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Biodegradation of Tetracycline Under Various Conditions and Effects on Microbial Community

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Abstract Five hundred tons of antibiotics are consumed yearly in the world. In this study, the biodegradation characteristics of tetracycline (TET) under nitrate-reducing, sulfate-reducing, and methanogenic conditions were determined by batch tests. Also, effects of TET on mixed microbial cultures were revealed by microbiological analysis. In this scope, gas generation and composition, dissolved organic carbon, and electron acceptor concentrations were monitored during 120 days. Additionally, changes on quantities of specific microbial groups were determined by Q-PCR. TET showed non-biodegradable behavior under nitrate- and sulfate-reducing conditions, whereas slightly biodegradable behavior under methanogenic conditions approximately 46 % degradation. The effects of TET on the abundance of mixed culture varied according to taxonomic units. Sulfate-reducing bacteria were inhibited by TET, while archaeal, bacterial, and methanogenic populations were not affected significantly.

Keywords Tetracycline · Anoxic · Anaerobic · Biodegradation test · Q-PCR · OECD 311

Introduction

Fate and occurrence of the human and veterinarian antibiotics in the environment have drawn great attention of researchers and public units because of the increasing concentrations of antibiotics within the natural and engineering ecosystems [1–3]. Average antibiotic concentrations in the receiving water bodies vary between nanograms per liter to micrograms per liter level, while their concentrations can be up to milligrams per kilogram level in the treatment

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sludges because of the accumulative behavior of the persistent compounds [4, 5]. Antibiotics usually cannot be fully metabolized by human and animal; thus, they and/or their metabolites are discharged to the sewage systems directly [6]. Also, hospitals, pharmaceutical industries, and livestock are counted as other facilities, which may discharge antibiotic-containing wastewaters to sewage systems [1]. During the transportation of antibiotics throughout treatment plants, elimination of these compounds can occur via biodegradation, photolysis, and sorption to sludge but treatment of these compounds cannot be achieved in conventional treatment plants and wastewaters are discharged to the receiving water bodies directly [6–8]. As a result of the introduction of metabolized and/or active antibiotics to the receiving water bodies caused an increase in the ratio of antibacterial-resistant pathogens [9, 10].

Tetracycline is one of the generally used antibiotics for treatment of infections of the respiratory tract. Also, this compound and its derivates are widely used as promoter in animal growth, so most of the studies have focused on the anaerobic digestion of manure, which contains this compound [11–16]. Tetracyclines inhibit the protein synthesis by binding 30S subunit of microbial ribosomes. The effects of the tetracycline can be reversible upon withdrawal of the drug. Thus, many bacterial strains have developed resistance to tetracycline [17]. Fate and occurrence of tetracycline have been investigated in activated sludge systems by many authors [1, 3, 6, 18]. Tetracyclines have been reported as non-biodegradable compounds that are easily sorbed to treatment sludges by binding to calcium and similar ions, thus forming stable complexes. [3, 6, 18]. Although biodegradability of tetracycline has been widely studied for aerobic systems, there are few studies about anaerobic biodegradability characteristics of tetracyclines wastewater treatment plants [19–21]. Most of the studies revealed that this compound is also almost non-biodegradable under anaerobic conditions. Cetecioglu et al. [21] showed the major fraction (>80 %) of the tetracycline introduced into the anaerobic reactor could be fully or partially biodegraded along with the organic substrate; however, it and its transformation products are still unknown. So, the authors could not speculate a complete mineralization. In the other studies about anaerobic degradation of manure, a wide range of degradation profile from 25 to 70 % was revealed [11, 16].

In this study, we aim to reveal the anoxic and anaerobic biodegradability characteristics of tetracycline and the effects of this compound on microbial community. In this scope, biodegradation capacity and the effects on the microorganisms were investigated by destructive batch tests based on a modified version of Anaerobic Biodegradability of Organic Compounds-OECD 311 protocol (OECD, 2006) under three different electron acceptor conditions, nitrate-reducing, sulfate-reducing, and methanogenic conditions. Quantification of defined microbial groups was also carried out to determine the effects of tetracycline on abundance of microbial community.

