İSTANBUL TECHNICAL UNIVERSITY ★ INSTITUTE OF SCIENCE AND TECHNOLOGY

DETERMINATION OF BIODEGRADATION CAPACITY OF ERTHROMYCIN UNDER ANAEROBIC AND ANOXIC CONDITIONS

M.Sc. Thesis by
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Programme : Environmental Biotechnology

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ERİTROMİSİNİN BİYODEGRADASYON KAPASİTESİNİN ANOKSİK VE ANAEROBİK KOŞULLARDA BELİRLENMESİ

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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
DOC	: Dissolved Organic Carbon
ERY	: Erythromycin
EtBr	: Ethidium Bromide
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
NC	: Non Carbon (Blank Control)
NTC	: No Template Control
P	: Phosphorus
Q-PCR	: Quantitative PCR
REF	: Reference Control
RNA	: Ribonucleic Acid
rRNA	: Ribosomal RNA
RT	: Reverse Transcription
RT-QPCR	: Real Time Quantitave Polymerase Chain Reaction
SIP	: Stable Isotype Probing
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 BIODEGRADATION CAPACITY OF ERYTHROMYCIN UNDER ANOXIC AND ANAEROBIC CONDITIONS

SUMMARY

Antibiotics, which are known to be xenobiotics substances, are widely used in medical care, veterinary and agriculture since 1950s and accumulate in the environment. Approximately 90% of the consumed antibiotics are excreted by urine and faeces, either as active substances or metabolites. Thus, the antibiotics reach the domestic wastewater plants, and the conventional plants are not able to treat efficiently xenobiotics like antibiotics which have adverse effects on the environment, both affecting aquatic and terrestrial organisms. Also antibiotics especially cause the increase of antibiotic resistance pathogens and these organisms threat firstly public health, then plants and animals. Humans also even might be threatened via drinking water that is contaminated by antibiotic resistance genes that induced by antibiotics. Thus the importance of degradation of antibiotics has been raised.

In the completed project, the biodegradation capacity and the effects on microbial culture have been investigated for Erythromycin (ERY) 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 degradation by Quantitative Polymerase Chain Reaction (Q-PCR), along with the Dissolved Organic Carbon (DOC), biogas measurement, electron acceptor and antibiotic concentration monitoring.

In this scope, batch tests were set-up, to be distracted at different sampling times. Gas generation and composition, DOC, antibiotic and electron acceptor concentrations were monitored for 120 days. In addition, the changes of the quantity of specific microbial groups were analysed 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 ERY was obtained. As a result, ERY was found out to be unable to utilizable as a carbon source under three different electron conditions; methanogenic, sulfate reducing and nitrate reducing.

ERİTROMİSİNİN BİYODEGRADASYON KAPASİTESİNİN ANOKSİK VE ANAEROBİK KOŞULLARDA BELİRLENMESİ

ÖZET

Zenobiyotik grubuna dahil olan antibiyotikler 1950ler'den beri tıp, veterinerlik ve tarımda aktif olarak kullanılmakta ve çevrede birikmektedir. Tüketilen antibiyotiğin yaklaşık %90'ı aktif veya metabolitlerine ayrışmış olarak dışkı ve idrar yoluyla evsel atıksu tesislerine karıştırmaktadır. Geleneksel arıtma tesisleri antibiyotikleri gidermekte yetersiz kalması sebebiyle antibiyotikler alıcı su ortamlarına deşarj olmakta ve antibiyotiklerin sucul ve karasal yaşama olan doğal dengeyi bozmak gibi negatif etkileri gözlenmektedir. Antibiyotikler özellikle antibiyotiğe dirençli patojenlerin yayılmasına sebep olmakta ve bu durum öncelikle toplum sağlığı ve de hayvan ve bitkilerin sağlığını tehdit etmektedir. İnsanlar nadir de olsa içme suyundan dahi gelen antibiyotik direnç genleriyle karşılaşabilmektedirler. Bu durum antibiyotiklerin giderimine verilen önemi artttırmaktadır.

Tamamlanan projede bir antibiyotik olan eritromisinin (ERM) biyodegradasyonu ve mikrobiyal kültürün üzerindeki etkileri metanojenik, nitrat indirgeyici ve sülfat indirgeyici koşullarda; degradasyondan sorumlu mikrobiyal komunitenin kantitatif polimeraz zincirleme reaksiyonuyla sayılması, çözünmüş organik karbon (ÇOK), biyogaz ölçümü, elektron alıcısı ve antibiyotik konsantrasyonu takibi yardımıyla yapılmıştır.

Bu kapsamda, kesikli reaktör şişeler, farklı numune alım zamanlarında bozulmak üzere kurulmuştur. Gaz üretimi ve kompozsiyonu, 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, ERM biyodegredasyonu hakkında genel bir sonuca varılmıştır. Sonuç olarak, ERM'nin metanojenik, sülfat indirgeyici ve nitrat indirgeyici ortamlarda karbon kaynağı olarak kullanılamadığı görülmüştür.

1. INTRODUCTION

Due to intensive use of antibiotics in medicine, veterinary and livestock since 1950s, (Summers, 2006); antibiotics are widely distributed among the environment, from surface and ground water to inhabited areas (Hirsch, et al., 1999; Martinez, 2008). Although the concentrations of antibiotic residues are as low as µg/l to ng/l (Louvet et al., 2010), antibiotics are concerned as xenobiotic pollutants, because of their negative impact on aquatic environments by disturbing its balance and inducing multiple antibiotic resistance genes among bacteria (Kümmerer, 2004).

As Martinez (2008) reported, microorganisms use antibiotics as signal substances and the accumulation of antibiotics threats the sensitive ecological balance although it is not able to be observed as apparently as heavy metal contamination. The variety of the distribution of antibiotics among the environment such as receiving water to soil highlights the importance of the treatment (Kümmerer, 2003; Godfrey et al., 2007).

Erythromycin (ERY) is a broad spectrum antibiotic that is typically used to treat respiratory infections (Zhanel et al., 2001). Erythromycin resistance can be tracked from untreated drinking water to airborne hospital bacteria with regard to its frequent use (Focazio et al., 2008; Gilbert et al., 2010), and the main ERY residue source is the domestic wastewater treatment plants which are inefficient in treating antibiotics (Godfrey et al., 2007). Literature on treatment of ERY is mostly focused on activated sludge (Alighardashi et al., 2009; Louvet et al., 2010; Li & Zhang, 2010), but the results indicated that an extensive research must be conducted. In this study, the potential of biodegradation of ERY is investigated both anaerobic (both methanogenic and sulfate reducing conditions) and anoxic conditions according to protocol of OECD 311 Anaerobic Biodegradability of Organic Compounds in Digested Sludge: by Measurement of Gas Production with Q-PCR, DOC, biogas production, electron acceptor and antibiotic measurements.

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 erythromycin (ERY) that is used commonly for the disease of human and animal. In this scope, biodegradation capacity and the effects on the microorganisms investigated by destructive batch tests based on Anaerobic Biodegradability of Organic Compounds-OECD 311 protocol under three different electron acceptor conditions; methanogenic, sulfate reducing, and nitrate reducing conditions. For the reability of the experimental results, experimental control tests were set-up. Gas generation and composition, DOC, 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). All of the results were statistically analyzed in order to view the connections between different parameters, affected by ERY.

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3. THEOROTICAL BACKGROUND

3.1 Xenobiotics

Xenobiotics are chemicals that are foreign to organisms or environment, they include both inorganic elements like heavy metals, metalloids and man-made organic compounds such as pesticides, surfactants, solvents, fragrances, flavours, and pharmaceuticals as well as endocrine disrupters, hormones, personal care products. But also a compound that is in much higher concentration than usual also called xenobiotic. The most common xenobiotics are antibiotics, although antibiotics are natural to certain microorganisms, human body does not contain or produce it. Xenobiotics have divided to two groups, biodegradable xenobiotics and non-biodegradable xenobiotics, recalcitrant. Although xenobiotics are able to biodegrade, this process is too slow and sometimes resistant (van der Meer et al., 1992).

By uprising discharge of xenobiotics to the environment, degradation of xenobiotics has become the spotlight because of their adverse effects like bioaccumulation, threating the ecological balance. In order to control the effects of xenobiotics on the environment, the information of the sources, flow paths, fate (transport, treatment, and natural attenuation) and impact on humans, livestock and the environment ought to be investigated.

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 waste 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 domestic 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 (Hlavinek et al., 2008).

Thus, there is a need to understand the xenobiotic biodegradation capacity of current urban wastewater treatment plants in order to improve the treatment of xenobiotics and prevent the adverse effects of them on the environment.

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, 2008a). 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, streptomycinsare used in fruit crops, while other antibiotics are used in beekeeping (Kümmerer, 2008b).

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, 2008a). 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, 2008a).

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, 2008a). Solubility, hydrophobicity and hydrophilicity are pH dependent. Thus along with pH, the physico-chemical and biological properties such as partition coefficient n-octanol/water, sorption behavior, photo reactivity and antibiotic activity may change with pH (Kümmerer, 2008a).

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 & Matheson, 1978; Alcock, Sweetman, & Jones, 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-Sorensen, 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 & 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, 2008b).

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 spiramycinewere 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, 2006).

3.2.3 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 & 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, 2008b).

3.2.3.1 Biodegradation

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

In general, biodegradability is poor for most of the compounds that were investigated so far in laboratory tests – even for some of the β-lactams. In a study carried out by Gartiser et al. (2007a), 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). 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 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, 2008a). 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, 2008a).

By the toxicity tests done on bacteria in the literature, it is shown that chronic exposure to antibiotics is critical rather than acute (Backhaus & 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 was found after short incubation time 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 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.

3.2.4.1 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-Sorensen, 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-Sorensen, 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 on inhibition. In another study, carried out by Dokianakis, Kornaros and Lyberatos (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 et al. (2006), the synergistic mixture effects of antibiotics against sewage sludge bacteria are found. 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 0.7 mg/l.

3.2.5 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, & Baquero, 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, 2002).

3.2.6 Chemical and physical properties of erythromycin

Erythromycin is belonged to macrolide antibiotics. The macrolide antibiotics contain 12- to 22-carbo lactone rings linked or more sugars. Erythromycin is usually bacteriostatic and binds to the 23S rRNA of the 50S (large) ribosomal subunit to inhibit peptide chain elongation during protein synthesis. Erythromycin is a relatively broad-spectrum antibiotic effective against gram-positive bacteria, mycoplasmas, and a few gram-negative bacteria. It is used with patients who are allergic to penicillins and in the treatment of whooping cough, diphtheria, diarrhea, caused by *Campylobacter,* and pneumonia from *Legionella* or *Mycoplasma* infections (Willey et al., 2008).

3.2.6.1 Derivatives

Originally isolated from Streptomyces species, the macrolides form probably the largest group of known natural products. Macrolides derive their name from their structure, a macrocyclic lactone ring to which various amino sugars are attached, and can be classified according to the number of carbon atoms in the macrocyclic ring. However, the addition of new compounds with original chemical moieties has made classification more complex. The most important macrolide antibiotics are 14-, 15- and 16-membered-ring compounds. These can be subdivided into two major groups —natural products and semisynthetic derivatives (Bryskier, 1999). The semisynthetic compounds can also be further divided into three subgroups according to the type of chemical modification of the core erythromycin A structure. The first subgroup comprises those compounds with substituent modifications, such as roxithromycin, clarithromycin and flurithromycin. Compounds obtained from modifications of the aglycone A, such as azithromycin, form the second subgroup. Finally, the compounds in the third subgroup, which includes the ketolides, are obtained by modification of the C-3 α-L-cladinose.

3.2.6.2 Mechanism of action

The classes of antibiotics clinically available can be differentiated according to how they inhibit bacterial growth. Macrolides are among the antibacterial agents that inhibit protein synthesis. Drugs with this mechanism of action have been the focus of much of the research and development of new antibacterial agents.

Protein synthesis is catalyzed by ribosomes and cytoplasmic factors. Bacterial ribosomes (70S) consist of two subunits, termed 50S and 30S, which contain ribosomal RNA (rRNA) and ribosomal proteins. The 30S subunit binds messenger RNA (mRNA) and begins the ribosomal cycle. The codon recognition between the mRNA and the transfer RNA (tRNA) occurs on the 50S subunit, then the 50S subunit binds tRNA and controls the elongation of the peptide. Peptide bond synthesis occurs in the peptidyltransferase centre of the 50S subunit. The growing peptide chain (peptidyl-tRNA) attached at the P-site undergoes transpeptidation with an aminoacyl-tRNA at the acceptor A site, followed by translocation to allow the next amino acid to be added.

