CHAPTER IV

BIODEGRADATION OF 17ALPHA-METHYLTESTOSTERONE BY MICROORGANISMS IN SEDIMENT UNDER DIFFERENT ELECTRON ACCEPTOR CONDITIONS

4.1 Introduction

To produce an all male population in aquaculture farming, 17alpha-methyltestosterone (MT), an anabolic androgenic steroid, is used widely in reversing the sex of Nile tilapia (*Oreochromis niloticus*), an important freshwater aquaculture fish in many developing countries. The production of a single sex population is advantageous as it overcomes the problem of overpopulation and stunting in the production of bi-sex population. In addition, male Nile tilapia is more desirable since the male has about twice the growth rate and a larger body size than the female (MacIntosh and Little, 1995; Green *et al.*, 1997). Masculinization of Nile tilapia fry can be achieved by simply feeding MT-impregnated food containing 60 mg of MT per kilogram of feed for 21 days after the fifteen day of post-fertilization (Fitzpatrick *et al.*, 1999).

It is possible that residual MT and its metabolites in the MT-impregnated food from the masculinizing process may accumulate in the ponds and be released into the receiving water body. MT is a suspect human carcinogen as it can induce production of nonmalignant tumors in the liver (Soe *et al.*, 1992). Nakata *et al.* (1997) found that MT caused prostate cancer in human when exposed to 6-9 mg of MT per day over a period of 30 years. In addition, MT can act as an endocrine disrupting compound interfering with the normal function of endocrine and reproductive systems of animals when exposed to low nanogram per liter levels of MT (Wason *et al.*, 2003; Masanori *et al.*, 2004; Schulte-Oehlmann *et al.*, 2004; Selzsam *et al.*, 2005; Andersen *et al.*, 2006; Korsgaard *et al.*, 2006). For example, when female eelpout (*Zoarces viviparous*) was exposed to 10-500 ng/L of MT for ten days, the circulation yolk-precusor protein vitellogenin that expresses the female characteristic was found to decrease (Korsgaard, 2006). A study by Schulte-Oehlmann *et al.* (2004) showed that imposex for the development of male sex organs such as penis and vas deferens was

stimulated in female freshwater ramshorn snail (*Marisa cornuarietis*) when exposed to 100-1000 ng/L of MT for six months. Kang *et al.* (2008) studied the effects of MT at an approximate concentration of 46.8 ng/L on adult medaka fish (both male and female) and found that the fecundity and fertility of paired medaka were significantly decreased. Above this concentration, gonadal development in female medaka was inhibited and reproduction of medaka was adversely affected (Kang *et al.*, 2008). For male fathead minnows (*Pimephales promelas*) exposed to a dose of about 20,200 μg/L, MT was found to be converted to 17α-methylestradiol (ME2) by aromatase enzyme which in turn had an estrogenic effect on the minnows (Hornung *et al.*, 2004). Furthermore, MT was found to inhibit the production of testosterone hormone in common crap as well as in rainbow trout (Fitzpatrick *et al.*, 1993). A study by Anderson *et al.* (2006) found that 11-ketotestosterone and testosterone levels in adult male zebrafish (*Danio rerio*) were decreased when exposed to MT at the lowest effective concentrations of 6.4 and 8.5 ng/L, respectively. MT does not only affect the aquatic organisms and invertebrates, but also birds. The egg-laying rate of female Japanese quails (*Coturnix cotumix japonica*) and the fertility in male Japanese quails were found to decrease when exposed to 50-110 mg/L of MT for 3 weeks (Selzsam *et al.*, 2005).

Thus far, an understanding on the occurrence and fate of MT in the environment has been limited. Hulak *et al.* (2008) reported the effects of residual MT in treated recirculated water from the masculinization process of common crap (*Cyprinus carpio* L.) and found that although the water was treated by a biological filter in the water recirculation system, the residual concentration of MT in the treated water was still high enough to achieve 81-100% sex inversion of common crap progeny. Only the work done by Fitzpatrick and Contreras-Sánchez, (2000) reported the concentrations of MT in a fish musculinization pond. They found that MT aqueous concentration of 3.6 μg/L at the end of the masculinization process decreased to background level (between non detectable and 0.02 μg/L) in a week. However, MT in the sediment persisted at concentrations between 2.8 and 2.9 ng/g for nearly three months after the end of the treatment (Fitzpatrick and Contreras-Sánchez, 2000). Based on the log K_{ow} (3.36) and vapor pressure (1.85 x 10^{-8} mmHg at 25 °C) of MT, MT is likely to be sorbed onto the solid particles rather than be in the aqueous phase (Yalkowsky and He, 2003).

To understand the fate of MT in soils and sediments, the biodegradation of MT by microorganisms in sediment from a masculinization pond of Nile tilapia fry under different electron acceptor conditions was investigated. Degradation conditions studied included aerobic, nitrate-reducing, iron (III)–reducing, sulfate- reducing, and methanogenic conditions. The androgenic potency of the degradation products of MT, if any, was assessed using the β-galactosidase assay and reported as testosterone equivalent (TEQ).

4.2 Materials and methods

4.2.1 Chemicals

MT (> 99% pure, HPLC grade) was purchased from Fluka (Buchs, Switzerland). Individual stock solutions of MT (500 and 5,000 mg/L) were prepared in methanol and store at -20°C.

4.2.2 Medium

Synthetic freshwater medium for biodegradation studies was prepared by dissolving 1.0 g of NaCl, 0.4 g of $MgCl_2.2H_2O$, 0.1 g of $CaCl_2.2H_2O$, 0.25 g of NH_4Cl, 0.2 g of KH_2PO_4, 0.5 g of KCl, 1 mL of trace element mixture, 30 mL of 1.0 M of $NaHCO_3$, and 7.5 mL of 0.2 M of Na_2S solution in one liter of Milli-Q water. The trace element mixture consisted of 12.5 mL HCl (25% or 7.7 M), 2.1 g $FeSO_4.7H_2O$, 30 mg H_3BO_3, 100 mg $MnCl_2.4H_2O$, 190 mg $CoCl_2.6H_2O$, 24 mg $NiCl_2.6H_2O$, 2 mg $CuCl_2.2H_2O$, 144 mg $ZnSO_4.7H_2O$ and 36 mg $Na_2MoO_4.2H_2O$ in 987 mL of Milli-Q water. The freshwater medium was sterilized by autoclaving and stored at 4 °C before use.

4.2.3 Sediment

Sediment was collected from a Nile tilapia fry masculinizing pond located in Pathumtani province, Thailand. Sediment from the topmost layer between the depth of 0 and 5 cm was taken and used for biodegradation tests. Samples were shipped under ice and stored in a refrigerator at 4 °C.

4.2.4 Biodegradation of 17alpha-methyltestosterone

For each electron acceptor condition, six batch experiments were conducted in parallel: T1- biodegradation test with MT (an initial concentration of 10 mg/L), T2 - biodegradation test without MT, to investigate the change in electron acceptor in the degradation of background organic compounds in the sediment, T3 - biodegradation test without MT, but with glucose (an initial concentration of 180 mg/L (1 mM)), to confirm the presence of microbial activity for each electron acceptor condition, T4 - control test with MT (an initial concentration of 10 mg/L) and sterilized sediment, to ensure that MT can be completely extracted from the sediment and aqueous solution, T5 - abiotic control test with MT (an initial concentration of 10 mg/L) without sediment, to observe abiotic transformation of MT, and T6 - abiotic control test with glucose (an initial concentration of 180 mg/L (1 mM)) but without sediment and MT, to observe abiotic transformation of glucose.

For only nitrate-reducing condition, the batch experiment was conducted to assess the ability of nitrate-reducing microorganisms to degrade MT in T7- biodegradation test with MT (at an initial concentration of 10 mg/l) and activated sludge from a denitrification tank of an industrial wastewater treatment plant (0.5 mL of 1,500 mg/L).

For aerobic condition, the biodegradation tests were conducted in 16 mL amber vials. For all tests, 100 µL of MT stock solution in methanol (500 mg/L) was added to a vial and the methanol evaporated using nitrogen gas. 4.5 mL of freshwater mineral medium and 0.5 mL of sediment slurry were added to the vial to give a final solution of 5 mL containing 10 % (vol/vol) of sediment slurry and an initial MT concentration of 10 mg/L. Suspended solids in the sediment slurry were 500 mg/mL. Final mass of sediment in the vial was 0.25 g. The vials were incubated at 25 °C in dark and rotated at a speed of 200 rpm. Between 200 and 300 vials were prepared and whole vials were sacrificed every 24 hr and analyzed for MT. Triplicate vials were sacrificed for each sampling event for the biodegradation tests with MT (T1) and biodegradation test with MT and denitrifying activated sludge (T7), while duplicate vials were sacrificed for the biodegradation tests without MT, with glucose, control, and abiotic control with MT and with glucose (T2–T6). In addition, vials sacrificed at the start, middle and end of the incubation period were used for androgenic activity analysis.

