İSTANBUL TECHNICAL UNIVERSITY ★ INSTITUTE OF SCIENCE AND TECHNOLOGY

DETERMINATION OF ANAEROBIC AND ANOXIC BIODEGRADATION CAPACITY OF SULFAMETHAXOSOLE AND THE EFFECTS ON MIXED MICROBIAL CULTURE

M.Sc. Thesis by
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Programme : Envrionmental Biotechnology

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SULFAMETOKSAZOLUN ANAEROBİK VE ANOKSİK BİYODEGREDASYON KAPASİTESİNİN BELİRLENMESİ VE MİKORBİYOLOJİK KÜLTÜR ÜZERİNDEKİ ETKLİLERİ

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FOREWORD

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ABBREVIATIONS

BOD	: Biological Oxygen Demand
COD	: Chemical Oxygen Demand
DDD	: Defined Daily Dose
ddH_2O	: Double Distilled Water
DNA	: Deoxyribonucleic Acid
dNTP	: Deoxyribonucleotide triphosphate
EtBr	: Ethidium Bromide
FEDESA	: European Federation of Animal Health
GC	: Gas Chromotograph
gDNA	: Genomic DNA
HPLC	: High Pressure Liquid Chromotography
ITS	: Internal Transcribed Spacer
MGB	: Minor Groove Binder
MPN-PCR	: Most Probable Number PCR
N	: Nitrogen
NTC	: No Template Control
P	: Phosphorus
Q-PCR	: Quantitative PCR
RNA	: Ribonucleic Acid
rRNA	: Ribosomal RNA
RT	: Reverse Transcription
RT-QPCR	: Real Time Quantitave Polymerase Chain Reaction
SIP	: Stable Isotype Probing
SMX	: Sulfamethaxosole
SRB	: Sulfate Reducing Organism
SS	: Suspended Solids
TOC	:Total Organic Carbon
TS	: Total Solids
TVS	: Total Volatile Solids
UASB	: Upflow Anaerobic Sludge Blanket
US	: United States of America
UV	: Ultra Violet Light

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DETERMINATION OF ANAEROBIC AND ANOXIC BIODEGRADATION CAPACITY OF SULFAMETHAXOSOLE AND THE EFFECTS ON MIXED MICROBIAL CULTURE

SUMMARY

Antibiotics which are known to be xenobiotics substances, that accumulate in the environment. Approximately 90% of the consumed antibiotics are excreted by urine and feaces, etiher as active substances or metabolites. Thus, the antibiotics reach the domestic sewage plants, and can't be treated in non-conventional systems, leading to the discarge of antibiotics to the receiving water bodies. They can have adverse effects on the environment, both affecting aquatic and terrestrial organisms. Humans can also be affected via drinking water that is contaminated by antibiotics even though there are no reports showing that antibiotics are present in drinking water.The microbial community in the sewage systems is prone to be affected by Antibiotics. If the microorganisms in the wastewater treatment, system is inhibited, the organic matter degradation may be seriously affected, therefore the inhibition effects of antibiotics on microbial population create a great risk. Also antibiotics especially cause the increase of antibiotic resistance pathogens and these organisms threat firstly public health, then plants and animals.

In the completed project, the biodegredation capacity and the effects on microbial culture have been investigated for Sulfamethaxasole (SMX) which is an antibiotic under methanogenic, sulfate reducing and nitrate reducing conditions, with the help of determination of quantities of the microbial communities that are responsible for the degredation, along with the TOC, gas composition, electron acceptor and antibiotic concentration monitoring.

In this scope, batch tests were set-up, to be distrupted at different sampling times. Gas generation and composition, TOC, antibiotic and electron acceptor concentrations were monitored for 120 days. In addition, the change of the quantity of specific microbial groups were analyzed by Quantitative PCR (Q-PCR).

By a profound examination of the results obtained by the experiments that have been carried out through-out the project, a general conclusion for the biodegradation of SMX was obtained. As a result, SMX was found out to be utilizable as a carbon source under three different electron conditions; methanogenic, sulfate reducing and nitrate reducing.

SULFAMETOKSAZOLUN ANAEROBİK VE ANOKSİK BİYODEGREDASYON KAPASİTESİNİN BELİRLENMESİ VE MİKORBİYAL KÜLTÜR ÜZERİNDEKİ ETKLİLERİ

ÖZET

Zenobiyotik olduğu bilinen antibiyotikler, çevrede birikmektedirler. Tüketilen antibiyotiklerin yaklaşık %90 kadarı dışkı ya da urin yoluyla dışarıya aktif halde ya da metabolitler halinde atılmaktadır. Bu nedenle antibiyotikler domestik arıtma sistemlerine ulaşmakta, ve konvensiyonel olarak arıtılamaması sayesinde, alıcı su ortamlarına ulaşmamaktadır. Antibiyotikler karada ve suda yaşayan canlıları etkileyerek, çevreyi negatif yönde etkilemektedir. İnsanlar da antibiyotiklerden içme suyu yoluyla etkilenebilirler, ancak şu ana kadar çeşitli sularda antibiyotik tespit edilse de, içme suyunda antibiyotiklerin varlığı tespit edilmemiştir. Arıtma sistemlerindeki mikrobiyal komunite antibiyotiklerin etkilerine maruz kalmaktadır. Eğer arıtma sistemlerindeki mikroorganizmalar inhibe olurduğunda, organik madde degredasyonu ciddi şekilde etkilenmektedir. Bu nedenle antibiyotiklerin mikrobiyal komunite üzerindeki etkileri büyük önem taşımaktadır. Ayrıca, antibiyotiklerin patojenlerdeki antibiyotik resistansını arttırması, ve patojenlerin öncelikle toplum sağlığı olmak üzere, bitki ve hayvan sağlığını negatif yönde etkilemesi, antibiyotiklere verilen önemi arttırmaktadır.

Tamamlanan projede, bir antibiyotik olan Sulfametaksazolün (SMX) metanojenik, sülfat indirgeyici, ve nitrat indirgeyici ortamlarda, biyodegredasyon kapasitesi ve etkileri, TOK, gas kompoziyonu, elektron alıcısı ve antibiyotik konsantrasyonlarının değişimi ve degradasyondan sorumlu mikrobiyal komunitenin Q-PCR yönetemiyle belirlenmesi ile saptanmıştır.

Bu kapsamda, kesikli reaktör şişeler, farklı numune alım zamanlarında bozulmak üzere kurulmuştur. Gaz üretimi ve kompoziyonu, TOK, antibiyotik ve elektron alıcı konsantrasyonları 120 gün boyunca izlenmiştir. Ayrıca spesifik mikrobiyal grupların populasyonlarındaki değişimler Q-PCR analizleriyle izlenmiştir.

Elde edilen sonuçların detaylı incelemesi sonucunda, SMX biyodegredasyonu hakkında genel bir sonuca varılmıştır. Sonuç olarak, SMXin metanojenik, sülfat indirgeyici ve nitrat indirgeyici ortamlarda karbon kaynağı olarak kullanılabildiği görülmüştür.

1. INTRODUCTION

Antibioitcs are chemotherapeutic agents that inhibit or abolish the growth mechanisms of microorganisms (Kümmerer, 2008). They are considered as xenobiotics, which are unfamiliar compounds to the existing enzyme systems, persistant in the environment, and resistant to biodegredation. In addition, antibiotics pose a risk of adverse effects to human health and the environment (van der Meer et al., 1992).

Not only antibiotics, but also many pharmaceuticals are classified as xenobiotic compounds. The global consumption of drugs (as total pharmaceutical formulation) produced and used by the humans is estimated to be 100,000 metric tons per year, which corresponds to a worldwide average consumption of approximately 15g per capita (Kümmerer, 2004). Antimicrobials are the most often discussed pharmaceuticals because of their potential role in the spread and maintenance of (multi-)resistance of bacterial pathogens. As xenobiotics, antibiotics are a major concern, due to the high amount of consumption and production.

Antibiotics haven't received attention until recently, even though antibiotics are in use for decades. In the recent years, investigations of antibiotic substances have been made, in order to find out the environmental risks they may create. Approximately 90% of the consumed antibiotics are excreted by urine and feaces, either as active substances or metabolites. In conventional sewage treatment systems, they are partially eliminated, thus the remaining amounts can reach surface waters, ground waters or sediments (Kümmerer, 2008a). For these reasons, antibiotics can reach the environment with no or little elimination, causing the effects of antibiotics on the environment to be more drastic.

The effects of antibiotics on the environment are adverse, both affecting aquatic and terrestrial organisms. In addition, humans can also be affected via drinking water that is contaminated by antibiotics even though there are no reports showing that antibiotics are present in drinking water. Furthermore, the microbial community in the sewage systems is prone to be affected by antibiotics. If the bacteria in the wastewater treatment, system is inhibited, the organic matter degradation may be seriously

affected, therefore the inhibition effects of antibiotics on microbial population create a great risk.

If antibiotics are not degraded, and enter the environment, it is possible that these residues will lead to development of antibiotic resistant microbial populations in the environment (Witte, 1998). The increase of antibiotic resistant pathogens and these organisms threat firstly public health, then plants and animals.

In this scope, sulfamethoxasole (SMX) was chosen to be the antimicrobial, for the investigation of the effects on microbial culture and biodegredation capacity. The main reason for chosing Sulfamethaxosle as the xenobiotic, in which the effect and biodegredation will be investigated, is the heavy usage of SMX both in human and veterinary medicine. Sulfamethoxasole is an antimicrobial that inhibits the growth of the organism by distrupting the folic acid cycle, under the group of sulfanomides.

2. AIM

Antibiotics are one of the most consumed and problematic pharmaceuticals in the world. The yearly consumption of antimicrobials worldwide is estimated between 100.000 and 200.000 tons (Wise, 2002). The main problem of antibiotic utilization is that approximately 90% of the consumed antimicrobials are excreted via urinary or fecal pathways from the human and animals after partial or no metabolism and they are transferred to the domestic sewage plants or directly to the environment. Antibiotics are classified as xenobiotic, which is resistant to biodegradation, so conventional biological treatment of domestic sewage provides very low – if any – reduction for the antimicrobials, which usually by-pass treatment and accumulate in the receiving waters. Direct discharge of these compounds to the environment cause the uncontrolled increase the (multi-)resistant pathogens.

For this reason, the aim of this thesis is to investigate the biodegradation profile of sulfamethoxasole (SMX) that is used commonly for the disease of human and animal. In this scope, biodegredation capacity and the effects on the microogranisms investigated by destructive batch tests based on Anaerobic Biodegradability of Organic Compounds-OECD 311 protocole under three different electron acceptor conditions; methanogenic, sulfate reducing, and nitrate reducing condtions. For the reability of the experimental results, experimental control test were set-up. Gas generation and composition, TOC, antibiotic and electron acceptor concentrations were monitored for 120 days. In addition, the change of the quantitiy of specific microbial groups were analyzed by Quantitative PCR (Q-PCR). All of the results were statisticly analyzed in order to view the connections between different parameters, effected by SMX.

3.THEOROTICAL BACKGROUND

3.1 Xenobiotics

Xenobiotics called as unfamiliar compounds, inside existing enzyme systems, which are persistent in the environment and resistant biodegradation like heavy metals, metalloids and man-made organic compounds. The reason why xenobiotics are in the spotlight and why they need to be treated is that these chemicals persist in the environment, bioaccumulate through food chain, and pose a risk of adverse effects to human health and the environment (van der Meer et al., 1992).

Because of the effects of Xenobiotics on the environment and the increase in usage and production, Xenobiotics are a rising concern in urban water cycle. Some inorganic elements like the heavy metals, metalloids and organic compounds such as pesticides, surfactants, preservatives, solvents, fragrances, flavors, endocrine disrupters and pharmaceuticals are defined as xenobiotic. In order to control the affects of xenobiotics on the environment, the information of the sources, flow paths, fate (transport, treatment, and natural attenuation) and impact on both humans, livestock and the environment must be followed.

Xenobiotics directly interfere with the urban water cycle, as a result of their high production and usage, since the water supply, urban drainage and the wastewater treatment systems were originally designed to solve conventional problems (protection of receiving water for downstream water supply, ecological integrity and sanitation) considering conventional parameters (oxygen situation, carbon, nutrients, hygienic parameters).

In the urban water systems, there are many different sources where xenobiotics can enter. The chemical pollution in rainfall-runoff and wastewaters resulting from atmospheric washout, erosion of building materials, traffic emissions, pesticides application, industrial production, and use of household chemicals, personal care products and pharmaceuticals are the main sources of pollution by xenobiotics. The use of rainwater and reuse of wastewater for industrial and domestics non-potable

purposes further increase the exposure to xenobiotics, however the conventional urban water cycle approaches are not designed to deal with xenobiotics.

Mainly the pollutants of interest have been the conventional parameters (BOD (Biological Oxygen Demand), COD (Chemical Oxygen Demand), N, P, SS (Suspended Solids) and microorganisms) which do not represent xenobiotic compounds. Thus, there is a need to understand, in an integrated manner, the sources, flow paths, fate (transport, treatment, natural attenuation) and impact of xenobiotics on both humans and environment in this technical system.

Innovative approaches and treatment technologies are therefore needed to prevent xenobiotics from being discharged into surface waters where they may give rise to impacts on the chemical water quality and ecological status of receiving waters.

In this context, determination of biodegradation characteristics of these refractory compounds and their effects on microbial community is substantial for environmental engineering. Anoxic and anaerobic degradation of these compounds have not been studied yet.

3.2 Antibiotics

Pharmaceuticals are classified as xenobiotic compounds. The global consumption of drugs (as total pharmaceutical formulation) produced and used by humans is estimated to be 100,000 metric tons per year, corresponding to a worldwide average pro capita consumption in the range of 15g/capita (Kümmerer, 2004). Antimicrobials are the most often discussed pharmaceuticals because of their potential role in the spread and maintenance of (multi-)resistance of bacterial pathogens as xenobiotics, antibiotics are a major concern, due to the high amount of consumption and production.

Antibiotics are chemotherapeutic agents that inhibit or abolish the growth mechanisms of microorganisms (Kümmerer, 2008b). Antibiotics are used in order to prevent and treat diseases in the field of medicine, veterinary medicine, farming and aquaculture. Some of the antibiotics are used for purposes other than human and veterinary medicine. For example, streptomycins are used in fruit crops, while other antibiotics are used in beekeeping (Kümmerer, 2008a).

The terms like chemotherapeutics or microbial are not synonymous. For example, an antimicrobial that has an effect on viruses is not an antibiotic. Chemotherapeutics are compounds that are used in order to treat a disease which kill cells. Antibiotics

originally referred to any compound that had biological activity against living organisms, today however all the substances that has antibacterial, anti-fungal, or anti-parasitical activity are referred as antibiotics (Kümmerer, 2008b). When the antibiotics were first used, they were naturally originated, like penicillin produced by fungi in the genus Penicillium, or streptomycin from bacteria of the genus Streptomyces. Today antibiotics are obtained by chemical synthesis, or by the chemical modification of compounds of natural origin. Definition of the antibiotics has been changed over the years. Initially it was defined as a compound produced by a microorganism, which inhibits the growth of another microorganism, then over the years the definition changed to a drug that kill or inhibit bacteria, fungi or viruses. (Kümmerer, 2008b).

3.2.1 Physical and Chemical Properties of Antibiotics

The antibiotics are grouped either by their chemical structure or by mechanism of action. They are subdivided into groups such as ß-lactams, quinolones, tetracyclines, macrolides, sulphonamides and others. Antibiotics may possess different functionalities within the same molecule, so under different pH conditions antibiotics can be neutral, cationic, anionic, or zwitterionic. For example ciprofloxacin, can possess both basic and acid molecule functions, depending on the pH. At a pH of 7.04, the isoelectric point of ciprofloxacin, it carries both a negative and a positive charge (Kümmerer, 2008). Solubility, hydrophobicity and hydrophilicity are pH dependent. Thus along with pH, the physico-chemical and biological properties such as Pow, sorption behavior, photo reactivity and antibiotic activity may change with pH. (Kümmerer, 2008b).

The main property of antibiotics is the excretion after administration. As an example many antibiotics that are being used in animal food producing industry are partially absorbed in the gut of the animals, so that the 30–90% of the parent compound is excreted directly (Elmund et al., 1971; Feinman and Matheson, 1978; Alcock et al., 1999). Most of the antibiotics are water-soluble so that up to 90% of one dose can be excreted in urine and up to 75% in animal feces (Halling-Sørensen, 2001).

Another property of antibiotics is the transformation of antibiotics metabolites to the parent compound after excretion, caused by the bioactivity of the antibiotic metabolites (Langhammer, 1989). This leads to the excretion of a significant percentage of the administered antibiotics into the environment in active forms (Warman and Thomas, 1981; Berger et al., 1986). For example, the excreted

sulfamethazine metabolite, glucoronide of N-4-acetylated sulfamethazine, is converted back to the parent form in liquid manure (Berger et al., 1986).In the liver, sulfamethazine undergoes conjugation with sugars present and thus inactivates the compound. After the metabolites are excreted, microbes can rapidly degrade the sugars, thereby allowing the compounds back to their bioactive forms (Renner, 2002). The antibiotics are excreted into the environment with no or little elimination. If they are not degraded, and enter the environment, it is possible that these residues will lead to development of antibiotic resistant microbial populations in the environment (Witte, 1998).

3.2.2 Antibiotics in the Environment

In sewage treatment plants, they are partially eliminated, thus the remaining amounts can reach surface waters, ground waters or sediments. Even though it has been decades, that the antibiotics are uses in large amounts, the existence of these substances in the environment did not receive any attention until recently. Today, a more detailed investigation of antibiotics in the environment and its effects on the environment is carried out (Kümmerer, 2008a).

There have been many studies, in order to determine if there are antibiotics that has been introduced to environment. In order examine, analyses were done on samples taken from the environment. Even though antibiotics are not found in all environmental conditions, there have been some cases, where different kinds of antibiotics were detected. In the rivers of Italy, tylosin, oleandomycin and spiramycine were found (Zuccato et al., 2000). In Swiss surface waters, Alder et al. (2001) detected sulfamethazine and some other groups of antibiotics used in veterinary medicine, and decided that the source was from a runoff of a land-applied manure. In addition, the USGS reported the occurrence of 21 antibiotic compounds in samples collected from 139 streams across a number of US sites (Sarmah et al., 2006).

3.2.3 Production and Consumption

The total consumption of antibiotics for humans vary from country to country (Mölstad et al., 2002). Antibiotic use ranges from 8.6 to 36 DDD per day and capita in Europe. ß-lactam antibiotics, including the sub-groups of penicillins, cephalosporins and, as a marginal fraction carbapenems and others, make up the largest share of

human use antibiotics in most countries. They account for approximately 50–70% of total antibiotic use. (Kümmerer, 2008a).

Even though antibiotics are sold over the counter, without any prescription consumption may still be higher. Reliable data providing information on the total use and the use patterns of antibiotics and the per capita consumption only exist for a few countries. In Germany, 250 different antibiotics and antimycotic substances are used in medicene ana veterinary medicine (Kümmerer and Henninger 2003). As for prescription rates, they vary from country to country (Mölstad et al. 2002).

It is expected to find out that the hospitals are the main source of antibiotics in municipal sewage, however comunity use is reported as 70% in the U.K. (United Kingdom) (House of Lords 1998) and 75% in the U.S. (Wise, 2002). In Germany, about 75% of antimicrobials are used in the community and 25% in hospitals (Kümmerer and Henninger, 2003). The percentage of the antimicrobial usage in hospitals, can get even more smaller in some countries. For example in Oslo it was found that less than 10% of certain analyzed antibiotics were from hospitals (Thomas et al., 2007).

A single antibiotic can have different usages, variying from one country to another. As an example, vancomycin is heavily used in the U.S., whereas in Germany it is only used in cases where all other possible compounds proven to be ineffective due to the resistance against the other antibiotics that were used. It is estimated that the antibiotic consumption worldwide lies between 100 000 and 200 000 t per annum (Wise ,2002). In 1996, about 10 200 t of antibiotics were used in the EU, about 50% were applied in veterinary medicine and as growth promoters. In 1999, 13 216 t of antibiotics were used in the European Union and Switzerland according to data supplied by the European Federation of Animal Health (FEDESA, 2001), of which 65% were applied in human medicine. For the E.U.(European Union) in total 22 g per capita and year would result from the use in medicine. For the U.S. about 17 g per capita and year can be calculated from the available data for use in human medicine.(Kümmerer, 2008a)

The excretion rates for the unchanged active compound cover a broad range . In general, if the amounts used and compound- specific excretion rates are observed three quarters of antibiotics that were used are excreted unchanged into wastewater (Kümmerer and Henninger, 2003). On average, if the volume for all the antibiotics used is totaled, the metabolic rate is 30% (Kümmerer and Henninger, 2003).

As for the veterinary use of antibiotics, antimicrobials are amongst the most widely used pharmaceutical compounds for animals (Boxall et al. 2003a,b). These drugs are used in animal husbandry for veterinary purposes or as growth promoters (particularly in large-scale animal farming and intensive livestock treatment) (Kümmerer,2008a).

3.2.4 Degradetion of Antibiotics

Antibiotics that are partially metabolized by humans and animals are excreted into the effluent and eventually reach the sewage treatment systems. Approximately 70% of the consumed antibiotics are excreted in an unchanged form and they are still active (Kümmerer and Henninger, 2003). In the sewage treatment plants, antibiotics are partially eliminated, and if they pass through the sewage system, they end up in the environment, mainly in the receiving waters.

Antibiotics can be eliminated in the environment bioticly or non-bioticly. They can face biodegradation by bacteria or fungi, or they can face non-biotic elimination by sorption, hydrolysis, photolysis, oxidation and reduction (Kümmerer, 2008).