Macrolides reversibly bind to the 50S subunit and inhibit the transpeptidation/translocation process. The actual mechanism by which the macrolides bind to the 50S ribosome has been the subject of a study by Hansen, et al. (1999). This study demonstrated that macrolides interact with bacterial 23S rRNA by connecting to the hairpin 35 in domain II of the rRNA and to the peptidyltransferase loop in domain V. It is thought that these two regions are probably folded close together in the 23S rRNA tertiary structure and form a binding pocket for macrolides and other agents that inhibit protein synthesis. Despite these advances in our understanding of the mechanism of action of macrolides, the exact process is still not known.

3.2.6.3 Mechanism of resistance

Susceptibility of *Streptococcus pneumoniae* to macrolides and β-lactams has declined in the last decade. Recent studies suggest this is primarily due to the dissemination of strains harbouring an efflux pump mechanism for 14- and 15 membered-ring macrolides in *S. pneumoniae* (mefE) and *S. pyogenes* (mefA). Over two-thirds of the macrolide-resistant S.pneumoniae isolates in the USA express the efflux mechanismmefA/E. The other third of the resistance strain harbour the ribosomal modification mechanism ermAM. This family of very similar genes codes for the23S-rRNA dimethyltransferase, which results in methylation of adenine 2058 of the 23S rRNA (*E. coli* numbering system). This methylation prevents the macrolidefrom binding to the ribosome. The erm mechanism yields high-level resistance to all 14- and 15-memberedring macrolides and, if the methylase enzyme is constitutively produced, this leads to 16-membered-ringmacrolide, clindamycin

and streptogramin B, and ketolide resistance. The frequency of strains harbouring the erm gene has not increased significantly in the USA, where about 99% of the macrolide-resistant *S. pneumoniae* have the erm or mef mechanism. In Belgium and Italy, most of the macrolide-resistant *S. pneumoniae* strains were also shown to contain the erm mechanism of resistance. A study conducted in Japan reported that approximately 60% of erythromycin-resistant *S. pneumoniae* isolates from paediatric patients carried the ermB gene, whereas 40% had only the mefE gene. Nishijima et al. (1999) found no correlation between the presence of a particular macrolide-resistant gene and resistance to penicillin G. However, the majority of erythromycin-resistant S. pyogenes isolates, at least in Western Europe, have the efflux mechanism (Retsema, & Fu, 2001).

3.3 Electron Acceptor Conditions

3.3.1 Methanogenesis

Methanogens are strict anaerobes that obtain energy by converting CO_2, H_2, formate, methanol, acetate and other compounds to either methane or methane and CO_2. They are the largest group in archaea domain. There are five orders (Methanobacteriales, Methanoccocales, Methanomicrobiales, Methanosarcinales and Methanopyrales) and 26 genera, which differ greatly in overall shape, 16S rRNA 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 Nitrate reducing conditions

Nitrate NO_3^-, can be reduced to N_2O, NO, and N_2. These products can easily be lost from the environment with a process called denitrification. Denitrrification is the process where gaseous N_2 is formed biologically (Madigan & Martinko, 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

(NO_2), and the enzyme nitrite reductase reduces to nitric oxide (NO) (Madigan & Martinko, 2006).

3.3.3 Sulfate reducing conditions

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 H_2S. The ability to use sulfate as an electron acceptor for energy-generating processes involves the large-scale reduction of SO_4 and it is restricted to the sulfate reducing bacteria. In assimilative sulfate reduction, the H_2S formed is immediately converted into organic sulfur in the form of amino acids and other organic sulfur compounds; however, in dissimilative sulfate reduction the H_2S is excreted (Madigan & Martinko, 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. 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(Madigan & Martinko, 2006).

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 et al., 1995; Hugenholtz et al., 1998; Muyzer et al., 1993; Muyzer, 1999; Lim et al., 1999; Guillou, et al., 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 unfavourable 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 & Ovreas, 2002; Torsvik et al., 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, et al., 2002; Ranjard et al., 2000).

3.4.2 The Importance of 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, et al., 1995; 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, et al., 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, et al., 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 et al., 1994; Choi et al., 1994; DeLong, 1992; Liesack & Stackebrandt, 1992; Schmidt et al., 1991; Ward et al., 1990). A scheme of approaches of microbial analyses for microbial diversity is shown at figure 3.1 (Dahllof et al., 2002).

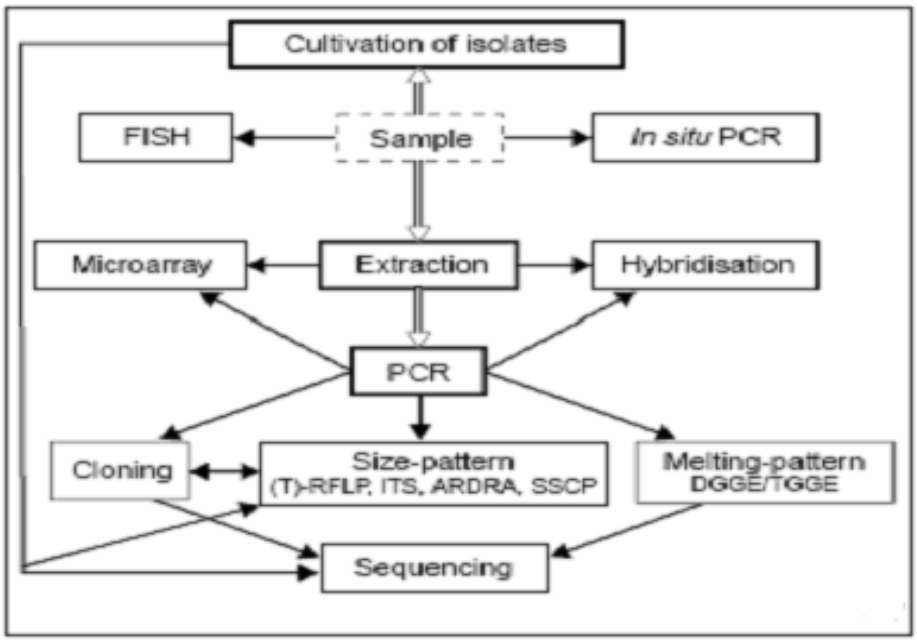


Figure 3.1: Approaches to the analysis of microbial diversity (Dahllof et al., 2002)

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 et al., 1986). Among the variable regions, V3 region is mostly used in molecular analysis (Neefs et al., 1990; Ovreas et al., 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 et al., 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 et al., 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 et al., 1991).

3.4.4 PCR based techniques used in molecular biology

3.4.4.1 Quantitative PCR

Using PCR technique after the extraction of nucleic acids (DNA and RNA) from environmental matrices, is highly important in the development of culture independent approaches in microbial ecology. These methods, which have been applied since the early 1990s (e.g. Giovannoni et al., 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 et al., 1998) driving the discovery of new microbial lineages and enabling the description of genetic diversity in a wealth of functional gene markers (Larkin et al., 2005). Although recently developed ultra-high-throughput sequencing technologies such as pyrosequencing (Margulies et al., 2005; Edwards et al., 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 et al., 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 et al. (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 et al., 2000; Suzuki et al., 2000; Takai, & Horikoshi, 2000). This development in Q-PCR technique had been facilitated by the earlier combination of the 50 nuclease assay developed by Holland et al. (1991) with fluorescence detection following cleavage of an internal (TaqManTM) DNA probe (Livak et al., 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

exponential 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 et al., 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 et al.,1992) and limiting dilutions or most probable number (MPN)-PCR (Skyes et al., 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 et al., 1997) and the TaqMan probe system (Holland et al., 1991; Livak et al., 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. 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 et al., 2003; Gonzalez-Escalona et al., 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 method utilizes a fluorescently labelled probe that hybridizes to an additional conserved region that lies within the target amplicon sequence. The TaqMan probe is fluorescently labelled at the 50 end and contains a quencher molecule at the 30 end (Livak et al., 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. 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 et al., 2007), or alternatively, probes can be labelled with different fluorophores, facilitating the development of multiplex Q-PCR protocols whereby different targets can be coamplified and quantified within a single reaction (Neretin et al., 2003; Baldwin et al., 2003, 2008). For example, Baldwin et al. (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 et al., 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 et al., 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 treshold (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 et al., 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 et al. (2007) nevertheless successfully utilized such an approach when confirming microarray-based determination of the transcriptional responses of Silicibacterpomeroyi 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 et al., 2003; Treusch et al., 2005; Kandeler et al., 2006). For example, Treusch et al. (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 et al., 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 et al., 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 et al., 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 et al., 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 et al., 2000; Suzuki et al., 2000; Takai & Horikoshi, 2000). Becker et al. (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 et al. (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 archaea 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 (Klappenbachet al., 2000).

Quantification of eukaryotes within environmental samples by Q-PCR can be carried out by targeting the 18S rRNA gene (Lueders et al., 2004; Zhu et al., 2005) or the internal transcribed spacer (ITS) region (Landeweert et al., 2003; Kennedy et al., 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 et al., 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 et al., 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 et al., 2004; Treusch et al., 2005; Leininger et al., 2006; Mincer et al., 2007), nitrate reduction and denitrification (Lopez-Gutierrez et al., 2004; Henry et al., 2006; Smith et al., 2007), sulfate reduction (Leloup et al., 2007), methanogenesis (Denman et al., 2007) and methane oxidation (Kolb et al., 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 archaea and bacteria to the first step of nitrification (ammonia oxidation) have been investigated both in soils (Leininger et al., 2006; He et al., 2007) and in seawater (Mincer et al., 2007) by determination of the abundance of archaeal and bacterial-related amoA genes. These studies have suggested that archaea 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 archaea (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

The seed is taken from the anoxic part of the Paşaköy Domestic Wastewater Treatment System for the nitrate reducing conditions, 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 m^3). The UASB reactor is he 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 58.000 mg/l and 44.000 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, nitrification and denitrification. 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. Erythromycin (ERY) from macrolide 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 2.000 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. Sodium azide (0,05 %) was used as an inhibitor.

Table 4.1: Vitamin solution (OECD, 2006)

CONSTITUTENT	AMOUNT (mg)
4-aminobenzoic acid	0,8
D(+)-biotin	0,2
Nicotinic acid	2
Calcium D(+)-pantothenate	1
Pyroxidinedihydrochloride	3
Thiamine	2
NaP Buffer (10 mM, pH 7,1)	to 20 ml

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.

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).

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.

Vitamin B12 Solution

cyanocobalamine (vit B12): 2,5 mg vit B12 + 50 ml MQ H_2O

Filter sterilise (0,2 μm filter) and store at 4^{o}C.

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 molybdatedihydrate ($Na_2MoO_4.2H_2O$)	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

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 were added to the medium in the anaerobic cabinet for each 1 ml of medium. The biodegradability test serum bottles that were setup 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-up for methanogenic conditions was given in Table 4.6, test set-up for nitrate reducing conditions was 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 ERY. Reference test bottles contain phenol, and in non-carbon test bottles in addition to ERY, test bottles contain sodium azide as inhibitor.

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

The biodegradability test serum bottles that were setup 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-up for methanogenic conditions was given in Table 4.6, test set-up for nitrate reducing conditions was 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 ERY. Reference test bottles contain phenol, and in non-carbon test bottles in addition to ERY, test bottles contain sodium azide as inhibitor.

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

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

The biodegradability test serum bottles that were setup 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-up for methanogenic conditions was given in Table 4.6, test set-up for nitrate reducing conditions was 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 ERY. Reference test bottles contain phenol, and in non-carbon test bottles in addition to ERY, test bottles contain sodium azide as inhibitor.

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 erythromycin. The initial DOC concentrations for reference test set is about 80 mg/l dissolved organic carbon (DOC), while the initial DOC concentration for experimental test set is 85 mg/l DOC.

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				+
ERY	+	+		
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.

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.

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				+
ERY	+	+		
Inhibitor(NaN3)		+		

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

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

4.3 Chemical Analyses

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

DOC measurements are done with Shimadzu ASI-V TOC analyzer (Japan). The wastewater samples that are taken from the test bottles are filtered through 0,45 µl filters before the samples are given to the TOC analyzer (Shimadzu ASI-V) for the

measurement of DOC concentrations. The measurements that were below the detection limits showed as zero value.

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 through 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 ERY concentrations, the samples were treated in order to be able to measure concentrations in the High Pressure Liquid Chromotography (HPLC) (Schimadzu LC-10 AD, Japan).

The sludge samples taken from the test bottles were freeze-dried and were grinded extensively. From the dried sludge samples ERY 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 5 minutes with 4 ml MetOH (methanol), 2 ml MetOH, and 4 ml acetone. This process enabled ERY to be extracted from the solid phase and introduced into the liquid phase. Supernatants were collected and evaporated to a volume of approximately 200 µl and then diluted with double distilled water (ddH_2O).