For nitrate-, iron (III)-, and sulfate-reducing conditions, 20 mM of $NaNO_3$ (1240 mg NO_3^-/L), Fe(III)NTA (1120 mg Fe(III)/L) and Na_2SO_4 (1920 mg SO_4^{2-}/L) were added to produce nitrate, iron(III), and sulfate-reducing conditions, respectively. All experiments were conducted in 10 mL amber serum vials. As in the aerobic experiments, 100 μL of MT stock solution (500 mg/L) was added to the vial and the methanol evaporated. 4.5 mL of freshwater mineral medium with the required electron acceptor and 0.5 mL of the sediment slurry were added to the vial to give a final solution of 5 mL containing 10 % (vol/vol) of sediment slurry. Rezasurine (Acros organics, Belgium) was added as an indicator for free dissolved oxygen. The vials were then sealed with butyl rubber stoppers and aluminum crimps and the headspace of the vials flushed with nitrogen gas (99.5%) for 3 min by inserting a syringe needle through the butyl rubber along with a second syringe needle to allow the flushed gas to escape. The vials were incubated at 25 °C in dark without shaking. Between 200 and 300 vials were prepared and whole vials were sacrificed every 48 hr for analysis of MT concentrations and electron acceptor concentrations. Triplicate vials were sacrificed for each sampling event for the biodegradation tests with MT (T1) and biodegradation test with MT and denitrifying activated sludge (T7), while duplicate vials were sacrificed for biodegradation without MT, with glucose, control and abiotic control with MT and with glucose (T2-T6). As for aerobic biodegradation tests, vials were sacrificed at the start, middle and end of the incubation period for androgenic activity analysis.

For biodegradation tests under methanogenic condition, MT was initially omitted and freshwater mineral medium and sediment slurry were added to the vials along with glucose at a concentration 180 mg/L (1 mM). The vials were purged with nitrogen gas and incubated until methane gas was produced. At this point, 10 μL of MT stock solution (5,000 mg/L) was injected into the vials. As for the other electron acceptor conditions, vials were sacrificed at various times over the incubation period.

4.2.5 Measurement of 17alpha-methyltestosterone concentration

An equivalent volume of methanol (HPLC grade, Merck Darmstadt, Germany) was added to each vial and the mixture agitated for 1 minute. The mixture was then filtered through a syringe filter (PTFE, 0.45 μm, Millipore Corp, Bedford, MA). Fifty μL of the filtered methanol mixture was injected directly into a high

performance liquid chromatography (HPLC 1100 series, Agilent Technologies, Palo Alto, CA) to measure the MT concentration (Marwah *et al.*, 2005).

A diode array detector (Agilent 1100 Series LC, Germany) was used at a wavelength of 245 nm along with a reverse phase C18 column (ODS Hypersil, 250 mm x 5 mm x 4.6 μm column, Hewlett Packard, Palo Alto, CA). The column temperature was set at 40.0 ± 0.5°C while the mobile phase was acetonitrile (ACN) (HPLC grade, Merck, Darmstadt, Germany) and water at a flow rate of 0.5 mL/min with the following gradient: at time 0 min - 20% ACN, at time 19 min - 96% ACN, and at time 20 min - 20% ACN and post run for 10 min at 20% ACN.

4.2.6 Analysis of androgenic activity

Androgenic activities of MT and its metabolites were tested using the β-galactosidase assay as described by Li *et al.* (2008). The yeast strain used was Y187 (*MATα, ura3-52, his3-200, ade 2-101, trp 1-901, leu 2-3, 112, gal4Δ, met–, gal80Δ, URA3 : GAL1*UAS-*GAL1*TATA-*lacZ*) which was a recombinant yeast of *Saccharomyces cerevisiae* provided by Prof. Dr. Zijian Wang (Research Center for Eco-Environment Sciences, Beijing, China). The AR plasmid coding for fusion proteins of GAL4 DNA-binding domain and the ligand binding domain of AR was pGBT9 AR. Yeast cells were co-transformed with the pGBT9 AR and a co-activator, pGAD424 GRIP1/FL. The AR plasmid was provided by Dr. Erik Jan Dubbink and Prof. Dr. J. Trapman (Department of Pathology, JNI, Erasmus University, Medical Centre, Rotterdam, Netherland). The recombinant yeast was grown overnight in a selective medium with vigorous vortex of 300 rpm at 30 ^{0}C until an optical density (OD_{600}) of 0.8-1.0 was reached. The selective medium for the recombinant yeast was a synthetic dextrose medium without leucine and tryptophane (SD/-Leu/-Trp) prepared by dissolving 6.7 g of nitrogen base without amino acid, 20 g of dextrose, 100 mL of 10x amino acid dropout mixture and 100 μL of 50 uM $CuSO_4$ in 1 liter of Milli-Q water. The 10x amino acid dropout mixture contained 800 mg of adenine, 800 mg of uracil, 800 mg of histidine, 800 mg of arginine, 800 mg of methionine, 1.2 g of tyrosine, 1.2 g of lysine, 2.0 g of phenylalanine, 8.0 g of threonine, 1.2 g of isoleucine, and 6.0 g of valine in one liter of Milli-Q water.

The yeast culture was then diluted to an OD_{600} of 0.03 with the selective medium. For each sample from the biodegradation tests, β-galactosidase assay was

prepared in triplicate. For each replicate, a sample was diluted into 7 dilutions (1x, 0.5x, 0.1x, 0.05x, 0.01x, 0.005x, and 0.001x) with methanol. Ten µL of each dilution was added into 2990 µL of selective medium containing the yeast culture with an OD_{600} of 0.03 (final solution <0.1 % methanol, Gaido *et al.*, 1997). The solution was incubated at 30 ^{0}C for 16-18 hrs with vigorous shaking at 300 rpm. After incubation, the OD_{600} of the solution was measured. Fifty µL of the solution was then transferred to a new vial and 120 µL of Z-buffer (16.1 g/L of $Na_2HPO_4.7H_2O$; 5.5 g/L $NaH_2PO_4.H_2O$; 0.75 g/L of KCl; 0.246 g/L of $MgSO_4.7H_2O$) and 20 µL of chloroform were added, mixed and incubated at 30 ^{0}C for 5 min. Forty µL of o-nitrophenyl-β-D-galactopyranoside (ONPG) (13.3 mM dissolved in Z-buffer) was then added to the solution to start the enzyme reaction. The solution was incubated for 2 hrs at 30 ^{0}C with mixing at 300 rpm. The reaction was stopped by adding 100 uL of 1 M Na_2CO_3. The solution was then centrifuged at 12,000 g for 15 min and the supernatant measured with a UV-Vis spectrophotometer at a wavelength of 420 nm. The β-galactosidase activity was calculated using the following equation:

$$u\ (U/min.mL) = [A_{420}/(t \times V \times A_{600})] \times 1000$$

where u = β-galactosidase assay activity in U (enzyme activity unit)/min.mL, A_{420} = absorbance at 420 nm, A_{600} = absorbance at 600 nm, t = time of incubation with ONPG in min, V = volume in mL of yeast culture used in the assay (Meng *et al.*, 2005).

Dose-response curves of the biodegradation test samples were obtained by plotting the β-galactosidase activity against the various volumes of the biodegradation samples. Similarly, dose-response curves were obtained by plotting the β–galactosidase response against various concentrations of testosterone. The EC_{50} for the biodegradation samples and samples containing testosterone were estimated.

The androgenic activity was reported in term of testosterone equivalent (TEQ) which was given by the ratio of EC_{50} of the β-galactosidase activity of the testosterone standard (in mass) and the EC_{50} of the β-galactosidase activity of the biodegradation sample (in volume) (Leusch *et al.*, 2006). In addition, the relative TEQ was determined by multiplying the MT concentration in the biodegradation samples as measured by the HPLC with the relative androgenic potency (RAP) of the

test compounds (Beck *et al.*, 2006). RAP was estimated by taking the ratio of EC_{50} of testosterone standard and EC_{50} of test compound standard which was MT in this case and was equal to 0.93.

4.2.7 Measurement of glucose, nitrate, iron (III), sulfate, and methane concentrations

Glucose concentration was measured by using UV-Vis spectroscopy (Helios Delta, Waltham, MA) at a wavelength of 490 nm in accordance to the Somogyi-Nelson method (Nelson, 1944)

NO_3^- and SO_4^{2-} concentrations were measured using ion chromatography (Dionex ICS 2500, Sunnyvale, CA) with a conductivity detector. An IonPac AS11-HC (4 x 250 mm) 9 μm particle size column and IonPac GS11-HC anion precolumn were used for anion separation with a Suprapur quality NaOH (12 mM/L) and 1.0 mL/min flow as eluent conditions and a 112 mA suppression current. The injection volume was 10 μL. Fe^{3+} concentration was measured using UV-Vis spectroscopy (Helios Delta, Waltham, MA) following the Standard Method 3500-Fe (Phenanthroline method) (APHA, 1995).

CH_4 was measured by gas chromatography (HP6890, Hewlett-Packard, Palo Alto, CA) with a thermal conductivity detector. The column used was HP-PLOT Q and helium gas was used as the carrier gas with a flow rate of 8.6 mL/min. The temperature of the detector was at 200 °C. The oven temperature was at 60 °C.

4.3 Results and discussions

4.3.1 Confirmation of microbial activity

Figure 4.1 presents the results of the biodegradation experiments with 1 mM glucose but without MT (T3) for each electron acceptor condition. For all electron acceptor conditions, glucose was reduced rapidly confirming the presence of microbial activity in the sediment. The low concentrations of glucose measured at the end of the experiments were probably due to the interference of the sediment on the transmission of light of the UV-vis spectrophotometer.