3.2.4.1 Biodegradation

There have been many studies on biodegradation of antibiotics under aerobic conditions, however most of the tested antibiotics were not biodegradable (Richardson and Bowron, 1985; Al-Ahmad et al., 1999; Wiethan et al., 2000; Kümmerer et al., 2000; Ingerslev et al., 2001; Ingerslev and Halling-Sørensen, 2001; Thiele-Bruhn, 2003; Alexy et al., 2003, 2004; Gartiser et al., 2007a; Li et al., 2008c).

In general, biodegradability is poor for most of the compounds that were investigated so far in laboratory tests – even for some of the □-lactams. In a study carried out by Gartiser and his friends, sixteen antibiotics were tested, only benzyl penicillin (penicillin G) was completely mineralized in a combination test (combination of two tests OECD 302B and OECD 301B) (2007a). By the trials made with radio labeled compounds, it was revealed that approximately 25% of benzyl penicillin was mineralized within twenty-one days, whereas ceftriaxone and trimethoprim were not mineralized at all (Junker et al. 2006) Antibiotics occurring in soil and sediment proved to be quite persistent in laboratory testing and in field studies. They do not biodegrade well under anaerobic conditions (Gartiser et al. 2007b).

3.2.4.2 Photolysis

Some of the antibiotics can be suspected to photolysis (e.g. tetracyclines, sulphonamides, tylosin) which is lysis by light, however not all of the antibiotics are photo-biodegradable (Turiel et al., 2005b). Incomplete photo-biodegradation can lead the creation of more toxic compounds, although it does not happen so often (Çokgör et al., 2006; Arslan-Alaton and Caglayan, 2006; González et al., 2007; Iskender et al., 2007; Paul et al., 2007).

The direct and the indirect photolysis of antibiotics in the aquatic environment are very significant. However, the incomplete photo transformation and photodegredation may lead to toxic compounds, but this is not always the case (Coqgor et al. 2006; Arslan-Alaton and Caglayan 2006; Gonzales et al. 2007; Iskender et al. 2007). The effectiveness of the photolysis depends on the light intensity and frequency (Hu and Coats 2007). The frequency relates to the absorption spectrum of a compound. Consequently, photo transformation may not occur when the compounds are present in turbid water or if the creek, river, or lake is shadowed by trees or covered in soil, sewage and sewage pipes. Thus, the photolysis varies with seasons and the latitude (Kümmerer, 2008a).

Photodecomposition may be the major factor in the elimination process, if the substance is sensitive towards light (Lunestad et al., 1995). It takes place mainly in the surface waters. Since soil and sediment prevent photochemical degradation of a substance, due to the lack of light that can go through these matrices. For example in a study by Samuelsen (1989), the persistence of oxytetracycline sensitivity towards light in seawater as well as in sediments was investigated. Oxytetracycline was found out to be more stable in sediments rather than in seawater.

3.2.4.3 Sorption

Antibacterial and detectability can be lost by binding of the particles, or the formation of complexes. For example, an aquaculture antimicrobial can be undetected in seawater, caused by the formation of complexes with the magnesium and calcium that are naturally present in the marine water.

Antibiotics that are applied in human medicine can reach the terrestrial environment with the aid of sewage sludge. In the case of sulfadiazine and other sulfonamides it has

been found that elimination by sorption to soil particles is a significant process (Tolls 2001; Kreuzig and Höltge 2005; Heise et al. 2006; Schmidt et al. 2008)

3.2.4.4 Hydrolysis

Hydrolysis is another mechanism where abiotic elimination of the substances is achieved. While some antibiotics were demonstrated to be instable in water, sulfonamides are stable against hydrolysis (Halling-Sørensen, 2000). Antibiotics like, oxytetracyline and □-lactams, have increased hydrolysis rates as pH deviates from pH 7 and as the temperature increases. The biodegradability testing with sewage sludge showed that □-lactams are hydrolyzed. This leads to deactivation of antibiotic activity (Längin et al., 2008).

3.2.5 Effects

If the antibacterial agent is not eliminated in any way, it can reach the environment with the potential to adversely affect aquatic and terrestrial organisms. Because of the fact that antibiotics are designed to affect microorganisms, bacteria, fungi and micro algae are the organisms primarily affected. In general, the effects of antibacterial agents on bacteria and micro algae are found to be two to three orders of magnitude below the toxic values for higher trophic levels (Wollenberger et al., 2000).

If antibiotics are not eliminated efficiently by sewage treatment plants, they can have adverse effects on the environment, both affecting aquatic and terrestrial organisms. Humans can be affected via drinking water that is contaminated by antibiotics even though there are no reports showing that antibiotics are present in drinking water (Kümmerer, 2008b). There are many effects of antibiotics on humans that are reported in the medical literature. The most known side effect of antibiotics is the allergic reactions that it may cause. Quinolones can increase light senility tetracylines can lead to negative interactions in developing teeth in young children, and because of all the antibiotics anti-microbial property they can lead to negative interaction within the gut (Kümmerer, 2008b).

By the toxicity tests done on bacteria in the literature, it is shown that chronic exposure to antibiotics is critical rather than acute (Backhaus and Grimme, 1999 ,2000; Froehner et al., 2000; Kümmerer et al., 2004). In the study by Thomulka and McGee (1993), the toxicity of a number of antibiotics (e.g., tetracycline, chloramphenicol, ampicillin, streptomycin) has been determined by using two bioassay method on Vibrio harveyi. Almost no toxic effects were found after short incubation times however, in the long term assays using a toxic effect for concentrations that are present in the environment could be detected for almost all the substances. Same results were obtained, by study done by Kümmerer et al. (2004), in which the tests were done on sewage sludge bacteria. The results of short and long-term bioassays with Vibrio fischeri demonstrate the risk of underestimating the severe effects of substances with delayed toxicity in acute tests.

3.2.5.1 Effects on Wastewater and Sewage Systems

The microbial community in the sewage systems has the potential to be affected by antibiotics. The effects of antibacterial agents on microbial population present in the sewage systems are of great interest, mainly because the inhibition of wastewater bacteria may seriously affect organic matter degradation. When model sewage treatment systems were applied with commonly used antibiotics in concentrations that may occur in hospital wastewater, a reduction in the number of bacteria together with alterations in microbial population were observed. (Stanislawska, 1979; Kümmerer et al., 2000; Al-Ahmad et al., 1999; Kümmerer et al., 2008). The inhibitory concentrations for a variety of antibiotics were found to be in the same order of magnitude, as the concentrations expected to be present in a typical hospital wastewater, thus the possibility of these substances affecting the microbial populations of hospitals' sewage systems could not be excluded.

Several antibiotics were found out to have low toxicity on nitrifying bacteria in acute tests, where nitrifications in an important step in wastewater purification, eliminating toxic ammonia. Even though higher concentration of these substances of what might be expected in the environment were tested, no effects upon nitrification were observed (Tomlinson et al., 1966; Gomez et al., 1996).However, the time period of the test significantly influences the results (Halling-Sørensen, 2000; Kümmerer et al., 2004). For example in a short term test (two to four hours), an antimicrobial was found to require high concentrations to inhibit the nitrification process, however a prolonged

test period over five days showed effects one order of magnitude below the inhibitory concentrations of the acute test (Tomlinson et al., 1966).

The microbial community in the sewage systems is prone to be affected by Antibiotics. If the bacteria in the wastewater treatment, system is inhibited, the organic matter degradation may be seriously affected, therefore the inhibition effects of antibiotics on microbial population create a great risk. Reduction of the number of bacteria, along with the shift of the microbial populations were observed in a model sewage treatment system when antibiotics which are commonly found in hospital wastewaters were added in different concentrations to the system (Stanislawska, 1979; Al-Ahmad et al., 1999; Kümmerer et al., 2000). For a variety of antibiotics the inhibitory concentrations were tested, and found out to be in the same order of magnitude, as the concentrations expected for the hospital wastewater.

Nitrification is a step in wastewater treatment, which eliminates toxic ammonia, which makes this step highly important. The second step of nitrification, oxidation of nitrite to nitrate is highly sensitive. Inhibition of this step under uncontrolled conditions leads to the accumulation of nitrite nitrogen, which is toxic. In the acute tests, several antibiotics had low toxicity to nitrifying bacteria. These antibiotics showed no inhibition on nitrification even used in higher concentrations than what might be environmentally expected (Tomlinson et al., 1966; Gomez et al., 1996). However, in longer incubation times, the test results are altered (Halling-Sørensen, 2000; Kümmerer et al., 2004). For example, in the study by Tomlinson et al., (1966), one of the antimicrobials required in high concentrations for the inhibition of the nitrification process in a short term test (2–4 h), but a prolonged test period over 5 days showed less effects of inhibition. In another study, carried out by Dokianakis et al. (2004) the effects of seven different pharmaceuticals on a culture of nitrite-oxidizing bacteria isolated from activated sludge were investigated. For ofloxacin and sulfamethaxazole, significant inhibition was observed. In the same study, triclosan showed to have a substantial inhibitory effect on the nitrite reduction rate.

In a study done by Christensen and co-workers, the synergistic mixture effects of antibiotics against sewage sludge bacteria are found (Christensen et al., 2006). In the anaerobic digestion process, acetoclastic methanogens are the most sensitive group of microorganisms. In the examinations, where pharmaceuticals were being tested, including antibiotics like sulfamethaxazole, mild inhibition of methanogens were seen

in most cases, which were directly related to the tendency on the compounds to adsorb on the anaerobic biomass (Fountoulakis et al., 2004).

In an another experiment, where ISO 13641 test was used on antibiotics primarily active against Gram negative bacteria, moderate inhibition effects were detected after a 7 day incubation period, with EC50 values between 24 mg/l and 1000 mg/l(Gartiser et al., 2007b). On the other hand, in the same test, it was found out that metronidazole was very toxic to anaerobic bacteria with an EC50 of 0.7mg/l.

3.2.6 Antibiotic Resistance

Antibiotics are agents that effective against bacteria. Microbial growth is controlled with the use of antimicrobials such as antibiotics, in order to eliminate the unwanted effects. Lots of research has been done about the emergence of resistance and the use of antimicrobials in medicine, veterinary medicine and animal husbandry.

The emergence of resistance is not yet fully understood, because it is a highly complex process. (Martinez et al., 2000; Björkman et al., 2000). The complexity rises from the interactions of bacterial populations and antibiotics. For example, antibiotics in sub-inhibitory concentrations can have an impact on cell functions and change the genetic expression of virulence factors or the transfer of antibiotic resistance (Ohlsen et al., 1998; Salyers et al., 2002).

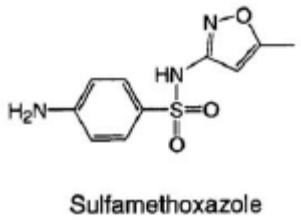
3.2.7 Chemical and Physical Properties of Sulfamethaxasole

Sulfamethaxasole is an antimicrobial, which is under the main group of antimicrobials called sulfonamides. The sulfonamides are synthetic antimicrobials with a wide spectrum against most gram-positive and many gram-negative organisms. Synthetic antimicrobial drugs have the selective toxicity, which is the ability to inhibit or kill the pathogenic microorganism without killing the host (Madigan et al., 2008). They inhibit the multiplication of bacteria, because they are competitive inhibitors of p-amino benzoic acid in the folic acid metabolism cycle (O'Neil et al., 2001), thus causes a decline in folate concentration. This decline is detrimental to the bacterium because folic acid is the precursor of purines and pyrimidines, the bases used in the construction of DNA, RNA and other important cell constituents (Nester et al., 2008). The resulting inhibition of purine and pyrimidine synthesis leads to cessation of protein synthesis and DNA replication, thus the pathogen dies. The sulfonamides

consist of a benzene ring, an amine moiety ($–NH_2$), and a sulfonamide group ($–SO_2NH_2$). The sulfonamide groups and the amine groups must be in para positions to each other in the antibiotic in order to possess antibacterial properties (Hardman et al., 2001; Beleh, 2003).The main compounds within this group are sulfadiazine-trimethoprim, sulfadimethoxine, sulfamethazine, sulfathiazole and sulfadimethoxine-ormetoprim (Beville, 1988).

Resistance against sulfonamides has been increasing because many formerly susceptible pathogens have developed an ability to take up folic acid from their environment while bacteria are initially are not able to synthesize their own folic acid, whereas most animals obtain folic acid from their own diet (Madison et al., 2006). Antimicrobial therapy with sulfamethaxasole plus trimetrophim, a related folic acid synthesis competitor, is still effective in many instances because the drug combination produces sequential blocking of the folic acid synthesis pathway (Madison et al., 2006).

Even though the sulfonamides are amphoteric, they generally function as weak acids at physiologic pH range. Because of this property, they are usually seen as sodium salts that have increased solubility as pH increases. The solubility of sulfonamides can range in the order of 0.1–8 g/l (Halling-Sørensen, pers. comm.) Most of the sulfonamides that are used for veterinary purposes, have at least two nitrogen functions, with the amide attached to the sulfur referred to as N1 and deprotonated at pH > 5.5–7. The amine attached to the aromatic cycle is referred to as N4 and is protonated at pH 2.5. For this reason, most sulfonamides are positively charged under acidic conditions, neutral between pH 2.5 and 6 and negatively charged at alkaline conditions (Haller et al., 2002).



Sulfamethoxazole

Figure 2.1: The chemical formula of sulfamethaxazole

3.3 Electron Acceptor Conditions

3.3.1 Methanogenesis

Methanogens are strict anaerobes that obtain energy by converting CO2, H2, formate, methanol, acetate and other compounds to either methane or methane and CO2. They are the largest group in Achaea domain. There are five orders (Methanobacteriales, Methanoccocales, Methanomicrobiales, Methanosarcinales and Methanopyrales) and 26 genera, which differ greatly in overall shape, 16srRNA sequence, cell wall chemistry and structure, membrane lipids, and other features. Methanogens thrive in anoxic environments rich in organic matter. For example, they are found in the rumen and intestinal system of animals, freshwater and marine sediments, swamps and marshes, hot springs, anoxic sludge digesters, and even within anerobic protozoa.(Nester et al., 2008)

3.3.2 Dissimilative Sulfate Reduction

Sulfate is the most oxidized form of sulfur and it is one of the major anions in seawater and is reduced by the sulfate-reducing bacteria. The end product of sulfate reduction is is hydrogen sulfide H2S. The ability to use sulfate as an electron acceptor for energy-generating processes involves the large-scale reduction of SO4 and it is restricted to the sulfate reducing bacteria. In assimilative sulfate reduction, the H2S formed is immediately converted into organic sulfur in the form of amino acids and other organic sulfur compounds; however, in dissimilative sulfate reduction the H2S is excreted. (Madison et al., 2006)

In dissimilative sulfate reduction, the sulfate in *Adenosine phosphosulfate(APS)* is reduced directly to sulfite ($SO_3^{=2}$) with the aid of the enzyme APS reductase with the release of AMP (Figure 3.1). In assimilative reduction, another phosphate is added to APS to form phosphoadenosine phosphosulfate (PAPS). In both cases the final product is sulfite ($SO_3^{=2}$). Once the sulfite is formed, sulfide is formed with the enzyme sulfite reductase. (Madison et al., 2006)

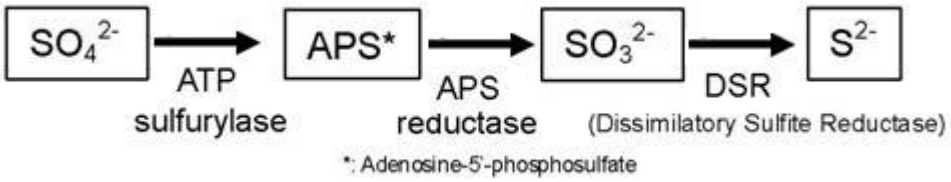


Figure 3.1: Biochemistry of Dissimilative Sulfate Reduction

3.3.3 Nitrate Reduction

Nitrate NO3, can be reduced to N2O, NO, and N2. These products can easily be lost from the environment with a process called denitrification. Denitrrification is the process where gaseous N2 is formed biologically (Madison et al., 2006).

The enzyme for nitrate reduction is nitrate reductase, which is a molybdenum containing membrane integrated enzyme whose synthesis is repressed by molecular oxygen. In addition to anoxic conditions nitrate must also be present in the environment for the nitrate reduction. The first product of nitrate reduction is nitrite (NO2), and the enzyme nitrite reductase reduces to nitric oxide (NO), as seen on figure 3.1 (Madison et al., 2006)

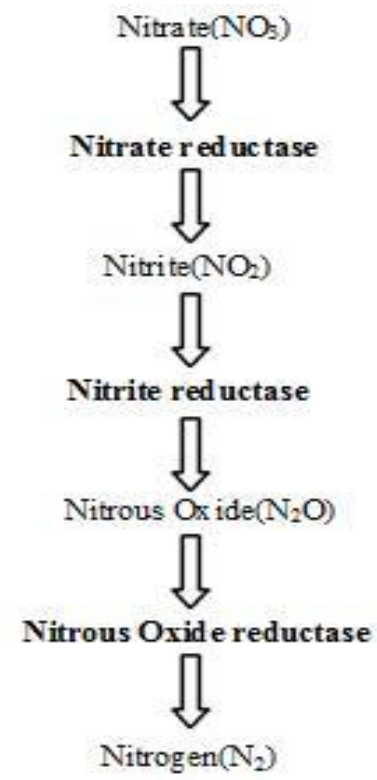


Figure 3.1: Nitrate reduction

3.4 Molecular Techniques Used In Molecular Ecology

3.4.1 The Benefits of Molecular Techniques

The techniques for identification of environmental microorganisms used in classical microbiology are generally based on cultivation dependent methods on selective growth media. The major handicap of these techniques is the prevention of efficient identification of the community. Not all the microorganisms can grow in cultures; it is not possible to identify all the microorganisms in cultures. The cultivable microorganisms makeup 0.1%-10% of all microorganisms on earth (Amann, 1995a; Hugenholtz, 1998; Muyzer, 1993; Muyzer, 1999; Lim, 1999; Guillou, 1999).

Culture dependents techniques were the most commonly used method for identification in the early years of microbiology. Microorganisms living in anaerobic environment are hard to grow because of low growth rates, syntrophic interactions and unknown growth requirements. In addition, cultivation dependent methods cause cultivation shift by favoring a normally unfavorable microorganisms by altering the flow of competitions. Therefore, it is not possible to culture all of the microbial community and the microorganisms that were able to be cultured do not represent the microbial community of the sample in question.

Another method for identification of microorganisms is microscopy. However, this technique has limitations, as well as the cultivation technique. These limitations are the small size of prokaryotic organisms, the absence of distinguishing phenotypic characters, and the fact that most of these organisms cannot be cultured (Pace, 1997; Torsvik and Øvreås, 2002; Torsvik, 2002). Because of these factors, evaluation of the biodiversity is limited in microscopy. In last 20 years, a significant number of studies dealing with microbial biodiversity involve the use of molecular tools and have often focused on investigating the dynamics of the composition and structure of microbial populations and communities in defined environments, and the impact of specific factors, such as pollution by xenobiotics on microbial diversity (Morris, 2002;Ranjard, 2000).

3.4.2 The Importance 16S rRNA

The methods that use 16S rRNA based techniques, were created as an alternative approach since a great percentage of the microorganisms were unable to detect with

culture dependent and microscopy dependent techniques. In 16S rRNA based techniques; a unique and distinct characteristic of each microorganism was used.

Ribosomal RNA (rRNA) molecules (16S and 23S) are used as phylogenetic markers. The main reason for selection of ribosome is, that it is an obligatory component each cell. In addition, ribosome is well abundant (10^3-10^5) in a cell. Because ribosomes are directly taking part in protein production, its number gives also clue about cell volume and growth rate (Amann, 1995b; Alcamo, 1996). By extraction DNA, and creation of a data bank for the specific genes, microorganisms can be identified without cultivation.

Both of the subunits can be used for analyses. The extracted 16S and 23S rDNA are amplified by specific primers using polymerase chain reaction (PCR) (Saiki, 1988). Amplified subunit coding sequences then can be used in cloning or in other molecular methods for identification or monitoring of the microbial community. There are more than 15000 16S rRNA sequences uploaded to the public databases. 23S rRNA database is smaller in size than the 16S rRNA database but it is growing rapidly with each day (Wilderer, 2002).

16S rRNA genes consist of highly conserved and highly variable regions (Lane, 1985) and thus the amplification of this gene with suitable primers makes it possible to identify all microorganisms. The comparison of amplified genes with known sequences in database helps to build a phylogenetic classification system. With the developments in analysis of 16S rRNA, the detection and identification of microorganisms in nature enhances greatly. The 16S rRNA analysis also shows the truth of the suspicions about inefficiency of culture dependent techniques (Barns, 1994; Choi, 1994; DeLong, 1992; Liesack and Stackebrandt, 1992; Schmidt, 1991; Ward, 1990).

