REACTIVATION POTENTIAL OF INDICATOR BACTERIA IN ANEROBICALLY DIGESTED SLUDGES AFTER DEWATERING PROCESSES

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submitted by **MÜGE ERKAN** in partial fulfillment of the requirements for the degree of **Master of Science in Biotechnology Department, Middle East Technical University** by,

Prof. Dr. Canan Özgen _____
Dean, Graduate School of **Natural and Applied Sciences**

Prof. Dr. İnci Eroğlu _____
Head of Department, **Biotechnology**

Prof. Dr. F. Dilek Sanin _____
Supervisor, **Environmental Engineering Dept., METU**

Assoc. Prof. Dr. Hilal Özdağ _____
Co-Supervisor, **Biotechnology Institute, Ankara University**

Examining Committee Members:

Prof. Dr. Gülay Özcengiz _____
Biology Dept., METU

Prof. Dr. F. Dilek Sanin _____
Environmental Engineering Dept., METU

Assoc. Prof. Dr. Hilal Özdağ _____
Biotechnology Institute, Ankara University

Assist. Prof. Dr. Tuba Hande Ergüder _____
Environmental Engineering Dept., METU

Gülsevim Şener (Environmental Engineer, M.Sc.) _____
Ministry of Environment and Urban Planning

Date: September 16th, 2011

I hereby declare that all information in this document has been obtained and presented in accordance with academic rules and ethical conduct. I also declare that, as required by these rules and conduct, I have fully cited and referenced all material and results that are not original to this work.

Name, Last name: Müge ERKAN

Signature :

ABSTRACT

REACTIVATION POTENTIAL OF INDICATOR BACTERIA IN ANEROBICALLY DIGESTED SLUDGES AFTER DEWATERING PROCESSES

Erkan Müge

M.Sc., Department of Biotechnology

Supervisor: Prof. Dr. F. Dilek Sanin

Co-Supervisor: Assoc. Prof. Dr. Hilal Özdağ

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Anaerobic digestion process which has long been known to successfully reduce the organic content of sludge is one of the most common alternatives to meet pathogen reduction requirements for particular classes of biosolids. However, it has recently been reported that, significantly higher densities of indicator bacteria have been measured in dewatered cake samples compared to samples collected right after anaerobic digestion. In addition, this increase in bacterial population has been commonly observed after centrifugation but not after belt filter dewatering. Even though several theories have emerged to explain this occurrence, with the use of molecular tools such as Quantitative Polymerase Chain Reaction (Q-PCR) in recent studies, much of the attention was given to the reactivation of the indicator bacteria which might enter a viable but non-culturable state (VBNC) during digestion process. The main objective of this research is to examine different treatment plants in Turkey to observe whether the aforementioned phenomenon is valid in these plants as well. Towards this end, the impact of dewatering processes

on indicator bacteria counts was investigated by performing both standard culturing methods (SCM) and Q-PCR on sludge samples collected after digestion and dewatering from selected full-scale treatment plants. Results indicated that, in treatment plants operating belt filter dewatering, reduced concentrations of indicator bacteria do not change after the dewatering process. However, indicator bacteria content of sludge increase immediately after centrifuge dewatering. Based on the results obtained by Q-PCR, reactivation of VBNC bacteria was speculated to be the main reason for the increases obtained.

Keywords: Anaerobic digestion, indicator bacteria, Quantitative PCR, standard culture methods