4.3.2 Biodegradation of 17alpha-methyltestosterone

Figure 4.2 presents the results for the biodegradation of MT in sediment from the masculinizing pond of Nile tilapia fry for the different electron acceptor conditions (aerobic, nitrate-reducing, iron (III)-reducing, sulfate-reducing, and methanogenic conditions). A lag period of 2-4 days was observed for all electron acceptor conditions except for iron (III)-reducing condition where no lag time was observed. MT was found to degrade rapidly under aerobic (Figure 4.2a), sulfate-reducing and methanogenic conditions (Figure 4.2b) after an initial lag time. MT degraded slowly under iron (III)-reducing condition and there were no observed degradation of MT under nitrate-reducing condition. The first order degradation rates along with their 95% confidence intervals and half-lives for each condition were estimated and presented in Table 4.1. Statistically, the degradation rate (0.51 ± 0.34 day^{-1}) under aerobic condition was similar to the degradation rates under methanogenic condition (0.69 ± 0.24 day^{-1}) and under sulfate-reducing condition (0.53 ± 0.01 day^{-1}). However, the half-lives of MT estimated from Figure 4.2 (a and b) for sulfate–reducing condition and methanogenic conditions were 5.3 and 5.1 days, respectively, as compared to 3.8 days for aerobic condition. The degradation rate estimated for nitrate-reducing condition (0.004 ± 0.00 day^{-1}) was similar to that of the control (0.002 ± 0.00 day^{-1}) meaning that no degradation of MT occurred under this condition. MT degraded slowly under iron (III)-reducing condition with a degradation rate of (0.007 ± 0.00 day^{-1}) which was statistically different from the degradation rate of the control.

Previous studies have shown that compounds with similar chemical structure as MT such as testosterone, natural estrogens (estrone, estradiol, and estriol) and synthetic estrogen (ethylnylestradiol) were readily degraded by microorganisms under aerobic conditions (Ying *et al.*, 2003; Lee *et al.*, 2003; Casey *et al.*, 2004; Das *et al.*, 2004; Ying and Kookana, 2003; Khanal *et al.*, 2006). For the aerobic tests, the HPLC chromatograms showed a peak of an unknown compound which appeared earlier than the MT peak (data not shown). The presence of this peak suggested a MT metabolite with higher polarity and/or with a lower molecular weight than MT. The MT metabolite was not identified but possible metabolites may be 17α-methyl-5α-androstan-3α, 17β-diol and 17α-methyl-5 β -androstan-3α, 17β-diol and their isomers which have higher polarity than MT and are the main MT metabolites found in urine

and feces of humans and animals (Rongone and Segaloff, 1962; Mosbach *et al.*, 1968; Shinohara *et al.*, 2000; Williams *et al.*, 2000 and McKinney *et al.*, 2007). The MT metabolite was observed to appear shortly after the lag period, with an increase in concentration during the middle of the incubation period followed by disappearance of the metabolite at the end of the incubation period.

The androgenic activities reported as TEQ at the initial, middle, and end of the incubation period for the aerobic biodegradation test are presented in Figure 4.3b. The TEQ provided an estimate of the potency of any remaining MT and its metabolites. The relative TEQ was also computed to provide the androgenic activity due to the MT residue in the biodegradation test samples. Under aerobic condition, the estimated relative TEQs were found to decrease with time and were directly proportional to the MT concentration present. The relative TEQ were similar to that of the TEQs measured. Also, plotted in Figure 4.3 for comparison purposes with the TEQs are the MT concentrations with time for the biodegradation test (see Figure 4.3a). The lack of androgenic activity at the end of the incubation indicated degradation of MT and its metabolites to residues that did not have androgenic properties.

Under methanogenic condition, MT was rapidly degraded (see Figure 4.4a) and the corresponding production of methane is presented in Figure 4.4b. The total amount of methane produced was 1.04 μmol which was less than the 2.20 μmol for theoretical methane production for the methaogenic degradation of MT. Also plotted in Figure 4.4 are the androgenic activities which remained at levels similar to the initial incubation levels throughout the incubation period (Figure 4.4c). This suggested that the degradation of MT resulted in the formation of MT metabolites which continued to maintain their androgenicity. Statistically, the TEQs for the initial incubation and the 7-day TEQ were similar. At the end of the incubation period of 25 days, the TEQ remained at levels similar to the initial levels even though the concentration of MT has been found to be close to zero. Extending the incubation period to 45 days, the TEQ was at the same level as that for 25 days. This result implied that the MT metabolites were recalcitrant or they are transformed to other compounds with androgenic properties. However, the unknown metabolite found in the aerobic bioassays was not found throughout the methanogenic experiment. Other metabolites may be present but were not detected by the HPLC. The production of

methane found for the biodegradation tests may not imply mineralization of MT but may be from other organics which were present in the sediment.

Under sulfate-reducing condition, MT was degraded within the first 15 days (see Figure 4.5a). During this period, the amount of sulfate (SO_4^{2-}) needed to degrade 10 mg/L of MT ranged from 0.52 – 0.73 g SO_4^{2-}/L (5.42 – 7.62 mM) (Figure 4.5b). In comparison, the theoretical amount of sulfate needed assuming complete mineralization and no production of biomass is 0.04 g SO_4^{2-}/L (0.44 mM). However, when the measured MT concentration in bioassays was zero, there was no significant difference in the concentrations of sulfate in the test with and without MT. As in the aerobic degradation test, the androgenic activity was found to reduce with time in proportion to the amount of MT remaining. This implies that the pathway of degradation under sulfate–reducing conditions may be similar to that under aerobic condition but different under methanogenic condition. However, as in the methanogenic bioassays, occurrence of the unknown metabolite for aerobic bioassay was not detected by HPLC throughout the sulfate-reducing experiment.

With iron (III)-reducing condition, MT was hardly degraded (see Figure 4.6a) but MT concentrations in the biodegradation tests were different from the control towards the end of the experiments. Iron (III) concentrations were found to decrease but slowly over 80 days with the same trend as for the test without MT (Figure 4.6b). The measured androgenic activities remained high throughout the incubation period indicating that MT was not degraded (Figure 4.6c). Similarly, the unknown metabolite detected in the aerobic bioassay was not detected by HPLC in the iron (III)-reducing condition. The results suggested that microorganisms that use iron (III) as a terminal electron acceptor do not degrade MT effectively. In contrast, Czajka and Londry (2006) found that estradiol was degraded under iron(III)–reducing condition.

In the case of nitrate-reducing condition, no biodegradation of MT was observed but the concentration of nitrate decreased by 53 % during the first 34 days of incubation (Figure 4.7b). During this period, low nitrite concentrations of 0.14 g NO_2^- /L (3.07 mM) were measured confirming the reduction of nitrate to nitrite (Figure 4.7c). Similar nitrate results were found over 34 days in the test without MT that was used to investigate the amount of electron acceptor used by microorganisms in degrading background organic compounds in the sediment (T2). On day 34, 50 µL of 124 g NO_3^-/L (2 M) nitrate solution was added to maintain unlimited electron

acceptor condition. After the addition of nitrate, no further reduction of nitrate was observed. Czajka and Londry (2006) found that estradiol which has a similar chemical structure as MT was degraded fastest by microorganism in sediment under iron(III)-reducing condition, followed by sulfate-reducing and methanogenic conditions but was the slowest under nitrate-reducing condition.

To further investigate the possible reasons for the lack of degradation of MT under nitrate-reducing condition, an experiment was conducted by exposing the sediment microorganisms to MT (initial concentration of 10 mg/L) under aerobic condition until MT was completely degraded to allow MT-degrading microorganisms to develop, and the vials were then immediately switched to nitrate-reducing condition by adding nitrate and MT to provide an initial concentration of 1240 mg NO_3^-/L (20 mM) and 10 mg/L, respectively. The approach taken assumed that in general, facultative bacteria responsible for nitrate reduction can shift their mode of use of electron acceptors from free oxygen to nitrate (Takahashi *et al.*, 1956). Results for this experiment (Figure 4.8) showed that MT was not degraded but remained in the vials while nitrate concentration gradually decreased to about 53% of the initial concentration within 34 days as in the previous nitrate-reducing experiment (Figure 4.7b).

In another set of experiments to assess the ability of nitrate-reducing microorganisms to degrade MT at an initial concentration of 10 mg/l, 0.5 mL of 1,500 mg/L activated sludge from a denitrification tank of an industrial wastewater treatment plant was used instead of the sediment (T7). Two sets of experiments were conducted: the first set established the nitrate-reducing condition immediately while the second one was conducted by enriching the MT degrading-microorganisms under aerobic condition, and then shifting the condition to nitrate-reducing condition. Results for the first set of experiment showed that MT was gradually degraded with a degradation rate of 0.030 day^{-1} and half life of 22.7 days (Figure 4.9). In the second set of experiments, MT was gradually degraded with a degradation rate of 0.061 day^{-1} and half life of 11.3 days (Figure 4.10). However, at the end of the incubation period, MT remained at about 26 % and 6 % of the initial concentration for the first and second set of experiments, respectively.

Based on the experiments using sediment and acclimatizing the microorganisms in the sediment to MT under aerobic conditions before changing to

nitrate-reducing conditions, it appeared that MT was not degraded by microorganisms in the sediment under nitrate-reducing condition. Possible reasons include: (1) nitrite may inhibit microorganisms under nitrate-reducing condition, (2) methyl group at C-17 position may block the transformation of MT by microorganisms, and (3) nitrate-reducing bacteria were unable to degrade MT.

In case of nitrite inhibition, some researchers reported that nitrite concentration in the range of 0.05 to 0.35 g NO_2^-/L (1.09 - 7.61 mM) did not inhibit denitrifying microorganisms (Dou *et al.*, 2008; Dou *et al.*, 2009). In this study, the maximum nitrite concentration measured was 0.14 g NO_2^-/ L (3.07 mM) which may not be high enough to inhibit the microorganisms under nitrate-reducing condition. Although it is probable that the localized nitrite concentration may be higher than the measured concentration.