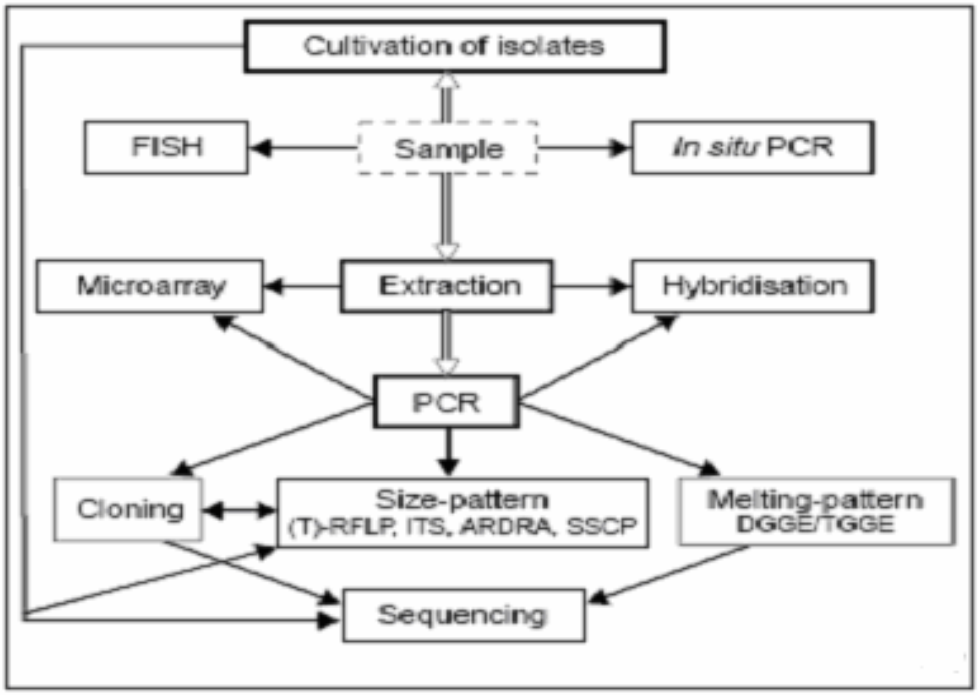


Figure 3.1: Approaches to the analysis of microbial diversity Dahlof (2002)

3.4.2.1 The Usage of Variable Regions in 16S rRNA

Because the vital function of ribosomes, the rRNA gene is highly conserved in nucleotide sequence as well as in secondary structure, since its function remains same through years of evolution. It has many variable regions in which random changes occur time to time. These changes reflect evolutionary relationships of the organisms. Conserved regions functions as binding places for PCR primers or hybridization probes. Even data from this analysis is sufficient to compare statistically significant phylogenetic relations (Olsen, 1986). Among the variable regions, V3 region is mostly used in molecular analysis (Neefs, 1990; Øvreas, 1997).

3.4.3 Polymerase Chain Reaction (PCR)

Polymerase Chain Reaction (PCR) is used in order to amplify specific regions of a DNA strand. This specific region can be a single gene, just a part of a gene, or a non-coding sequence. PCR is based on three main steps: Denaturation, Annealing, and Extension. In the denaturation step, the double stranded DNA templates are melted and separated by using a high enough temperature for the breakage of hydrogen bonds. In the annealing step, the temperature is taken to lower temperatures so that the specific primers can attach to the single-stranded DNA template. Then the temperature is taken to temperature where Taq polymerase can elongate the chain by adding

nucleotides (dNTPs). The cycle that consists of denaturation, annealing and extension is repeated up to 30-40 times to obtain enough DNA segment of interest. The addressed sequence amplified in order of two. (2^n where n is the cycle number) The resulted product will be run on an agarose gel to monitor efficiency of the PCR. Mostly Ethidium Bromide (EtBr) is used to stain DNA which makes the DNA fragments that are amplified, visible under UV light.

According to the aimed DNA fragment that is aimed to be amplified, changes in the steps of PCR are done, such as enzyme concentration, dNTP concentrations, magnesium concentration, annealing and extension temperatures and times, cycle number and other reaction components.

3.4.3.1 Limitations and Biases of PCR

PCR is a powerful tool in molecular biology; however, it has its limitations. The biggest problem of PCR is that DNA polymerase is not 100% trustworthy in transcribing DNA. Approximately 0.02-0.3% incorrect nucleotides incorporated during amplification (Bej, 1991). The contamination present in template like humic acids, phenolic compounds or chelating agents will decrease efficiency and fidelity of *Taq* polymerase. In order to solve this problem, the DNA purification methods were developed. Due to processive characteristics of *Taq* polymerase, the depletion of nucleotides increases the error rate. Primer dimer formation is possible when primers complement each other at 3' end (Bej, 1991). Creation of recombinant or chimeric products is another problem. This problem mostly arises when target sequence of primers was shared in other DNAs other than template.

Another limitation of PCR is the problems caused by its power to amplify DNA fragments. An extreme sterilization and care needed in performing PCR. A negative control without a DNA template or DNAse treatment of reagents can be done to prevent contamination caused by a foreign DNA (Schmidt , 1991).

3.4.4 PCR Based Techniques used in Molecular Ecology

3.4.4.1 Quantitative PCR

Using PCR techinique after the extraction of nucleic acids (DNA and RNA) from environmental matrices, is higly important in the development of culture independent approaches in microbial ecology. These methods, which have been applied since the

early 1990s (e.g. Giovannoni, 1990), enabling the analysis of the total microbial communities present within environmental systems, have revolutionized our understanding of microbial community structure and diversity within the environment. Coupling environmental nucleic acid isolation to subsequent PCR amplification of both taxonomic (i.e. rRNA) and functional gene markers and in combination with DNA fingerprinting- and sequencing-based analyses has enabled description of the hitherto uncharacterized majority of environmental microorganisms (Head, 1998) driving the discovery of new microbial lineages and enabling the description of genetic diversity in a wealth of functional gene markers (Larkin, 2005). Although recently developed ultra-high-throughput sequencing technologies such as pyrosequencing (Margulies, 2005; Edwards ,2006) now dwarf PCR-based sequence studies in terms of sequence coverage, the ability of the PCR to specifically target particular taxonomic or functional markers from domain – down to strain – or phylotype levels means that PCR will remain an invaluable method in the molecular microbial ecologist's toolbox. Nevertheless, PCR has inherent limitations (Von Wintzingerode, 1997), particularly those that result in biases in the template to product ratios of target sequences amplified during PCR from environmental DNA (Suzuki & Giovannoni, 1996; Polz & Cavanaugh, 1998), with such amplification biases found to increase with increasing numbers of PCR cycles. These limitations presented a significant challenge to microbial ecologists who were interested in determining the abundance of individual genes present in environmental samples. In order to answer the problems, an adaptation of the PCR method developed by Holland (1991). He utilized the so-called '50 nuclease assay' method that was used for the quantification of target 16S rRNA genes which were amplified from environmental DNA by PCR (Becker, 2000; Suzuki , 2000; Takai & Horikoshi, 2000). This development in qpcr technique had been facilitated by the earlier combination of the 50 nuclease assay developed by Holland (1991) with fluorescence detection following cleavage of an internal (TaqManTM) DNA probe (Livak, 1995), which enabled the accumulation of amplicons that are monitored after each cycle thus it facilitated quantitative determination of the initial template gene (or transcript) numbers.

Q-PCR has been shown to highly reproducible and sensitive method to quantitatively track phylogenetic and functional gene changes across temporal and spatial scales under varying environmental or experimental conditions. Moreover, the quantitative

data generated can be used to relate variation in gene abundances and/or levels of gene expression (in terms of transcript numbers) in comparison with variation in abiotic or biotic factors and/or biological activities and process rates. The provision of Q-PCR data sets that describe the abundance of specific bacteria or genes to complement other quantitative environmental data sets is of increasing importance in microbial ecology as it furthers understanding of the roles and contributions of particular microbial and functional groups within ecosystem functioning. Furthermore, reverse transcription (RT) analyses are now increasingly combined with Q-PCR methods in RT-Q-PCR assays, offering a powerful tool for quantifying gene expression (in terms of numbers of rRNA and mRNA transcripts) and relating biological activity to ecological function.

Quantitative-PCR or Q-PCR (real-time PCR) is now widely used in microbial ecology to determine gene and/or transcript numbers present within environmental samples. The target specificity of any Q-PCR assay is determined by the design of the primers (and in some cases an internal probe), allowing quantification of taxonomic or functional gene markers present within a mixed community from the domain level down to the quantification of individual species or phylotypes.

Q-PCR combines the detection of target template with quantification by recording the amplification of a PCR product via a corresponding increase in the fluorescent signal associated with product formation during each cycle in the PCR. The xponential phase helps the determination of quantification of gene (or transcript) numbers of the PCR amplification when the numbers of amplicons detected are directly proportional to the initial numbers of target sequences present within the environment. Quantification of the target gene during exponential amplification avoids problems that are related with the so-called ‘end-point’ PCR (in which amplicons are only analyzed after completion of the final PCR cycle). In end-point PCR, the proportions of numerically dominant amplicons do not necessarily reflect the actual abundances of sequences present within the environment due to the inherent biases of PCR that are associated with amplification of targets from mixed template community DNA (Reysenbach, 1992; Suzuki & Giovannoni, 1996; Polz & Cavanaugh, 1998). Moreover, Q-PCR that uses fluorescence-based detection offers greater sensitivity and enables discrimination of gene numbers across a wider dynamic range than is found with end-point PCR; for example twofold changes in target concentration can be discriminated using Q-PCR.

Before the development of fluorescence-based Q-PCR-based methods, two alternative PCR-based methods for gene number quantification had been developed, namely competitive PCR (Diviacco , 1992) and limiting dilutions or most probable number (MPN)-PCR (Skyes , 1992). However, these methods are time- and resource-consuming, requiring post-PCR analysis, and have now largely been replaced by fluorescence-based Q-PCR methods.

3.4.4.2 Fluorescence detection chemistries used to detect template amplification during Q-PCR

Quantitative real-time PCR works in essentially the same manner as end-point PCR, i.e. multiple amplification cycles in which template DNA is initially denatured, followed by annealing of oligonucleotide primers targeting specific sequences, followed by subsequent extension of a complementary strand from each annealed primer by a thermostable DNA polymerase, resulting in an exponential increase in amplicon numbers during the PCR. However, in contrast to end-point PCR, the increase in amplicon numbers is recorded in 'real-time' during the PCR via detection of a fluorescent reporter that indicates amplicon accumulation during every cycle. Two reporter systems are commonly used, namely, the intercalating SYBR green assay (Wittwer, 1997) and the TaqMan probe system (Holland, 1991; Livak, 1995).

SYBR green binds to all double-stranded DNA via intercalation between adjacent base pairs. When bound to DNA, a fluorescent signal is emitted following light excitation (Fig. 1a). As amplicon numbers accumulate after each PCR cycle, there is a corresponding increase in fluorescence. Because SYBR green binds to all double-stranded DNA, it is essential to use primer pairs that are highly specific to their target sequence to avoid generation of nonspecific products that would contribute to the fluorescent signal, resulting in an overestimation of the target. Extensive optimization of primer concentrations used in SYBR green Q-PCR assays may be required to ensure that only the targeted product is formed. Primer pairs that exhibit self-complementarity should also be avoided to prevent primer–dimer formation. A post-PCR dissociation (melting) curve analysis should be carried out to confirm that the fluorescence signal is generated only from target templates and not from the formation of nonspecific PCR products. During a dissociation curve, the double-stranded template is heated over a temperature gradient and fluorescence levels are measured at each discrete temperature point. As the double-stranded template is heated, it

denatures, resulting in a corresponding decline in fluorescence due to SYBR green dissociation from the double-stranded product (Giglio, 2003; Gonzalez- Escalona, 2006). The temperature at which 50% of the double-stranded template is denatured can be used to confirm that the template being targeted is present, along with the presence of other nonspecific template and primer dimers in much the same way as agarose gel electrophoresis of an end-point PCR product is used.

The TaqMan probe methodutilizes a fluorescently labeled probe that hybridizes to an additional conserved region that lies within the target amplicon sequence. The TaqMan probe is fluorescently labeled at the 50 end and contains a quencher molecule at the 30 end (Livak, 1995). The close proximity on the probe of the quencher molecule to the fluorophore prevents it from fluorescing due to fluorescent resonance energy transfer. During the annealing step of each cycle of the PCR, primers and the intact probe bind to their target sequences. During subsequent template extension, the 50-exonuclease activity of the Taq polymerase enzyme cleaves the fluorophore from the TaqMan probe and a fluorescent signal is detected, as the fluorophore is no longer in close proximity to the quencher (Figure 5.2b). Amplification of the template is thus measured by the release and accumulation of the fluorophore during the extension stage of each PCR cycle. The additional specificity afforded by the presence of the TaqMan probe ensures that the fluorescent signal generated during Q-PCR is derived only from amplification of the target sequence. Multiple TaqMan probes and primer sets can be used in different Q-PCR assays to differentiate between closely related sequences (Smith, 2007), or alternatively, probes can be labeled with different fluorophores, facilitating the development of multiplex Q-PCR protocols whereby different targets can be coamplified and quantified within a single reaction (Neretin, 2003; Baldwin, 2003, 2008). For example, Baldwin (2003) developed a multiplex Q-PCR assay targeting a number of different aromatic oxygenase genes using bacterial strains and then subsequently applied the assay to simultaneously quantify aromatic oxygenase genes in contaminated groundwater (Baldwin, 2008). TaqMan probes are, however, a more expensive option than using SYBR green chemistry and the former requires the presence of an additional conserved site within the short amplicon sequence to be present. Identification of three conserved regions within a short region (typically 100 bp) may not always be possible, especially when primer/probe combinations are being designed to target divergent gene sequences. More recent

advances in TaqMan probe technology have involved the introduction of the minor groove binder (MGB) probe (Kutyavin, 2000). The MGB molecule is attached to the 30 end of the probe and essentially folds back onto the probe. This not only increases the stability of the probe, but also allows the design of shorter probes (13–20 bp) than are required for traditional TaqMan probes (20–40 bp), while at the same time, maintaining the required hybridization annealing temperature.

3.4.5 Target quantification using the cycle threshold (Ct) method

Irrespective of the fluorescence chemistry used, quantification of the target template DNA is carried out in essentially the same manner. There are a number of different commercially available instruments to carry out Q-PCR, each with its own associated software. Currently, there is considerable debate as to which algorithms are the best used to analyze Q-PCR data (Rebrikov & Trofimov, 2006). All the Q-PCR platforms collect fluorescent data from every amplification cycle and the increase in fluorescence is plotted against the cycle number, resulting in the typical amplification curve. The Q-PCR amplification curve can be subdivided into four stages, namely background noise, where the background fluorescence still exceeds that derived from initial exponential template accumulation, exponential amplification, linear amplification and a plateau stage. During the exponential phase of the amplification, the amount of target amplified is proportional to the starting template and it is during these cycles that gene numbers are quantified using the Ct method. The Ct is reached when the accumulation of fluorescence (template) is significantly greater than the background level (Heid, 1996). During the initial cycles, the fluorescence signal due to background noise is greater than that derived from the amplification of the target template. Once the Ct value is exceeded, the exponential accumulation of product can be measured. When the initial concentration of the target template is higher, the Ct will be reached at an earlier amplification cycle.

Quantification of the initial target sequences of an unknown concentration is determined from the Ct values and can be described either in relative or in absolute terms. In relative quantification, changes in the unknown target are expressed relative to a coamplified steady state (typically housekeeping) gene. Any variation in the presence (or expression) of the housekeeping gene can potentially mask real changes or indicate artificial changes in the abundance of the gene of interest. While this approach is commonly applied for studying eukaryotic gene expression, it is more

difficult to apply this method for studying prokaryotic genes where the identification of a valid steady-state reference gene is problematic. Burgmann (2007) nevertheless successfully utilized such an approach when confirming microarray-based determination of the transcriptional responses of Silicibacter pomeroyi to dimethylsulphoniopropionate additions. From microarray experiments, they identified a gene whose expression was not altered by experimental conditions and used the expression of this gene to normalize levels of expression of the target genes of interest in RT-Q-PCR assays. In a number of other studies, gene and transcript numbers of the target gene of interest have been normalized to the numbers of 16S rRNA gene or transcripts (Neretin, 2003; Treusch, 2005; Kandeler, 2006). For example, Treusch (2005) normalized the number of amoA transcripts to numbers of 16S rRNA gene transcripts in RNA extracted from ammonia-amended orunamended soils. They reported a statistically significant increase in amoA transcript numbers in the ammonia-amended soils. However, although 16S rRNA genes and transcripts are now commonly used in this manner, the application of such an approach is controversial, especially when studying genes/ transcripts amplified from nucleic acids extracted from complex environmental samples. This is, in particular, because 16S rRNA gene copy and transcript numbers are highly variable, with the number of 16S rRNA genes per operon varying dramatically between species (1–15 copies) while 16S rRNA gene transcription rates are regulated primarily by resource availability (Klappenbach, 2000). The 16S rRNA genes and transcripts cannot therefore be considered as a steady-state (housekeeping) gene, especially when studying genes/transcripts in environmental samples.

In absolute quantification protocols, the numbers of a target gene or transcript are determined from a Standard curve generated from amplification of the target gene present at a range of initial template concentrations, and then the Ct values for each template concentration are determined. Subsequently, a simple linear regression of these Ct values is plotted against the log of the initial copy number. It should be ensured that the Ct value of the most diluted template DNA used to construct the Standard curve is at least a log fold lower (3.3 cycles) than the Ct value of the no template control (NTC). Quantification of the unknown target template is determined by comparison of the Ct values of the target template against the Standard curve. However, in reality, this ‘absolute’ quantification of the target gene represents

quantification of the target in comparison against a constructed standard curve, rather than as an absolute measurement of the number of target genes present within an environmental sample. Any number of factors involved in the construction of the standard curve including the initial quantification of the standard curve template, serial dilution of the template and the algorithmic determination of the Ct value (Love, 2006) contribute to the final quantification of the environmental sample. Therefore, it is recommended that the following descriptors of the standard curve be reported for each Q-PCR amplification: amplification efficiency (E), the linear regression coefficient (r^2) and especially the y-intercept value, which uniquely describes the standard curve and indicates the sensitivity of the reaction (Smith. 2006). Furthermore, the Ct value of the NTC and its equivalent value in terms of gene numbers should be reported. Moreover, we have previously demonstrated that even highly reproducible standard curves may result in statistically significant differences in gene numbers for the same template (with equivalent Ct values) when gene numbers are quantified within different Q-PCR assays (Smith, 2006) due to the log nature of the curve, where by minor differences in Ct values and standard curves result in large differences in gene copy numbers.

3.4.5.1 Application of Q-PCR for investigating the microbial genetic potential within the Environment

The first applications of Q-PCR in microbial ecology were reported in three papers published in November 2000, which used TaqMan-based assays to target 16S rRNA genes (Becker, 2000; Suzuki, 2000; Takai & Horikoshi, 2000). Becker (2000) demonstrated the ability of TaqMan probes to determine the abundance of a specific ecotype of Synechococcus sp. BO 8807 against a mixed background of phylogenetically related bacteria using artificial mixed communities. Suzuki (2000) exploited the specificity and the sensitivity of TaqMan Q-PCR assays to determine spatial and temporal quantitative differences in the distributions of Synechococcus, Prochlorococcus and Achaea in marine waters, while Takai & Horikoshi (2000) quantified archaeal 16S rRNA gene numbers within samples from a deep-sea hydrothermal vent effluent, hot spring water and from hot spring and freshwater sediments. By targeting highly conserved regions of the 16S rRNA gene, Q-PCR assays have been designed to quantify ‘total’ bacterial and or archaeal, numbers while targeting of taxa-specific sequences within hyper variable regions within the gene

enables quantification of sequences from phylum to species levels, provided that there are sequence data available that enable the design of primers and probes. A caveat of this approach must be stressed; 16S rRNA gene numbers from environmental samples cannot be converted to cell numbers as the exact number of copies of the 16S rRNA gene in any given bacterial species varies (Klappenbach 2000).

Quantification of eukaryotes within environmental samples by Q-PCR can be carried out by targeting the 18S rRNA gene (Lueders, 2004; Zhu, 2005) or the internal transcribed spacer (ITS) region (Landeweert, 2003; Kennedy, 2007). The ITS region is often targeted for the design of taxon-specific Q-PCR assays as it provides a greater degree of sequence differentiation between species and lower within-species variability (Kennedy, 2007) than is seen for the 18S rRNA gene. As with quantification of 16S rRNA gene numbers, Q-PCR-derived ITS region and 18S rRNA gene numbers cannot be directly equated to cell numbers. However, numbers of fungal rRNA gene or ITS numbers per volume of sample can be used to compare the relative numbers of fungi between different environmental samples (Guidot, 2002).

In addition to quantitative data on taxonomic markers, Q-PCR has also been applied to quantify functional genes within the environment. By targeting functional genes that encode enzymes in key metabolic or catabolic pathways, the (genetic) potential for a particular microbial function within a particular environment can be assessed. To understand microbial functioning in the environment at a molecular level, it is essential not only to know what genes are present and the diversity of these genes but also to determine their abundance and distribution within the environment

To this end, Q-PCR assays have been designed to target microbially mediated biogeochemical processes in the environment. Quantification of functional genes involved in ammonia oxidation (Hermansson & Lindgren, 2001;Okano, 2004; Treusch, 2005; Leininger, 2006; Mincer, 2007), nitrate reduction and denitrification (Lopez-Guti´errez , 2004; Henry, 2006; Smith, 2007), sulfate reduction (Leloup, 2007), methanogenesis (Denman, 2007) and methane oxidation (Kolb, 2003) have been investigated . In a particularly striking example of the value of such functional gene Q-PCR assays, the relative contributions of ammonia-oxidizing Achaea and bacteria to the first step of nitrification (ammonia oxidation) have been investigated both in soils (Leininger, 2006; He, 2007b) and in seawater (Mincer,2007) by determination of the abundance of archaeal- and bacterial-related amoA genes. These

studies have suggested that Achaea and not bacteria are the numerically dominant ammonia oxidizers in both environments. The results of such studies are therefore encouraging a re-evaluation of our basic understanding of nitrogen cycling and the relative importance of bacteria and Achaea (or specific taxa or functional guilds within the domains) within key environmental processes. While these studies have greatly enhanced our understanding of gene numbers in the environment, the next step to further our understanding is to link variation in genetic potential (i.e. gene numbers) within a system in relation to variation in rates and activity of the biologically driven environmental processes in question, and hence enabling improved understanding of the underpinning factors that influence microbial functioning within the environment.

