 9 mL of 30% methanol in acetone and 9 mL of 20% methylene chloride (dichloro methane [DCM]) in iso-octane. The C-18 extracts were brought to dryness using a gentle nitrogen gas stream, while the tubes were submerged in a bath at 40°C. The extracts were reconstituted in 1 mL of 20% DCM in iso-octane and quantitatively transferred to the cartridges. The cartridges were then washed with 9 mL of hexanes and eluted with 9 mL 30% methanol in acetone. The cleaned extracts were then concentrated to approximately 1 mL under nitrogen and transferred quantitatively to 2 mL reaction vials for derivatization.

The sex hormones were derivatized before injection in the GC-MS. The procedure consists of two derivatization steps in series, based on a previously published method (Keith et al., 2000). The extracts were brought to dryness under a gentle nitrogen gas stream at 40°C. The dried residue was reconstituted with 50 µL of 15% (w/v) methoxyamine hydrochloride in pyridine. The reaction took place in a heating block maintained at 70°C for 4 hours. After completion of the first reaction, the vials were allowed to cool down, and excess reactant was evaporated under a nitrogen stream. Pyridine (50 µL) and 100 µL of 10% trimethylchlorosilane in bis(trimethylsilyl) trifluoroacetamide (BSTFA) were added, and the vials were returned to the reaction block. The conversion of the hydroxyl groups required 15 hours at 70°C. The content of the vials were brought once more to dryness, and the derivatized target compounds were reconstituted in 200 µL of injection solvent, 20% DCM in hexanes, plus 5% (v/v) BSTFA. Before injection, 250 ng of

each of the internal standards, 5 $\alpha$ -androstane and 5 $\alpha$ -cholestane, were added to each vial. The internal standards were selected because of structural similarities with the target compounds.

The sludge was collected from both pilot plants using silanized bottles, and the pH of the mixture was adjusted to 2 for inactivation. The sludge was concentrated by centrifugation and then freeze-dried. The freeze-dried sludge was extracted with methanol and tumbled for 12 hours. The surrogates were added to the solid before the first volume of methanol was added. The extract was then cleaned using neutral alumina and the same procedure detailed in the procedure for the liquid phase. After SPE cleanup, the extract was further cleaned by injection to a bare silica high-pressure liquid chromatography (HPLC) preparatory column. Three fractions were collected, which contained the target compounds and surrogates. After HPLC fractionation, the extract was derivatized and injected to the GC-MS.

Triplicate samples were taken and analyzed for both liquid and solid matrixes at each sampling point. No sex hormones were detected in any of the liquid or solid blanks run with the samples. Continuing calibration checks were prepared and derivatized with the samples. All work was done in accordance with a U.S. EPA approved quality assurance-quality control plan.

The target concentration of each of the seven sex hormones in the synthetic wastewater is 100 ng/L. The concentrations of these compounds in the primary effluent ranged from a low of 11 ng/L for E3 to a high of 91 ng/L for testosterone (Table 4). The sex hormones were further reduced in the activated-sludge process, with complete removal of testosterone, androstenedione, and progesterone and E2. Removal efficiencies for E1 and EE2 were only 85 and 60%, respectively. The presence of E1 in the influent may be because of the fact that it is a degradation byproduct of E2. Ternes et al. (1999a) and Lee et al. (2003) reported good degradability for E2 and E1 under aerobic conditions. Both studies report data from batch studies performed using activated sludge from full-scale wastewater treatment plants. In both cases, the authors report the generation of E1 as a result of the biodegradation of E2. Ternes et al. (1999b) did not report any degradation for EE2 under the experimental conditions investigated. They directly extracted the slurry sampled from the reactor (liquid and sludge). Therefore, in their case, only degradation is responsible for the disappearance of the hormones. Baronti et al. (2000) investigated the removal (influent minus effluent) of E2, E3, and EE2 from six full-scale wastewater treatment plants in the Rome, Italy, area. The average removal for E2 and EE2, considering both physical absorption and degradation, was approximately 85%. In the case of E3, they reported that 95% of the influent concentration persisted in the effluent. The general findings reported in these investigations are very similar to those observed in the present study, with the exception of EE2.