Since MT has a similar structure to estrogen, degradation studies on estrogens may provide clues on probable reasons for the lack of degradation under nitrate-reducing conditions. Estradiol was found to degrade by microorganisms under all electron acceptor conditions (oxygen, nitrate-reducing, iron (III)-reducing, sulfate-reducing, and methanogenic condition) whereas ethynylestradiol was not degraded under all conditions except for aerobic condition (Shi *et al.*, 2002; Czajka and Londry, 2006; Haiyan *et al.*, 2007). The difference in structure between estradiol and ethynylestradiol is the ethynyl group at the C-17 position of ethynylestradiol. This would suggest that the ethynyl group may block the formation of a ketone as observed in estradiol (Czajka and Londry, 2006). In the case of MT, the methyl group at C-17 position of MT molecule may block transformation by microorganisms under nitrate-reducing condition.

Another possible reason is that nitrate–reducing microorganisms in the sediment may lack the enzyme system to degrade MT. The degradation of nitrate using activated sludge suggests that the bacteria communities in denitrifying sludge were different from that in the sediment from the masculinizing pond.

4.4 Conclusion

MT was biodegraded under aerobic, sulfate-reducing and methanogenic conditions but was hardly degraded under iron (III)-reducing condition and there was no degradation under nitrate-reducing condition. The estimated first-order

biodegradation rates for aerobic, sulfate-reducing and methanogenic conditions were found to be statistically similar. However, the androgenic activity of the final solution under methanogenic conditions was found to persist even though the concentrations of MT were reduced which implied that MT was transformed under methanogenic condition to androgenic-like metabolites. A possible reason for the lack of degradation of MT under nitrate-reducing condition may be due to the methyl group at C-17 position of MT molecule which may block transformation by microorganisms. The results from this study suggest that MT and its metabolites can potentially accumulate in the environment under certain electron acceptor conditions such as iron (III)-reducing, nitrate-reducing and methanogenic conditions. The information obtained from this study showed that clean-up or remediation of MT-contaminated sediments from masculinizing ponds should be conducted under aerobic and sulfate-reducing conditions for maximum removal of MT and reduction of androgenic activity.

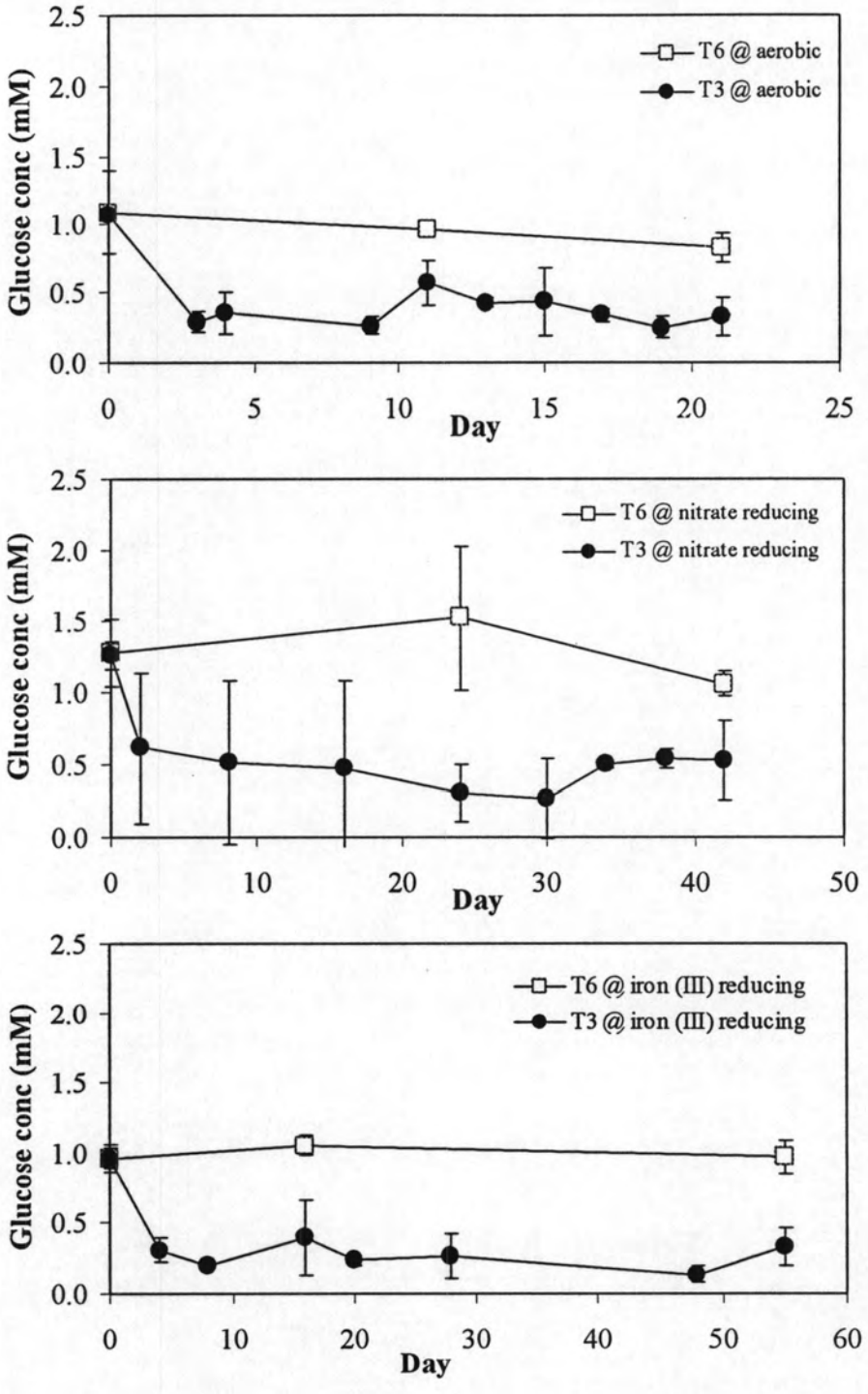

Figure 4.1 Biodegradation of glucose at initial concentration of 1 mM under different electron acceptors (T3: biodegradation test without MT, but with glucose (an initial concentration of 180 mg/L (1 mM) and T6: abiotic control test with glucose (an initial concentration of 180 mg/L (1 mM)) but without sediment and MT)

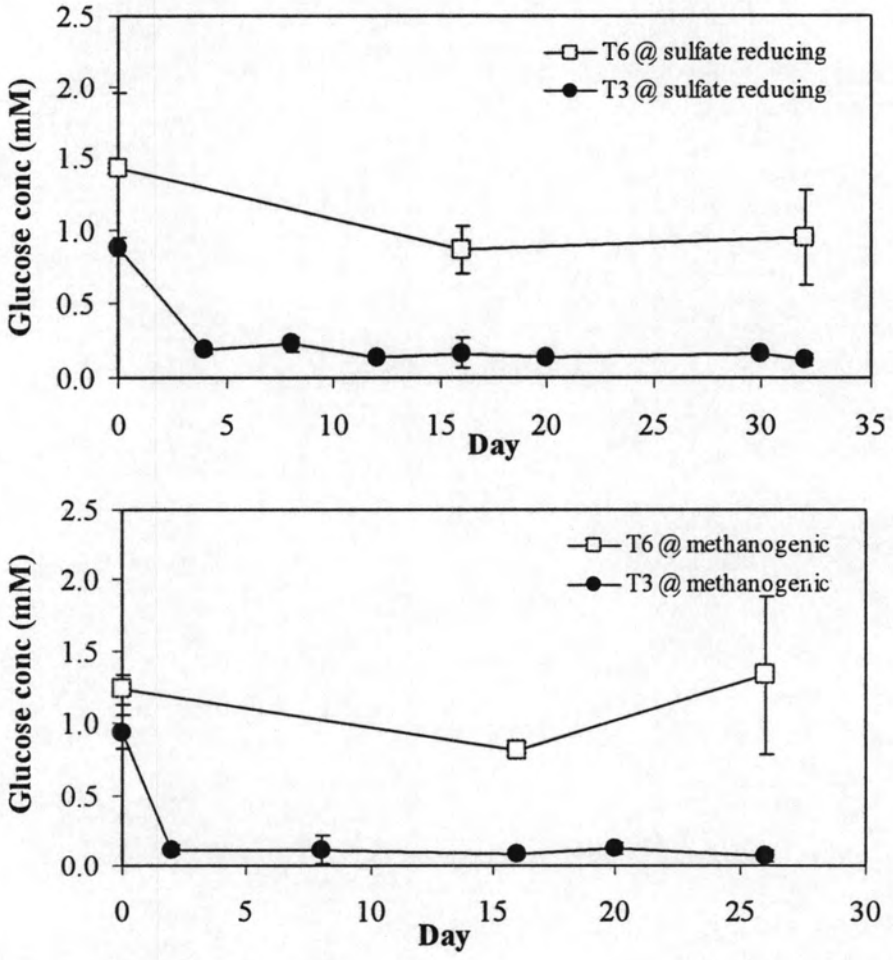

Figure 4.1(cont') Biodegradation of glucose at initial concentration of 1 mM under different electron acceptors (T3: biodegradation test without MT, but with glucose (an initial concentration of 180 mg/L (1 mM) and T6: abiotic control test with glucose (an initial concentration of 180 mg/L (1 mM)) but without sediment and MT)