4 MATERIALS AND METHODS

4.1 Seed Sludge Characteristics

For the nitrate reducing conditions, the seed is taken from the anoxic part of the Paşaköy Domestic Wastewater Treatment System, where areas, for the sulfate reducing and methanogenic conditions; the seed is taken from the anaerobic treatment reactor of a local alcohol distillery (Mey İçki).

The seed that was taken from Mey İçki was obtained from a full-scale UASB reactor (with a volume of 490 m3). The UASB reactor is the first stage of a two-stage anaerobic-aerobic biological treatment plant. The temperature and pH in the UASB(Upflow Anaerobic Sludge Blanket) reactor were maintained within the ranges of 32-35°C and 7.2-7.7, respectively. Total solid (TS) and total volatile solid (TVS) concentrations of the granular sludge was 58000 mg/l and 44000 mg/l, respectively.

Paşaköy Domestic Wastewater Treatment System contains pre-treatment, biological phosphorus removal, denitrification, nitrification and final settling. Biological treatment is done in two stages, nitrifcation and denitrfication. In nitrification stage the necessary nitrate as an electron acceptor for biological carbon removal in denitrification step, is obtained.

4.2 Biodegrability Test Tube Set-Up

The test set-ups were prepared in 120 ml serum bottles, having 100 ml of active volume, using OECD 311 protocol, "Anaerobic Biodegradability of Organic Compounds in Digested Sludge: by Measurement of Gas Production", with minor modifications. Sulfamethaxasole (SMX) from sulfonamide group is chosen as the model carbon source.

The test tubes were set up as duplicates in an anaerobic cabinet (Coy Laboratory Products, U.S.), in nitrate reducing, sulfate reducing and methanogenic conditions, in 4 sets that were planned to be destructed in 4 different sampling times. The first of the

four sets are destructed after all the test tubes were set-up, the other three sets are spoiled in day 20, day 60 and day 120. The sampling times were chosen, by monitoring of the gas pressure weekly. When there was a major change in the gas pressure, a set of test tubes were destructed for sampling. In every test tube, the seed that is taken from the wastewater treatment facility, is introduced having 2000 mg/l TVS.

For the reliability of the experiments, three control groups were set-up, the first one that inhibits the biological activity, the second one having no carbon source, and the third one having only phenol as the carbon source. The test tubes that contain inhibitor were set-up in order to control biological activity in the experimental test sets. Furthermore, the second control set, having no carbon source, was the negative control, while the control sets having phenol, as the carbon source was the positive control. The negative control demonstrates the base-line result obtained when a test does not produce a measurable positive result, while the positive control is for the conformation of the basic conditions of the experiment were able to produce a positive result, even if none of the actual experimental samples produce a positive result. As an inhibitor, sodium azide (0.05%) was used.

For the setting up of the batch tests, medium for all different electron-accepting conditions, trace element solution and vitamin solution were prepared. The constituent of the vitamin solution is given in table 4.1, while the constituents for the trace element solution were given in table 4.2.

Table 4.1: Vitamin solution (OECD, 2006)

CONSTITUENT	AMOUNT (mg)
4-aminobenzoic acid	0,8
D(+)-biotin	0,2
Nicotinic acid	2
Calcium D(+)-pantothenate	1
Pyroxidine dihydrochloride	3
Thiamine	2
NaP Buffer (10 mM, pH 7.1)	to 20 ml

Table 4.2: Stock solution for trace elements (OECD, 2006)

TRACE ELEMENTS	AMOUNT (mg)
Manganese chloride tetrahydrate ($MnCl_2.4H_2O$)	25
Boric acid (H_3BO_3)	2,5
Zinc chloride ($ZnCl_2$)	2,5
Copper (II) chloride ($CuCl_2$)	1,5
Disodium molybdate dihydrate ($Na_2MoO_4.2H_2O$)	0,5
Cobalt chloride hexahydrate ($CoCl_2.6H_2O$)	50
Nickel chloride hexahydrate ($NiCl_2.6H_2O$)	5
Disodium selenite (Na_2SeO_3)	3
$Na_2WO_4 \cdot 2H_2O$	4
Add de-oxygenated water	to 500 ml

The constituents for the medium methanogenic conditions is given in Table 4.3, while the constituents for nitrate reducing conditions is given in Table 4.4, and for sulfate reducing conditions the constituents are given in Table 4.5 (OECD 311).

Table 4.3: Medium for methanogenic conditions (OECD, 2006)

CONSTITUENT	AMOUNT (g)
Anhydrous potassium dihydrogen phosphate (KH_2PO_4)	0,27
Disodium hydrogen phosphate dodecahydrate ($Na_2HPO_4.12H_2O$)	1,12
Ammonium chloride (NH_4Cl)	0,53
Calcium chloride dihydrate ($CaCl_2.2H_2O$)	0,075
Magnesium chloride hexahydrate ($MgCl_2.6H_2O$)	0,1
Iron (II) chloride tetrahydrate ($FeCl_2.4H_2O$)	0,02
Resazurin (oxygen indicator)	0,001
Sodium sulphide nonahydrate ($Na_2S.9H_2O$)	0,1
Stock solution of trace elements	10 ml
Add de-oxygenated water	to 1 liter

Table 4.4: Medium for nitrate reducing conditions (OECD, 2006)

CONSTITUENT	AMOUNT (g)
Anhydrous potassium dihydrogen phosphate (KH_2PO_4)	0,27
Disodium hydrogen phosphate dodecahydrate ($Na_2HPO_4.12H_2O$)	1,12
Ammonium chloride (NH_4Cl)	0,53
Potassium Nitrate (KNO_3)	1
Calcium chloride dihydrate ($CaCl_2.2H_2O$)	0,075
Magnesium chloride hexahydrate ($MgCl_2.6H_2O$)	0,1
Iron (II) chloride tetrahydrate ($FeCl_2.4H_2O$)	0,02
Resazurin (oxygen indicator)	0,001
Sodium sulphide nonahydrate ($Na_2S.9H_2O$)	0,1
Stock solution of trace elements	10 ml
Add de-oxygenated water	to 1 liter

For the nitrate ion source, in nitrate reducing conditions, potassium nitrate was used in the medium of nitrate reducing conditions while potassium sulfate was used as sulfate ion source, in sulfate reducing conditions. In order to adjust pHs, HCl and NaOH was used.

Table 4.5: Medium for sulfate reducing conditions (OECD, 2006)

CONSTITUENT	AMOUNT (g)
Anhydrous potassium dihydrogen phosphate (KH_2PO_4)	0,27
Disodium hydrogen phosphate dodecahydrate ($Na_2HPO_4.12H_2O$)	1,12
Ammonium chloride (NH_4Cl)	0,53
Potassium Sulfate (K_2SO_4)	1,8
Calcium chloride dihydrate ($CaCl_2.2H_2O$)	0,075
Magnesium chloride hexahydrate ($MgCl_2.6H_2O$)	0,1
Iron (II) chloride tetrahydrate ($FeCl_2.4H_2O$)	0,02
Resazurin (oxygen indicator)	0,001
Sodium sulphide nonahydrate ($Na_2S.9H_2O$)	0,1
Stock solution of trace elements	10 ml
Add de-oxygenated water	to 1 liter

Vitamin B12 Solution

cyanocobalamine (vit B12): 2.5 mg vit B12 + 50 ml MQ H_2O

Filter sterilise (0.2 um filter) and store at 4^{o}C.

The medium solutions were sterilized in the autoclave at 121 ^{0}C for 20 minutes. After the sterilization of the mediums, they were degassed with Nitrogen for 10 minutes, in order to eliminate oxygen in the solutions. The test tubes were set-up in anaerobic cabinet. 1 ml of vitamin mixture and 1 ml of vitamin B12 mixture was added to the mediums in the anaerobic cabinet for each 1 ml of medium.

The biodegradability test serum bottles that were set—up in methanogenic and sulfate reducer conditions were kept at 35±2 ^{0}C, on the other hand the test tubes having nitrate reducing conditions were kept at room temperature. All of the test tubes were kept in dark, with the aid of thick plastic bags, and they were shaken by hand 2 times a week. The test set-ups for methanogenic conditions were given in table 4.6, test set-up for

nitrate reducing conditions were given in table 4.7, while the components for sulfate reducing conditions are given in table 4.8. In each electron acceptor conditions, four different sets are prepared: experimental set, inhibited set, reference set and, sets with no carbon source. All of the test sets contain seed and medium, while both experimental and inhibited sets contain SMX. Reference test bottles contain phenol, and in non-carbon test bottles in addition to SMX, test bottles contain inhibitor, sodium azide.

The carbon sources that were given as the active ingredient to the test tubes had the concentrations as such: 111 mg/l for phenol and 176 mg/l for sulfamethaxasole. The initial TOC concentrations for reference test set is about 80 mg/l TOC, while the initial TOC concentration for experimental test set is 300 mg/l TOC.

Table 4.6: Test tubes that are set-up in methanogenic conditions and their components

	Methanogenic Conditions			
		Control Sets		
	Experimental	Inhibited	With no carbon source	Reference
Components	ME	MI	NC	MR
Seed	+	+	+	+
Medium	+	+	+	+
Phenol				+
SMX	+	+		
Inhibitor (NaN3)		+		

Table 4.7: Test tubes that are set-up in nitrate reducing conditions and their components

	Sulfate Reducing Conditions			
		Contol Sets		
	Experimental	Inhibited	With no carbon source	Reference
Components	SE	SI	NC	SR
Seed	+	+	+	+
Medium	+	+	+	+
SO4-2	+	+	+	+
Phenol				+
SMX	+	+		
Inhibitor(NaN3)		+		

Table 4.8: Test tubes that are set-up in sulfate reducing conditions and their components

	Nitrate Reducing Conditions			
		Control Set		
	Experimental	Inhibitorty	With no carbon source	Reference
Components	NE	NI	NC	NR
Seed	+	+	+	+
Medium	+	+	+	+
NO3-	+	+	+	+
Phenol				+
SMX	+	+		
Inhibitor (NaN3)		+		

In the scope of the project, totally 48 test bottles were set-up, containing, duplicate test bottles that will be destructed at days 0, 20, 60 and 120, as 4 equal test set-ups, having 3 different electron acceptor conditions, along with the control groups. All of these test bottles were destructed in the chosen times for sampling, and the samples were used for the chemical analyses, antibiotic concentration measurements in the liquid and solid phase and for the microbial analyses.

4.3 Chemical Analyses

Total Organic Carbon (TOC) and anion analyses are done on the batch tests that are destructed at 4 different times including the start of the experiment.

TOC measurements are done with Shimadzu ASI-V TOC analyzer (Japan). The wastewater samples that are taken from the test bottles are filtered from 0.45 µl filters before the samples are given to the TOC machine for the measurement of TOC concentrations.

Nitrate and sulfate concentrations are measured by DIONEX ICS 1500 ion chromatograph (U.S.) The samples that are measured in the ion chromatograph were filtered by 0,22 µl after they were diluted in the ratio of 1/10. The filtered samples are placed into the ion chromatograph, and the concentrations are determined.

4.4 Gas Measurements

Gas composition and gas pressure were observed in four different times including the start of the experiment, in order to monitor the changes in the chemical and microbiological parameters. Excluding the start-up set, gas samples were taken weekly, and the changes in gas pressure were monitored. The gas analyses were done directly from the test bottles, by a gas chromatograph (Perichrom, France). For the gas pressure monitoring, a monometer (Lutron PM-9107, U.S.) was used weekly for the observation of the pressure changes.

4.5 Antibiotic Measurements

Antibiotic measurements were done on both liquid samples, and sludge samples. Before measuring SMX concentrations, the samples were treated in order to be able to measure concentrations in the HPLC(High Pressure Liquid Chromotography).

The sludge samples taken from the test bottles, were freeze-dried and were grinded extensively. From the dried sludge samples SMX was crossed over to the liquid phase, with the aid of methanol and acetone in the ultrasonic bath. 500 mg of the dried sample, was ultrasonicated for 5minutes with 4ml MetOH(methanol), 2ml MetOH, and 4 ml acetone. This process enabled SMX to be extracted from the solid phase and introduced into the liquid phase. Supernatants were collected and evaporated to a volume of approximately 200ul and then diluted with ddH2O

On the other hand, liquid phase samples of the test-bottles, were filtered with 0.45 μl filters, and diluted with ddH_2O(double distilled water) . All the antibiotic samples, including the sludge samples and the liquid phase samples were adjusted to pH 4 with sulfuric acid (H_2SO_4).

The cartridges that were going to be used for liquid solid phase extraction were conditioned. Firstly, they were washed with 1,5 ml MetOH-Ethyl acetate (1:1) 2 times and 1,5 ml MetOH containing 1% (v/v) ammonia 2 times, in order to clean the cartridges from the residues that could interfere with antibiotic measurements. In order to get rid of the MetOH, the cartridges were washed with 1,5 ml H_2O that was adjusted to pH 4 with H_2SO_4, 2 times.

All of the samples were percolated through cartridges at flow rate of less than 5ml/minute. After percolation, the cartridges were washed with 1,5 ml water-MetOH

(95:5) and eluent was discarded. Then, the cartridges were dried completely in a nitrogen flow for 1 h.

The analytes then will be eluted with,1,5 ml methanol- ethyl acetate (1:1) 2 times and then 1,5 ml methanol containing 1% ammonia into 10 ml graduated glass vessels.

Elutes were be reduced to 50 µl by a gentle flow of nitrogen at room temperature. The samples' volumes were adjusted to 0,5 ml with water and they were stored in amber glass at -15 Cº.

Both the liquid samples taken from the sludge and the wastewater samples were filtered from 0.45 µl filters. The filtered samples were purified and cleaned by solid phase extraction. Antibiotic concentration measurements were done in High Pressure Chromatography (HPLC), using c18 colon and UV detector. Method was optimized by having the detection limit 1mg/l as the lower limit and 200 mg/l as the upper limit in HPLC. SMX measurements done in the light of the protocol that Karcı and Balcıoğlu (2009), have applied.

4.6 Microbiological Analyses

The test sets that were stopped at 4 different times including the start-up time of the experiments, and from the genomic DNA samples that were taken from the test bottles, microbiological quantification was achieved. Triplicate samples were collected for DNA extraction from each point and these samples were stored at -20 ºC until DNA extraction.

4.6.1 DNA Extraction

From the samples that were taken specifically for the purpose genomic DNA was extracted with FAST DNA Spin Kit for Soil (MP Biomedical, France), which combines chemical and physical disruption for efficient genomic DNA extraction. The DNA was extracted according to the protocol supplied with the kit. The concentration of the extracted gDNA was measured with Qubit Fluoremeter(Invitrogen, U.S.) and the DNA was diluted to 25ng/µl, and stored at -20 ºC.

4.6.2 Q-PCR

4.6.2.1 Preperation of the Q-PCR Standards

For the preparation of the standards that will be used in the Q-PCR analysis, specific primers that would be used in the analysis were cloned.

Firstly, the extracted GDNAs were used as templates for the amplification of 16S gene sequences by specific primers using polymerase chain reaction (PCR). The primers used, and their annealing temperatures were given in Table 4.1.

The amplification of specific oligonucleotide sequences was achieved by setting up a PCR that had 50□l reaction volume containing 200 ng of DNA, 10 pmol of each primer, 10 mM of each deoxynucleoside triphosphate, 1.5 mM $MgCl_2$, 5 □l of 10×*Taq* buffer and 4u of *Taq* DNA polymerase (Fermentas, Latvia). The reactions were carried out in Techne TC-412 thermal cycler (Barloworld Scientific Ltd.,U.K.). All the reactions had an initial denaturation step at 94°C for 5 min, than had 30 cycles containing a denaturation step at 94°C for 1 min, an annealing step for 1 min and an extension step at 72°C for 2 min and a final extension step at 72°C for 10 min. Each specific primer couple, had their own specific annealing temperature.

After the amplification of the oligonucleotide sequences by PCR, in order to confirm that the correct oligonucleotide sequence is amplified, the PCR products are visualized by electrophoresis (Thermo-Scientific Ltd.,U.K.). The gel was prepared as 1% (w/v) agarose gel, and was run in 1× Tris–borate–EDTA buffer (89 mM Tris, 89 mM boric acid, 2 mM EDTA, pH 8.3), at 90 Volts for 15 minutes. The gel was screened by Smart 3000 gel documentation system (Vilber Lourmat, France) after staining with ethidium bromide. The positive PCR results that had the expected length for the oligonucleotide sequences were chosen for the cloning.

One of positive PCR product result was chosen for cloning, and the PCR product was cloned by the protocol supplied with the TOPO TA Cloning Kit. The colonies were grown on agar plates containing 50 □g/ml kanamycin.

Table 4.1: Bacterial and archaeal oligonucleotide primers used for PCR amplification

Target Microorganism	Primer	Sequence of the Primer	Target gene	Annealing Temprature	Reference
Bacteria	Bac519f	5′ - CAGCMGCCGCG GTAANWC-3′	16S rRNA	53	Lane, 1991
	Bac907r	5′ - CCGTCAATTCMT TTRAGTT-3′			
Archaea	Arc349f	5′ - GYGCASCAGKCG MGAAW-3′	16S rRNA	55-60	Takai et al,2000
	Arc806r	5′ - GGACTACVSGGG TATCTAAT-3′			
Methanogen	Met348f	5′ - GYGCAGCAGGC GCGAAA-3′	16S rRNA	55	Sawayama et al., 2006
	Met786r	5′ - GGACTACVSGGG TATCTAAT-3′			
Sulfate Reducing Bacteria	DSRp2060F	5′ - CAACATCGTYCA YACCCAGGG-3′	Sulfite Reductase Beta Subunit (dsrB)	55	Geets et al., 2005
	DSR4R	5′ -GTG TAG CAG TTA CCG CA-3′			

4.6.3 Q-PCR Analyses

Ten standards of serial different dilutions were prepared to quantify the number of gene expression of each gene in question. A calibration curve was drawn by using these standards by the program used.

The procedure recommended by Roche was followed and Light Cycler Master Kit (Roche) was used to set up the reaction (2.0 ❐l master mix, 1.6 ❐l $MgCl_2$ 1.0 ❐l Primer F and R, 13.4 ❐l H_2O, 1 ❐l sample). All the Qpcr reactions were utilized in LightCycler (Roche Diagnostics GmbH, Mannheim, Germany)

To observe the results of the reaction, Light Cycler Software 4.05 program provided by Roche was used. The program consists of four sections; denaturation (95^0C), amplification (95^0C, 56^0C, 72^0C), melting (95^0C, 53^0C, 95^0C) and cooling (40^0C). The view of the software that is used, is given in figure 4.1.

Colonies were picked from plate and transferred into 200 µl PCR tubes containing 50 µl TE buffer (10mM Tris-HCl, 1mM EDTA pH 8.0). Colonies were boiled at 95^0 C for 5 minutes then frozen at -20^0 C overnight. Thawed solution was used as templates for PCR. The DNA fragments were isolated from vector by PCR with primers M13f-M13r (M13 Forward 5′-GTA AAA CGA CGG CCA G-3′/ M13 Reverse 5′-CAG GAA ACA GCT ATG AC-3′)

The PCR products were purified with Invitrogen PCR product purification Kit, according to the manufacturer's specifications. The yield of purified dsDNA has been estimated by agarose gel electrophoresis. To estimate the yield, agarose gel electrophoresis of the purified PCR product and known quantities of DNA fragment of the same size was performed. The band intensity of the purified PCR product with the standard DNA fragments was compared. So the purified PCR product was used as Q-PCR standards. The standards concentrations were determined using a fluorometer (Qubit, Invitrogen) according to the manufacturer's specifications. Application ready standards were diluted in 1/100 ratio for Q-PCR experiments.

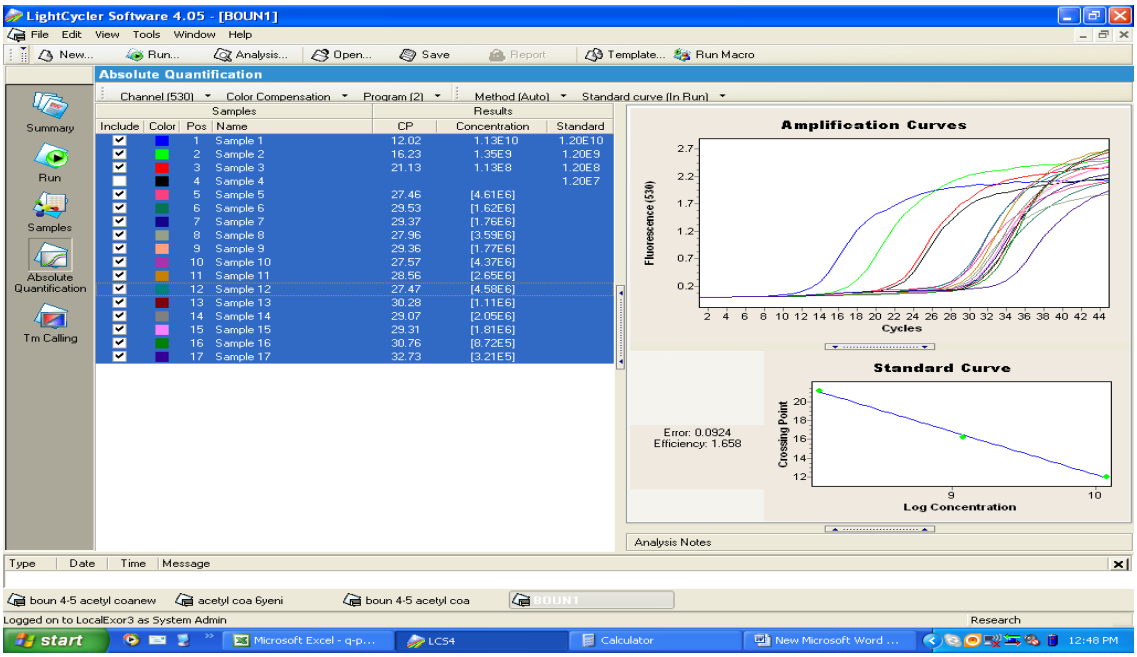


Figure 4.1. View of Light Cycler Software 4.05

To convert the detected gene targets into cell numbers, averages of 3.6 and 1.6 copies of the 16S rRNA gene were estimated for Bacteria and Achaea, respectively (Klappenbach,2001). Copy numbers of all other genes were directly correlated to cell numbers (Phillippot, 2002; Da Silva and Alvarez, 2002; Beller, 2002).