On the other hand, liquid phase samples of the test-bottles, were filtered through 0,45 µl filters, and diluted with ddH_2O. 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 2 times that was adjusted to pH 4 with H_2SO_4.

All of the samples were percolated through cartridges at flow rate of less than 5 ml/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.

The volume of elute was reduced to 50 µl by a gentle flow of nitrogen at room temperature. The sample volume was adjusted to 0,5 ml with water and they were stored in amber glass at -15 Cº.

The liquid samples both taken from the sludge and the wastewater samples were filtered through 0,45 µl filters. The filtered samples were purified and cleaned by solid phase extraction. Antibiotic concentrations were measured by HPLC, using c18 colon and UV detector. Method was optimized by setting the detection limit 1mg/l as the lower limit and 200 mg/l as the upper limit in HPLC. ERY concentrations were measured in the light of the protocol that Karcı and Balcıoğlu (2009), have applied. Due to lack of time of absorption of ERY to sludge at the 0^{th} time, ERY concentrations from sludge for 0^{th} time were not measured

4.6 Microbial 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 fluorometer (Qubit 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 rRNA 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 μl reaction volume containing 200 ng of DNA, 10 pmol of each primer, 10 mM of each deoxynucleoside triphosphate, 1,5 mM $MgCl_2$, 5 μl of 10×Taqbuffer and 4 μl of TaqDNA 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 (VilberLourmat, France) after staining with ethidium bromide. The positive PCR results that had the expected length for the oligonucleotide sequences were chosen for the cloning.

Table 4.9: Bacterial and archaeal oligonucleotide primers used for PCR amplification

Target Microorganism	Primer	Sequence of the Primer	Target gene	Annealing Temprature	Reference
Bacteria	**Bac519f**	**5′-CAGCMGCCGCGGTAANWC-3′**	**16S rRNA**	**53**	**Lane, 1991**
	Bac907r	**5′-CCGTCAATTCMTTTRAGTT-3′**			
Archaea	**Arc349f**	**5′-GYGCASCAGKCGMGAAW-3′**	**16S rRNA**	**55-60**	**Takai & Horikoshi, 2000**
	Arc806r	**5′-GGACTACVSGGGTATCTAAT-3′**			
Methanogen	**Met348f**	**5′-GYGCAGCAGGCGCGAAA-3′**	**16S rRNA**	**55**	**Sawayama et al., 2006**
	Met786r	**5′-GGACTACVSGGGTATCTAAT-3′**			
Sulfate Reducing Bacteria	**DSRp2060F**	**5′-CAACATCGTYCAYACCCAGGG-3′**	**SulfiteReductase Beta Subunit (dsrB)**	**55**	**Geets et al., 2005**
	DSR4R	**5′-GTG TAG CAG TTA CCG CA-3′**			

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 μg/ml kanamycin.

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 µl master mix, 1,6 µl $MgCl_2$ 1,0 µl Primer F and R, 13,4 µl H_2O, 1 µl sample). All the Q-PCR 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 output of the software that 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 concentrations of standard was 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.

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 archaea, respectively (Klappenbach et al., 2001). Copy numbers of all other genes were directly correlated to cell numbers (Philippot, 2002; Beller, 2002).

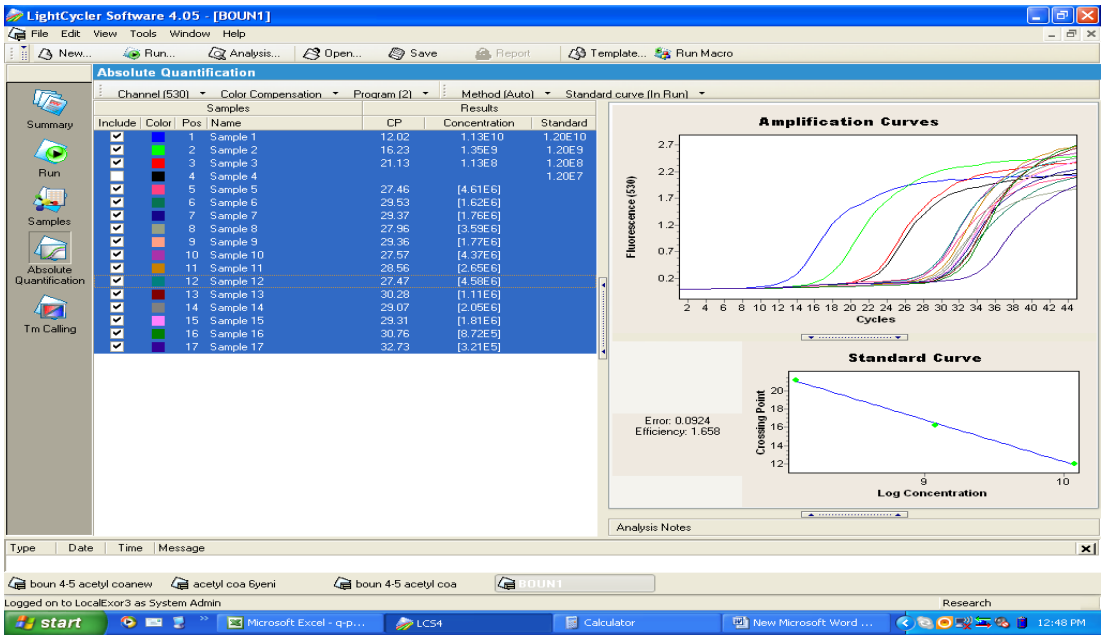


Figure 4.1. View of Light Cycler Software 4.05

4.7 Statistical Analyses

The experimental results were analyzed statistically by SPSS program. Independent samples *t-test* was used for significance analyses of counting of archaea and bacteria cells for all conditions. Pearson correlation was used for correlation analyses of methanogenic, nitrate reducing and sulfate reducing conditions with parameters of DOC, electron acceptor concentrations and cell countings.

5. RESULTS AND DISCUSSION

The biodegradation capacity and the effects on microbial culture have been investigated for Erythromycin (ERY) 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 degradation by Quantitative Polymerase Chain Reaction (Q-PCR), along with the Dissolved Organic Carbon (DOC), biogas measurement, electron acceptor and antibiotic concentration monitoring.

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

5.1 Experimental Control Tests

According to OECD 311 protocol, three different control groups were designed; blank control group (NC), reference substance control group (REF) and inhibited control group (I). In blank control group there was no addition of carbon source for allowance of the sludge and monitoring the endogenous decay. To check biodegrability test tube set up; phenol, a carbon source with well-known degradation pathway, used as reference substance control group which corresponds to positive control. Sodium azide used as inhibition control in order to check the toxicity level of ERY. Because of insignificancy, the results of inhibition control are not presented. With respect to blank control group DOC analyses, the rest of the DOC analyses were normalized.

5.1.1 Dissolved organic carbon (DOC) and ion chromatography

In order to observe carbon removal during the experiment, dissolved organic carbon analyses carried out for blank control group (NC) and reference control group (REF) at four different sampling times; the first day, 20^{th} day, 60^{th} day and 120^{th} day. For

nitrate and sulfate reducing conditions, respectively nitrate and sulfate concentrations were measured. The change in DOC concentration and percentage of removed DOC in methanogenic conditions of NC and REF are given in figure 5.1 and 5.2.

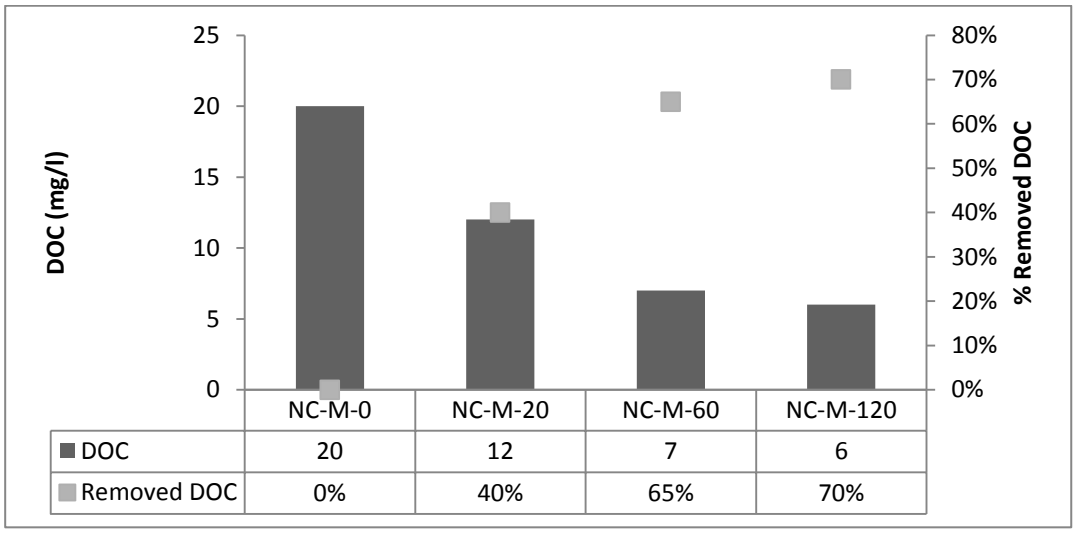


Figure 5.1: DOC removal of NC in methanogenic conditions

Although blank control was designed as having no carbon source, the DOC analyse showed that there was an initial 20 mg/l DOC value in NC at the 0^{th} day for methanogenic conditions. 65 percentage of total DOC were removed at the 60^{th} day of the experiment, and 60^{th} to 120^{th} day, only 5 percentage of total DOC were removed. At the end of the experiment, 70 percentage of total DOC were removed which might indicated that after the 60^{th} day, the biological activity decreased.

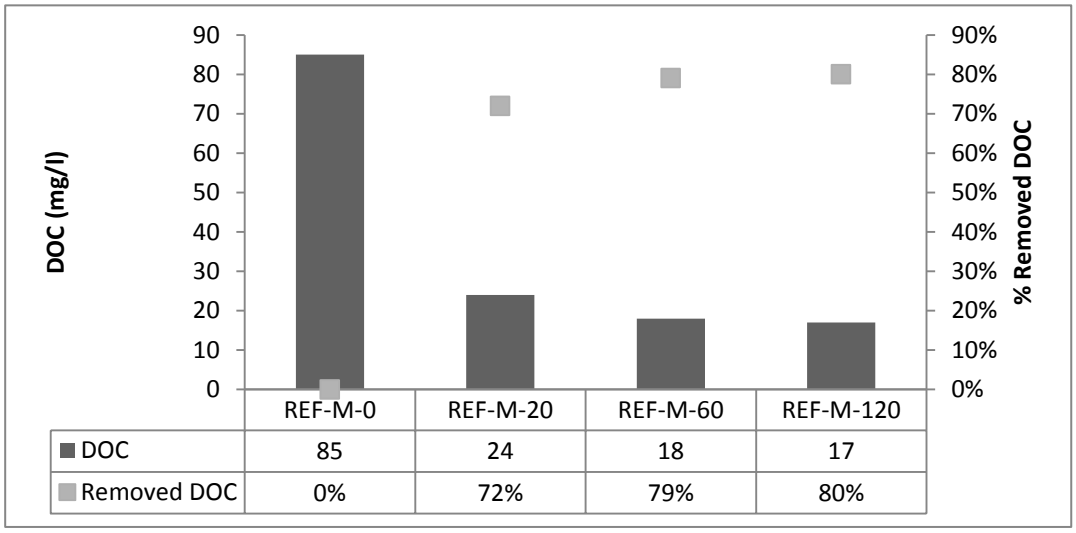


Figure 5.2: DOC removal of REF for methanogenic conditions

The initial DOC value of reference control group was 85 mg/l as consistent with experimental design. 72 percentage of total DOC were removed at 20th day and at the end of the 60th day 79 percentage of total DOC were already removed which confirmed the anaerobic sludge requirements with respect to OECD 311 protocol.

The change in DOC and final electron concentrations in nitrate and sulfate reducing conditions of NC and REF are given in figure 5.2 to 5.6.

For nitrate reducing conditions, depletion of nitrate was observed at 60th day for NC and at 20th day for REF. The unexpected beforetime depletion of nitrate amount might be related with the anoxic sludge characteristics. For NC, the initial DOC concentration was 20 mg/l, 55 percentages of both DOC and nitrate concentrations were removed at the 20th day. Total DOC was removed at the 60th day and also nitrate concentration decreased from 200 mg/l to 1,4 mg/l for NC. For REF, the initial concentration of DOC was 80 mg/l and by the 20th day 75 percentage of total DOC was already removed, and nitrate concentration decreased from 260 mg/l to 2 mg/l. Nitrate concentration was limited for NC and REF after the 60th day and 20th day, respectively. Anoxic sludge also confirmed the requirements of the OECD 311 protocol by reaching more than 60 percentage removal before the 60th day.