ÖZ

ANAEROBİK OLARAK ÇÜRÜTÜLMÜŞ ÇAMURLARDAKİ İNDİKATÖR BAKTERİLERİN YENİDEN AKTİFLEŞME POTANSİYELİ

Erkan, Müge

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Uzun zamandır arıtma çamurlarının organik içeriğini azaltmadaki başarısıyla bilinen anaerobik çürütme süreci, belirli biyokatı sınıflarının patojen giderim gerekliliklerini yerine getirmede kullanılan en yaygın alternatiflerden biridir. Fakat yakın zamanda, anaerobik çürütme sonrası toplanan örneklerdeki indikatör bakteri miktarlarının susuzlaştırılmış kekte ölçülenlere kıyasla hayli yüksek olduğu bildirilmiştir. Ayrıca, bakteri populasyonundaki bu artışlar genellikle belt filtreden sonra değil, santrifüj susuzlaştırmadan sonra gözlenmektedir. Bu durumu açıklamak için çeşitli teoriler ortaya atılmış olsa da, son çalışmalarda Kantitatif PCR gibi moleküler araçların kullanılması ile birlikte, dikkatler anaerobik çürütme

sürecinde canlı fakat kültüre edilemeyen bir evreye girmiş olan bakterilerin yeniden aktifleşmesi üzerinde toplanmıştır. Bu çalışmanın amacı ise bahsedilen artışların ülkemizdeki arıtma tesislerinde de geçerli olup olmadığını incelemek amacıyla Türkiye'deki farklı arıtma tesislerinin değerlendirilmesidir. Bu amaçla, seçilen tam ölçekli arıtma tesislerinden çürütme ve susuzlaştırma sonrası toplanan çamur örneklerine hem standart kültür metodları hem de Kantitatif PCR analizleri uygulanarak, susuzlaştırma tekniğinin indikatör bakteri sayıları üzerindeki etkisi incelenmiştir. Sonuçlar, belt filtre ile susuzlaştırma yapan tesislerde indikatör bakterilerin azalan konsantrasyonlarında susuzlaştırma sonrasında bir değişiklik olmadığını göstermektedir. Fakat, santrifüj susuzlaştırmanın hemen akabinde çamurdaki indikatör bakteri seviyeleri artmaktadır. Kantitatif PCR'dan elde edilen sonuçlara dayanarak, canlı fakat kültüre edilemeyen bakterilerin yeniden aktifleşmesinin bu artışların başlıca sebebi olduğu tahmin edilmektedir.

Anahtar Kelimeler: Anaerobik çürütme, Kantitatif PCR, indikatör bakteri, standart kültür metodları.

to my family and family soon to be… :)

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LIST OF ABBREVIATIONS

BOD: Biological Oxygen Demand

CFU: Colony-forming Unit

COD : Chemical Oxygen Demand

Cp: Crossing point

DGGE: Denaturating Gradient Gel Electrophoresis

DS: Dry Solids

E.coli: Escherichia coli

FC: Fecal Coliform

MF : Membrane Filter

MPN: Most Probable Number

PCR: Polymerase Chain Reaction

Q-PCR: Quantitative PCR

SRT: Solids Retention Time

TGGE: Temperature Gradient Gel electrophoresis

TS: Total Solids

U.S. EPA: United States Environmental Protection Agency

VBNC: Viable but Not Culturable

WERF: Water Environment Research Foundation

WWTP: Wastewater Treatment Plant

CHAPTER 1

INTRODUCTION

One of the main research areas related with the management of sewage sludge all over the world is to promote the beneficial reuse of sludge by taking necessary precautions to protect the environment and human health. Sewage sludge contains high levels of organic matter and nutrients as well as a variety of microorganisms (Kinney et al., 2006b). Typical sewage treatment processes reduce the number of microorganisms in sewage but these microorganisms are concentrated in the sewage (Gerba and Smith, 2005). Therefore, one of the most vital issues to be considered about the land application of sewage sludge is the investigation and quantification of pathogenic microorganisms in sludge.