Materials and Methods

Experimental Approach

This study involved setting up batch biodegradation test to investigate biodegradation characteristics of tetracycline (TET) under anoxic and anaerobic conditions. The biodegradation microcosms were set up under nitrate-reducing conditions (NRC), sulfate-reducing conditions (SRC), and methanogenic conditions (MC). Experiment was carried out for 120 days. During the experiment, gas production was monitored daily. Wet chemical analysis [dissolved organic carbon (DOC) and electron acceptor measurements] and microbiological analysis [quantitative real-time PCR (Q-PCR)] were carried for four sampling time as days 0, 20, 60, and 120.

Batch Biodegradation Bottle Tests Set-Up

In this study, two different seed sludges were used for setting up of the batch tests. For NRC, the seed was taken from anoxic part of the domestic wastewater treatment plant, whereas test tubes for the SRC and MC were inoculated by anaerobic sludge from a full-scale UASB reactor treating alcohol distillery effluents.

The batch tests were conducted in 120 mL serum bottles and 100 mL of active volume according to modified OECD 311 protocol (Anaerobic Biodegradability of Organic Compounds in Digested Sludge: by Measurement of Gas Production) [22]. TET was chosen as the model carbon source.

The test tubes were set up as duplicates including positive and negative controls. Phenol was chosen as slowly biodegradable carbon source for positive control set. Negative control sets were constructed without any carbon source to determine endogenous decay. All sets were set up in an anaerobic cabinet (Coy Laboratory Products, USA). Experimental sets were destructed in four different sampling times. The first set was destructed immediately after all the test tubes were set up; the other three sets were sacrificed in days 20, 60, and 120. In each test tube, after inoculation, 2,000 mg/L TVS was maintained. Phenol and TET concentrations were adjusted to 90 ppm within the all experimental groups. Constituents of medium were given in the Table 1. All solutions were deoxygenated and adjusted to pH 7. Biodegradation microcosms were incubated at 20 °C and 35 °C for NRC and MC/SRC, respectively. All microcosms were stored at dark chambers to ensure occurring only biodegradation and sorption mechanisms during the experiment. The test tubes were shaken daily by hand. All microcosms were flushed by nitrogen gas to provide oxygen-free conditions. Also, resazurine was used in the medium to check anoxic/anaerobic conditions.

Headspace pressure was measured by handheld pressure transducer (Lutron PM-9107, U.S.A.) everyday. At each sampling time, biogas composition of the samples was determined via gas chromatography (Perichrom, France). DOC concentration of each sample was measured by Shimadzu ASI-V TOC analyzer (Japan). Nitrate and sulfate concentrations were measured by DIONEX ICS 1500 ion chromatograph (USA).

Table 1 Medium constituents for SRC, NC, and MC

Constituent	Amount for NC (g)	Amount for SRC (g)	Amount for MC (g)
Anhydrous potassium dihydrogen phosphate (KH_2PO_4)	0.27	0.27	0.27
Disodium hydrogen phosphate dodecahydrate ($Na_2HPO_4{\cdot}12H_2O$)	1.12	1.12	1.12
Ammonium chloride (NH_4Cl)	0.53	0.53	0.53
Calcium chloride dihydrate ($CaCl_2{\cdot}2H_2O$)	0.075	0.075	0.075
Magnesium chloride hexahydrate ($MgCl_2{\cdot}6H_2O$)	0.1	0.1	0.1
Iron (II) chloride tetrahydrate ($FeCl_2{\cdot}4H_2O$)	0.02	0.02	0.02
Resazurin (oxygen indicator)	0.001	0.001	0.001
Sodium sulfide nonahydrate ($Na_2S{\cdot}9H_2O$)	0.1	0.1	0.1
Stock solution of trace elements (prepared as suggested by OECD 311)	10 mL	10 mL	10 mL
Vitamin solution (prepared as suggested by OECD 311)	5 mL	5 mL	5 mL
Potassium nitrate (KNO_3)	1	–	–
Potassium sulfate (K_2SO_4)	–	1.8	–
Diluted with deoxygenated water	to 1 L	to 1 L	to 1 L