For sulfate reducing conditions, the initial sulfate concentrations for both NC and REF were 490±10 mg/l and at the end of the experiment, the final sulfate concentrations were 155±5 mg/l. The decrease in sulfate concentration was parallel with DOC removal and sulfate concentration was not limited during the experiment. The initial DOC concentration of NC was 20 mg/l like other electron conditions, and only 55 percentage of total DOC removed at the end of the experiment. For REF, the initial concentration of DOC was lesser than other electron acceptor conditions as 76 mg/l, that most possibly caused by experimental errors, but the removal percentage at the 20th day was similar to other conditions as 72 percentage. Anaerobic sludge for sulfate reducing conditions also confirmed the OECD 311 protocol by reaching more than 60 percentage removal before the 60th day.

For all three different electron acceptor conditions, the initial DOC concentrations were 20 mg/l and for all conditions at least 55 percentage of total DOC were removed at the end of the experiment. The initial DOC concentration of REF for all electron acceptor conditions were 80±5 mg/l, for methanogenic and nitrate reducing

conditions the final removal ratios were 80 percentage of total DOC and for sulfate reducing conditions it was 75 percentage. Both blank control group and reference control group met the requirements of the OECD 311 protocol.

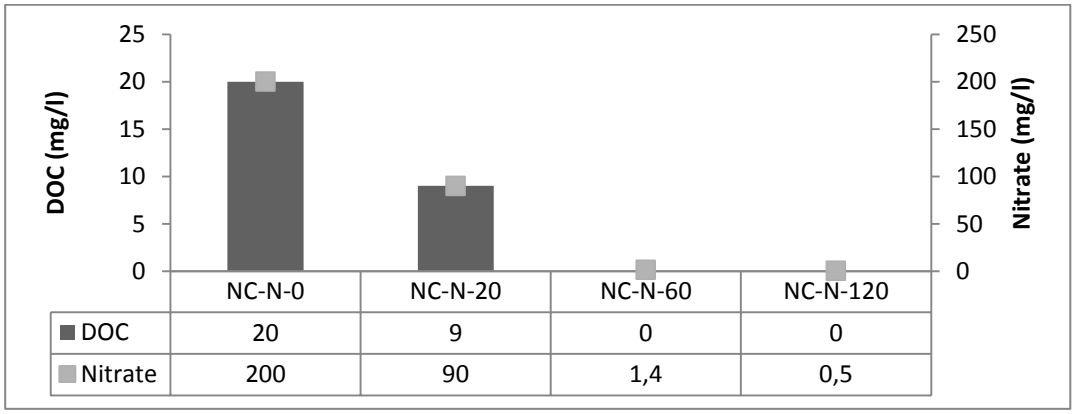


Figure 5.3: DOC and nitrate concentration change of NC in nitrate reducing conditions

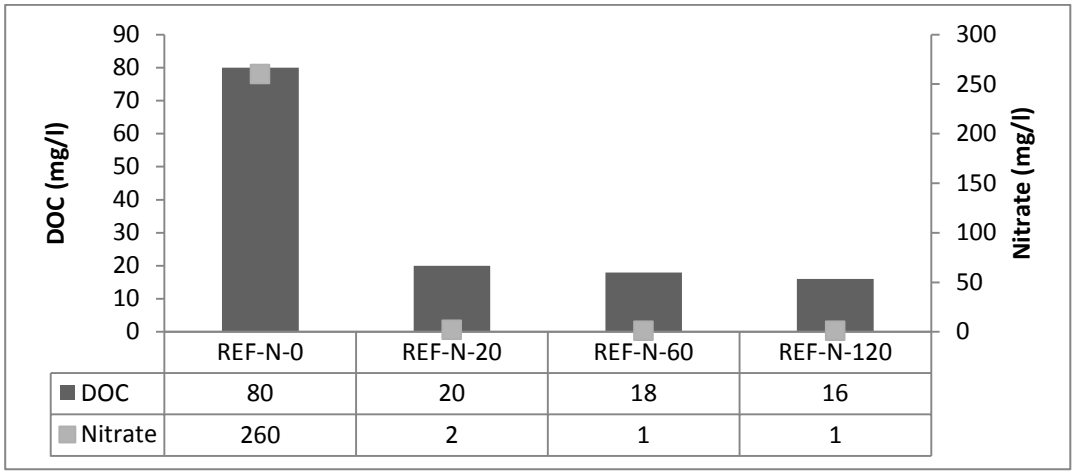


Figure 5.4: DOC and nitrate concentration change of REF in nitrate reducing conditions

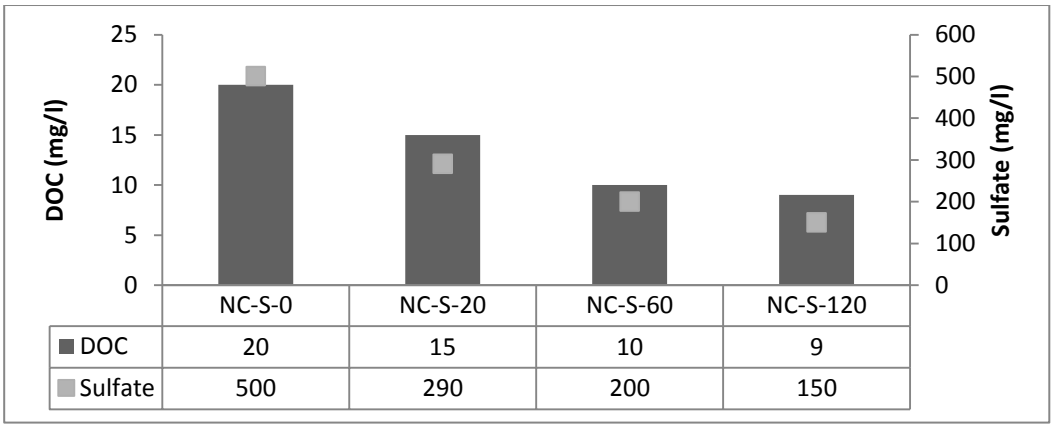


Figure 5.5: DOC and sulfate concentration change of NC in sulfate reducing conditions

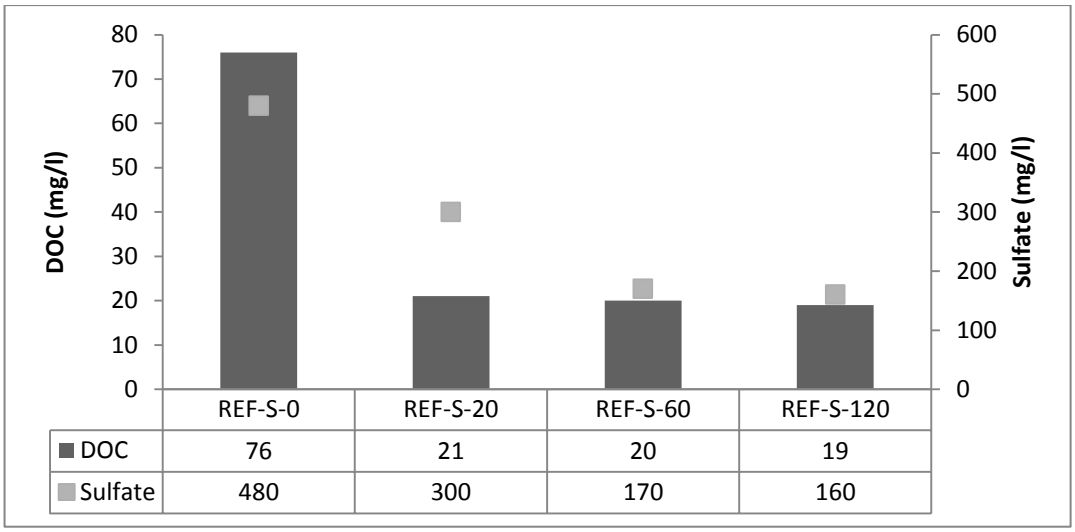


Figure 5.6: DOC and sulfate concentration change of REF in sulfate reducing conditions

5.1.2 Quantitative PCR (Q-PCR)

In order to observe the changes of microorganisms of blank control group (NC) and reference control group (REF) with phenol; bacteria, archaea, methanogen and sulfate reducing bacteria amounts were quantified with Quantitative-PCR. Microorganisms count for methanogenic conditions of NC and REF are given in figure 5.7, and 5.8, respectively.

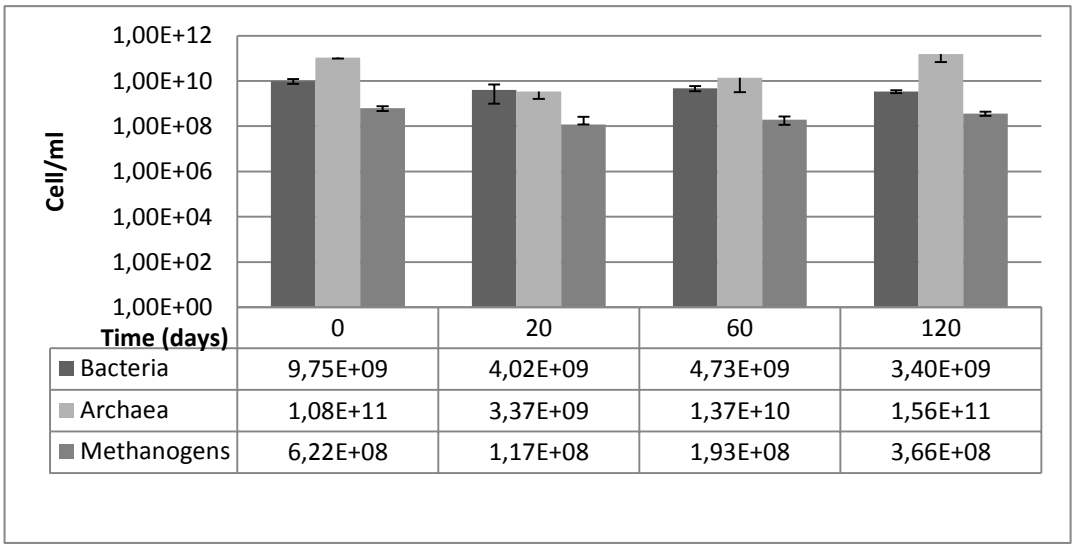
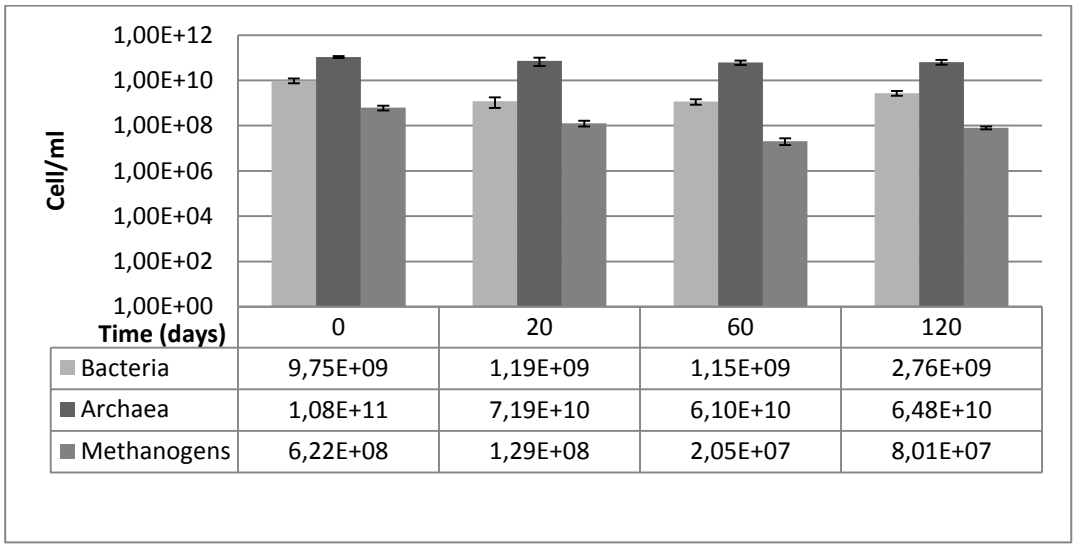


Figure 5.7: Bacteria, archaea and methanogen count of NC for methanogenic conditions.