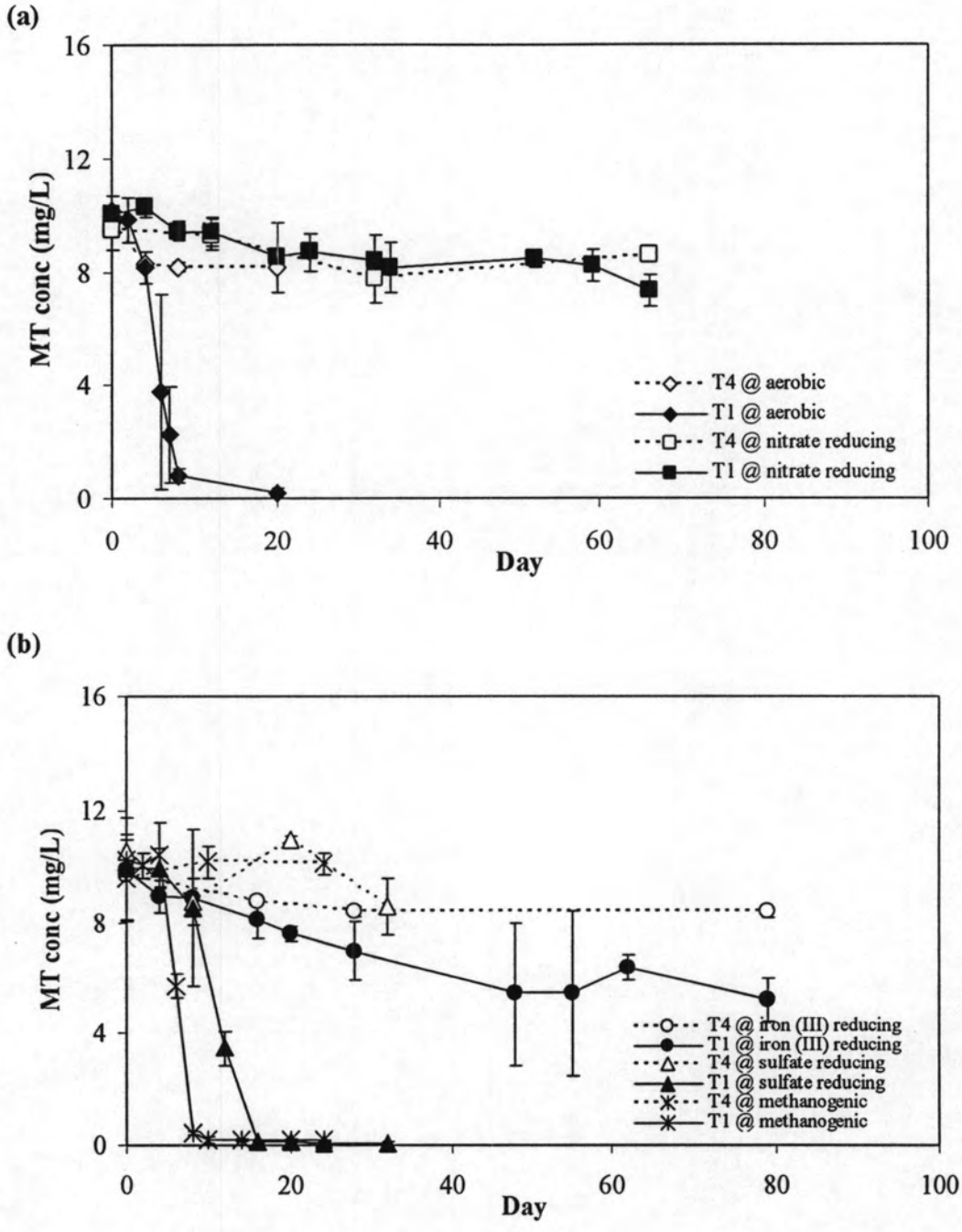

Figure 4.2 Biodegradation of MT under (a) aerobic, nitrate-reducing conditions and control and (b) iron (III)-reducing, sulfate-reducing, methanogenic conditions and control (T1: biodegradation test with MT (an initial concentration of 10 mg/L) and T4: control test with MT (an initial concentration of 10 mg/L) and sterilized sediment)

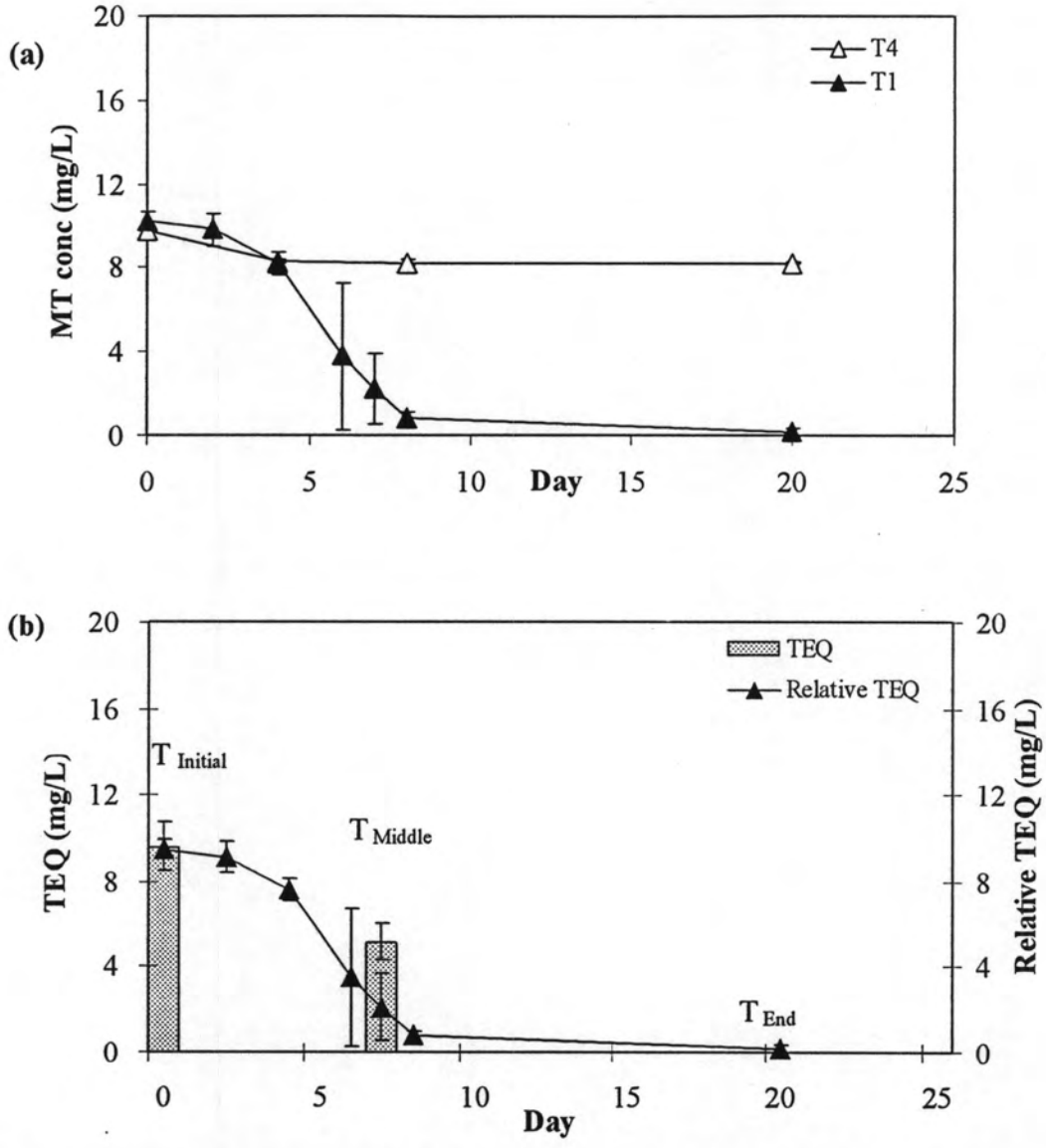

Figure 4.3 (a) Biodegradation of MT and (b) change in androgenic activity in terms TEQ and relative TEQ under aerobic condition (T1: biodegradation test with MT (an initial concentration of 10 mg/L) and T4: control test with MT (an initial concentration of 10 mg/L) and sterilized sediment)