Calculation of Mass Balance

Theoretical CO_2 (Th CO_2) and theoretical biogas (Th biogas), which were used for evaluation of biodegradation, were calculated according to DOC and gas and ion chromatography results. Mass balances were calculated by the assumptions, which were described by Ritmann and Mc Carty [23]. Simplified mass balances were given in Equations 1–3 for NRC, SRC and MC, respectively.

$$\mathrm{TET} + \mathrm{NO_3^-} \rightarrow \mathrm{Biomass} + \mathrm{CO_2} + \mathrm{N_2} + \mathrm{H_2O} \quad (1)$$

$$\mathrm{TET} + \mathrm{SO_4^{2-}} \rightarrow \mathrm{Biomass} + \mathrm{CO_2} + \mathrm{H_2S} + \mathrm{HS^-} + \mathrm{H_2O} \quad (2)$$

$$\mathrm{TET} + \mathrm{H_2O} \rightarrow \mathrm{Biomass} + \mathrm{CO_2} + \mathrm{CH_4} \quad (3)$$

Biodegradation ratios were estimated with two different approaches:

(a) ThCO_2 and Th biogas production (which were assumed to be produced as a result of 100 % biodegradation of tetracycline) were compared to actual CO_2 and biogas production within the batch tests.
(b) Evaluation of DOC removal.

Microbiological Analysis

Genomic DNA (GDNA) was extracted from 0.5 g sludge using the Fast DNA Spin Kit for Soil (Qbiogene Inc., UK) following the manufacturer's instructions.

Q-PCR procedure recommended by Roche was followed and a Light Cycler Master Kit (Roche, Applied Science, Switzerland) was used to set up the reaction (2.0 μl master mix, 1.6 μl MgCl2 1.0 μl primer F and R, 13.4 μl H2O, and 1 μl sample). Absolute quantification analysis of the GDNA was carried out with a Light Cycler 480 Instrument (Roche Applied Science, Switzerland). Bac 519f- Bac 907r [24], Arc 349f- Arc 806r primers [25], and Met 348f- Met 786r [26] and DSRp 2060f- 4r [27] were used for quantification of bacteria, Archaea and methanogens, respectively. Triplicate experiments were carried out for each extracted GDNA during Q-PCR analysis.

Statistical Analyses

Significant differences between positive control and TET microcosms were determined according to independent sample *t* test. Pearson correlation was used for the interactions between variables between chemical and microbiological analysis. The mean values and standard errors in Q-PCR experiments were also calculated. All the statistical analyses were conducted by using SPSS (IBM, USA), and $p<0.05$ level was used for significance.

Results and Discussions

Reliability of the Test Procedure

Biodegradation tests were performed under three different electron acceptor conditions with positive and negative control groups during 120 days. In all three electron-accepting conditions, phenol that was organic carbon source in positive control microcosm was ultimately biodegraded

(74–78 % in 120 days). Therefore, biomass used in test procedure could be assumed as metabolically active.

The CO_2 productions in the negative control microcosms reached a total of 4–12 mL in 120 days corresponding to 55–100 % of the Th CO_2 production while biogas production reached 40 mL corresponding to 100 % of the Th biogas occurred via degradation of biomass completely. In this case, measured CO_2 and biogas production subtracted as blanks to reduce the inaccuracy of the biodegradation percentage as suggested by OECD 311 protocol [22].

Biodegradation of Tetracycline

Figure 1 shows ultimate biodegradation (evaluated according to gas production only derived from TET biodegradation) and DOC removal (percent DOC) for each electron-accepting condition throughout the operating period. Tetracycline showed non-biodegradable behavior under SRC and NRC, whereas slightly biodegradable behavior under MC.