For NC, at the end of the experiment, there were a significant decrease of bacteria and methanogen populations, unlike the insignificant change of archaea population

with regard to independent samples *t-test*. Bacteria population decreased regularly during the experiment. Archaea population decreased at 20th day, but then increased up to initial level until 120th day. Also methanogens decreased until 20th day and then increased at the rest of the experiment. The regular decrease of bacteria and the increase of archaea and methanogens after 20th day might be related as endogenous decay of bacteria. Archaea can be the dominant group with time due to microbial interactions.



Time (days)	0	20	60	120
Bacteria	9,75E+09	1,19E+09	1,15E+09	2,76E+09
Archaea	1,08E+11	7,19E+10	6,10E+10	6,48E+10
Methanogens	6,22E+08	1,29E+08	2,05E+07	8,01E+07

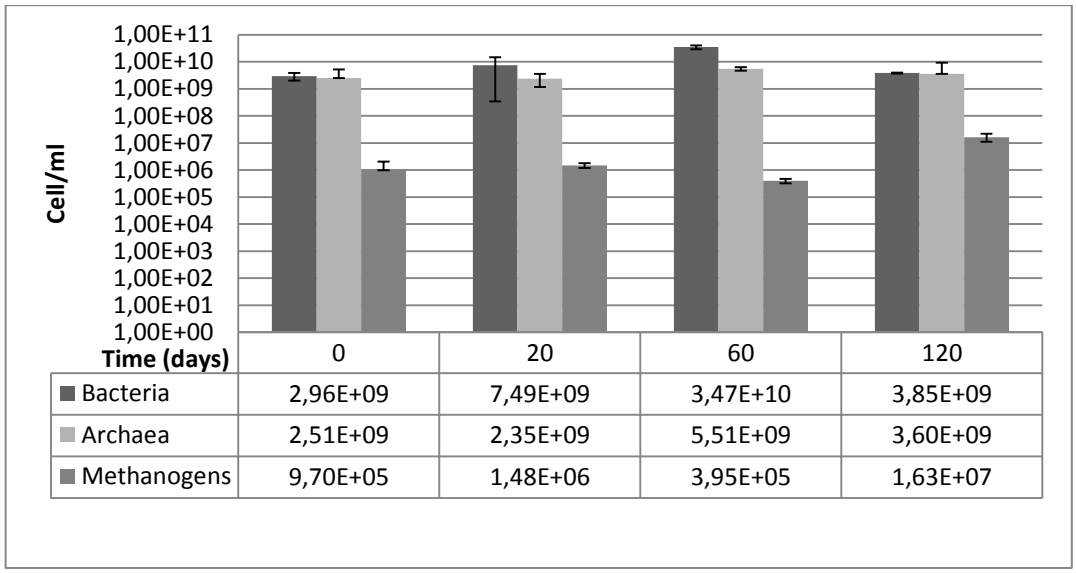
Figure 5.8: Bacteria, archaea and methanogen count of REF for methanogenic conditions.

For REF; bacteria, archaea and methanogen populations decreased significantly from 0th day to end of the experiment with regard to independent samples *t-test* analyses. But decreasing trend continued until the 60th day, then bacteria and methanogens increased significantly. It might be caused that after acclimation period to phenol, the advantageous microorganisms started to increase.

Bacteria, archaea and methanogen count for nitrate reducing conditions of NC and REF are given in figure 5.9, and 5.10 respectively.

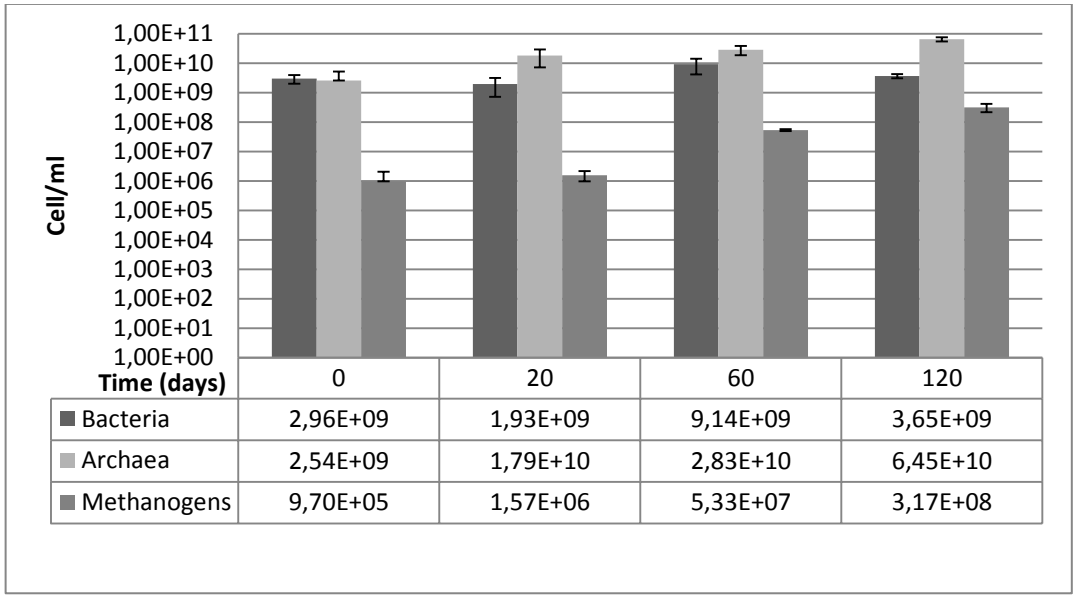
For NC at nitrate reducing conditions, there were no significant change for bacteria and archaea populations. But there was an irregular change for methanogens, the population increased and decreased unexpectedly. Because of methanogen population was lesser than the other populations; this irregularity might be caused due to insensitivity of QPCR.

For REF at nitrate conditions, there were significant increase for archaea and methanogens, and no significant change for bacteria. As the nitrate reducing conditions inoculated with anoxic sludge, the increase of archaea and methanogens might refer to transition anaerobic conditions to anoxic conditions. The observed biogas and methane production also confirm this transition.



Time (days)	0	20	60	120
Bacteria	2,96E+09	7,49E+09	3,47E+10	3,85E+09
Archaea	2,51E+09	2,35E+09	5,51E+09	3,60E+09
Methanogens	9,70E+05	1,48E+06	3,95E+05	1,63E+07

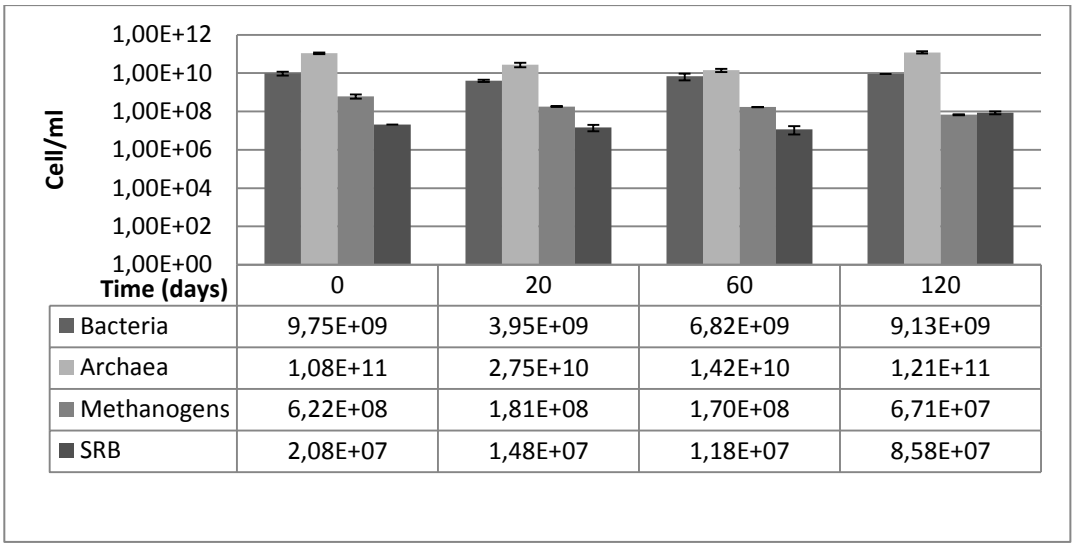
Figure 5.9: Bacteria, archaea and methanogen count of NC for nitrate reducing conditions.



Time (days)	0	20	60	120
Bacteria	2,96E+09	1,93E+09	9,14E+09	3,65E+09
Archaea	2,54E+09	1,79E+10	2,83E+10	6,45E+10
Methanogens	9,70E+05	1,57E+06	5,33E+07	3,17E+08

Figure 5.10: Bacteria, archaea and methanogen count of REF for nitrate reducing conditions.

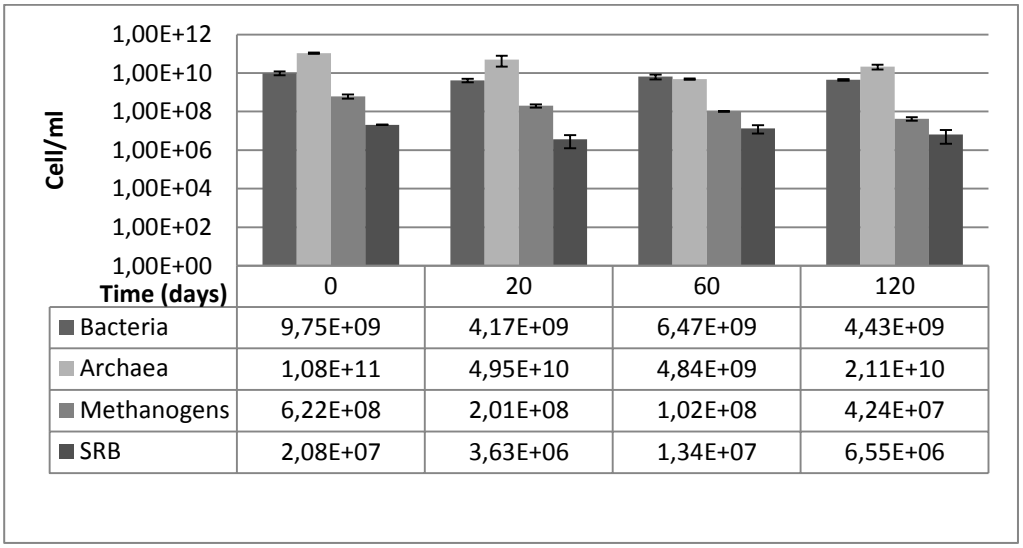
Bacteria, archaea, methanogen sulfate reducing bacteria (SRB) count for sulfate reducing conditions of NC and REF are given in figure 5.11, and 5.12 respectively.



Time (days)	0	20	60	120
Bacteria	9,75E+09	3,95E+09	6,82E+09	9,13E+09
Archaea	1,08E+11	2,75E+10	1,42E+10	1,21E+11
Methanogens	6,22E+08	1,81E+08	1,70E+08	6,71E+07
SRB	2,08E+07	1,48E+07	1,18E+07	8,58E+07

Figure 5.11: Bacteria, archaea, methanogen and SRB count of NC for sulfate reducing conditions.

While bacteria and archaea populations did not change significantly, population of their subgroups, methanogens and SRB changed significantly from the beginning to end of the experiment for NC at sulfate reducing conditions. While methanogen population was decreasing, SRB population increased during the experiment. The SRB increase was expected due to SRB favouring conditions and methanogen decreasing most probably caused by unfavourable conditions.



Time (days)	0	20	60	120
Bacteria	9,75E+09	4,17E+09	6,47E+09	4,43E+09
Archaea	1,08E+11	4,95E+10	4,84E+09	2,11E+10
Methanogens	6,22E+08	2,01E+08	1,02E+08	4,24E+07
SRB	2,08E+07	3,63E+06	1,34E+07	6,55E+06

Figure 5.12: Bacteria, archaea, methanogen and SRB count of REF for sulfate reducing conditions.

For REF at sulfate reducing conditions, all of the microorganism groups decreased significantly. These results were unexpected because sulfate reducing conditions are known as favourable conditions for biodegradation of complex organic compounds

(Aitiken et al., 2004). However the results indicated that in REF group, microorganisms were altered negatively and it explains why ERY and REF had similar biogas production.

Except nitrate reducing conditions, NC microorganism count was more than REF microorganism count. Apparently it is related with inoculum sludge and correlated with biogas measurements. The reason for that might be caused while phenol had inhibitory effect on anaerobic sludge, blank control with no carbon source might have increase of population due to endogenous decay.