**Table 4—Removal of sex hormones from the aqueous phase in aeration tank.**

Sex hormones	Primary effluent (ng/L)	Final effluent (ng/L)	Change (%)
Testosterone	91 (10)	Not detected	-100
Androstenedione	80 (7)	Not detected	-100
Progesterone	77 (5)	Not detected	-100
Estrone (E1)	43 (5)	5 (1)	-88
Estradiol (E2)	46 (9)	Not detected	-100
Estradiol + Estrone	89	5	-99
Estriol (E3)	11 (3)	1 (1)	-90
Ethinylestradiol (EE2)	44 (8)	17 (1)	-61

**Table 5—Concentration of sex hormones in the primary and waste-activated sludge.**

Sex hormones	Primary sludge concentration (ng/g)*	Activated sludge concentration (ng/g)*
Testosterone	22 (11)	Not detected
Androstenedione	Nd	Not detected
Progesterone	41 (12)	Not detected
Estradiol (E2)	56 (1)	Not detected
Estrone (E1)	32 (6)	Not detected
Estriol (E3)	4 (2)	Not detected
Ethinylestradiol (EE2)	121 (5)	6 (1)

\* Note: Standard deviations are shown in parenthesis (n = 3).

The concentration of sex hormones measured in the primary and waste-activated sludge is given in Table 5. It is apparent from these data that a significant fraction of these hormones partition to the primary sludge, with the exception of testosterone, androstenedione, and progesterone.

What is very surprising is the absence of these compounds from waste-activated sludge, with the exception of EE2. The E1 and E3 were present in the aqueous effluent at concentrations of 5 and 1 ng/L, respectively. This makes their absence from the waste-activated sludge solids very surprising. Andersen et al. (2003) sampled a full-scale wastewater treatment plant to determine the fate of E1, E2, EE2, and mestranol. They analyzed several liquid and sludge samples along the plant. They concluded that adsorption of the hormones in question was very slow, and the liquid and solid phases may not be in equilibrium.

### Summary and Conclusions

Biological treatment of low influent concentrations of MTBE is feasible if sufficiently long SRTs are maintained. The achievement of such long SRT values was achievable only through the use of membrane retention of biomass, because gravity separation does not permit harvesting of biomass when its generation per liter of water treated is smaller than what can be expected in clarifier effluents. A very small value of  $K_s$  is needed to achieve effluent concentrations of MTBE that meet or exceed the U.S. EPA drinking water advisory of 20 to 40 µg/L.

Municipal biological wastewater treatment plants appear to affect complete removal of four of the seven sex hormones studied, while E1, E3, and EE2 were respectively removed at efficiencies of 95, 99, and 83%. Analysis for these compounds in the sludge matrix is very difficult and requires extensive cleanup and handling.

### Acknowledgments

**Credits.** This research was supported by the National Risk Management Research Laboratory of the U.S. Environmental Protection Agency, Washington, D.C.

**Authors.** M. T. Suidan is a professor of environmental engineering in the Department of Civil and Environmental Engineering, University of Cincinnati, Ohio. M. Esperanza and M. M. Zein are doctoral candidates in environmental engineering, University of Cincinnati, Ohio. P. McCauley, R. C. Brenner, and A. D. Venosa are research scientists with the U.S. Environmental Protection Agency, NRMRL, Cincinnati, Ohio. Correspondence should be addressed to M. T. Suidan, Department of Civil and Environmental Engineering, University of Cincinnati, Cincinnati, OH 45221-0071; e-mail: makram.suidan@uc.edu.

Submitted for publication May 14, 2004; revised manuscript submitted August 19, 2004; accepted for publication September 27, 2004.

The deadline to submit Discussions of this paper is May 15, 2005.