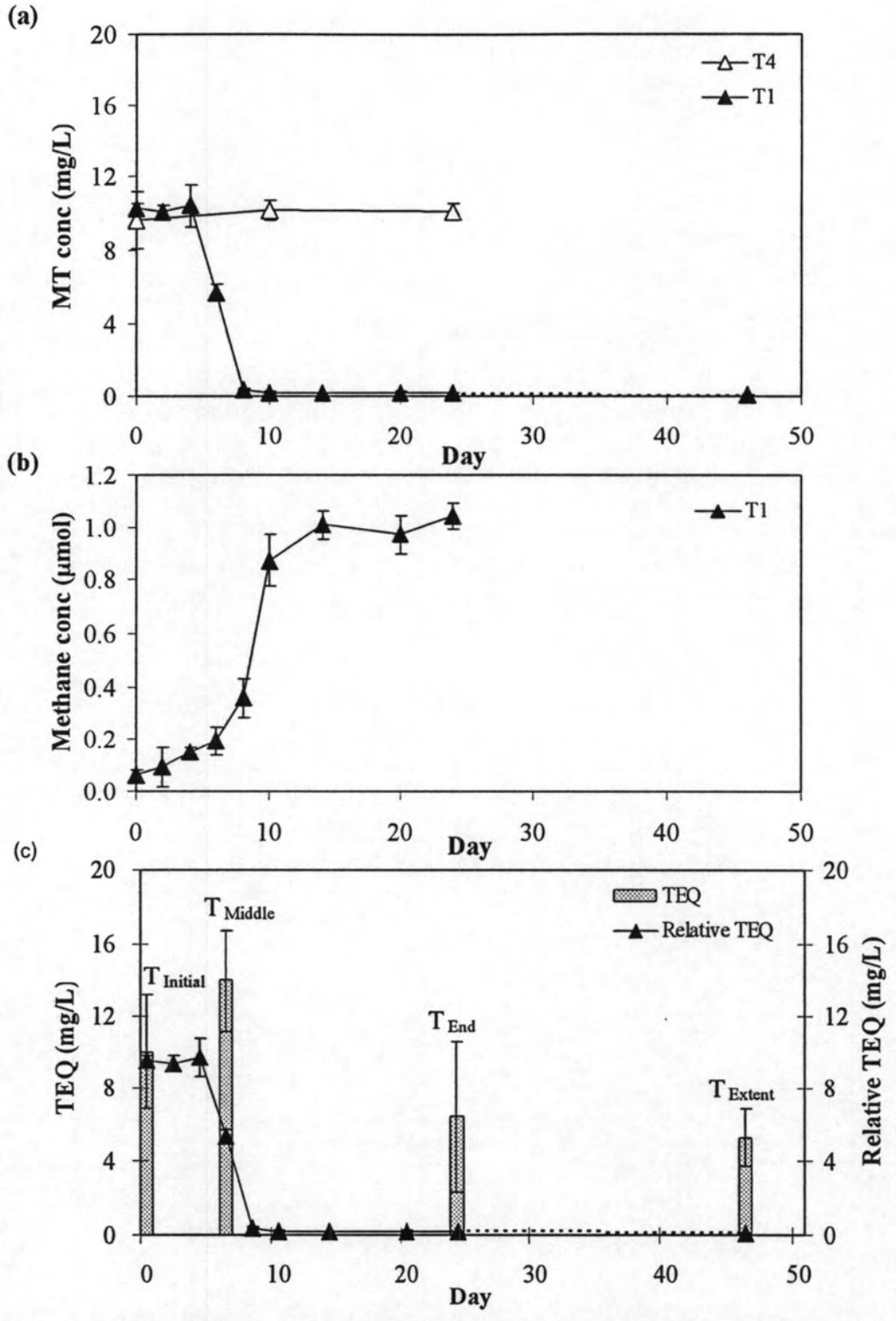

Figure 4.4 (a) Biodegradation of MT, (b) methane production, and (c) change in androgenic activity in terms of TEQ and relative TEQ under methanogenic condition (T1: biodegradation test with MT (an initial concentration of 10 mg/L) and T4: control test with MT (an initial concentration of 10 mg/L) and sterilized sediment)

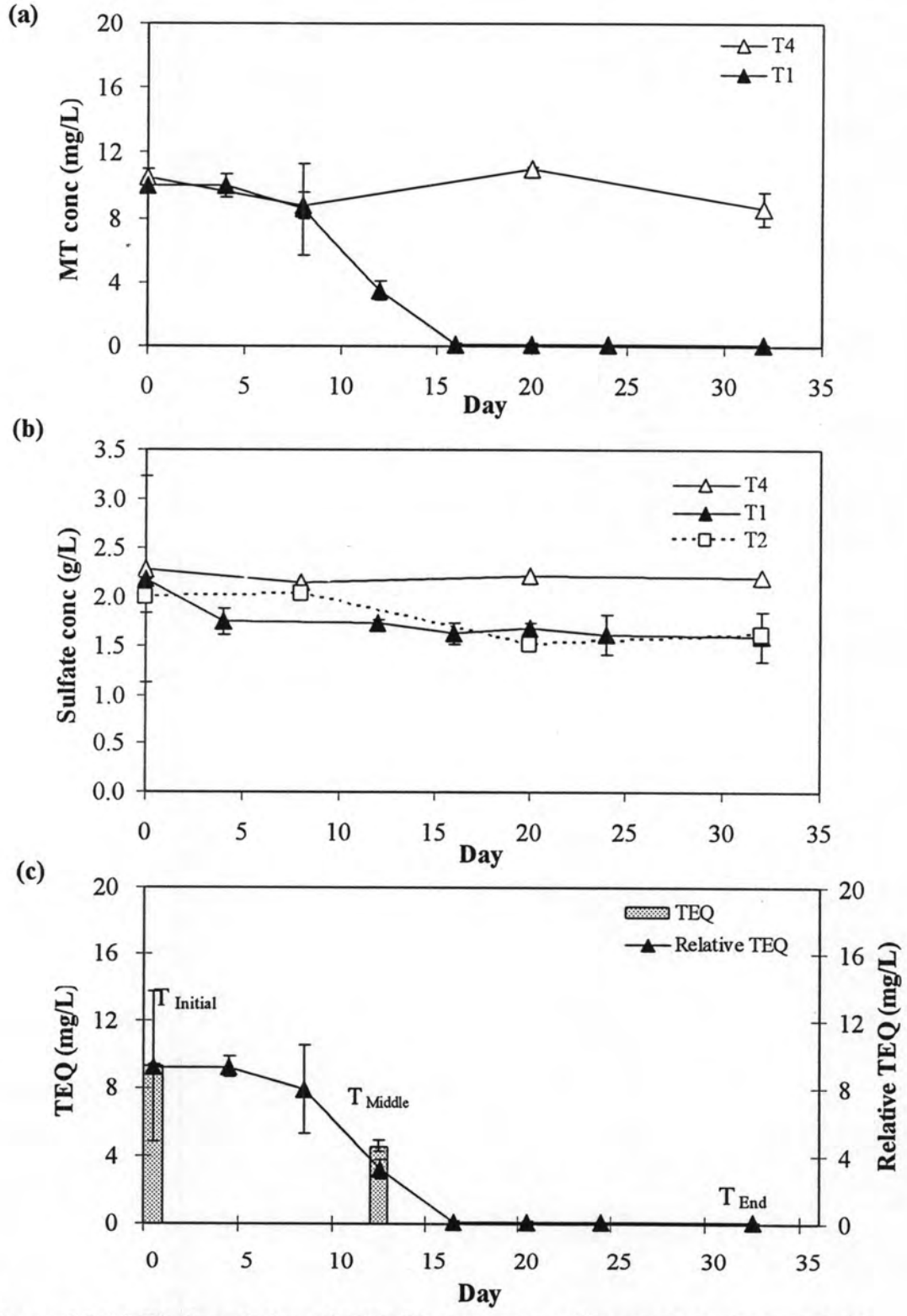

Figure 4.5 (a) Biodegradation of MT, (b) change in sulfate concentrations, and (c) change in androgenic activity in terms of TEQ and relative TEQ under sulfate-reducing condition (T1: biodegradation test with MT (an initial concentration of 10 mg/L), T2: biodegradation test without MT and T4: control test with MT (an initial concentration of 10 mg/L) and sterilized sediment)