5.2 Methanogenic Conditions

In order to assess the biodegradation capacity of erythromycin in methanogenic conditions, a biodegradation test according to OECD 311 protocol was designed as batch tests with having erythromycin as the only carbon source. During 120 days; the biogas and methane production, dissolved organic carbon (DOC) removal, erythromycin concentration, population change of bacteria, archaea, and methanogenic microorganisms were observed.

5.2.1 Biogas measurements

The cumulative biogas measurements of blank control with no carbon source (NC), reference control with phenol (REF) and the test substance that had erythromycin (ERY) as the only carbon source for 120 days is given in figure 5.13.

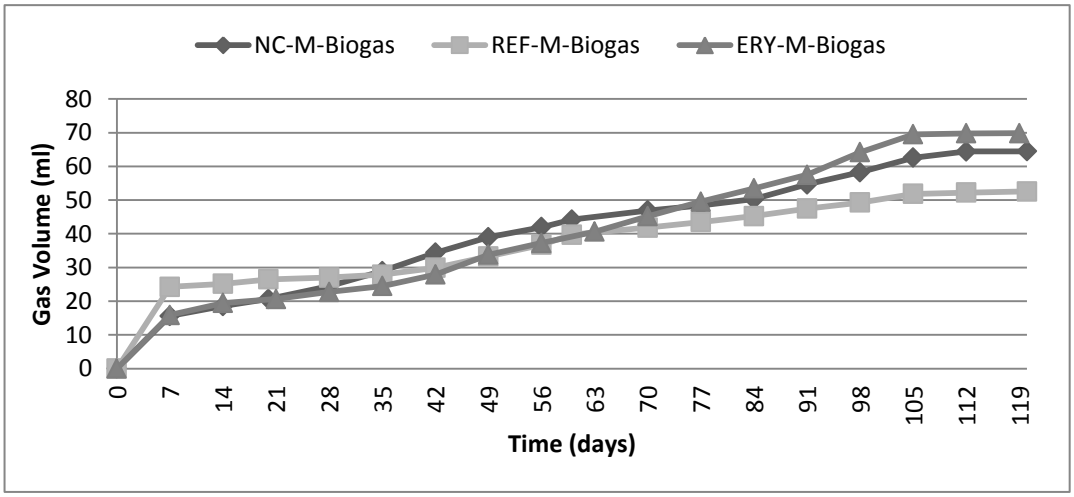


Figure 5.13 : Change in biogas during 120 days for blank control (NC), reference control (REF) and the test substance (ERY)

During the experiment, the final biogas volumes of NC, REF and ERY were 64 ml, 52 ml and 69 ml. REF having less biogas volume than NC was expected; because while phenol has known adverse effects on anaerobic digestion, NC acted like anaerobic digester and had more biogas production. Also the count of microorganisms showed that REF had lesser microorganisms than NC, which supports it. ERY had the highest biogas volume with 69 ml, but it was slightly more than control groups and it was equal to only 0,055 mmol which indicated that erythromycin was not able to biodegraded in this experiment.

5.2.2 Dissolved organic carbon (DOC)

The DOC removal of ERY during four sampling times as 0^{th}, 20^{th}, 60^{th} and 120^{th} with removal percentage is shown in figure 5.14.

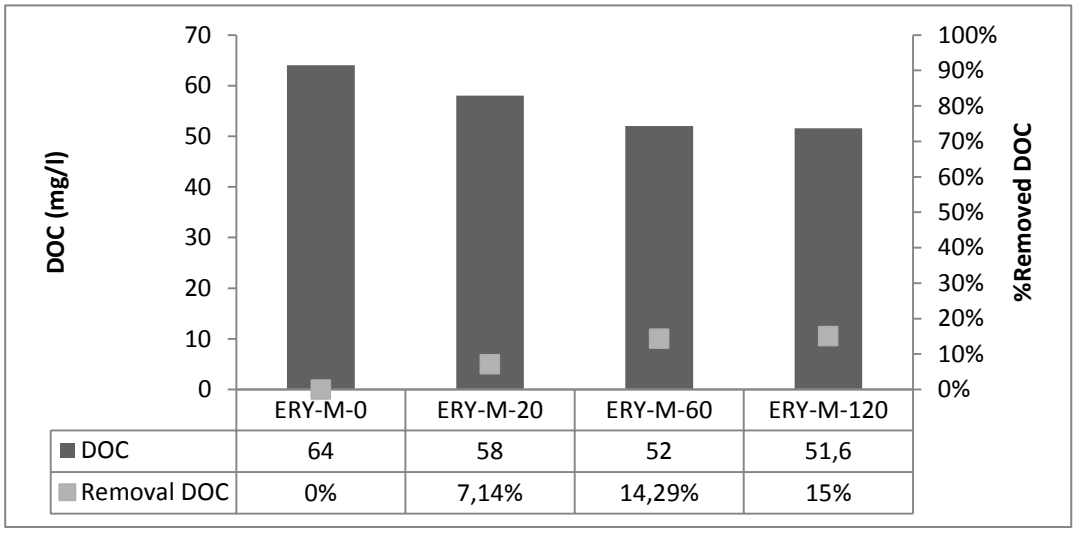


Figure 5.14:DOC removal with percentage of ERY

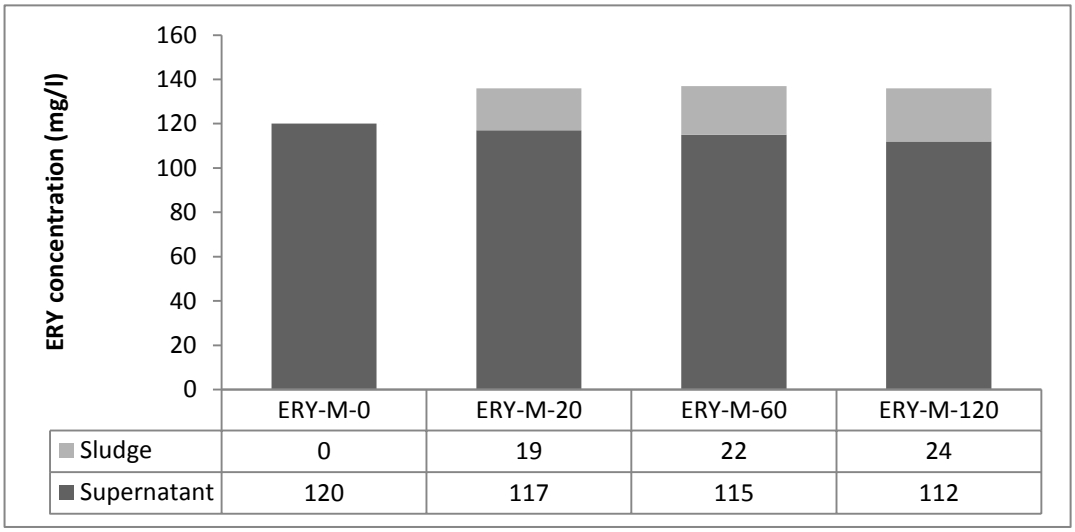
The initial DOC concentration was 64 mg/l and the final concentration was 51,6 mg/l. At the end of the experiment, only 15 percentage of total DOC removed, that clearly indicated erythromycin biodegradation did not meet the requirements of OECD 311 and degradation was minimal.

5.2.3 Antibiotic measurement

The change of erythromycin concentration in wastewater and sludge during four sampling times; 0^{th}, 20^{th}, 60^{th} and 120^{th} is shown in figure 5.15.

The experimental group was designed as having 138 mg/l erythromycin concentration that was calculated according to theoretical carbon need of the selected

microorganisms. The samples were measured 82% recovery and erythromycin standards were measured with p=0,01 regression analyse value. During the experiment, absorption of erythromycin to sludge was observed, with 24 mg/l final concentration at the sludge. The initial concentration of erythromycin was 120 mg/l at the wastewater and the final concentration was 112 mg/l. The insignificant removal of erythromycin is consistent with biogas and DOC analyses.

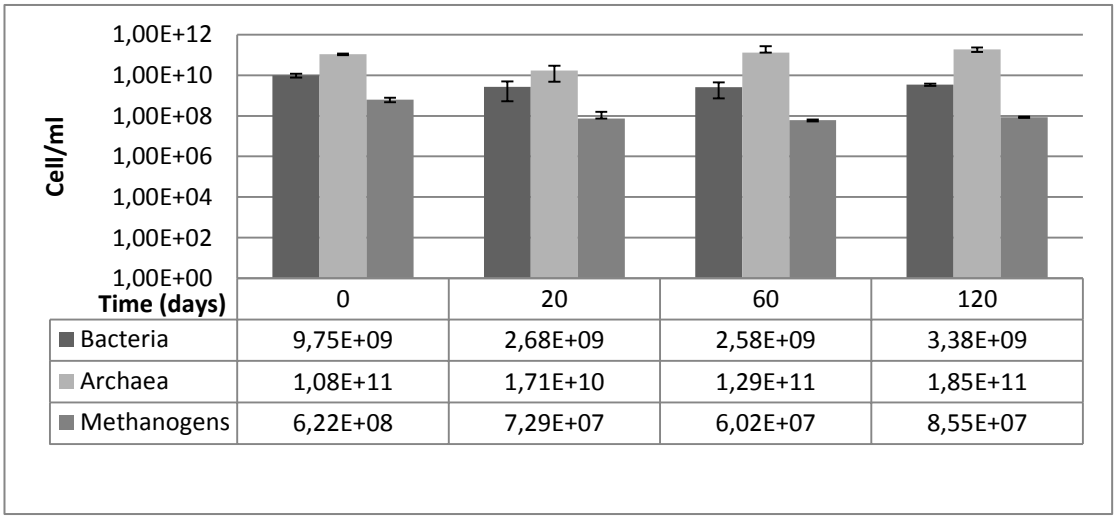


	ERY-M-0	ERY-M-20	ERY-M-60	ERY-M-120
Sludge	0	19	22	24
Supernatant	120	117	115	112

Figure 5.15: The change of erythromycin concentration in wastewater and sludge during four sampling times; 0^{th}, 20^{th}, 60^{th} and 120^{th}

5.2.4 Quantative PCR (Q-PCR)

Bacteria, archaea and methanogen count for methanogenic conditions of ERY is given in figure 5.16.



Time (days)	0	20	60	120
Bacteria	9,75E+09	2,68E+09	2,58E+09	3,38E+09
Archaea	1,08E+11	1,71E+10	1,29E+11	1,85E+11
Methanogens	6,22E+08	7,29E+07	6,02E+07	8,55E+07

Figure 5.16: Bacteria, archaea and methanogen count of ERY for methanogenic conditions.

In methanogenic conditions, significant decreasing of bacteria and methanogens and significant increasing of archaea population were observed. The inhibition of erythromycin on anaerobic digestion process cannot be relate to the decreasing of bacteria and methanogens, because biogas production of ERY was higher than NC and also the microorganism count of ERY and NC were similar. These results lead us that although erythromycin was not able to biodegrade, also it did not have significantly adverse effects on the microorganisms.

5.2.5 Correlation analyses

The correlation analysis of bacteria, archaea, methanogens and DOC over 4 sampling times 0^{th}, 20^{th}, 60^{th} and 120^{th} days is given in Table 5.1.

Table 5.1: Correlation analyses for methanogenic conditions

	Time	Bacteria	Archaea	Methanogen	DOC
Time	1	-0,494	0,511*	-0,585*	-0,844**
Bacteria	-0,494	1	0,202	0,805*	0,668**
Archaea	0,511*	0,202	1	-0,015	-0,414
Methanogen	-0,585*	0,805**	-0,015	1	0,791**
DOC	-0,844**	0,668**	-0,414	0,791**	1

Correlation coefficient pearson two tailed is used, * denotes correlation is significant at the level 0,05 and ** denotes correlation is significant at the level 0,01. Archaea is moderately correlate and increased with time unlike moderately correlated methanogens decreased with time. Also DOC strongly correlated with time negatively. Bacteria had strong correlation with methanogens and moderate correlation with DOC, they all decrease. Methanogens had strong correlation with DOC. Overall only archaea population increased while other parameters were decreasing. Apparently archaea favoured these conditions more than other microorganisms.

5.3 Nitrate Reducing Conditions

In order to assess the biodegradation capacity of erythromycin in nitrate reducing conditions, a biodegradation test according to OECD 311 protocol was designed as batch tests with having erythromycin as the only carbon source. During 120 days; the biogas and methane production, dissolved organic carbon (DOC) removal, nitrate concentration, population change of bacteria, archaea, and methanogenic microorganisms were observed.

The erythromycin concentration was not able to be measured. The unproblematic calibration and trustable measurements of the groups that were inoculated with anaerobic sludge (methanogenic and sulfate reducing conditions) indicate that it was caused most probably because of the anoxic sludge characteristics.