The presence of pathogens in sewage sludge has been determined by indicator microorganisms for years and limit values of corresponding regulations have been set for these microorganisms. In the United States which has one of the most detailed regulations concerning pathogenic microorganisms, biosolids are divided into two classes as Class A and Class B, based on the indicator microorganism content of sludge (U.S. EPA, 1993). Class A is defined as the sludge which has a lower concentration than 1000 MPN/g TS for Fecal Coliforms or 3 MPN/4 g TS for Salmonella immediately after the stabilization processes. The criteria for Class B biosolids require the concentration of Fecal Coliforms to be less than 2,000,000 MPN/g TS. Despite the council directive applicable in EU countries (86/278/EEC) has no limitations related to pathogens, most of the member countries such as France, Italy and Luxembourg have pathogen limits in their own regulations. Similarly a new regulation concerning the use of domestic and municipal sewage sludge in the soil published on August 3, 2010 by the Ministry of Environment and

Forests in Turkey (Bylaw on the Use of Domestic and Urban Treatment Sludge on the Soil, 2010). This new regulation obligates 2log10 removal of *E. coli* in the sludge by the stabilization processes applied.

Among all the stabilization alternatives involved in above mentioned regulations, anaerobic digestion is known to be widely adopted by many wastewater treatment facilities all over the world. Biological activity of biosolids is reduced along with the organic matter content of sludge during anaerobic digestion process. However, it has recently been reported in the literature that significantly higher densities of Fecal Coliform and/or *E.coli* have been measured in dewatered cake samples compared to samples collected after anaerobic digestion (Hendrickson et al., 2004, Iranpour et al., 2003; Cheung et al., 2003; Monteleone et al., 2004; Qi et al., 2004). This finding was strikingly more pronounced in the centrifuge dewatered sludge cakes compared to the belt filter press dewatered cakes (Erdal et al., 2003; Qi et al., 2007). Moreover, densities of indicator bacteria has been observed to further increase in case of storage of the cake samples (Erdal et al., 2003; Qi et al., 2007; Higgins et al., 2007).

Several predictions have been emerged to explain this occurrence including false positive results associated with enumeration techniques (Qi et al., 2004), contamination from the centrifuge (Hendrickson et al., 2004), effect of floc break up on improved culturability, (Cheung et al., 2003), removal of growth inhibitors during dewatering and effect of day-light throughout the storage of biosolids (Gözen and Örmeci, 2010). Since some conditions are known to induce pathogenic and nonpathogenic organisms to enter dormancy state, the ability of indicator bacteria persisting in Viable but Not Culturable (VBNC) state during anaerobic digestion process was also investigated in the same manner. Along with the common use of molecular tools for environmental samples which provide an approach to enumerate bacteria without relying on standard culturing techniques, research all over the world intensified in order to investigate that phenomena of

VBNC state for the purpose of elucidating the observed increases (Higgins 2007, Dunaev 2008, Higgins 2008).

In this context, the purpose of this study is to examine selected wastewater treatment plants in Turkey by considering these findings in order to investigate whether similar increases exist in the number of indicator bacteria after the implementation of dewatering processes. Selection of the plants were done in such a way that the differences, if there is any, between centrifuge dewatering and belt filter press dewatering can be observed satisfactorily. Samples taken from anaerobic digester influent, anaerobic digester effluent and dewatered cake were examined for their indicator bacteria content by standard culturing methods (SCM). Similarly for all the samples collected Quantitative PCR analyses were also conducted to examine the treatment operations by a culture independent technique which might also provide a new perspective for the reactivation and regrowth phenomena through results obtained by a molecular enumeration approach.

CHAPTER 2

LITERATURE SURVEY

2.1. Municipal Wastewater and Wastewater Sludge

Municipal wastewater is the waste orginating from a community which composed of domestic wastewaters and/or industrial discharges. It typically consists of different types of solids, organics, inorganic chemicals, metals and microorganisms (Metcalf and Eddie, 2003). Owing to the wide range of potential contaminants it contains, municipal wastewater poses a significant threat to human health and environment. Therefore, it should be treated before the final discharge. Wastewater treatment systems operated in plants can differ in some technical aspects. Yet, since the desired result is to obtain a clean effluent, in a typical wastewater treatment plant a number of physical, chemical and biological treatment processes are commonly applied to wastewater (Tchobanoglous et al.,2003). However, although treatment of wastewater is a solution for prevention of water pollution, a similar source of pollution arise from this treatment operation which is vast quantity of sewage sludge produced at the end of the water treatment process.