Under NRC, DOC concentrations decreased from 90 to 38 ppm, while nitrate concentration fell dramatically in the first 20 days from 220 to 3 ppm. Rest of the experiment, the change in the nitrate concentration was determined as only 1.5 ppm. DOC removal of 58 % was achieved. CO_2 production derived from TET biodegradation reached 1.5 mL within 60 days, which comprise

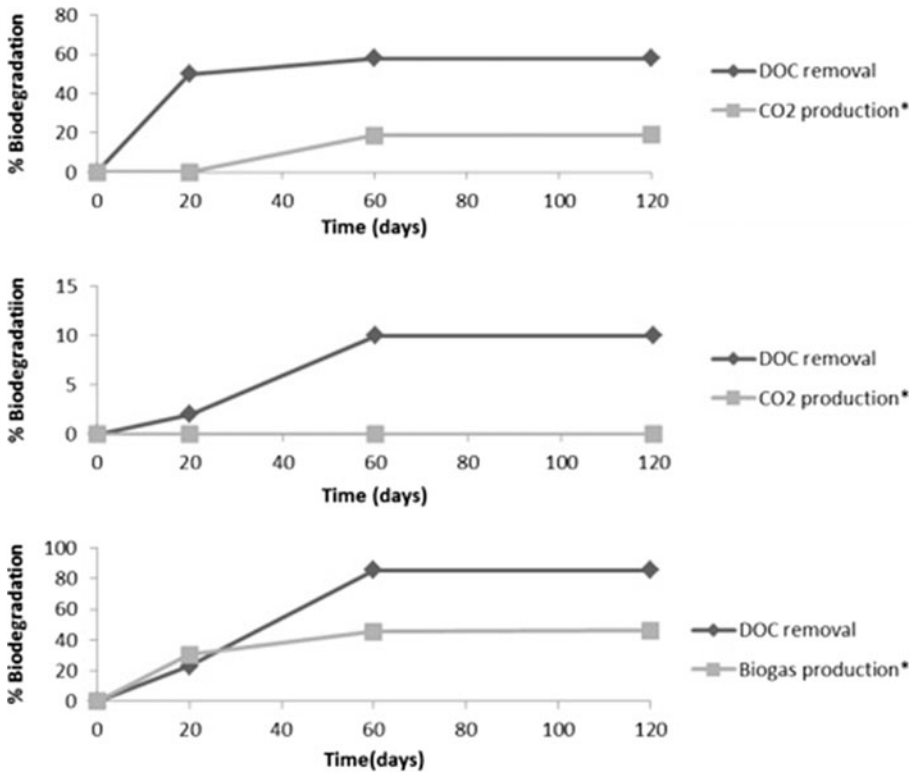

Fig. 1 Biodegradation of TET under different e^- accepting conditions **a** NRC, **b** SRC, **c** MC (*gas production was calculated with the amount of the gas produced as a result of a TET biodegradation, excluding endogenous decay)

19 % of the Th CO_2 production. Difference between CO_2 production and DOC removal can be interpreted by the sorption of the tetracycline into the treatment sludge. In this concept, 39 % of the observed DOC removal derived from the sorption rather than biodegradation.

Under SRC, according to CO_2 production, no biodegradation occurred. Gas production was mainly caused by the endogenous decay during 120 days. DOC concentrations decreased from 90 to 80 ppm. During 120 days, sulfate concentrations in the microcosms decreased from 43 to 34 ppm. 1Ten percent DOC removal was observed. Results confirmed that biodegradation of TET under SRC and NRC cannot be achievable.

Results from NRC and SRC experimental tests showed similarity with the studies that involves aerobic biodegradation characteristics of TET. Gartiser et al. [28] used inherit tests to assess the biodegradability of several antibiotics. In this study, TET proved to be non-biodegradable, whereas DOC removal reached up to 84 % in 28 days. Alexy et al. [29] also reported TET as a non-biodegradable substance. They also concluded that the removal of TET (30 %) to abiotic factors.