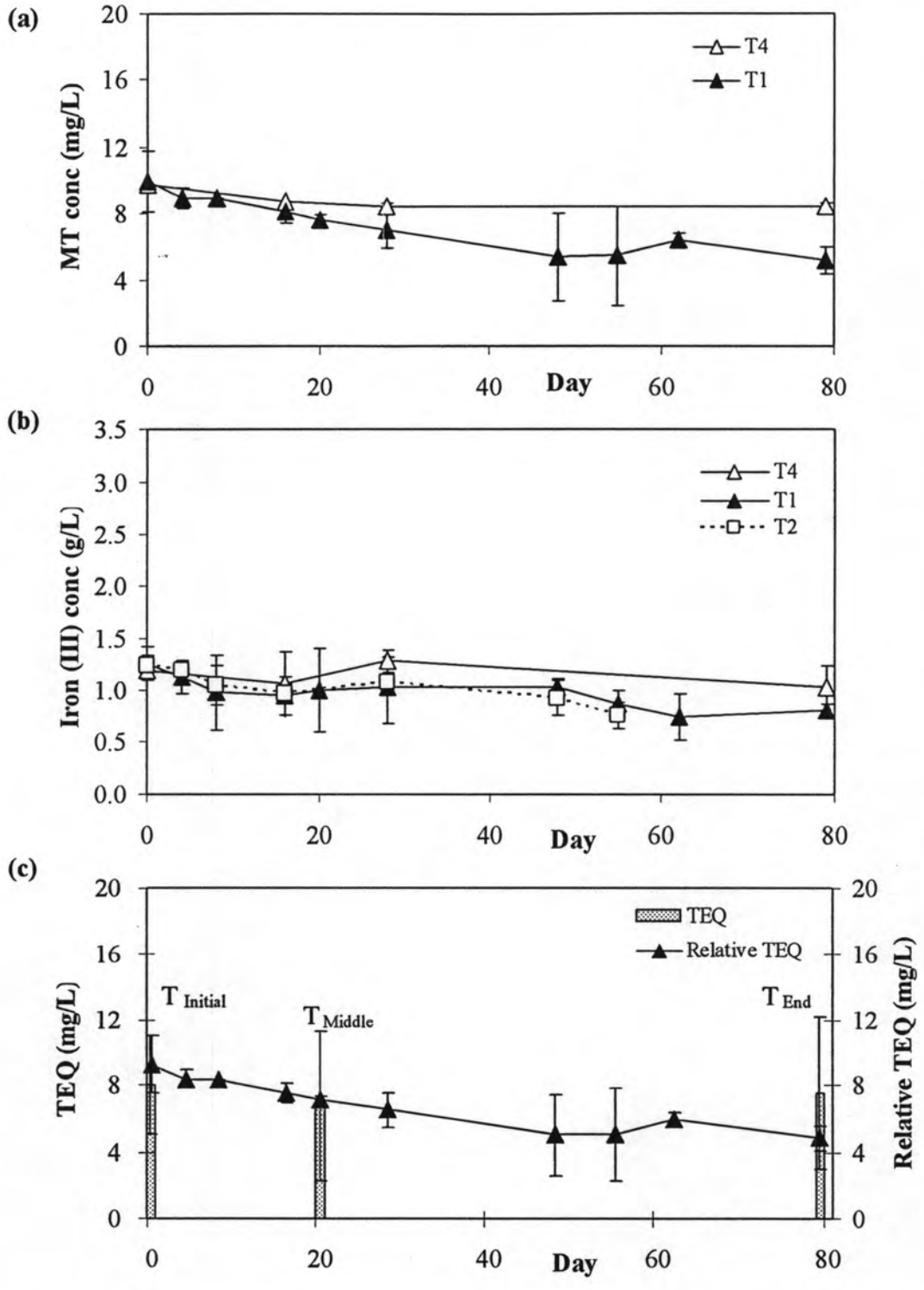

Figure 4.6 (a) Biodegradation of MT (b) change in iron (III) concentrations and (c) changes in androgenic activity in term of TEQ and relative TEQ under iron (III)-reducing condition (T1: biodegradation test with MT (an initial concentration of 10 mg/L), T2: biodegradation test without MT and T4: control test with MT (an initial concentration of 10 mg/L) and sterilized sediment)

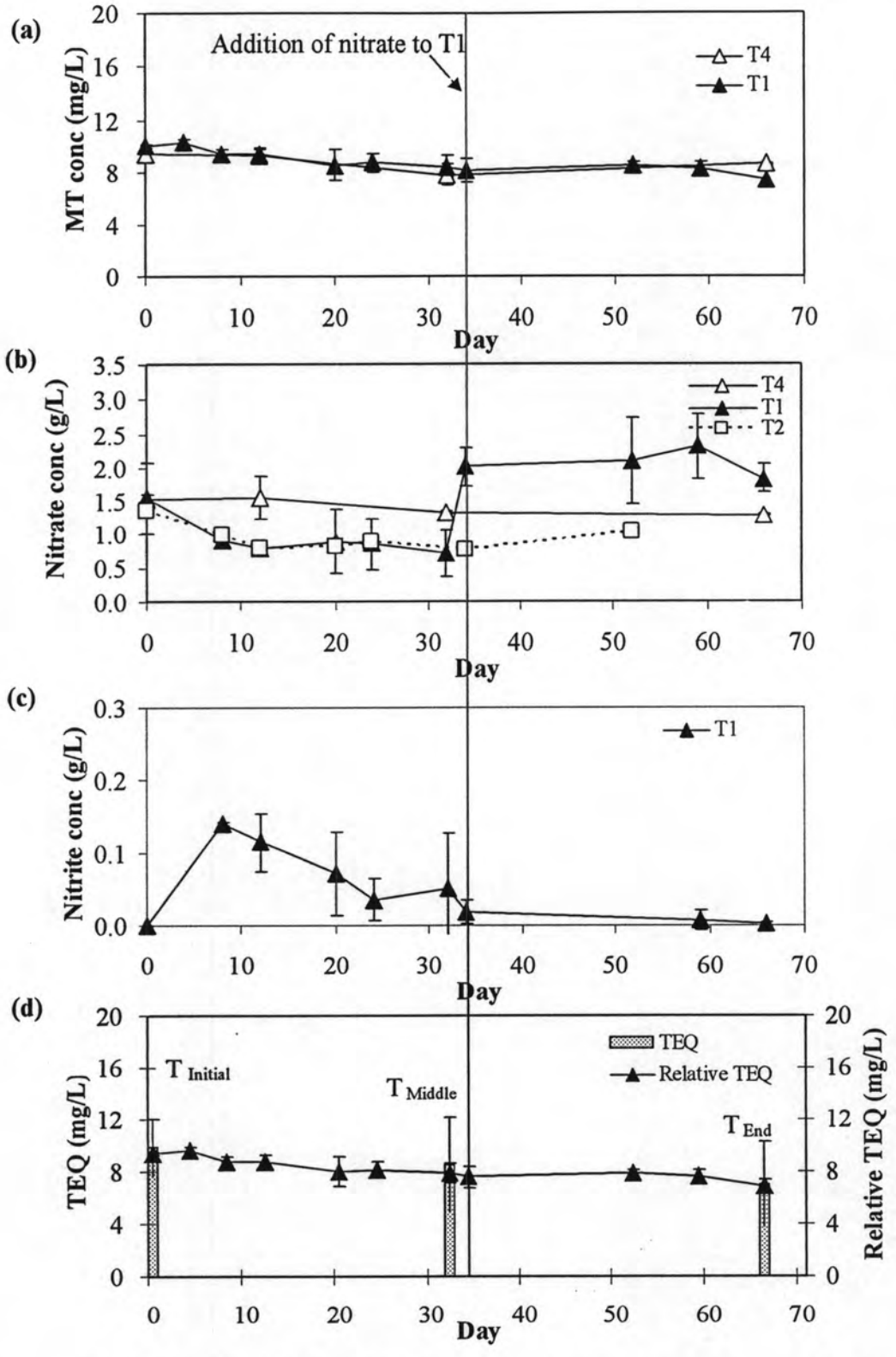

Figure 4.7 (a) Biodegradation of MT, changes in (b) nitrate concentrations, (c) nitrite concentrations and (d) androgenic activity in term of TEQ and relative TEQ under nitrate-reducing condition (T1: biodegradation test with MT (an initial concentration of 10 mg/L), T2: biodegradation test without MT and T4: control test with MT (an initial concentration of 10 mg/L) and sterilized sediment)

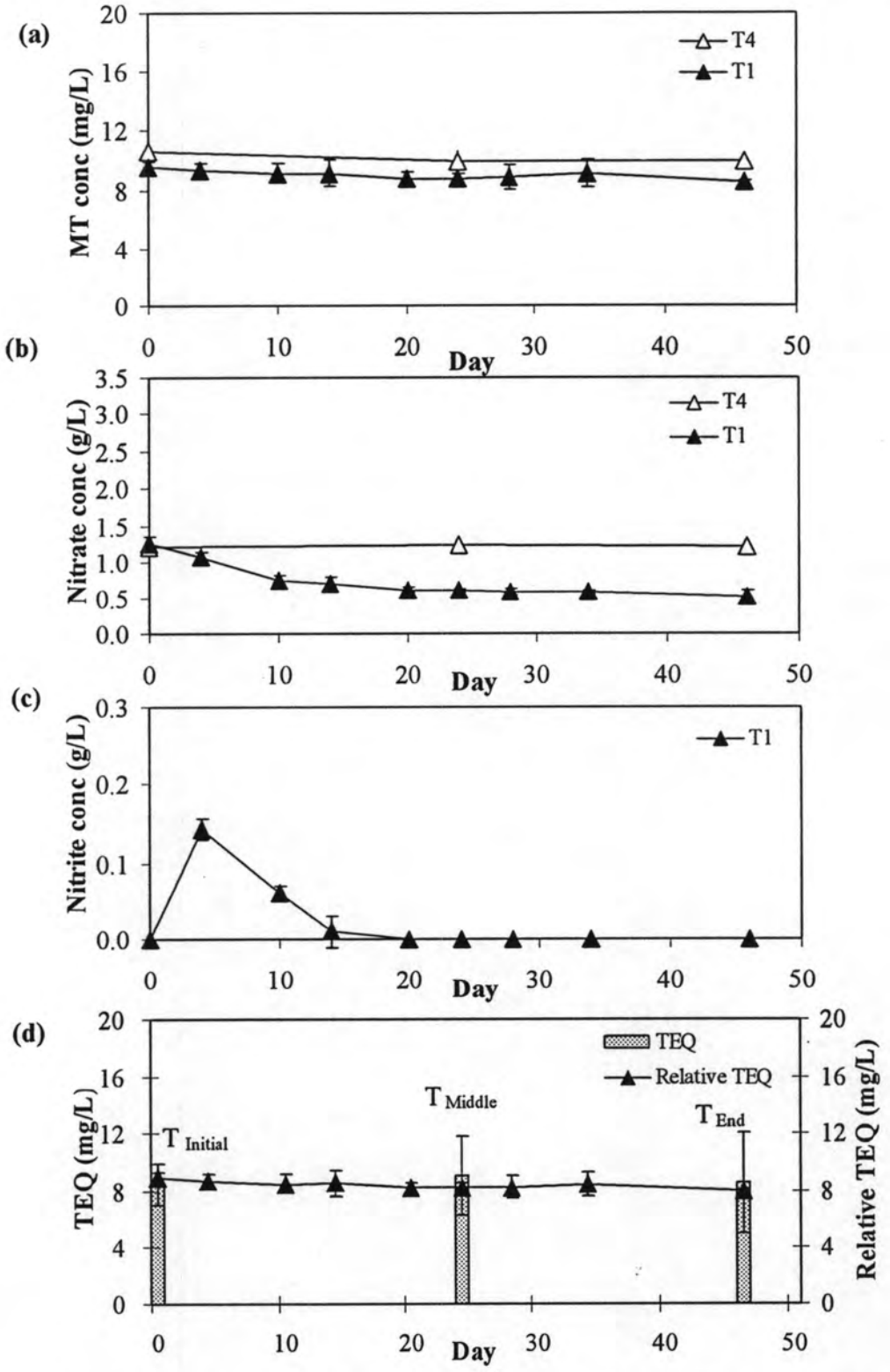

Figure 4.8 (a) Biodegradation of MT, changes in (b) nitrate concentrations, (c) nitrite concentrations and (d) androgenic activity in term of TEQ and relative TEQ under nitrate-reducing condition by firstly enriching with MT under aerobic condition (T1: biodegradation test with MT (an initial concentration of 10 mg/L) and T4: control test with MT (an initial concentration of 10 mg/L) and sterilized sediment)