Sewage sludge is mainly defined as a semi solid material which is odiferous and difficult to manage (Sanin et al., 2011). According to Environmental Protection Agency (EPA), sewage sludge is the residual by-product produced during municipal wastewater treatment (U.S. EPA, 1999). Sludge is typically in the form of a liquid or semisolid, which contain approximately 0.25 to 12 percent solids by

weight (Metcalf & Eddy, 2003). Although types of the produced sludges vary in accordance with the treatment options applied in treatment plants, typically two prominent types of sludge are produced in a wastewater treatment plant which are primary and secondary sludge. A major part of solids are produced after gravitational settling of the incoming wastewater in primary treatment and named as primary sludge. This type of sludge is known to have high concentration of pathogens along with high percentage of water which make it hard to manage (Sanin et al., 2011). Wastewater generally enters a secondary treatment after primary treatment. In the secondary treatment, the removal of organics typically quantified as Biochemical Oxygen Demand (BOD) is aimed by the suspended biomass. The process results with an excess growth of biomass called which is called as waste activated sludge or secondary sludge or sometimes called as biological sludge. Even though the pathogen concentration of the waste activated sludge is not as high as the primary sludge, secondary sludge is more difficult to be dewatered because of the attachment of water particles to the microorganisms found in secondary sludge (Spinosa and Vesilind 2001).

Primary and secondary sludge formed this way, can not be utilized or deposited on land without an additional due to several constraints. First of all, sludge may contain toxic substances and pathogenic microorganisms which can pose a health hazard. The concentrated organic matter present in both primary and secondary sludge might cause adverse environmental effects (Spinosa and Vesilind 2001; Metcalf & Eddy, 2003; Sanin et al., 2011). Thus, sewage sludge generated in a treatment plant must be further stabilized in order to overcome the problems associated with the ultimate usage and disposal.

2.2. Sewage Sludge Treatment and Disposal Methods

Sewage sludge as it is produced contains high amounts of pathogenic organism and chemicals. Owing to the biodegradable nature, sewage sludge also degrades and rapidly causes an unpleasant smell. It also contains very high amount of (99%)

water. The alternatives available for sewage sludge treatment and disposal are given in Figure 2.1 which also summarizes the route for a typical wastewater goes through after generation. The three unit operations that wastewater sludge goes through are stabilization, thickening and dewatering. The purpose of sludge stabilization is to stabilize the sludge so that the organic content and microbial content of sludge is reduced. On the other hand, the purpose of thickening and dewatering operations is to remove the excess water from sludge.

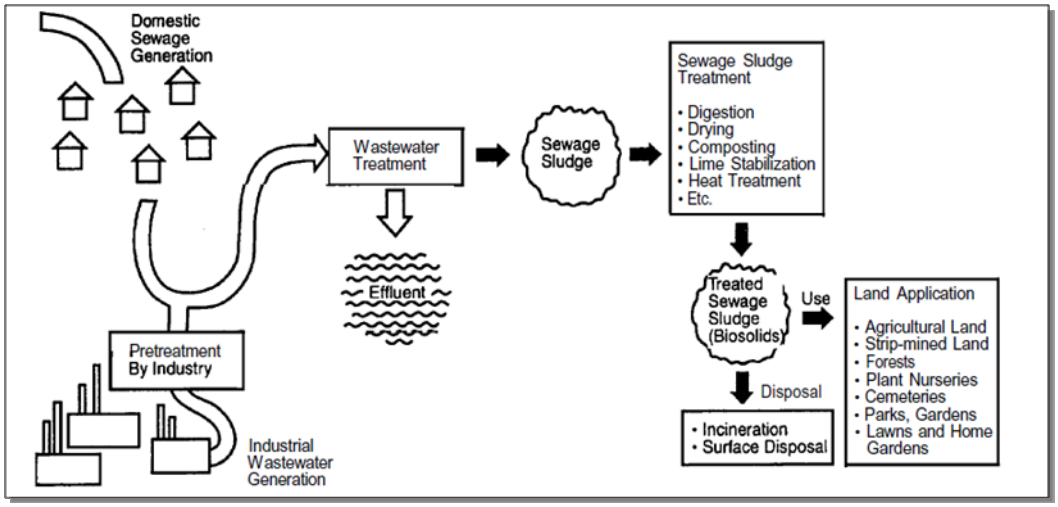

Figure 2.1. Generation, treatment, use, and disposal of sewage sludge

(U.S. EPA, 2003)