### References

- Andersen, H.; Siegrist, H.; Halling-Sorensen, B.; Ternes, T. A. (2003) Fate of Estrogens in a Municipal Sewage Treatment Plant. *Environ. Sci. Technol.*, **37**, 4021–4026.
- Baronti, C.; Curini, R.; D'Ascenzo, G.; Di Corcia, A.; Gentili, A.; Samperi, R. (2000) Monitoring Natural and Synthetic Estrogens at Activated Sludge Sewage Treatment Plants and in a Receiving River Water. *Environ. Sci. Technol.*, **34**, 5059–5066.
- Belfroid, A. C.; Van der Horst, A.; Vethaak, A. D.; Schäfer, A. J.; Rijs, G. B. J.; Wegener, J.; Cofino, W. P. (1999) Analysis and Occurrence of Estrogenic Hormones and their Glucuronides in Surface Water and Waste Water in The Netherlands. *Sci. Total Environ.*, **225**, 101–108.
- Borghoff, S. J.; Murphy, J. E.; Medinsky, M. A. (1996) Development of Physiologically Based Pharmacokinetic Model for Methyl tertiary-Butyl Ether and tertiary-Butanol in Male Fischer-344 Rats. *Fundam. Appl. Toxicol.*, **30**, 264–275.
- Bradley, P. M.; Landmeyer, J. E.; Chappelle, F. H. (1999) Aerobic Mineralization of MTBE and tert-Butyl Alcohol by Stream-Bed Sediment Microorganisms. *Environ. Sci. Technol.*, **33**, 1877–1879.
- Braids, O. C. (2001) MTBE-Panacea or Problem. *Environ. Forensics*, **2**, 189–196.
- Colborn, T.; Dumanoski, D.; Myers, J. P. (1996) *Our Stolen Future: Are We Threatening our Fertility, Intelligence, and Survival?* Dutton: New York.
- Deeb, R. A.; Scow, K. M.; Alvarez-Cohen, L. (2000) Aerobic MTBE Biodegradation: An Examination of Past Studies, Current Challenges and Future Research Directions. *Biodegradation*, **11**, 171–186.
- Desbrow, C.; Routledge, J.; Brighty, G.; Sumpter, J.; Waldock, M. (1998) Identification of Estrogenic Chemicals in STW Effluent. I. Chemical Fractionation and in Vitro Biological Screening. *Environ. Sci. Technol.*, **32**, 1549–1558.
- Esperanza, M.; Suidan, M. T.; Nishimura, F.; Wang, Z. M.; Sorial, G. A.; Zaffiro, A.; McCauley, P.; Brenner, R. C.; Sayles, G. D. (2004) Determination of Sex Hormones and Nonylphenol Ethoxylates in the Aqueous Matrices of Two Pilot-Scale Municipal Wastewater Treatment Plants. *Environ. Sci. Technol.*, **38**, 3028–3035.
- Finneran, K. T.; Lovley, D. R. (2001) Anaerobic Degradation of Methyl tert-Butyl Ether (MTBE) and tert-Butyl Alcohol (TBA). *Environ. Sci. Technol.*, **35**, 1785–1790.
- François, A.; Mathis, H.; Godefroy, D.; Piveteau, P.; Fayolle, F.; Monot, F. (2002) Biodegradation of Methyl tert-Butyl Ether and Other Fuel Oxygenates by a New Strain, *Mycobacterium austroafricanum* IFP 2012. *Appl. Environ. Microbiol.*, **68**, 2754–2762.
- Garnier, P. M.; Auria, R.; Augur, C.; Revah, S. (1999) Cometabolic Biodegradation of Methyl t-Butyl Ether by *Pseudomonas aeruginosa* Grown on Pentane. *Appl. Microbiol. Biotechnol.*, **51**, 498–503.
- Hanson, J. R.; Ackerman, C. E.; Scow, K. M. (1999) Biodegradation of Methyl tert-Butyl Ether by a Bacterial Pure Culture. *Appl. Environ. Microbiol.*, **65**, 4788–4792.
- Hardison, L. K.; Curry, S. S.; Ciuffetti, L. M.; Hyman, M. R. (1997) Metabolism of Diethyl Ether and Cometabolism of Methyl tert-Butyl Ether by a Filamentous Fungus, a *Graphium* sp. *Appl. Environ. Microbiol.*, **63**, 3059–3067.
- Hartley, W. R.; Englande, A. J.; Harrington, D. J. (1999) Health Risk Assessment of Groundwater Contaminated with Methyl tertiary Butyl Ether. *Water Sci. Technol.*, **39** (10/11), 305–310.
- Hatzinger, P. B.; McClay, K.; Vainberg, S.; Tugusheva, M.; Condee, C. W.; Steffan, R. J. (2001) Biodegradation of Methyl tert-Butyl Ether by a Pure Culture. *Appl. Environ. Microbiol.*, **67**, 5601–5607.
- Hernandez-Perez, G.; Fayolle, F.; Vandecasteele, J.-P. (2001) Biodegradation of Ethyl t-Butyl Ether (ETBE), Methyl t-Butyl Ether (MTBE) and