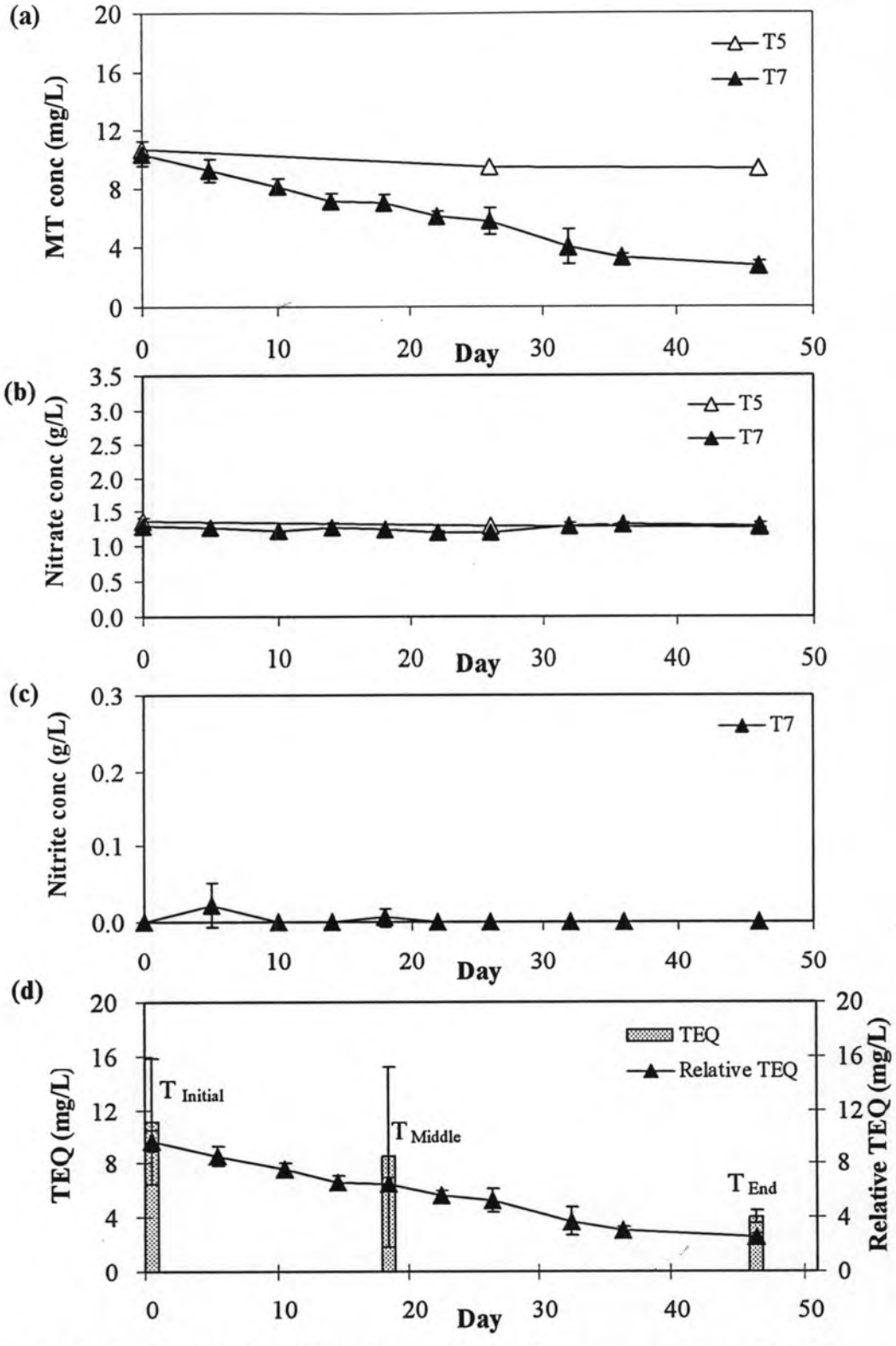

Figure 4.9 (a) Biodegradation of MT, changes in (b) nitrate concentrations, (c) nitrite concentrations and (d) androgenic activity in term of TEQ and relative TEQ under nitrate-reducing condition (T5: abiotic control test with MT (an initial concentration of 10 mg/L) without sludge) and T7: biodegradation test with MT (an initial concentration of 10 mg/L) by microorganism in sludge)

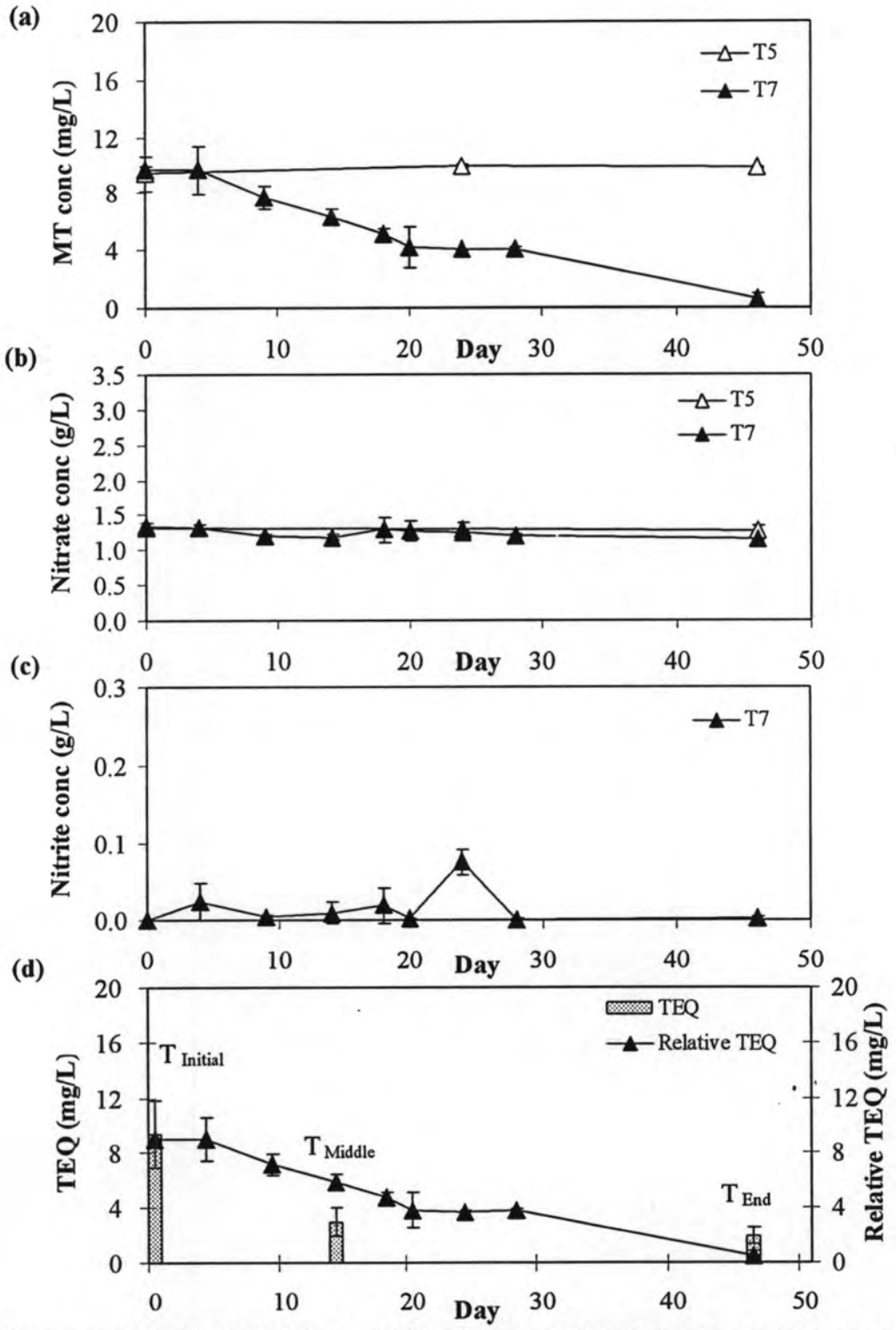

Figure 4.10 (a) Biodegradation of MT, changes in (b) nitrate concentrations, (c) nitrite concentrations and (d) androgenic activity in term of TEQ and relative TEQ under nitrate-reducing condition by firstly enriching with MT under aerobic condition (T5: abiotic control test with MT (an initial concentration of 10 mg/L) without sludge) and T7: biodegradation test with MT (an initial concentration of 10 mg/L) by microorganism in sludge)

Table 4.1 Lag time, first-order degradation rate (± 95% CI), and half life (± 95% CI) of MT under different electron acceptor conditions.

Electron acceptor condition	Lag time (day)	Degradation rate (d^{-1})	Half life (day)
Aerobic	2	0.51±0.34	1.8±1.4
Nitrate-reducing	4	0.004±0.00	181.0±52.8
Iron (III)-reducing	0	0.007±0.00	96.3±18.9
Sulfate-reducing	4	0.53±0.01	1.3±0.0
Methanogenic	4	0.69±0.24	1.1±0.4