4.7 Statistical Analysis

The experimental results were analyzed statistically by SPSS program.

5 RESULTS AND DISCUSSION

5.1 Biogas, Methane and Antibiotic

In figures 5.1, 5.2 and 5.3, biogas and methane production that is observed for the test period of 120 days in reference test bottles (REF) which contain phenol as carbon source and control test bottles (NC) that has no carbon source is shown for all the different electron acceptor conditions. There was no gas production detected in test bottles that contained inhibitor, thus the results are not shown.

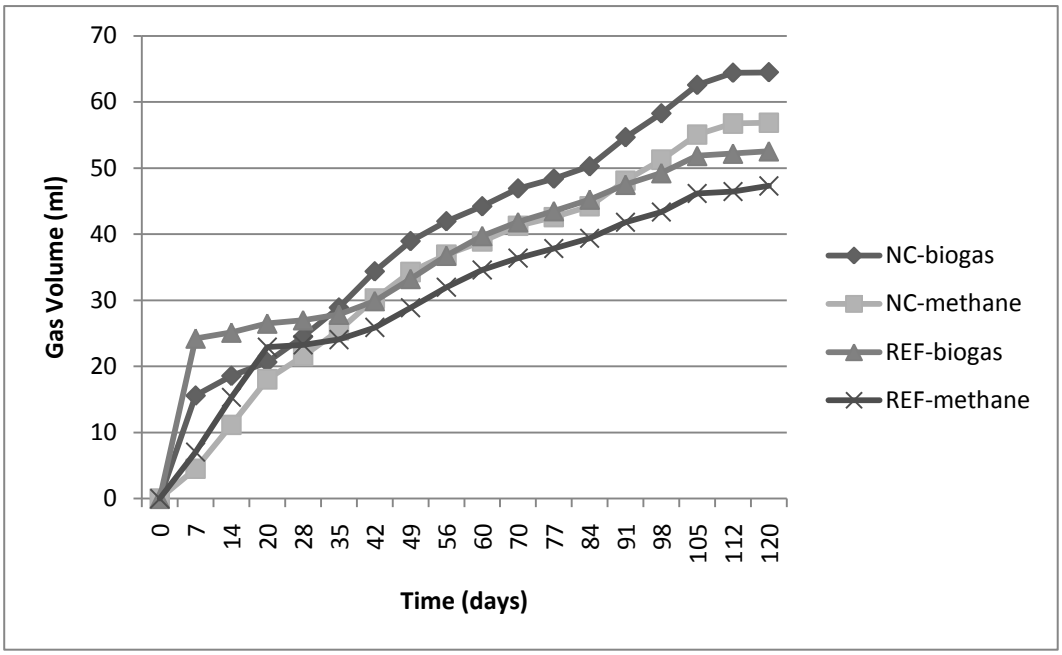


Figure 5.1: Total biogas and methane production in REF and NC test bottles in methanogenic conditions

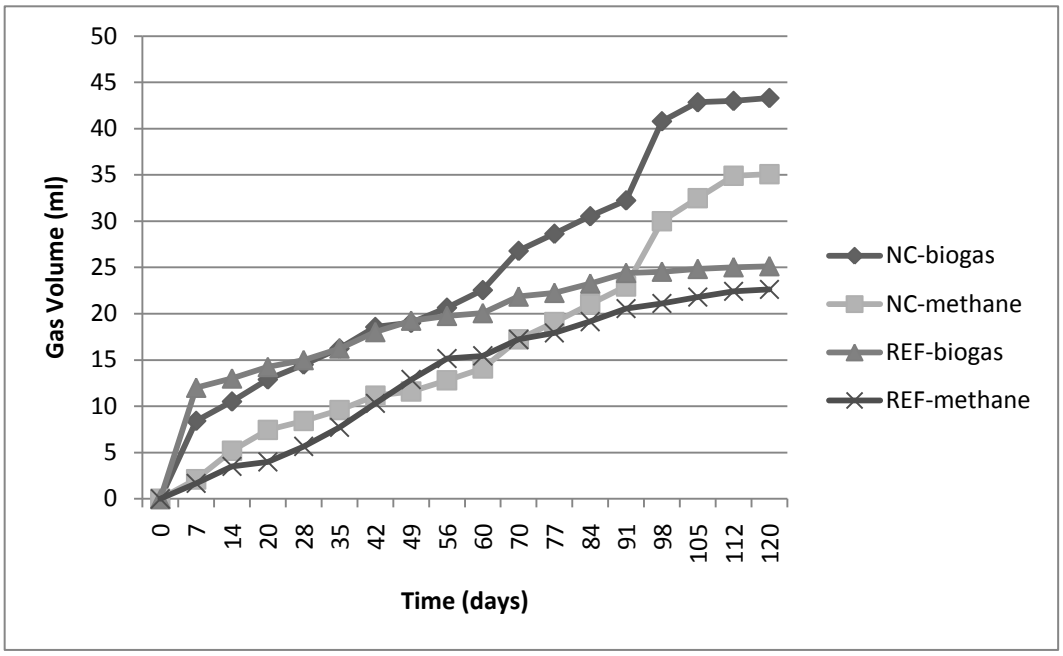


Figure 5.2: Total biogas and methane production in REF ve NC test bottles in nitrate reducing conditions

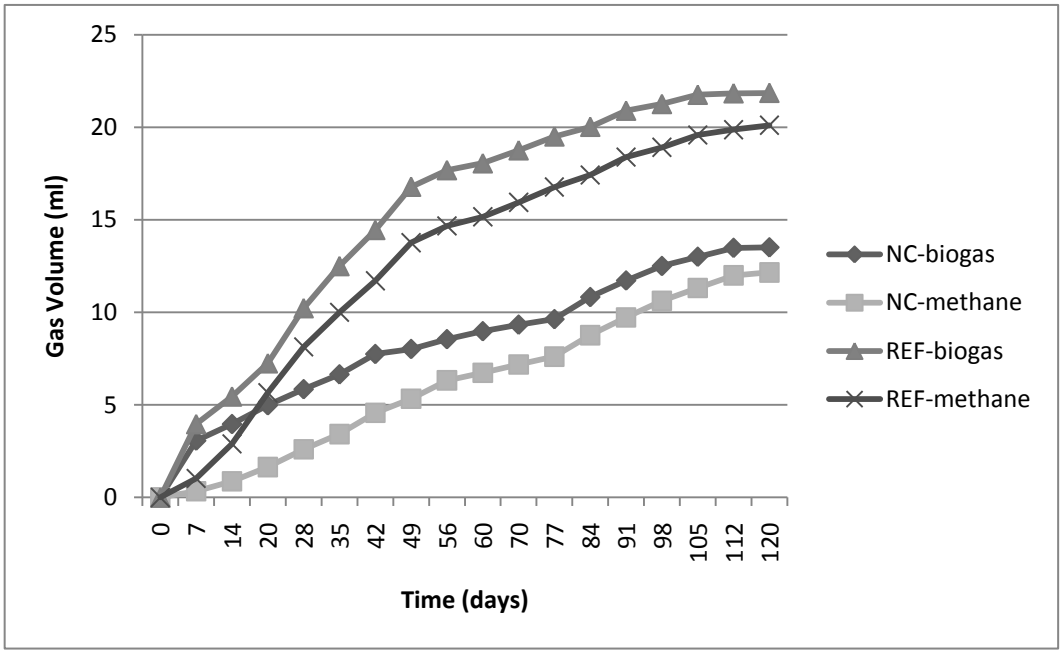


Figure 5.3: Total biogas and methane production in REF ve NC test bottles in sulfate reducing conditions

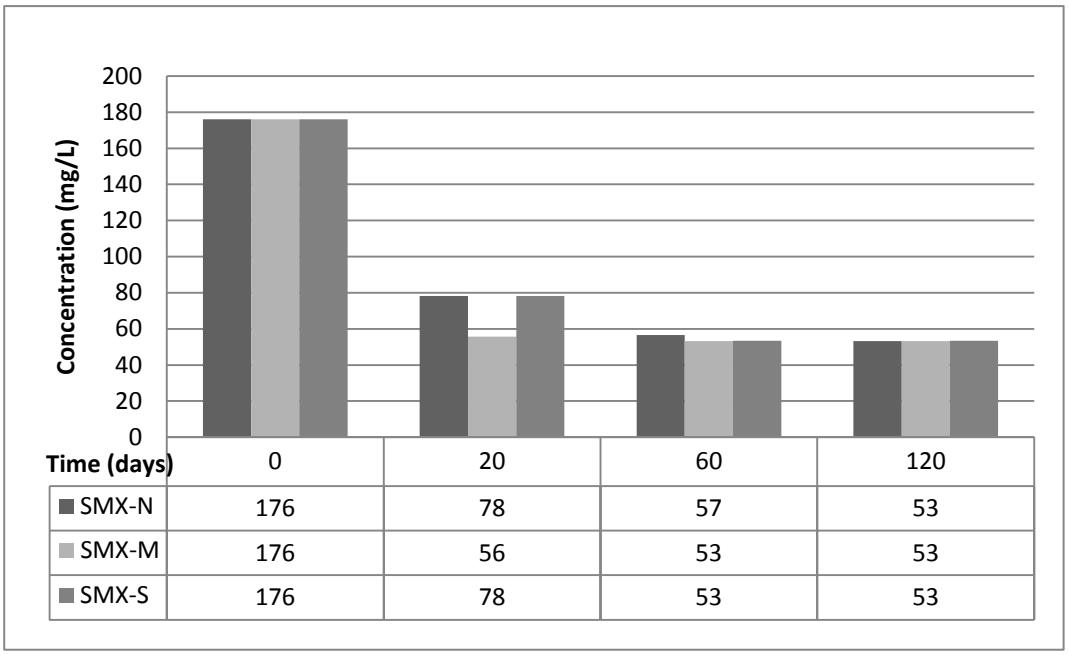
Gas data is given cumulatively for 120 days. For methanogenic conditions, non-carbon test tubes have 64 ml biogas and 56ml methane production at the end of 120 days, whereas for reference test tubes there is 52 ml biogas and 47 ml methane production in total. If a nitrate-reducing condition is observed for non-carbon and reference test tubes, 43 ml biogas and 35 ml methane in non-carbon test tubes, and 25 ml biogas and

22 ml methane can be seen at the end of the test period. Finally, in sulfate reducing conditions, 13 ml biogas and 12 ml methane observed in non–carbon test tubes, while 21 ml biogas and 20 ml methane was observed in reference test tubes.

The fact that the biogas and methane production in reference test tubes, in methanogenic and nitrate reducing conditions, leads to the conclusion that the GC analysis that were carried out were problematic. Therefore, the biogas and methane data for these two electron-accepting conditions are not reliable.

Antibiotic concentration is measured both is sludge and liquid phase samples taken from the test bottles. All the samples that were taken from the test bottles were cleaned by solid-phase extraction and they were measured by %92 recovery. The antibiotic concentrations of the sludge samples of the test bottles, showed no change over time, for all the three different electron acceptor conditions. The antibiotic concentration of the sludge samples were 50.4±3 mg/l.

One of the characteristics of antibiotics are sorption quality, so the fact that the antibiotic concentration that was measured over time in the sludge samples were unchanging, shows that the antibiotic that was adsorbed and/or absorbed to the sludge had no biological and/or photolytic degradation. The antibiotic concentrations that change over time for three electron acceptor conditions are given in figure 5.4.



Time (days)	0	20	60	120
SMX-N	176	78	57	53
SMX-M	176	56	53	53
SMX-S	176	78	53	53

Figure 5.4: SMX concentration change over time in nitrate reducing, sulfate reducing and methanogenic conditions

The antibiotic removal efficiency is nearly %98 in the liquid phase samples, in the three different electron acceptor conditions. If the antibiotics that are absorbed to the sludges are taken into consideration, the total antibiotic removal efficiency decreases to levels of %70.

Most of antibiotic removal continues to day 60, however in between days 60 and 120 no significant change is observed in antibiotic concentrations. In nitrate reducing conditions, the sudden decrease of the final electron acceptor concentration between days 40 and 60 may affect activity negatively (Thomas et al., 1998).

In the figures 5.5, 5.6 and 5.7, the graphs representing the biogas production from the test bottles are shown in 7-day intervals for 120 days in which antibiotic is degraded microbiologically. In the correlation analyses, it is shown that there is a strong correlation between biogas production and antibiotic removal. According to Pearson correlation, for nitrate-reducing conditions there is +0,828, for methanogenic conditions +0,935 and for sulfate reducing conditions +0,855 correlations is found between biogas production and antibiotic removal. Similar results are obtained in graphs, showing biogas production along with antibiotic removal.

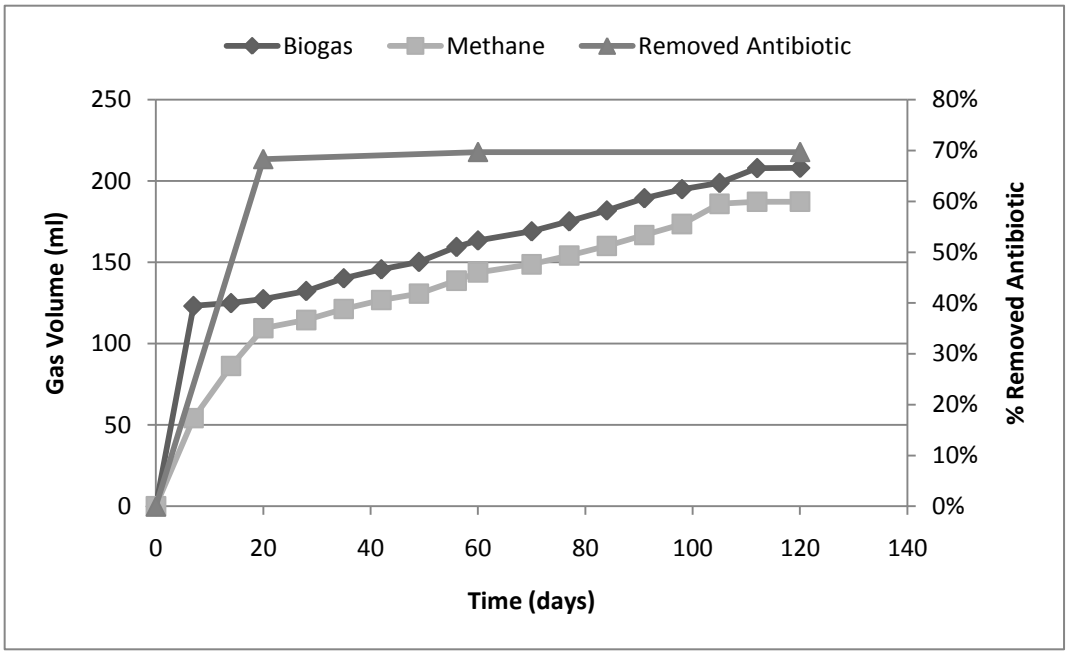


Figure 5.5: Change in biogas, methane volume and antibiotic removal percentage over time for 120 days in methanogenic conditions

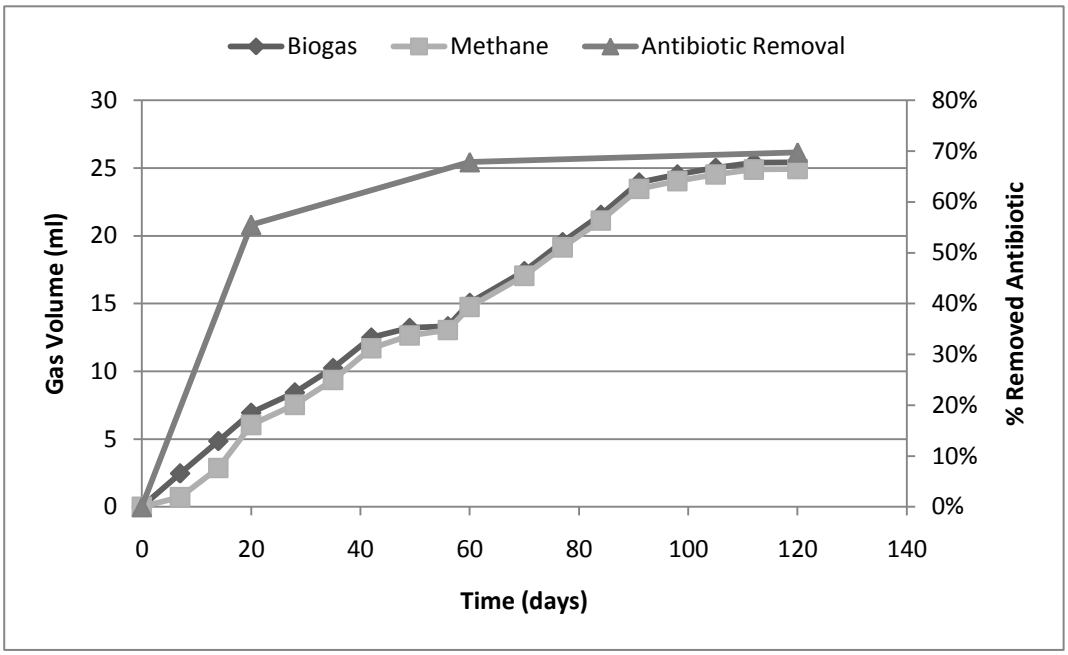


Figure 5.6: Change in biogas, methane volume and antibiotic removal percentage over time for 120 days in nitrate reducing conditions

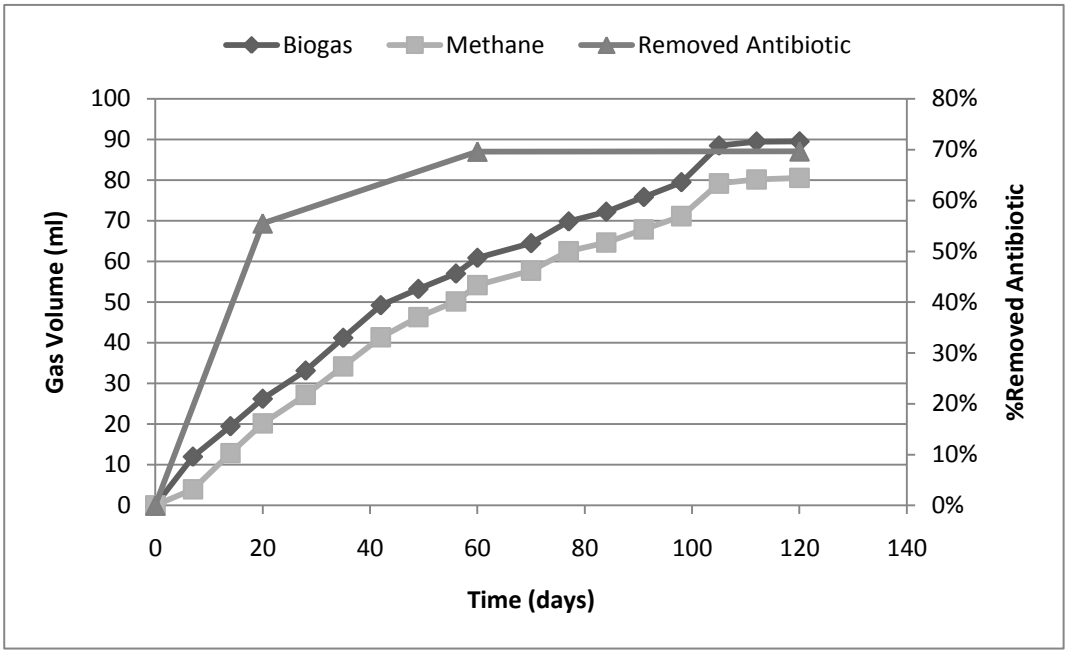


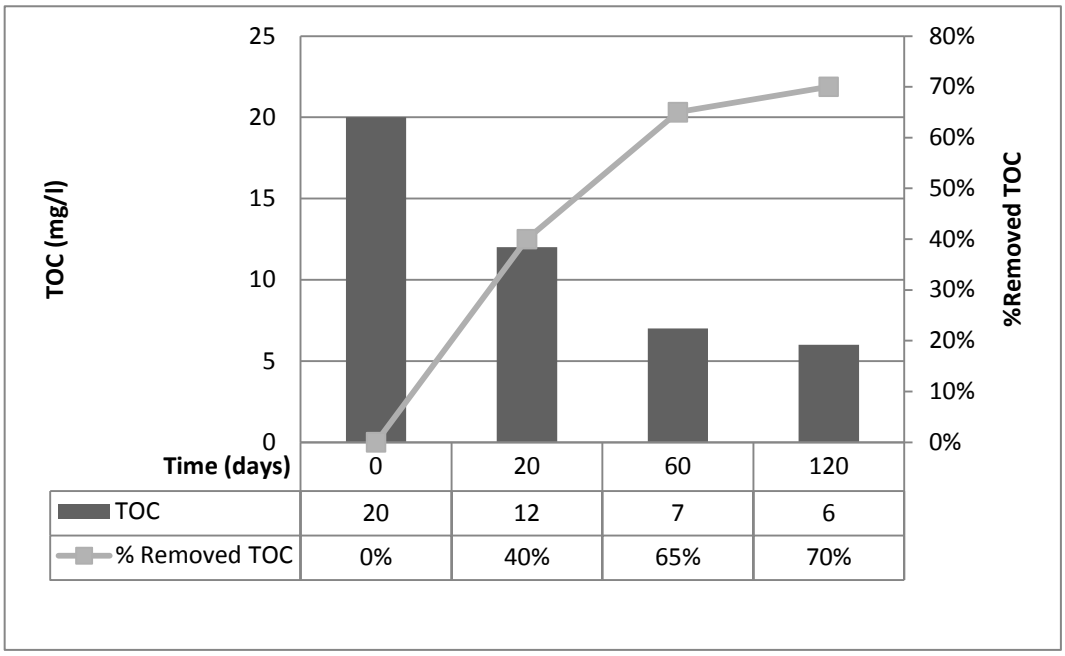
Figure 5.7: Change in biogas, methane volume and antibiotic removal percentage over time for 120 days in sulfate reducing conditions

As expected, the most biogas and methane production was observed in test bottles that were set up in methanogenic conditions. The low nitrate and sulfate concentrations in those test bottles, may have made methanogens advantageous compared to the other microorganisms. In addition, the antibiotic removal in methanogenic conditions is relatively faster than the other two electron acceptor conditions. In the methanogenic

condition test tube samples taken in day 20, %68 of the antibiotic was degraded, while in the samples taken in day 120, %70 of the antibiotic were degraded. In addition, the rapid increase, of the biogas production, also supports the rapid degradation of SMX in methanogenic conditions.