Under MC, DOC concentration decreased from 90 to 10 ppm. Biogas production derived from TET biodegradation reached up to 35 mL in 120 days. The amount of the produced biogas comprised 46 % of the Th biogas production. DOC removal increased up to 89 %. This major difference between two evaluation methods may be derived from sorption behavior of tetracyclines as observed in the case of biodegradation of this compound under NRC. Forty percent of the TET may be sorbed into the anaerobic sludge. However, Cetecioglu et al. [21] showed that sorption mechanism during TET removal in the anaerobic systems can be negligible. Removed TET (46 %) by biodegradation was higher than the findings from aerobic biodegradation studies [28, 29].

Biodegradation of TET under anaerobic conditions is not properly documented. There are considerably limited studies about the relevant subject. One of them showed the biodegradation of four veterinary antibiotics in aerobic and anaerobic surface water simulation systems by analytical detection. In this study, the selected antibiotics were found as slowly biodegradable substances [19]. Another study belongs to Gartiser et al. [20] who investigated the inhibition and biodegradation of several antibiotics. Thus, the study focused on inhibition; no decisive conclusion was gathered. The most comprehensive study about TET behavior during long-term methanogenic operation in the literature belongs to Cetecioglu et al. [21]. The authors mentioned that more than 80 % of TET can be mineralized in terms of mother compound. Also, the inhibitory effect of TET under long-term operation is higher than short-term test on anaerobic consortium [21, 30].

Biodegradation characteristics of TET in the wastewater treatment plants (WWTP) show similar results with the data from SRC and NRC of this study. Many authors indicated the non-biodegradability and high sorption behaviors (up to 90 %) [3, 6, 29]. In our study, sorption rates were relatively lower than the observed ratios in the WWTP. This may cause from the higher amounts of hardness ions within the activated sludge processes that can cause chelation [3].

Microbiological Analysis

Q-PCR analyses have been carried out for four sampling times. Four different taxonomic groups were quantified. These were bacteria, Archaea, methanogenic Archaea, and sulfate-reducing bacteria (SRB). There was no significant change in the amount of these populations during 120 days. Also, no correlation found between DOC removal rate and the amount of these groups. Figure 2 shows the changes in the bacterial, archaeal, and SRB populations during the experiment, respectively.