5.3.1 Biogas measurements

The cumulative biogas measurements of blank control with no carbon source (NC), reference control with phenol (REF) and the test substance that had erythromycin (ERY) as the only carbon source for 120 days is shown in figure 5.17. During the experiment, the final biogas volumes of NC, REF and ERY were 43 ml, 25 ml and 22 ml. The REF had produced less biogas than NC much probably caused by same reasons as mentioned in methanogenic conditions in 5.2.1 section. The observed biogas and methane productions in anoxic sludge conditions indicate that the anoxic conditions might be turned into methanogenic conditions. Total biogas production of ERY was equal to 0,031 mmol, which was also meant that there was no significant removal of erythromycin.

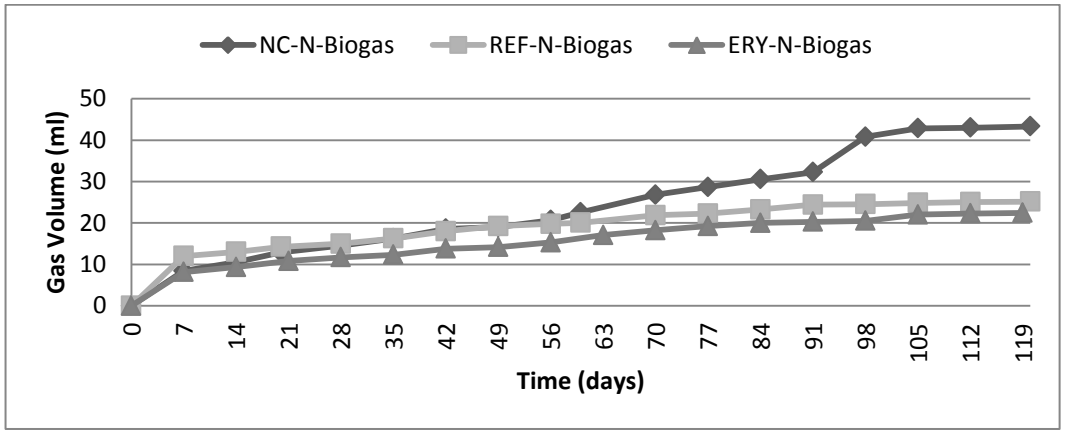


Figure 5.17: Change in biogas during 120 days for blank control (NC), reference control (REF) and the test substance (ERY)

5.3.2 Dissolved organic carbon (DOC) and ion chromatography

In figure 5.18, the DOC removal of ERY during four sampling times as 0^{th}, 20^{th}, 60^{th} and 120^{th} with change in nitrate concentration.

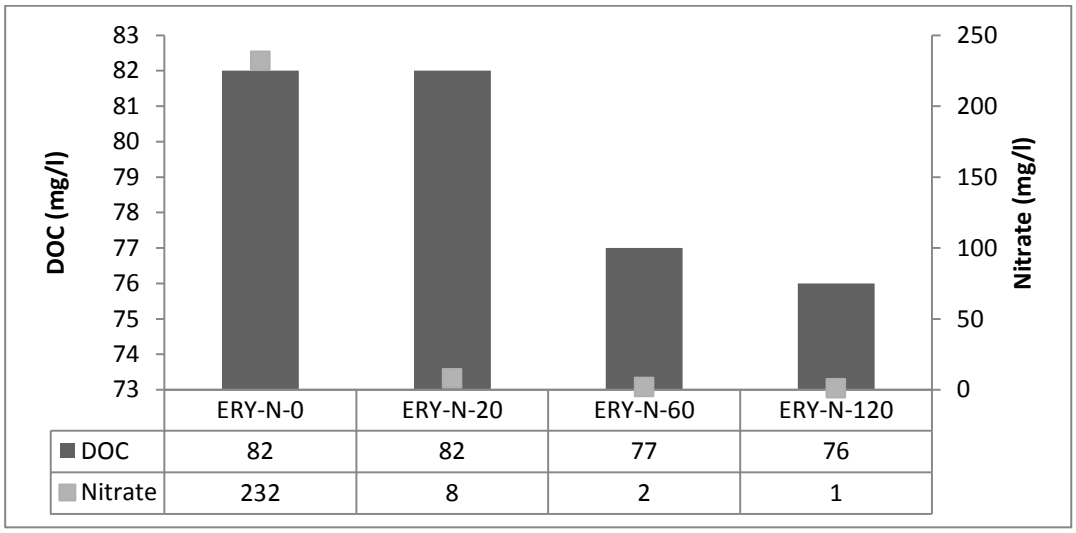


Figure 5.18: DOC removal with change in nitrate concentration of ERY

The nitrate concentration depleted at the 20^{th} day but removal of DOC continued until the 60^{th} day. The initial DOC concentration was 82 mg/l and the final concentration was 76 mg/l. Total removal percentage was only 7, an insignificant total DOC removal.

5.3.3 Quantitative PCR (Q-PCR)

Bacteria, archaea and methanogen count for nitrate reducing conditions of ERY is given in figure 5.19.

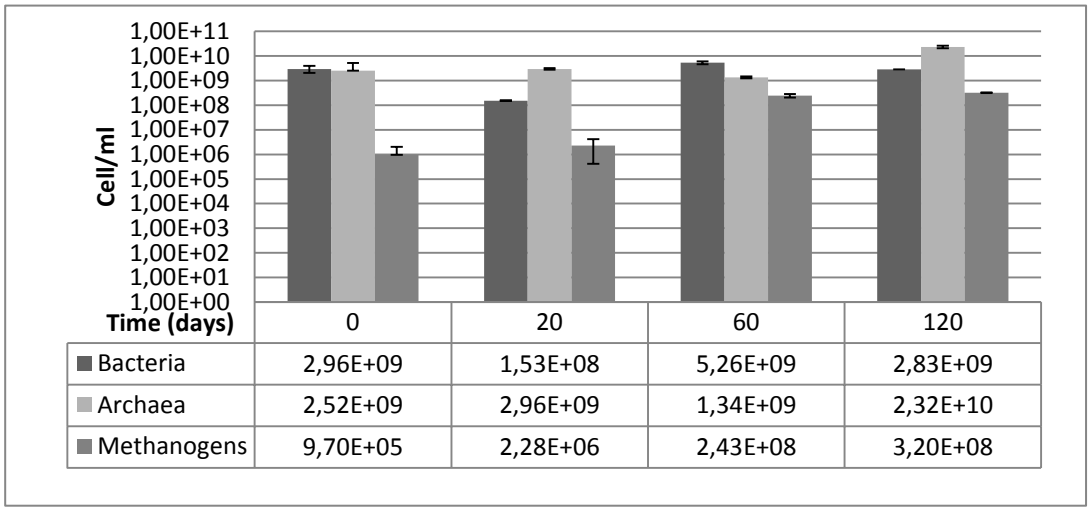


Figure 5.19: Bacteria, archaea and methanogen count for nitrate reducing conditions.

While archaea and methanogens significantly increased, bacteria population did not change significantly. Due to inoculum sludge was an anoxic sludge and batch test conditions were anaerobic, shifting anoxic conditions to anaerobic conditions can be observed from microorganism counting. Also methanogen population percentage over archaea population dramatically increased.

5.3.4 Correlation analyses

The correlation analysis of bacteria, archaea, methanogens, DOC and nitrate concentration over 4 sampling times 0^{th}, 20^{th}, 60^{th} and 120^{th} days is given in Table 5.2.

Table 5.2: Correlation analyses for nitrate reducing conditions

	Time	Bacteria	Archaea	Methanogen	DOC	Nitrate
Time	1	0,285	0,839**	0,939**	-0,858**	-0,648**
Bacteria	0,285	1	-0,037	0,570*	-0,574*	0,027
Archaea	0,839**	-0,037	1	0,664**	-0,568*	-0,326
Methanogen	0,939**	0,570*	0,664**	1	-0,911**	-0,587*
DOC	-0,858**	-0,574*	-0,568*	-0,911**	1	0,532*
Nitrate	-0,648**	0,027	-0,326	-0,587*	0,532*	1

Correlation coefficient pearson two tailed is used, * denotes correlation is significant at the level 0,05 and ** denotes correlation is significant at the level 0,01. Over time, while archaea and methanogens were increasing, DOC and nitrate concentrations decreased. Bacteria population moderately correlated with methanogens that indicate although there was no significant increase of bacteria, over methanogens there were an increase of bacteria population. And also there was another moderate correlation between bacteria and DOC, while DOC decreasing bacteria population increased. As the other correlations indicate, archaea and methanogens moderately correlated and archaea had a negative moderate correlation with DOC. Methanogens had a strong

negative correlation with DOC and a moderate negative correlation with nitrate. And also DOC and nitrate had a positive moderate correlation. Overall, while microorganisms increased, DOC and nitrate concentrations decreased.

5.4 Sulfate Reducing Conditions

In order to assess the biodegradation capacity of erythromycin in sulfate reducing conditions, a biodegradation test according to OECD 311 protocol was designed as batch tests with having erythromycin as the only carbon source. During 120 days; the biogas and methane production, dissolved organic carbon (DOC) removal, sulfate concentration, erythromycin concentration, population change of bacteria, archaea, and methanogenic microorganisms were observed.

5.4.1 Biogas measurements

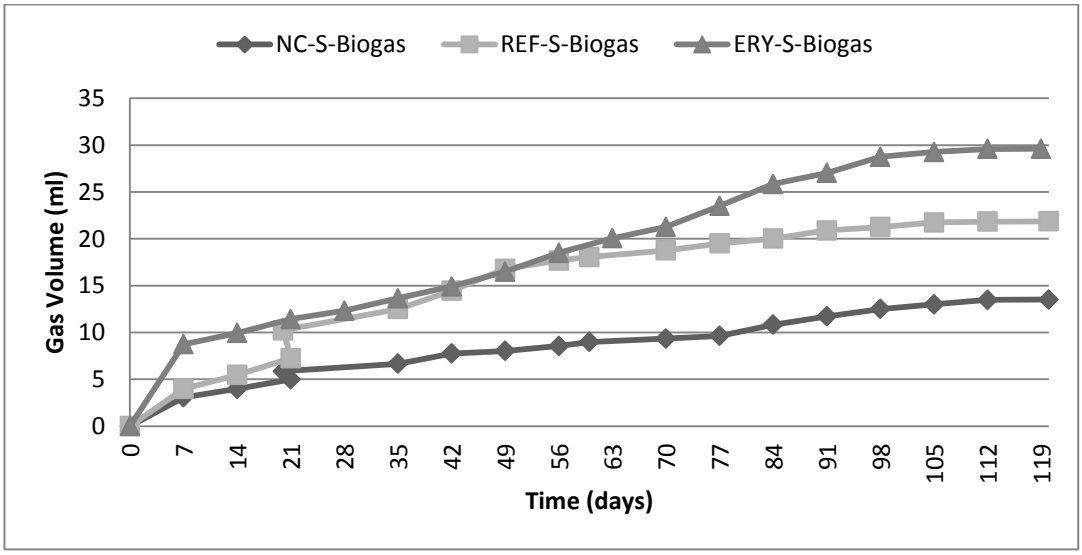


Figure 5.20: Change in biogas during 120 days for blank control (NC), reference control (REF) and the test substance (ERY)

The change in biogas during 120 days for blank control (NC), reference control (REF) and the test substance (ERY) is given in figure 5.20. During the experiment, the final biogas volumes of NC, REF and ERY were 13 ml, 21 ml and 29 ml. For sulfate reducing conditions, biogas measurements were consistent with the acceptance as blank control group NC had lowest amount of biogas and test group ERY had highest, unlike other conditions. But total biogas amount was very low as 0,031 mmol. Like the other conditions, in sulfate reducing conditions there was no evidence for biodegradation of erythromycin.

5.4.2 Dissolved organic carbon (DOC) and ion chromatography

The DOC removal of ERY during four sampling times as 0^{th}, 20^{th}, 60^{th} and 120^{th} with change in sulfate concentration is given in figure 5.21.