5.2 Total Organic Carbon (TOC) and Ion Chromatography

In Figures 5.8 and 5.9, the change in TOC concentration and TOC removal efficiency are given for test tubes that have no carbon source, and the test tubes that are containing reference carbon source in methanogenic conditions. On the other hand, in figures 5.10 to 5.13, changes in TOC and final electron acceptor concentration over time are given for non-carbon and reference test bottles under nitrate and sulfate reducing conditions. For all the test tubes, the initial nitrate concentrations were given as 230±20 mg/l, while the initial sulfate concentrations were given as 470±30 mg/l. There was no change observed over time for inhibitor containing test bottles in TOC analysis.



Time (days)	0	20	60	120
TOC	20	12	7	6
% Removed TOC	0%	40%	65%	70%

Figure 5.8: TOC Removal for NC in methanogenic conditions

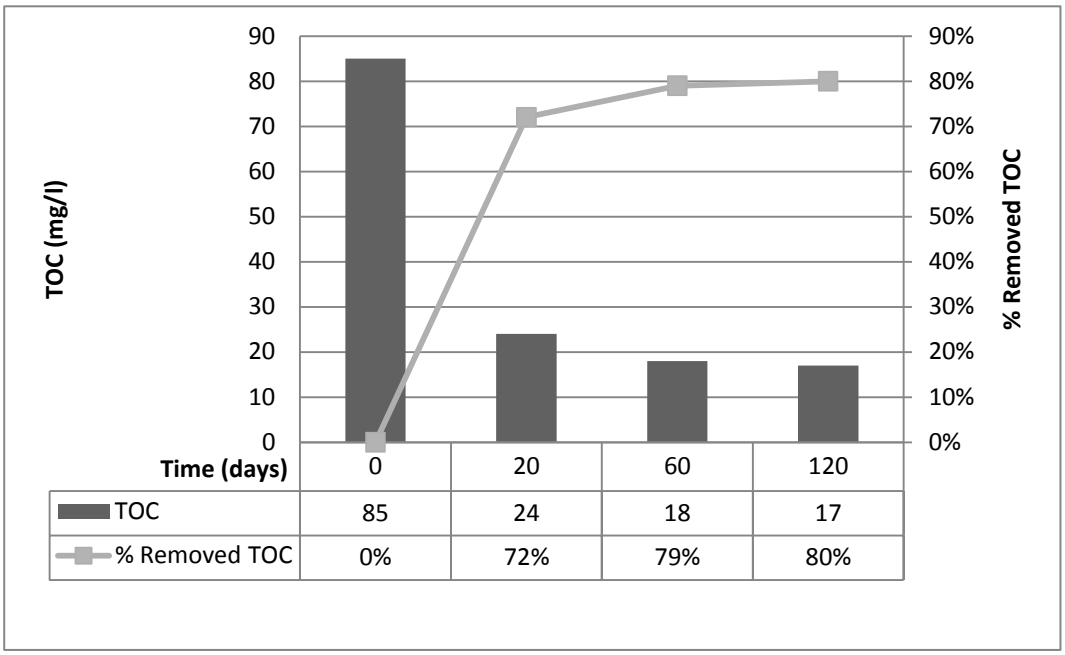


Figure 5.9: TOC Removal for REF in methanogenic conditions

In the test tubes containing no carbon source in methonogenic conditions, 20 mg/l TOC was measured in the start of the experiment, while 85 mg/l TOC was measured in reference (REF) test tubes having phenol as the only carbon source.

In non-carbon test tubes set-up in nitrate reducing conditions, TOC was totally eliminated when the day 60 was reached, while in methanogenic condition, the removal was %70 and in sulfate reducing condition the removal was %52 at the end of the 120 days. Even though the starting TOC amount for non-carbon containing test tubes in all the electron-reducing conditions is nearly the same about 20±1 mg/l, in nitrate reducing conditions the TOC removal percentage at the end of 120 days, is higher than the other two electron reducing conditions.

In the phenol containing reference (REF) test-bottles, the TOC removal is about 80% for all the electron accepting conditions. The reference organic compound, phenol that is hard to degrade biologically, verified results fit results according to the OECD 311 protocol, and showed the realibility of the test set-up.

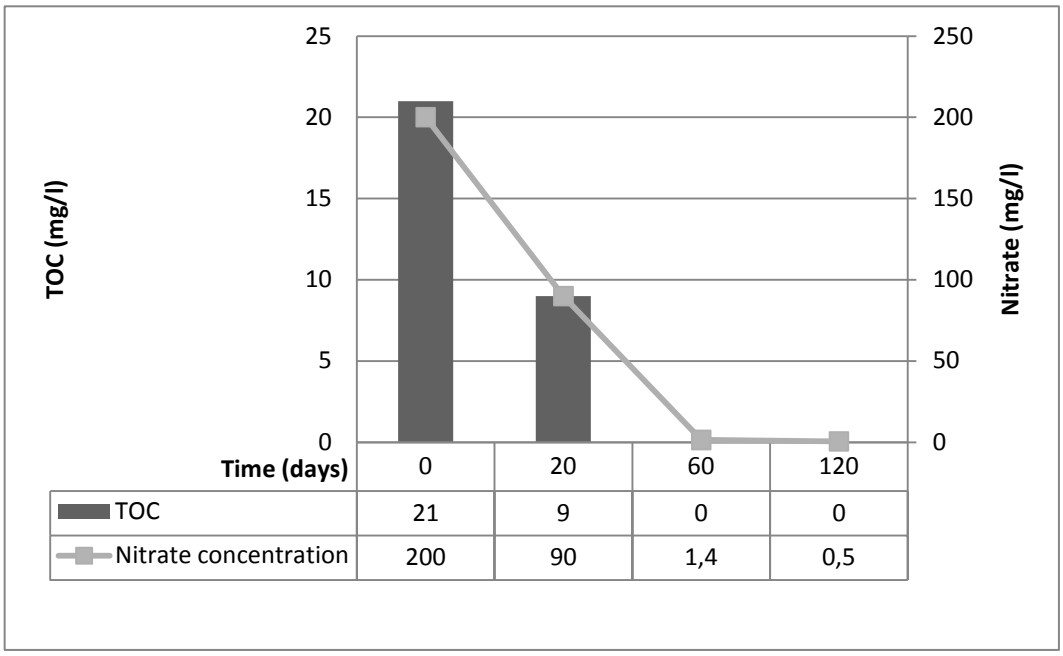


Figure 5.10: TOC removal and nitrate concentration change over time in NC test tubes for nitrate reducing conditions

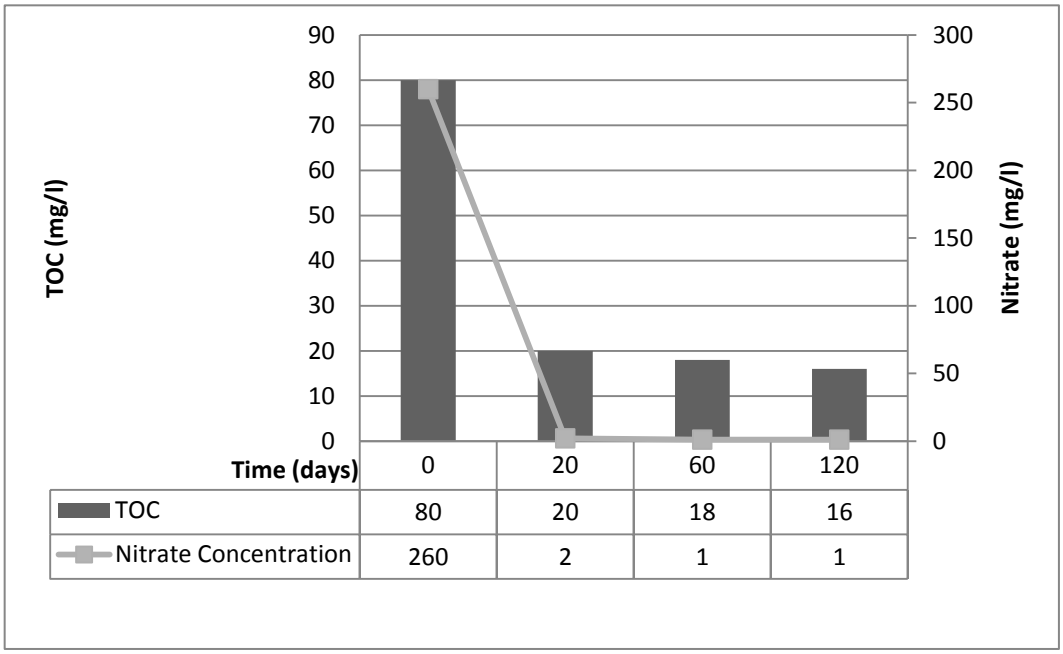
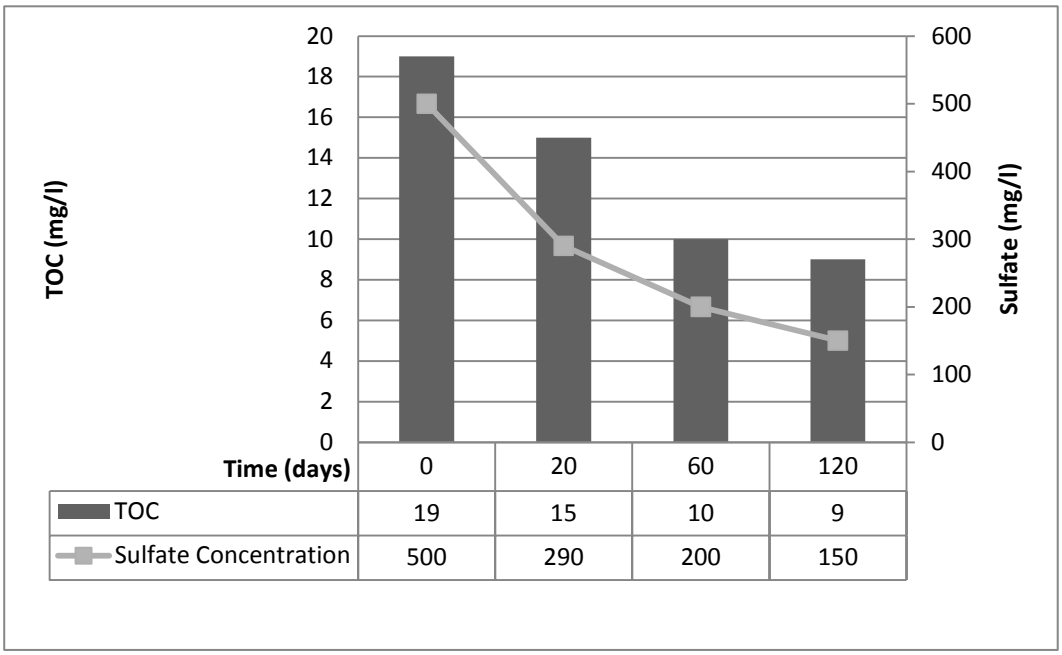
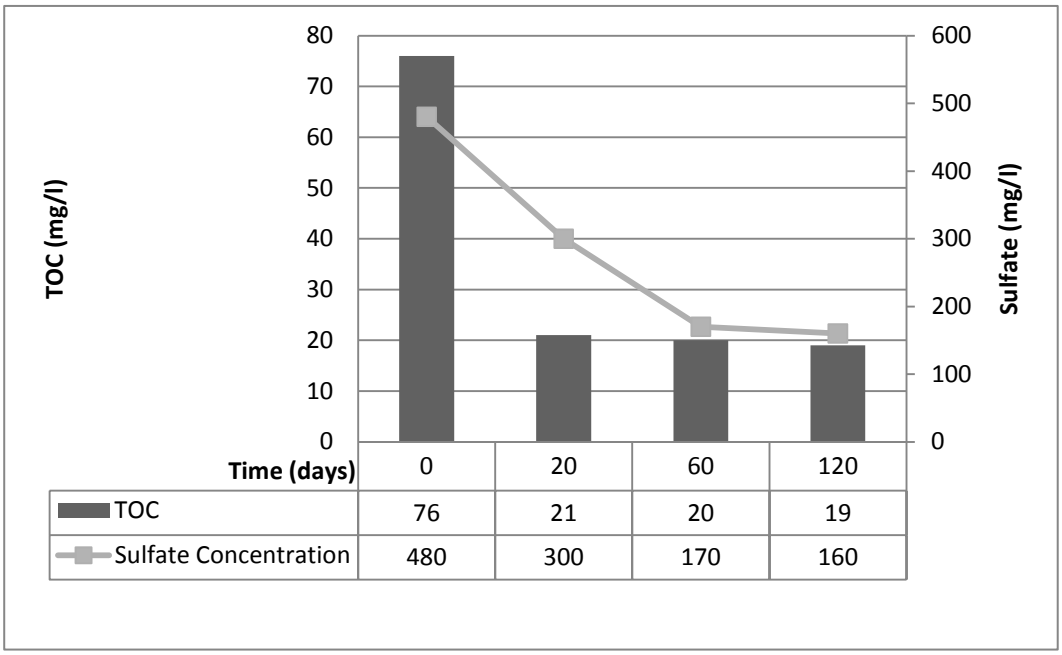


Figure 5.11: TOC removal and nitrate concentration change over time in REF test tubes for nitrate reducing conditions



Time (days)	0	20	60	120
TOC	19	15	10	9
Sulfate Concentration	500	290	200	150

Figure 5.12: TOC removal and sulfate concentration change over time in NC test tubes for sulfate reducing conditions



Time (days)	0	20	60	120
TOC	76	21	20	19
Sulfate Concentration	480	300	170	160

Figure 5.13: TOC removal and sulfate concentration change over time in REF test tubes for sulfate reducing conditions

The non-carbon (NC) test tubes in which nitrate was used as the final electron acceptor, it is observed that nitrate in the test bottles was nearly consumed during 60 days, while in REF test bottles which contained phenol as the carbon source, nitrate utilized in first 20 days. These results are parallel to the results of TOC removal in REF test bottles. In REF test bottles, it is seen that the nitrate concentrations retained

very low after 20th day, while the TOC elimination after this time nearly stopped. When the nitrate diminished in the test bottles, the TOC removal was also limited in REF test bottles.

Under sulfate reducing condition, the sulfate concentration became 155 mg/l on average at the end of 120-day test period in both REF and NC test bottles. The sulfate concentration utilized with time, is parallel to the TOC removal results, thus sulfate was not seen as limiting factor for TOC removal.

In figure 5.14, the change of average TOC values of test bottles set-up in methanogenic conditions over time, and TOC removal efficiency is given. In figures 5.15 and in figures 5.16, TOC values for nitrate and sulfate reducing conditions are given. along with the change of final electron acceptor concentration change over time.

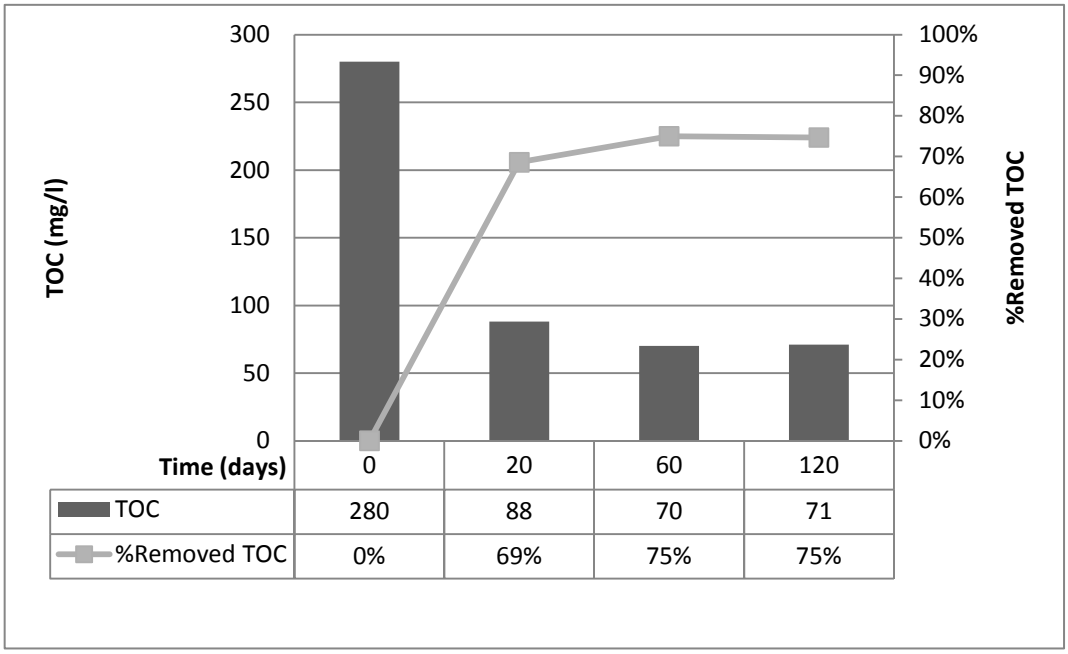


Figure 5.14: TOC removal in methanogenic conditions

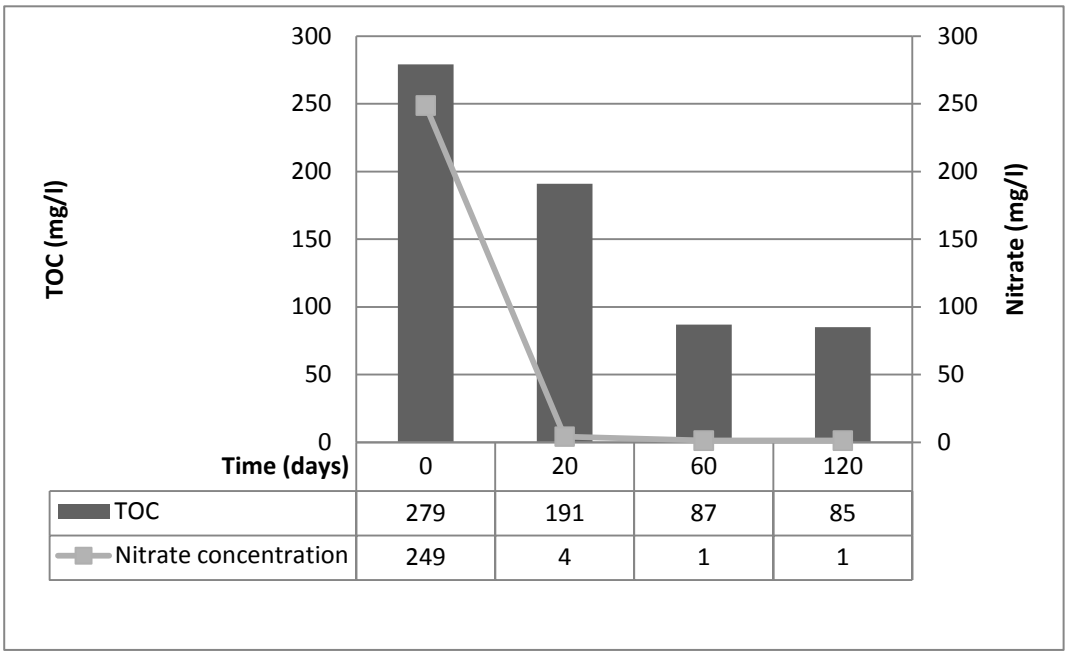


Figure 5.15: TOC removal in nitrate reducing conditions and nitrate concentration change

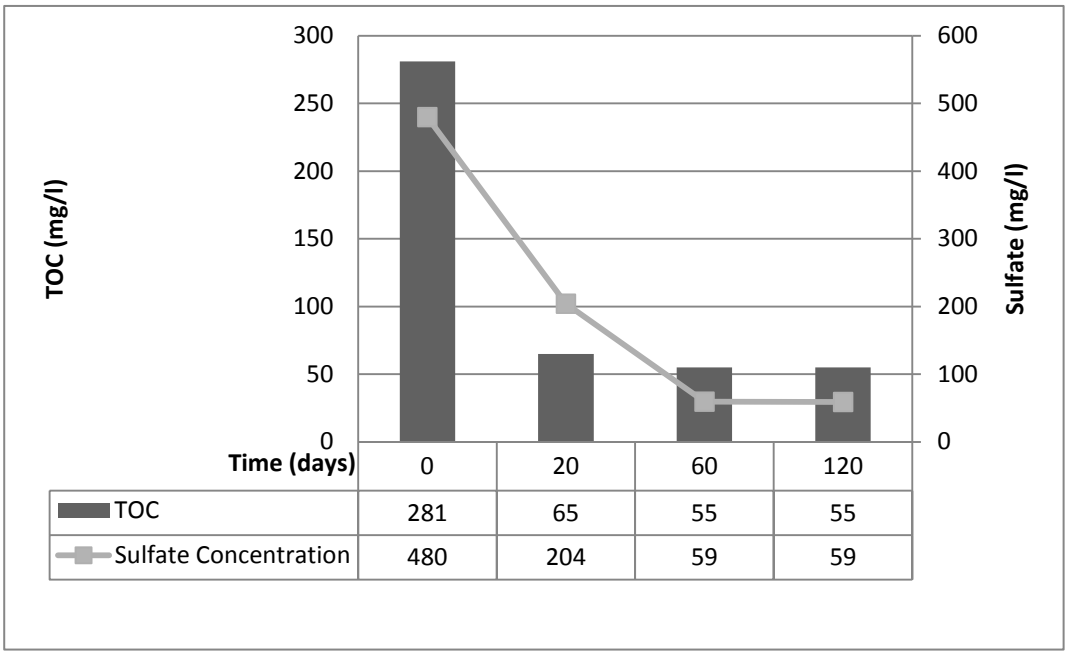


Figure 5.16: TOC removal in sulfate reducing conditions and sulfate concentration change

In the test tubes that were set-up using OECD311 protocol with slight modifications, TOC removal reaches its highest point in day 60 for all the three electron reducing conditions. In addition, between days 60 and 120 no change is observed in TOC values.