- t*-Amyl Methyl Ether (TAME) by *Gordonia terrae*. *Appl. Microbiol. Biotechnol.*, **55**, 117–121.
- Fraser, I. S.; Jansen, R.; Lobo, R.; Whitehead, M.; Fraser, J. S.; Lobo, R. A. (1998) *Estrogens and Progestogens in Clinical Practice*. Churchill Livingstone Publishing: Edinburgh, United Kingdom.
- Johnson, A. C.; Belfroid, A.; Di Corcia, A. (2000) Estimating Steroid Oestrogen Inputs into Activated Sludge Treatment Works and Observations on their Removal from the Effluent. *Sci. Total Environ.*, **256**, 163–173.
- Keith, L. H.; Jones-Lepp, T. L.; Needham, L. L. (2000) *Analysis of Environmental Endocrine Disruptors*. American Chemical Society: Washington, D.C.
- Kolpin, D. W.; Furlong, E. T.; Meyer, M. T.; Thurman, E. M.; Zaugg, S. D.; Barber, L. B.; Buxton, H. T. (2002) Pharmaceuticals, Hormones, and Other Organic Wastewater Contaminants in U.S. Streams, 1999–2000: A National Reconnaissance. *Environ. Sci. Technol.*, **36**, 1202–1211.
- Kröner, W.; Bolz, U.; Süßmuth, W.; Hillet, G.; Schuller, W.; Hanf, V.; Hagenmaier, H. (2000) Input/Output Balance of Estrogenic Active Compounds in a Mayor Municipal Sewage Plant in Germany. *Chemosphere*, **40**, 1131–1142.
- Layton, A. C.; Gregory, B. W.; Seward, J. R.; Schulz, T. W.; Saylor, G. S. (2000) Mineralization of Steroidal Hormones by Biosolids in Wastewater Treatment Systems in Tennessee, U.S.A. *Environ. Sci. Technol.*, **34**, 3925–3931.
- Lee, L. S.; Strock, T. J.; Sarma, A. K.; Rao, P. S. C. (2003) Sorption and Dissipation of Testosterone, Estrogens and their Primary Transformation Products in Soils and Sediments. *Environ. Sci. Technol.*, **37**, 4098–4105.
- Lopez de Alda, M. J.; Barceló, D. (2001) Review of Analytical Methods for the Determination of Estrogens and Progestogens in Waste Waters. *J. Anal. Chem.*, **371**, 437–447.
- Mehlman, M. A. (2001) Methyl-*tertiary*-Butyl-Ether (MTBE) Misclassified. *Am. J. Ind. Med.*, **39**, 505–508.
- Metzler, M. (Ed) (2001) *Endocrine Disruptors Part I. The Handbook of Environmental Chemistry*, Vol. 3: Anthropogenic Compounds, Part L. Springer-Verlag: Berlin, Germany.
- Mo, K.; Lora, C. O.; Wanken, A. E.; Javanmardian, M.; Yang, X.; Kulpa, C. F. (1997) Biodegradation of Methyl *t*-Butyl Ether by Pure Bacterial Cultures. *Appl. Microbiol. Biotechnol.*, **47**, 69–72.
- Morrisson, J. R.; Suidan, M. T.; Venosa, A. D. (2002) Use of Membrane Bioreactor for Biodegradation of MTBE in Contaminated Water. *J. Environ. Eng.*, **128**, 836–841.
- Morrison, J. (2002) *Bioremediation of Methyl tert-Butyl Ether Using a Membrane Bioreactor*. M.S. Thesis, University of Cincinnati, Ohio.
- Nihlén, A.; Wälinder, R.; Löf, A.; Johanson, G. (1998) Experimental Exposure to Methyl *tertiary*-Butyl Ether II. Acute Effects in Humans. *Toxicol. Appl. Pharmacol.*, **148**, 281–287.