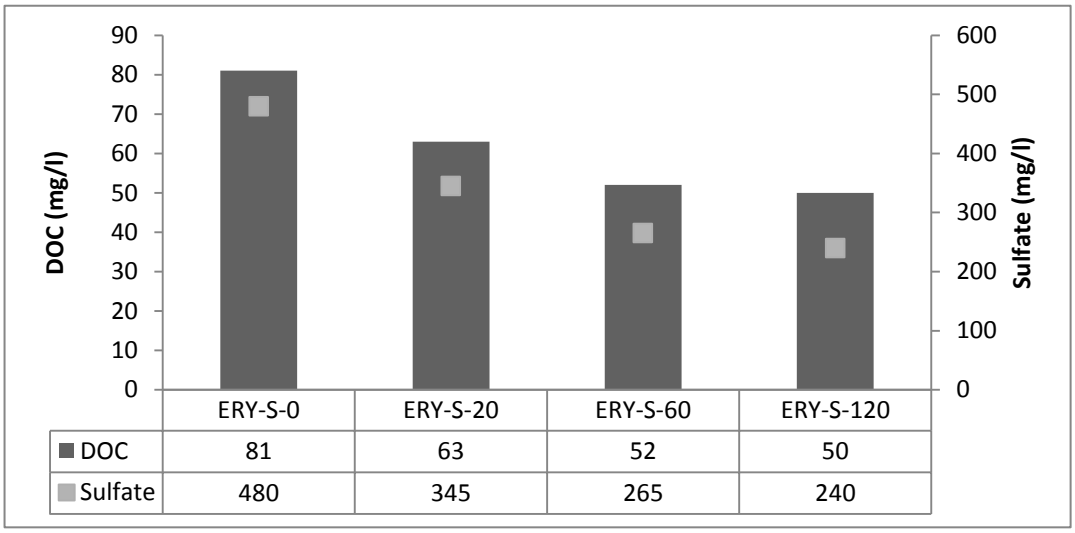


Figure 5.21: DOC removal with change in sulfate concentration of ERY

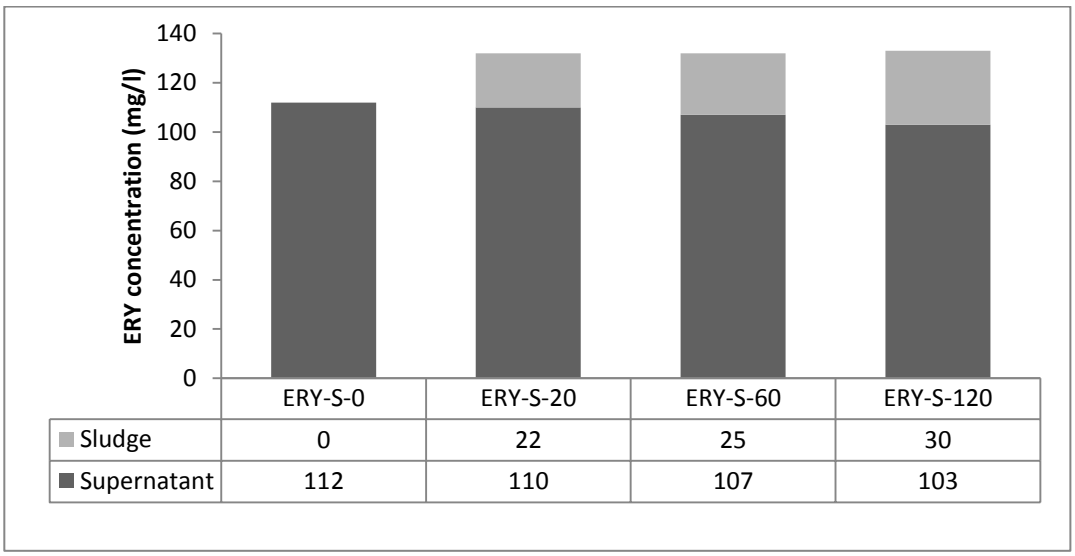
The initial concentration of sulfate was 480 mg/l and the final was 240 mg/l. The removal of sulfate was highly correlated with DOC removal ($p<0,01$), and the sulfate concentration was not limited at the end of the experiment. The DOC concentration decreased 81 mg/l to 50 mg/l and only 38 percentage of total DOC was removed. At the 60^{th} day, 35 percentage of total DOC was already removed. Although DOC removal was the highest among all of the electron acceptor conditions, it was unsatisfactory.

5.4.3 Antibiotic measurements

The change of erythromycin concentration in wastewater and sludge during four sampling times; 0^{th}, 20^{th}, 60^{th} and 120^{th} is shown in figure 5.22.

The experimental group was designed as having 138 mg/l erythromycin concentration. The samples were measured 82% recovery and erythromycin standards were measured with $p=0,01$ regression analyse value. The initial erythromycin concentration was 112 mg/l at the wastewater and it decreased to 103 mg/l. During 120 days, erythromycin absorbed to sludge with final concentration 30 mg/l. Erythromycin concentration measurements indicated that in sulfate reducing

conditions erythromycin has an inert character. The insignificant removal of erythromycin is consistent with biogas and DOC analyses.

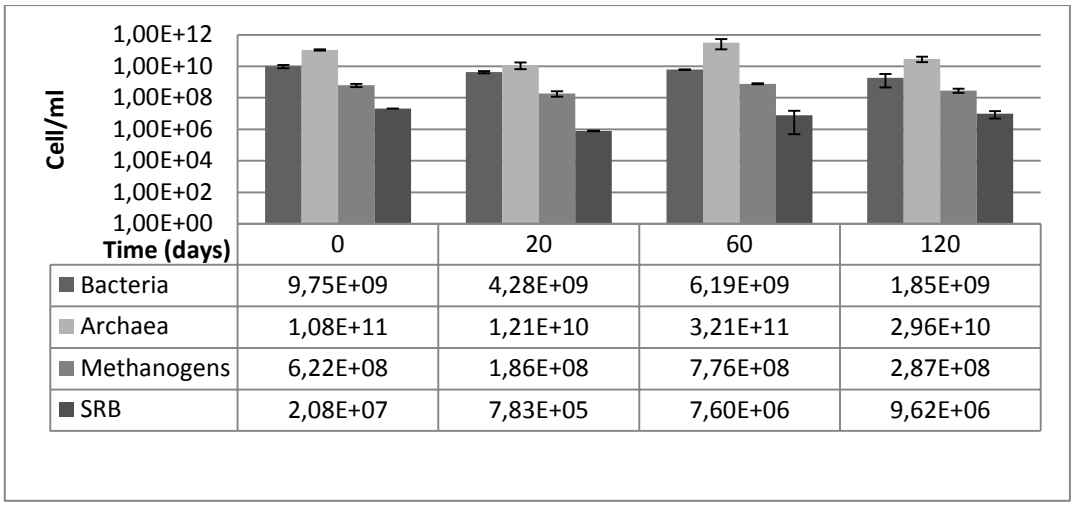


	ERY-S-0	ERY-S-20	ERY-S-60	ERY-S-120
Sludge	0	22	25	30
Supernatant	112	110	107	103

Figure 5.22: Erythromycin concentration in wastewater and sludge

5.4.4 Quantative PCR (Q-PCR)

Bacteria, archaea, methanogen sulfate reducing bacteria (SRB) count for sulfate reducing conditions is given in figure 5.23.



Time (days)	0	20	60	120
Bacteria	9,75E+09	4,28E+09	6,19E+09	1,85E+09
Archaea	1,08E+11	1,21E+10	3,21E+11	2,96E+10
Methanogens	6,22E+08	1,86E+08	7,76E+08	2,87E+08
SRB	2,08E+07	7,83E+05	7,60E+06	9,62E+06

Figure 5.23: Bacteria, archaea, methanogen and SRB count for sulfate reducing conditions.

For all counts, significant decrease is observed like reference control group. These results indicate the adverse effects of phenol and erythromycin on sulfate reducing conditions. But it also conflicts with DOC results because among all of the conditions, the highest DOC removal percentage with 38% is belong to sulfate

reducing conditions. And significant decrease for all microorganisms is only observed for sulfate reducing conditions. These results might be related with DNA based Q-PCR counting, if we accept that sulfate reducing conditions had higher activity than the other conditions, the decay of DNA of dead microorganisms would be faster than other conditions. So although it seemed like there are no change for microorganisms at other conditions, actually we would be counting the dead microorganisms too. But also it might be caused by microorganism decrease because of adverse effects and DOC removal to endogenous decay.

5.4.5 Correlation analyses

Table 5.3: Correlation analyses for sulfate reducing conditions

	Time	Bacteria	Archaea	Methanogens	SRB	DOC	Sulfate
Time	1	-0,744**	-0,017	-0,190	-0,210	-0,847**	-0,869**
Bacteria	-0,744**	1	0,269	0,583*	0,591*	0,755**	0,774
Archaea	-0,017	0,269	1	0,652**	0,299	-0,175	-0,151
Methanogens	-0,190	0,583*	0,652**	1	0,417	0,141	0,158
SRB	-0,210	0,591*	0,299	0,417	1	0,544*	0,549*
DOC	-0,847**	0,755**	-0,175	0,141	0,544*	1	0,995**
Sulfate	-0,869**	0,774	-0,151	0,158	0,549*	0,995*	1

The correlation analysis of bacteria, archaea, methanogens, DOC and sulfate concentration over 4 sampling times 0^{th}, 20^{th}, 60^{th} and 120^{th} days is given in Table 5.3.

Correlation coefficient pearson two tailed is used, * denotes correlation is significant at the level 0,05 and ** denotes correlation is significant at the level 0,01. Over time, bacteria population, DOC and sulfate concentration decreased as negatively correlated. Bacteria population moderately correlated with methanogens and SRB positively, and strongly positive correlated with DOC. Archaea only correlated with methanogens, they both decreased. SRB moderately correlated with DOC and

sulfate, they decreased during the experiment. Also DOC and sulfate had a strong positive correlation.

5.5 Comparasion the results with literature

Many authors concluded that erythromycin is non-biodegradable and domestic wastewater plants failed to treat them. Giger et al. (2003) reported that macrolide antibiotics were not fully eliminated and therefore residual amounts occur in the receiving surface waters. Göbel, et al. (2005) agreed that erythromycin persist in water phase and no significant sorption process takes place on biological sludge. Avella (2010) reported the inhibition of chemical oxygen demand and nitrogen removal by erythromycin. Another inhibition of nitrifiers by erythromycin was reported by Alighardashi et al. (2009). Although Louvet et al. (2010) indicated that the effect of erythromycin on nitrification is variable and depend on sludge origin. Alexy et al. (2004) also reported that erythromycin is not readily biodegradable which do not interfere the results of this project.

6. CONCLUSION

In order to assess the biodegradation capacity of erythromycin, a biodegradation test is designed according to the OECD 311 test with modifications. Three electron acceptor conditions were investigated, methanogenic conditions, nitrate reducing conditions and sulfate reducing conditions. The biogas production, DOC, nitrate, sulfate, erythromycin concentration, and the change of bacteria, archaea, methanogen and sulfate reducing bacteria populations were observed.

The biogas production was highest at the methanogenic conditions among nitrate and sulfate reducing conditions as expected. In nitrate reducing conditions, biogas production observed that might be the indicator of shifting anoxic conditions to anaerobic conditions. Sulfate reducing conditions also did not favour biogas production and had the lowest biogas production.

The highest DOC removal was for sulfate reducing conditions with 38%, for methanogenic conditions 15% and for nitrate reducing conditions 10%. Since the biodegradation criteria is more than 60% removal before the 60^{th} day, none of the electron acceptor conditions fulfilled the requirements. With respect to other analyses, sulfate reducing conditions cannot be referred as the best condition.

Erythromycin concentration was able to be measured for methanogenic and sulfate reducing conditions. Nitrate reducing conditions much probably could not be measured because of anoxic sludge characteristics. Erythromycin was inert for both methanogenic and sulfate reducing conditions. With DOC and biogas results, it is cleared that erythromycin could not be biodegraded in this experiment.

Microorganism counts indicate the transition of anaerobic conditions from anoxic conditions for nitrate reducing conditions, and a dramatic decrease for sulfate

reducing conditions. With comparing control groups, microorganism counts enlightened the other analyses.

This study had shown that erythromycin was not biodegradable for chosen anaerobic and anoxic sludge and three electron acceptor conditions. But also literature indicates that the biodegradability of antibiotics strongly related with the concentration and sludge characteristics. Lower concentrations and acclimated sludge with antibiotics might improve the results.

7. RECOMMENDATIONS

Although the OECD 311 protocol offers the information about the biodegradation capacity of the erythromycin, it is an indirect method and it cannot be determined with full confidence that ERY was biodegraded. In order to assess biodegradation and the microbial community responsible for it, Stable Isotope Probing (SIP) must be conducted for further research. But since this project concluded with no biodegradation, it can be assumed that SIP result would be similar.

Erythromycin was proven to be unable to biodegraded and erythromycin resistance genes were tracked in many different environments in literature and this project also showed that erythromycin was unable to be biodegraded. Since erythromycin is widely used, non-biodegradable and its resistance can be tracked hospitals to inhabited surroundings; it would be wise to switch erythromycin to new generation macrolide antibiotics in long term.

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