Nitrate concentration change over time in nitrate reducing conditions in consistent with the TOC results. Nitrate is depleted at the end of day 60, thus the TOC removal slows down, reaching day 60.

The sulfate concentration in the test tubes set-up in sulfate reducing conditions, were 480 mg/l at the start of the experiment, after 60 days, sulfate concentration decreases to 59 mg/l.

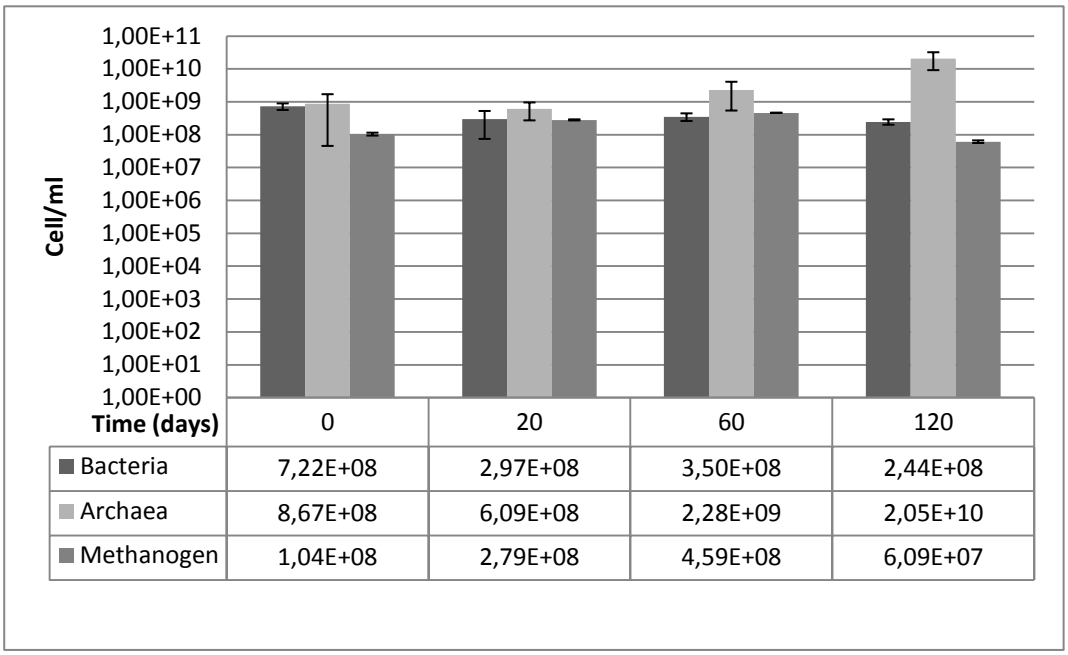
The highest TOC removal, in the test tubes that Sulfamethaxasole is used as the only carbon source, was in sulfate reducing conditions with %78 removal. While %74 removal is observed in methanogenic conditions, the lowest removal efficiency is observed in nitrate reducing conditions with %71 removal.

5.3 Quantitative PCR (Q-PCR)

By Quantitative PCR method, the bacterial and archaeal populations in NC, REF and SMX (Experimental) bottles that were set-up under three different electron acceptor conditions were determined, thus endogenous decay and the effects of the reference organic compound which the degradation characteristics is well-known on bacterial and archeal populations were examined. In addition, metanogenic population count was done because the methane production data was also available for three different electron acceptor conditions. Finally, in sulfate reducing conditions, where sulfate was used and a change in sulfate concentration observed, so sulfate reducing bacteria (SRB) amount was determined. The microbial organisms were not counted by Q-PCR for inhibitor containing test bottles, for there was no change over time observed for biogas and TOC results.

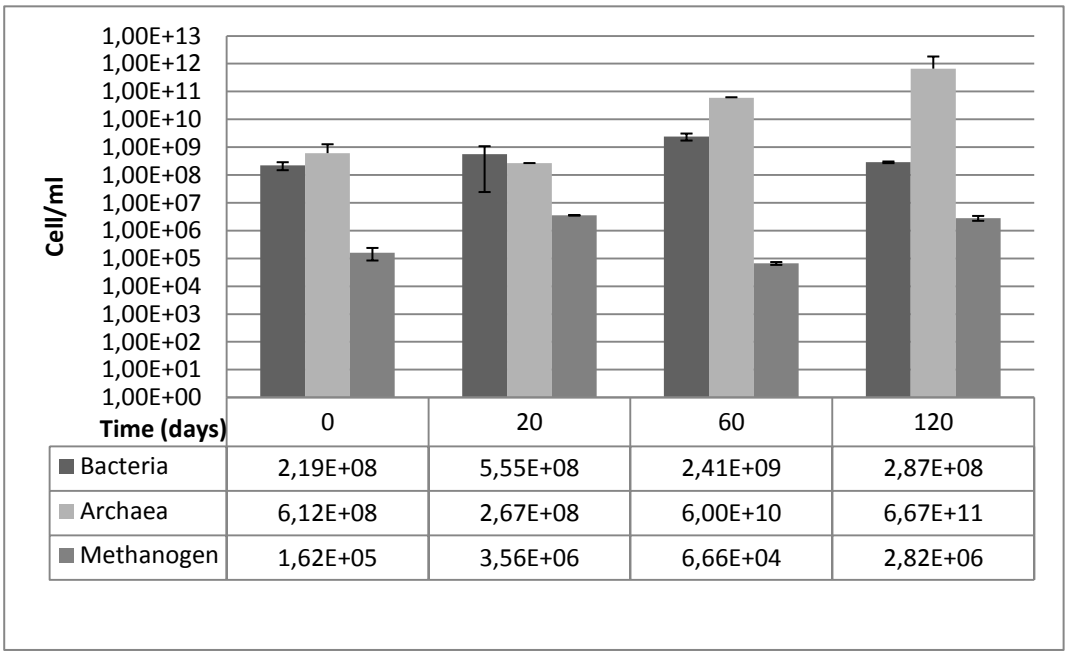
The seed was taken from an anaerobic reactor treated wastewater of an alcoholic drinks industry for methanogenic and sulfate reducing conditions, while the seed for nitrate reducing condition was taken from an aerobic-anoxic reactor of domestical wastewater treatment plant.

In Figures 5.17, 5.18 and 5.19 bacterial, archaeal, methanogenic and SRB population changes are given over time for NC in the order; methanogenic, nitrate and sulfate reducing conditions.



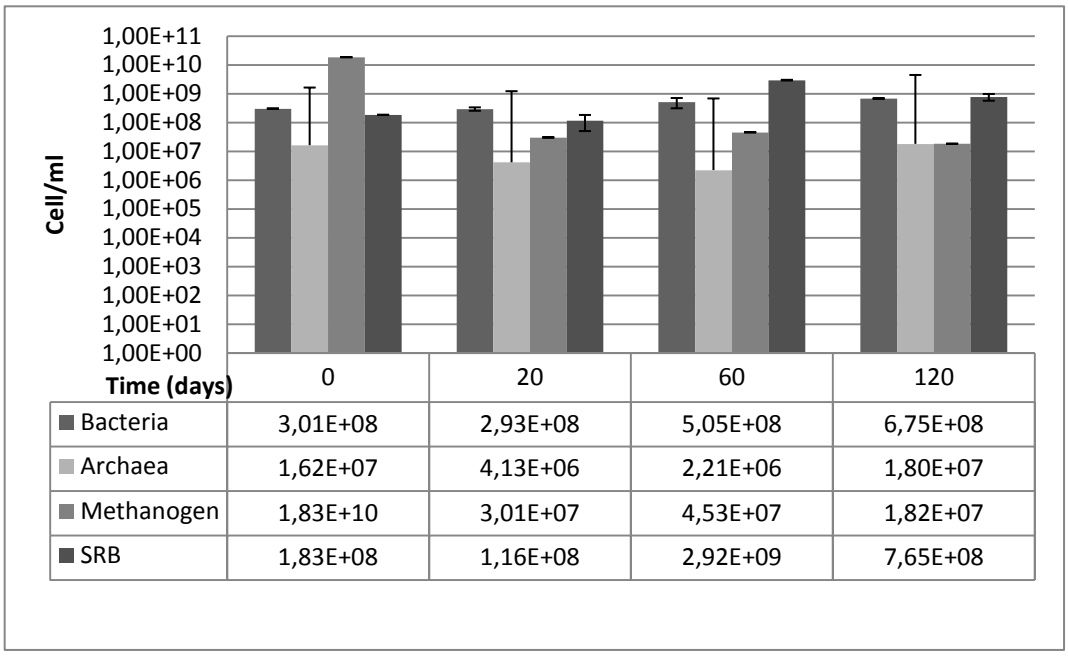
Time (days)	0	20	60	120
Bacteria	7,22E+08	2,97E+08	3,50E+08	2,44E+08
Archaea	8,67E+08	6,09E+08	2,28E+09	2,05E+10
Methanogen	1,04E+08	2,79E+08	4,59E+08	6,09E+07

Figure 5.17: Bacteria, archaea, and methanogen count for NC in methanogenic conditions



Time (days)	0	20	60	120
Bacteria	2,19E+08	5,55E+08	2,41E+09	2,87E+08
Archaea	6,12E+08	2,67E+08	6,00E+10	6,67E+11
Methanogen	1,62E+05	3,56E+06	6,66E+04	2,82E+06

Figure 5.18: Bacteria, archaea, and methanogen count for NC in nitrate reducing conditions



Time (days)	0	20	60	120
Bacteria	3,01E+08	2,93E+08	5,05E+08	6,75E+08
Archaea	1,62E+07	4,13E+06	2,21E+06	1,80E+07
Methanogen	1,83E+10	3,01E+07	4,53E+07	1,82E+07
SRB	1,83E+08	1,16E+08	2,92E+09	7,65E+08

Figure 5.19: Bacteria, archaea, sulfate reducing bacteria and methanogen count for NC in sulfate reducing conditions

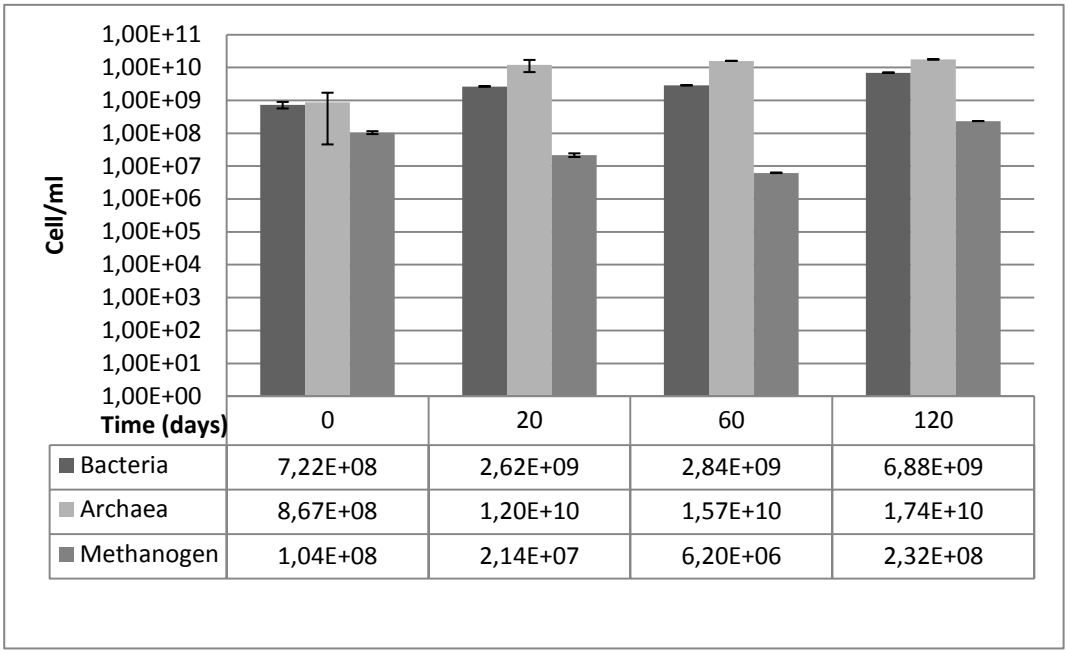
The bacteria population increased until 60th day than a decrease was observed under methanogenic conditions for NC bottles. Amounts of methanogen and Achaea increased in the while bacterial population decreased.

Both in nitrate reducing conditions and methanogenic conditions, the bacteria population started to decrease in 60th day, while the increase in Achaea population still continued. Under nitrate reducing conditions, methanogens showed a significant increase between 20th and 40th days, and also methanogens increased over time, having -0,700 correlation with time ($p<0,05$). The reason for this increase of methanogens in non-supporting conditions may depend on the depletion of nitrate when day 20 is reached in the experiments. It is acceptable to see methanogens, in sulfate and nitrate-reducing conditions, while there has been methanogens found in active sludge of real scale domestic wastewater treatment plants. The methanogens and Achaea found in Q-PCR results verify the methane production found in nitrate and sulfate reducing conditions in the gas data.

As seen in Figure 5.19, in sulfate reducing conditions, archaeal and methanogenic communities were declining, while the bacterial community increased until day 60, than declined to day 120. Even though the sulfate reducing bacteria were less, compared to the other microorganism communities, it is seen that sulfate-reducing

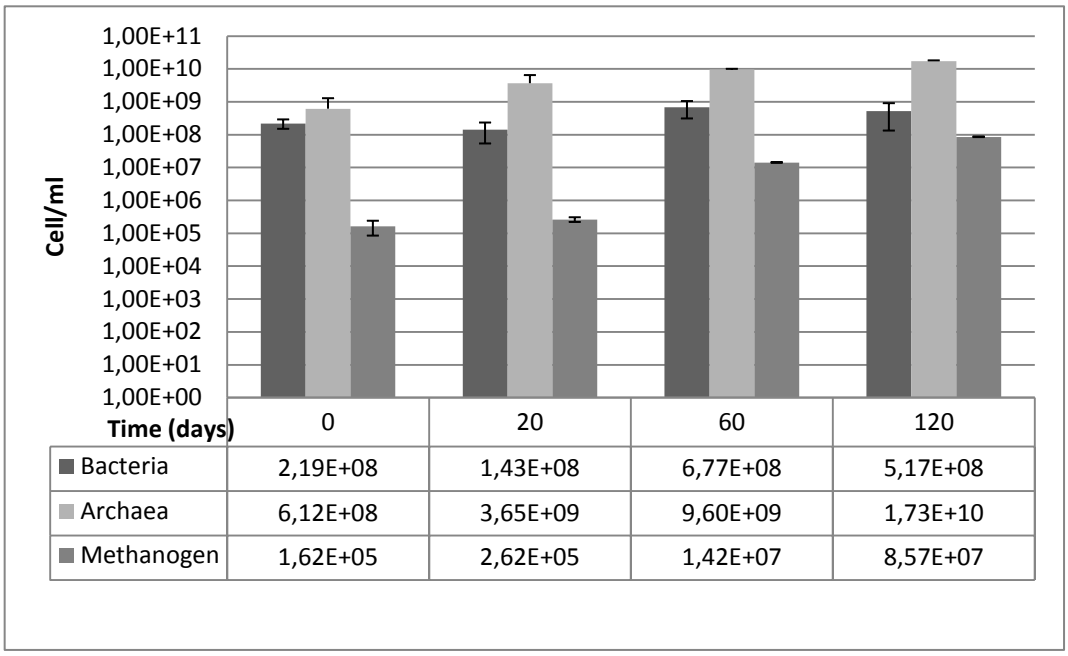
bacteria were increasing over time. The increase in sulfate reducing bacteria was in correlation with time, having +0,614 correlation ($p<0.05$). In addition, bacterial community increases having +0.766 correlation with time ($p<0.05$), while methanogens have -0,687 correlation with time. Overall, from the Q-PCR results for NC test bottles for sulfate reducing conditions, the knowledge in the literature is supported. Sulfate reducing conditions do not support archaeal community while it favors sulfate-reducing organisms as it is pointed out in a study by Stams and his friends (2003).

In figure, 5.19, 5.20 and 5.21 the Q-PCR results for reference (REF) test bottles that contain phenol as the only carbon source are given. The microbial count of different microbial groups under different electron acceptor conditions in the reference test bottles; show that the positive control tests show biological activity.



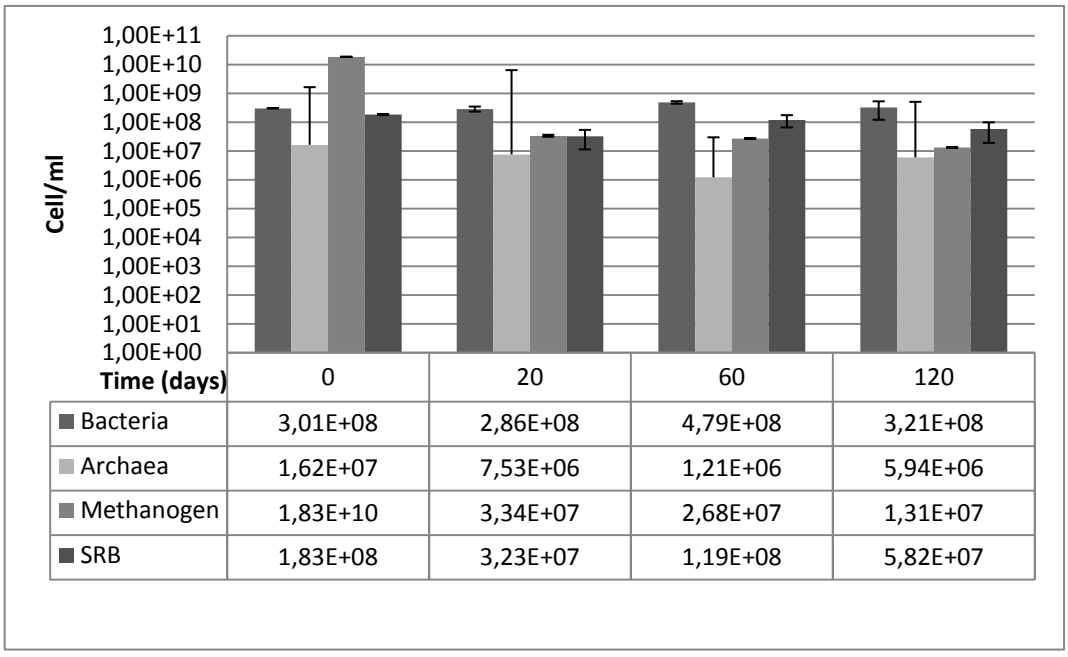
Time (days)	0	20	60	120
Bacteria	7,22E+08	2,62E+09	2,84E+09	6,88E+09
Archaea	8,67E+08	1,20E+10	1,57E+10	1,74E+10
Methanogen	1,04E+08	2,14E+07	6,20E+06	2,32E+08

Figure 5.20: Bacteria, archaea, and methanogen count for REF in methanogenic conditions



Time (days)	0	20	60	120
Bacteria	2,19E+08	1,43E+08	6,77E+08	5,17E+08
Archaea	6,12E+08	3,65E+09	9,60E+09	1,73E+10
Methanogen	1,62E+05	2,62E+05	1,42E+07	8,57E+07