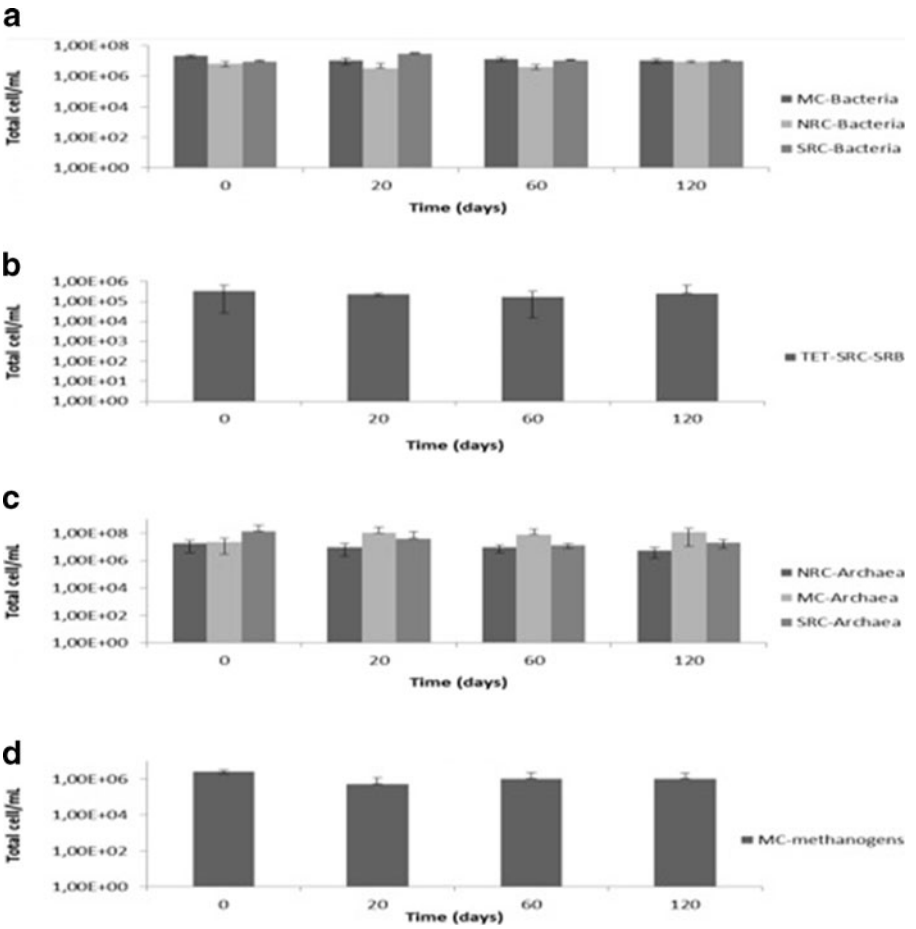

Fig. 2 Changes in the microbial groups within the batch tests through 120 days **a** bacteria, **b** SRB, **c** Archaea, **d** methanogens

In this case, there are two possible approaches to explain the situation for bacterial community: (a) Tetracycline may have an inhibition on bacterial growth as described by Loftin et al. [31] and Gartiser et al. [20, 28]. (b) Iwane et al. [32] reported approximately 8 and 6.7 % of tetracycline-resistant bacteria to be found in domestic wastewater treatment plants. Based on that knowledge, bacterial populations in the batch tests might have gained resistance to TET with time but would not able to use TET efficiently. In this case, bacterial populations lowered their metabolic functions to survive rather than grow. For SRB, despite of no important changes occurred in batch tests amount of the SRB was correlated by the gas production derived from biodegradation of TET under SRC. Since there was no biodegradation under SRC, TET might have inhibited the activity of the SRB.

Figure 2c shows there was no significant change in the archaeal population during 120 days. This can be mainly explained by the fact that TET does not affect the Archaea excluding only Halobacteriales [33].

Figure 2d shows the changes in the amount of methanogenic population under MC. Significant changes were observed in the numbers of methanogens between the 0th and 20th

days on the contrary of other microbial groups. Total methanogenic cell numbers decreased from 2.76E6 to 5.53E5 total cell/mL. Eighty percent decrease in the numbers of methanogenic population occurred after start up of the experiment. However, the abundance of the methanogens increased by 10 % within the archaeal population during this period. Decrease in the amount of population can be derived from the microbial interactions. Thus, methanogens were not affected by TET. Otherwise, biogas production would not have occurred or would have been inhibited because of their susceptibility to toxic compounds [33]. However, Sanz et al. [34] indicated that chlortetracycline, which is an antibiotic from the same group of TET, inhibited acetoclastic methanogens and was considered a potential inhibitor of archaea. Furthermore, Stone et al. [15] speculated that chlortetracycline might have contributed to inhibition of acetoclastic methanogens. In the same study, the VFA concentration during the operation of anaerobic digestor increased.

Conclusion

While TET showed a non-biodegradable behavior under SRC (0 %) and NRC (19 %), a slight biodegradation was determined under MC (46 %). However, the biodegradation rate under MC can be lover in real full-scale anaerobic reactors because of the higher ionic strength within the treatment systems.

Effects of TET on microbial mixed cultures varied between the microbial groups:

- SRB was inhibited by TET.
- Methanogens and Archaea were not affected by TET.
- Bacterial populations were either inhibited or gained resistance to TET but they were unable to utilize TET efficiently.

Further antibiotic and metabolite measurements are planned for future studies to gather additional information on fate of TET. Also, resistance genes can be monitored by Q-PCR to get decisive knowledge about the population dynamics whether the population was inhibited or gained resistance to TET. Additionally, expression of 16S rRNA gene can be investigated to clarify activity of the microorganisms.

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