- Pruden, A.; Sedran, M. A.; Suidan, M. T.; Venosa, A. D. (2004) Anaerobic Biodegradation of Methyl *tert*-Butyl Ether Under Iron-Reducing Conditions in Batch and Continuous-Flow Cultures. *Biodegradation, Water Environ. Res.*, **76**, 47–55.
- Pruden, A.; Suidan, M. T.; Venosa, A. D.; Wilson, J. (2001) Biodegradation of Methyl *tert*-Butyl Ether under Various Substrate Conditions. *Environ. Sci. Technol.*, **35**, 4235–4241.
- Pruden, A. J. (2002) *Biodegradation of Methyl tert-Butyl Ether*. Ph.D. Thesis, University of Cincinnati, Ohio.
- Rittmann, B. E.; McCarty, P. L. (2001) *Environmental Biotechnology: Principles and Applications*. McGraw-Hill: New York.
- Rong, Y. (2001) The MTBE Paradox of Groundwater Pollution. *Environ. Forensics*, **2**, 9–11.
- Salanitro, J. P.; Diaz, L. A.; Williams, M. P.; Wisniewski, H. L. (1994) Isolation of a Bacterial Culture that Degrades Methyl *tert*-Butyl Ether. *Appl. Environ. Microbiol.*, **60**, 2593–2596.
- Sedran, M. A.; Pruden, A.; Wilson, G. J.; Suidan, M. T.; Venosa, A. D. (2002) Effect of BTEX on Degradation of MTBE and TBA by Mixed Bacterial Consortium. *J. Environ. Eng.*, **128**, 830–835.
- Snyder, S. A.; Keith, T. L.; Verbrugge, D. S.; Snyder, E. M.; Gross, T. S.; Kannan, K.; Gyesy, J. P. (1999) Analytical Methods for Detection of Selected Estrogenic Compounds in Aqueous Mixtures. *Environ. Sci. Technol.*, **33** (16), 2814–2820.
- Solé, M.; Lopez de Alda, M. J.; Castillo, M.; Porte, C.; Ladegaard-Pedersen, K.; Barceló, D. (2000) Estrogenicity Determination in Sewage Treatment Plants and Surface Waters from the Catalanian Area (NE Spain). *Environ. Sci. Technol.*, **34**, 5076–5083.
- Somsamak, P.; Cowan, R. M.; Häggblom, M. M. (2001) Anaerobic Biotransformation of Fuel Oxygenates under Sulfate-Reducing Conditions. *FEMS Microbiol. Ecol.*, **37**, 259–264.
- Squillace, P. J.; Zogorski, J. S.; Wilber, W. G.; Price, C. V. (1996) Preliminary Assessment of the Occurrence and Possible Sources of MTBE in Groundwater in the United States, 1993–94. *Environ. Sci. Technol.*, **30**, 1721–1735.
- Steffan, R. J.; McClay, K.; Vainberg, S.; Condee, C.; Zhang, D. (1997) Biodegradation of the Gasoline Oxygenates Methyl *tert*-Butyl Ether, Ethyl *tert*-Butyl Ether, and *tert*-Amyl Methyl Ether by Propane-Oxidizing Bacteria. *Appl. Environ. Microbiol.*, **63**, 4216–4222.
- Steffan, R. J.; Vainberg, S.; Condee, C.; McClay, K.; Hatzinger, P. B. (2000) Biotreatment of MTBE with a New Bacterial Isolate. In *Bioremediation and Phytoremediation of Chlorinated and Recalcitrant Ether Bonds*. Wickramanayake, G. B.; Gavaskar, A. R.; Alleman, B. C.; Magar, V. S. (Eds.); Battelle Press: Columbus, Ohio, p 165–173.