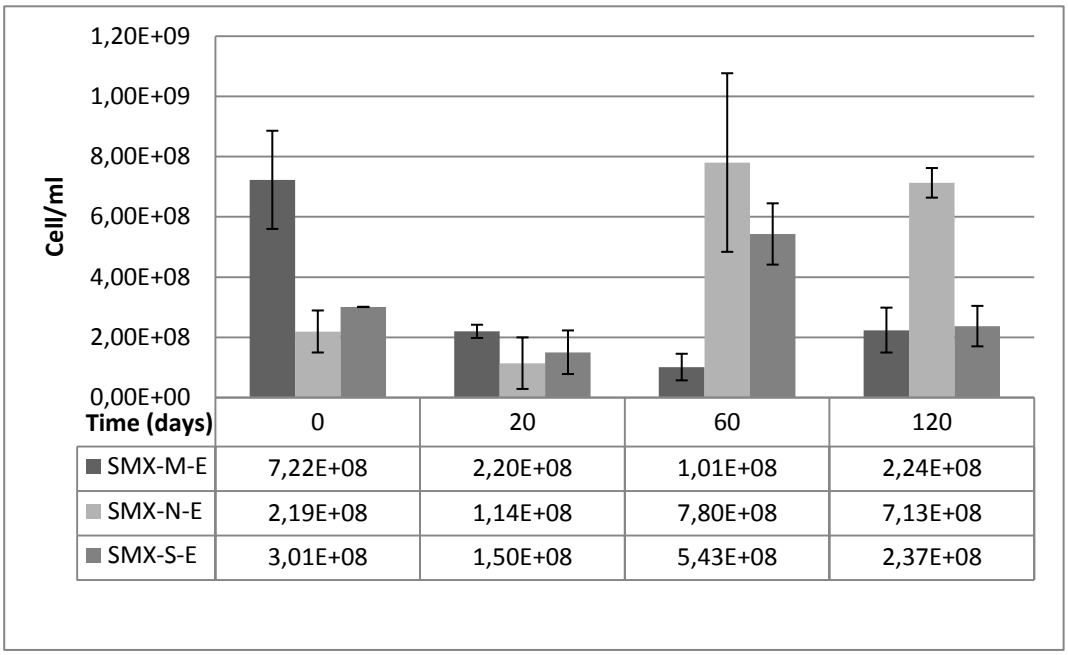
Figure 5.21: Bacteria, archaea, and methanogen count for REF in nitrate reducing conditions conditions



Time (days)	0	20	60	120
Bacteria	3,01E+08	2,86E+08	4,79E+08	3,21E+08
Archaea	1,62E+07	7,53E+06	1,21E+06	5,94E+06
Methanogen	1,83E+10	3,34E+07	2,68E+07	1,31E+07
SRB	1,83E+08	3,23E+07	1,19E+08	5,82E+07

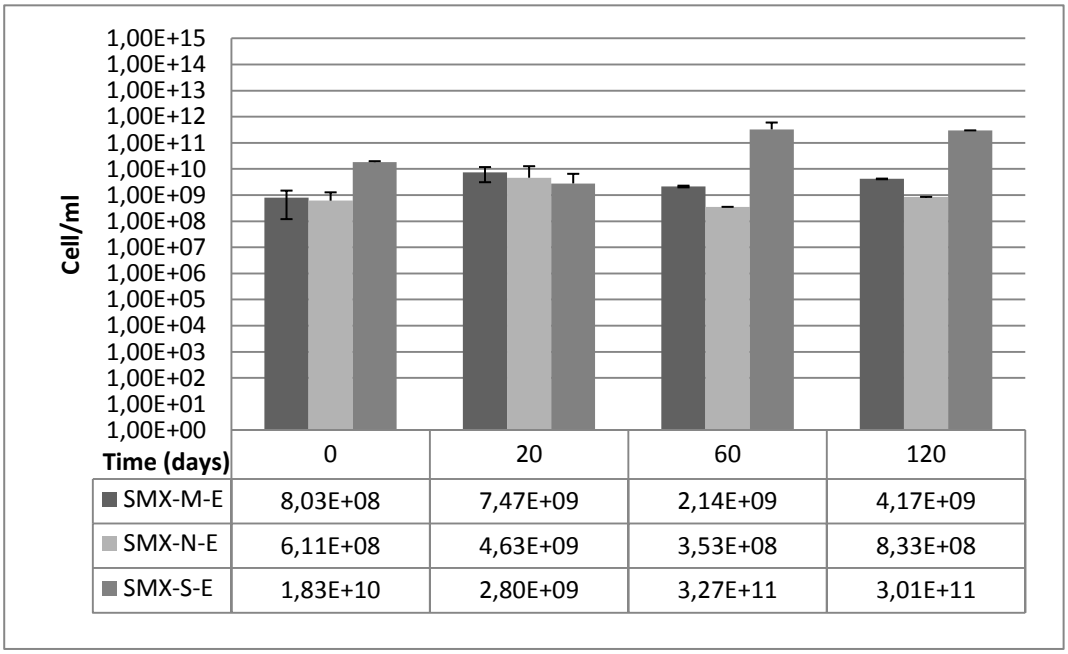
Figure 5.22: Bacteria, archaea, sulfate reducing bacteria and methanogen count for REF in sulfate reducing conditions

As for the quantitative PCR test results for the experimental test bottles containing SMX as the only carbon source, the results are given in figures 5.23, 5.24 and 5.25 and 5.26 which show bacterial, archaeal, methanogenic, and SRB count over time, for all the three electron reducing conditions.



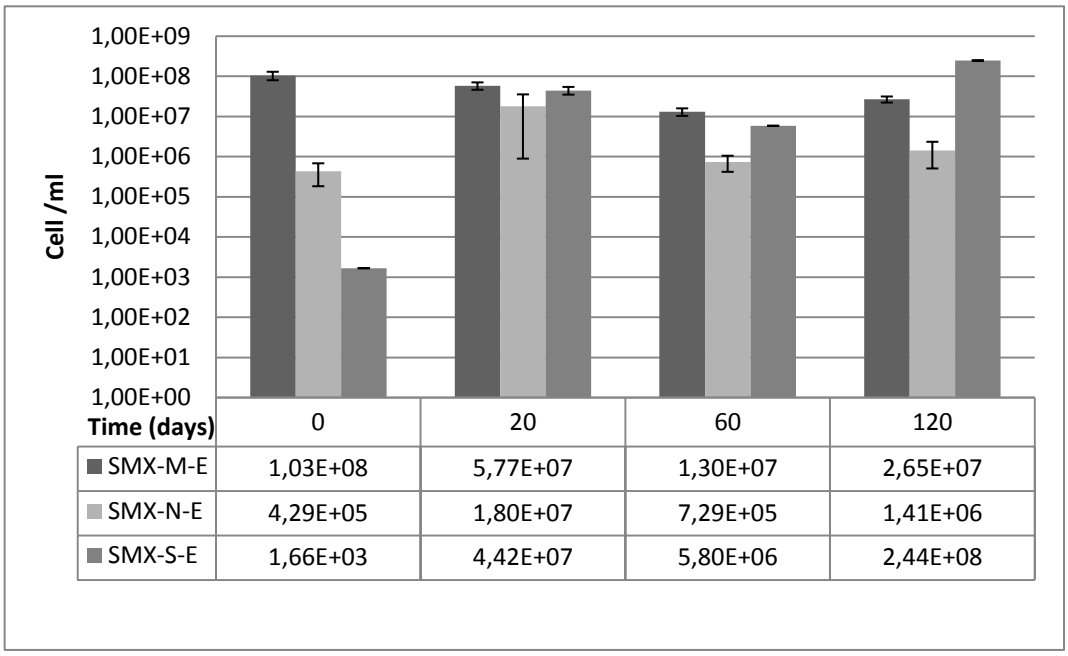
Time (days)	0	20	60	120
SMX-M-E	7,22E+08	2,20E+08	1,01E+08	2,24E+08
SMX-N-E	2,19E+08	1,14E+08	7,80E+08	7,13E+08
SMX-S-E	3,01E+08	1,50E+08	5,43E+08	2,37E+08

Figure 5.23: Bacterial count in methanogenic, nitrate reducing and sulfate reducing conditions



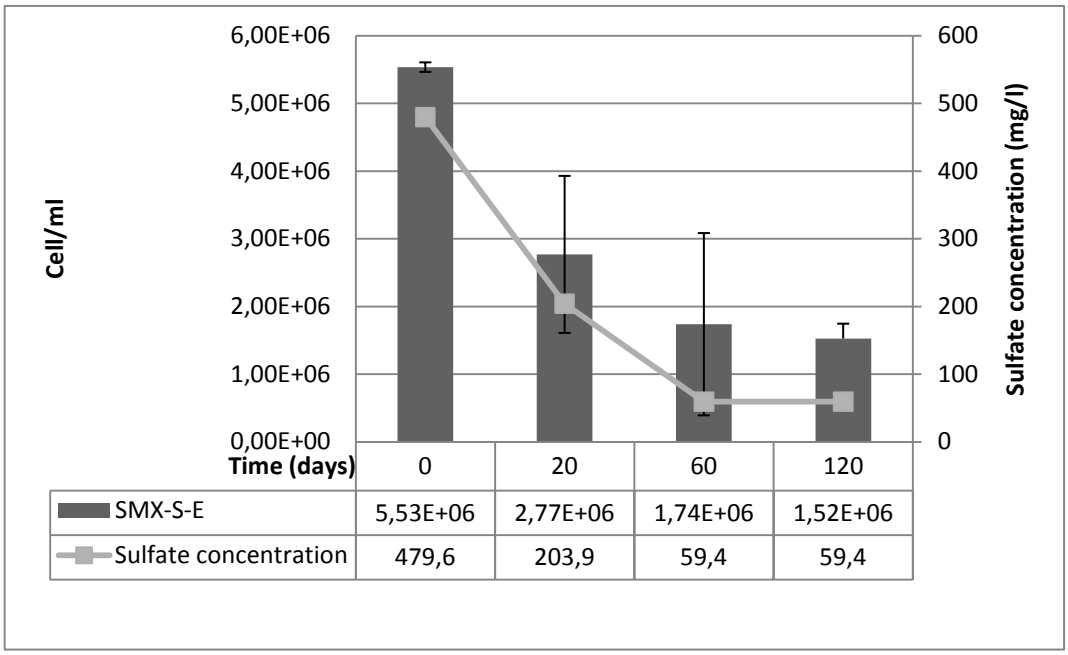
Time (days)	0	20	60	120
SMX-M-E	8,03E+08	7,47E+09	2,14E+09	4,17E+09
SMX-N-E	6,11E+08	4,63E+09	3,53E+08	8,33E+08
SMX-S-E	1,83E+10	2,80E+09	3,27E+11	3,01E+11

Figure 5.24: Archaeal count in methanogenic, nitrate reducing and sulfate reducing conditions



Time (days)	0	20	60	120
SMX-M-E	1,03E+08	5,77E+07	1,30E+07	2,65E+07
SMX-N-E	4,29E+05	1,80E+07	7,29E+05	1,41E+06
SMX-S-E	1,66E+03	4,42E+07	5,80E+06	2,44E+08

Figure 5.25: Methanogen count in methanogenic, nitrate reducing and sulfate reducing conditions



Time (days)	0	20	60	120
SMX-S-E	5,53E+06	2,77E+06	1,74E+06	1,52E+06
Sulfate concentration	479,6	203,9	59,4	59,4

Figure 5.26: Sulfate reducing organism count, and sulfate concentration change over time in methanogenic, nitrate reducing and sulfate reducing conditions

In all three-electron acceptor conditions, the amount of the bacterial population, which is the major target of the antimicrobial agent SMX, has decreased in the first 20 days. This was an expected reaction, while antibiotics inhibit the growth of bacteria, which does not have the specific resistance for that specific antibiotic.

After the 20th day, there was a significant increase in bacterial population in sulfate and nitrate reducing conditions after the decrease of bacteria amount until day 20 ($p<0.05$). The bacterial population may have gained resistance in the first 20 days against the antimicrobial agent (Skold, 2000; Perretene, 2003 and Boerlin, 2003). It is possible that the bacteria, who have gained resistance against SMX, have increased their amount between days 20 and 60. The decrease of bacterial population that is observed between days 60 and 120 may have been caused by the lack of antibiotics present in the liquid phase. The significant increase ($p<0,05$) of bacterial population in methanogenic conditions between days 60 and 120 may be related to the slow gaining of resistance of methanogens against SMX. However, this hypothesis can't be proved without the analyses of microbial studies, done with the aid of antibiotic resistance genes.

The increase of antibiotic removal efficiency, despite the decrease of bacteria amount in methanogenic conditions, shows that there may be a syntrophic relationship between Achaea population and bacteria population. Until day 20, there is a decrease observed in bacterial population while there is an increase in archaeal population in methanogenic conditions. There can be 3 different explanations to this observation, because there is no study done on the metabolic pathways of degradation of SMX.

First explanation can be the survival of a specific bacterial population, which haven't encountered SMX before, by using the non-toxic by-product of SMX degradation. This specific bacterial population may have survived without gaining resistance against SMX. The non-toxic by-products maybe produced by the archaeal population that remains unaffected by the antimicrobial agent. Archaeal population may have used SMX as a carbon source, and by using enzymes, they may have degraded SMX to its non-toxic by-products.

The second explanation assumes that archaeal population did not use SMX as carbon source, and the bacterial population gained resistance to sulfamethaxasole. In order to have the bacterial population gain resistance against SMX, the bacteria that does not have resistance against SMX, it could be assumed that those bacteria activated their resistance genes, with the stress condition caused by the inner respiration mechanism. In a study by Teresa and her friends, experiments showed that stress conditions activated the stress genes (2000). The small amount of bacteria population that gained resistance against SMX may have increased its metabolic activity and degraded the

SMX present in the test bottles, and thus the Achaea population increased by using the metabolites that are produced by the bacteria. This hypothesis, explains the change in archaeal and bacterial population in sulfate reducing conditions.

The final explanation is that the archeal population has increased because they are advantageous against bacteria at the start of the experiment. The main reason for that is that Achaea remains unaffected by the presence of SMX. The bacteria, which gain resistance against SMX, may get more active than Achaea population over time, and degrade most of SMX present in the test tubes. The decrease of Achaea population in nitrate reducing and methanogenic conditions between days 20 and 60 supports this hypothesis.

The archaeal population change in the test bottles, are parallel to the change in final electron acceptor over time. While the change of archaeal population in nitrate reducing and methanogenic conditions show a similar profile, the archaeal populations show a different profile for sulfate reducing conditions. Most highest Achaea amount is counted as 9,82E+09 at day 60 in sulfate reducing conditions.

The methanogen amount change in the samples, are consistent with the archaeal change over time. In addition, according to the analysis depending on Pearson coefficient, there is a strong correlation between methane production and methanogens, which are responsible for the methane production. This correlation supports that the quantitative PCR analysis are correct.

The sulfate reducing organisms show a decline over time in sulfate reducing conditions. The competition between sulfate reducing organisms and merhanogens have continued until, the sulfate concentration of 200 mg/l which is an advantageous concentration for sulfate reducing conditions. Even though a decrease in both populations are observed, in between days 60 and 120 where sulfate concentration has decreased to 60 mg/l , an increase in methanogens is observed in sulfate reducing conditions.

For a better understanding of results, and for an overall picture comparing all the analysis that were carried out, correlation analysis using Pearson Correlation, for different experimental results were correlated. The correlation analysis for the results of the experiments for methanogenic conditions are given in Table 5.1.

Table 5.1: Correlation analysis for methanogenic conditions

	Time	Bacteria	Methanogen	Achaea	TOC	SMX
Time	1	-0,742	-0,755	-0,034	-0,614	-0,596
Bacteria	-0,742	1	0,935	-0,519	,984(*)	,981(*)
Methanogen	-0,755	0,935	1	-0,228	0,919	0,896
Achaea	-0,034	-0,519	-0,228	1	-0,593	-0,637
TOC	-0,614	,984(*)	0,919	-0,593	1	,998(**)
SMX	-0,596	,981(*)	0,896	-0,637	,998(**)	1

For methanogenic conditions, there was a strong positive correlation between methanogenic count and bacterial count; also, they had a positive correlation with TOC and SMX concentration change over time. This observation shows that there may be syntrophic relationship between bacteria and methanogens in methanogenic conditions. In addition, SMX concentration is strongly correlated with bacterial and methanogenic count. In addition, the correlation analysis for the results of experiments in sulfate reducing and nitrate reducing conditions are given in table 5.2 and table 5.3.

Table 5.2: Correlation analysis for sulfate reducing conditions

	Time	Bacteria	Achaea	Methanogen	SRB	TOC	SMX	Sulfate
Time	1	0,77	0,611	-0,167	-0,612	-0,601	-0,636	-0,654
Bacteria	0,77	1	0,621	-0,738	-0,187	-0,057	-0,163	-0,257
Achaea	0,611	0,621	1	-0,145	-0,762	-0,581	-0,703	-0,801
Methanogen	-0,167	-0,738	-0,145	1	-0,482	-0,625	-0,524	-0,417
SRB	-0,612	-0,187	-0,762	-0,482	1	,967(*)	,995(**)	,997(**)
TOC	-0,601	-0,057	-0,581	-0,625	,967(*)	1	,987(*)	,953(*)
SMX	-0,636	-0,163	-0,703	-0,524	,995(**)	,987(*)	1	,989(*)
Sulfate	-0,654	-0,257	-0,801	-0,417	,997(**)	,953(*)	,989(*)	1

In sulfate reducing conditions, there were +0,967 correlations between TOC concentration and SRB count, +0,995 correlations between SMX concentration and

SRB count, and +0,997 correlations between sulfate concentration and SRB count. Relating to these correlations, sulfate-reducing bacteria may be responsible for the decrease in SMX concentration over time.

Nitrate concentration and SMX concentration was strongly correlated in nitrate reducing conditions, thus nitrate concentration strongly effects biodegradation of SMX by microorganisms. The negative correlation between TOC and bacteria amount, refers that as bacteria increase over time, the TOC concentration in the test tubes gets lower in the nitrate reducing conditions.

Table 5.3: Correlation analyses for nitrate reducing conditions

	Time	Bacteria	Achaea	Methanogen	TOC	SMX	Nitrate
Time	1	0,591	-0,152	-0,078	-0,647	-0,612	-0,593
Bacteria	0,591	1	-0,682	-0,65	-0,863	-0,622	-0,477
Achaea	-0,152	-0,682	1	,997(**)	0,221	-0,148	-0,317
Methanogen	-0,078	-0,65	,997(**)	1	0,183	-0,186	-0,356
TOC	-0,647	-0,863	0,221	0,183	1	0,932	0,853
SMX	-0,612	-0,622	-0,148	-0,186	0,932	1	,984(*)
Nitrate	-0,593	-0,477	-0,317	-0,356	0,853	,984(*)	1

All three electron-reducing conditions can be used for SMX degradation in biological treatment plants for antibiotic removal efficiency in three electron-reducing conditions are nearly the same. However, the high correlation between SMX concentration and sulfate reducing organisms refers that sulfate-reducing organisms can be responsible for SMX biodegradation. This result was expected, since sulfamethaxasole is an antimicrobial that belong to the group of sulfonamides, and like any other sulfonamide, SMX contains sulfate group. However, this hypothesis cannot be confirmed without SIP (Stable Isotope Probing) which is a method that confirms that the substance in question is metabolized with a specific organism.

For the degradation of SMX, biologically it is not necessary to have high sludge ages in treatment plants. The main reason for that is the cessation of the decrease in both TOC and SMX concentrations in the 60^{th} day. Thus, the degradation of SMX does not necessarily require high sludge ages. Having lower sludge ages reduces the chronic

effects of toxic substances on microbial community present in the biological treatment plants.

As the results of the experiments that have been carried out, biological degradation of sulfamethaxasole is shown. When the antibiotic removal in liquid phase is observed, it is seen that antibiotic removal efficiency reaches as high as %98 in all the three electron acceptor conditions, while the final antibiotic removal efficiency is about %70 because of the antibiotic s that is absorbed to the sludge. In the studies that are done on antibiotics using the similar test set-ups to the OECD 311 protocol, the final antibiotic removal efficiency is reported as %2, however there was no antibiotic concentration measurements carried out (Gartiser, 2007). In another study by Siegrun and his friends (2009), the antibiotic removal in the liquid phase is reported as %99. This study supports the results found in these experiments that have been carried out.

6 CONCLUSION

This study focuses on the evaluation of biodegradability of sulfamethaxasole (SMX) under anoxic and anaerobic conditions and the effect of the microbial community, which have a potential role of the degradation process. It showed that high removal efficiency was achieved for sulfamethaxasole (SMX) under all electron-accepting conditions; methanogenesis sulfate and nitrate reduction in general aspects.

Under methanogenic conditions, the biogas, TOC, antibiotic concentration and microbial quantification data indicate that there is a strong correlation between antibiotic concentration, bacteria and methanogens. This correlation is a strong proof of the biodegradability of SMX and shows that the bacterial and archaeal community continue to work together while this compound is only carbon source.

A high correlation was found between TOC concentration and sulfate reducers under sulfate reducing conditions and also nitrate and SMX concentrations under nitrate reducing conditions. Results obtained from chemical and microbiological analysis showed that biological degradation mechanism processes for SMX removal.

The antibiotic removal efficiency in three electron-reducing conditions are nearly the same, thus all three electron-reducing conditions can be used for SMX degradation in biological treatment plants. Because of the high correlation between SMX concentration and sulfate reducing organisms, sulfate-reducing condition is optimal for SMX biodegradation. This result was expected, since sulfamethaxasole is an antimicrobial that belong to the group of sulfonamides, and like any other sulfonamide, SMX contains sulfate group.

Even though sulfate-reducing condition is the most suitable condition for SMX biodegradation, today in treatment of wastes, high-energy yielding treatment systems are preferred. Thus, when engineering prospects and economics are considered, anaerobic treatment systems are preferred. Although the decrease in bacterial population over time in methanogenic conditions creates a concern for the degradation

of SMX in anaerobic treatment plants, the increase of bacterial population between days 60 and 120, leads to conclusion of gaining of resistance in bacterial population.

It is seen from the TOC and SMX concentration results, SMX is degradation stops when day 60 is reached, thus the treatment plant that is trying to degrade SMX doesn't require high sludge age. Having lower sludge ages reduces the chronic effects of toxic substances on microbial community present in the biological treatment plants.

Operating lab scale reactors under different operating conditions to get the higher degradation efficiency and further microbiological analysis such as cloning-sequencing to identify and DGGE to reveal microbial pattern will be done to examine the system completely in the future.

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