- Sulfitia, J. M.; Mormile, M. R. (1993) Anaerobic Biodegradation of Known and Potential Gasoline Oxygenates in the Terrestrial Subsurface. *Environ. Sci. Technol.*, **27**, 976–978.
- Ternes, T.; Stumpf, M.; Mueller, J.; Haverer, K.; Wilken, R.; Servos, M. (1999a) Behavior and Occurrence of Estrogens in Municipal Sewage Treatment Plants—I. Investigation in Germany, Canada and Brazil. *Sci. Total Environ.*, **225**, 81–90.
- Ternes, T. A.; Kreckel, P.; Mueller, J. (1999b) Behaviour and Occurrence of Estrogens in Municipal Sewage Treatment Plants—II. Aerobic Batch Experiments with Activated Sludge. *Sci. Total Environ.*, **225**, 91–99.
- Ternes, T. A.; Andersen, H.; Gilberg, D.; Bonerz, M. (2002) Determination of Estrogens in Sludge and Sediments by Liquid Extraction and GC/MS/MS. *Anal. Chem.*, **74**, 3498–3504.
- U.S. Environmental Protection Agency (1997a) *Drinking Water Advisory: Consumer Acceptability Advice and Health Effects Analysis on Methyl tertiary-Butyl Ether (MTBE)*, EPA-822/F-97-009. U.S. Environmental Protection Agency: Washington, D.C.
- U.S. Environmental Protection Agency (1997b) *Special Report on Environmental Endocrine Disruption: An Effects Assessment and Analysis*, EPA-630/R-96-012. Prepared for the Risk Management Forum, U.S. Environmental Protection Agency: Washington, D.C.
- U.S. Environmental Protection Agency (1998) *Oxygenates in Water: Critical Information and Research Needs*; EPA-600/R-98-048. U.S. Environmental Protection Agency: Washington, D.C.
- U.S. Geological Survey (1999) Effects of Environmental Conditions on MTBE Degradation in Model Column Aquifers. Water Resources Investigations, Report 99-4018C. U.S. Geological Survey: Washington, D.C.
- U.S. Geological Survey (2001) MTBE and Other Volatile Organic Compounds—New Findings and Implications on the Quality of Source Waters Used for Drinking-Water Supplies, FS-105-01. U.S. Geological Survey: Washington, D.C.
- Wilson, G. J.; Richter, A. P.; Suidan, M. T.; Venosa, A. D. (2001) Biodegradation Kinetics of MTBE in Laboratory Batch and Continuous Flow Reactors. *Water Sci. Technol.*, **43**, 277–284.
- Wilson, G. J.; Richter, A. P.; Suidan, M. T.; Venosa, A. D. (2002) Aerobic Biodegradation of Gasoline Oxygenates MTBE and TBA. *J. Environ. Eng.*, **128** (2), 824–829.
- Yeh, C. K.; Novak, J. T. (1994) Anaerobic Biodegradation of Gasoline Oxygenates in Soils. *Water Environ. Res.*, **66**, 744–752.
- Ying, G.; Kookana, R. S.; Dillon, P. (2003) Sorption and Degradation of Selected Five Endocrine Disrupting Chemicals in Aquifer Material. *Water Res.*, **37**, 3785–3791.
- Zein, M.; Suidan, M. T.; Venosa, A. D. (2004) MTBE Biodegradation in a Gravity Flow, High-Biomass Retaining Bioreactor. *Environ. Sci. Technol.*, **38**, 3449–3456.