2.2.1. Sludge Stabilization

The stabilization term corresponds to any approach that reduce the detrimental effects of sewage sludge, pathogens, offensive odors and the potential for further biodegradation after the disposal (Sanin et al., 2011). Several definitions have been used for stabilization but considering the common features, it could be defined as a process which was developed for the degradation of biodegradable fraction of organic matter in sludge in order to prevent public health and environmental risk when disposed or used for a beneficial purpose (Sanin et al., 2011).

The stabilization technologies can be divided into three basic processes according to the mechanism of concern; biological stabilization, chemical stabilization and thermal stabilization (Andreoli et al., 2007).

Regardless of the mechanism applied, the efficiency of a stabilization process can be followed by the examination of several parameters. Reduction of odor, volatile solids and pathogen content of sludge are the basic indications of stability.

2.2.1.1. Biological Stabilization

Aerobic digestion, anaerobic digestion and composting are the most commonly used biological stabilization methods which rely on the consumption of biodegradable fraction of organic matter in sludge by a specific group of bacteria. Details of each stabilization method are provided below.

Anaerobic Digestion

Anaerobic sludge digestion is the destruction of organic matter by a mixed consortium of bacteria which function in the absence of oxygen. The final products of the degradation process are a mixture of mainly methane (CH_4), carbondioxide (CO_2) and new cells as well. The purposes of anaerobic digestion process are the stabilization of organic solids, reduction of sludge volume and odor, destruction of pathogenic organisms, production of gases to be used as energy sources and the improvement of sludge dewaterability (Dohanyos and Zabranska, 2001; Epstein, 2003).

The simplified degredation reaction can be shown as:

$$\text{Organic matter} + H_2o \longrightarrow CH_4 + CO_2 + \text{new cells}$$

In a conventional secondary wastewater treatment plant, mixture of primary sludge and secondary sludge are stabilized in anaerobic digesters which is presented as organic matter in the above reaction.

Throughout the anaerobic digestion process, organic matter is biologically converted into stable end products by a series of metabolic reactions. Methane, as a valuable product of anaerobic digestion process, can be used in heating the treatment units and/or producing electricity (Romano and Zhang, 2008).

In terms of temperature, anaerobic digesters perform under two different modes of operation; mesophilic (30–35 ◦C) and thermophilic conditions (50–60 ◦C). Since the rate of biochemical reactions accelerates with the increased temperature, thermophilic digesters are known to achieve higher organic solids reduction efficiency (Rubia et al., 2005). The other significant advantage of the thermophilic anaerobic digestion over mesophilic digestion is known as the higher efficiency obtained in the pathogen destruction under higher temperatures (Smith et al., 2005). However, mesophilic anaerobic digesters are advantegous in terms of their specific methane yield, effluent quality and process stability (Song et al., 2004).

Time is a significant parameter for anaerobic digestion process regarding the contact duration (Sanin et al., 2011). Solid retention time (SRT) is the average time that bacteria are retained in the digester. SRT must be kept long enough (higher than 15 days) to ensure sufficient residence, especially for slow-growing methanogens. Depending on the required treatment, efficiencies and/or operational conditions such as temperature, waste characteristics and mixing, different SRT values may suit specific treatment needs (Gerardi 2003).

Since anaerobic digestion is a biological process, pH of the system should be kept between the pH requirement of the specific bacteria involved in the digestion process. Methanogens operate optimum at a range of 6.5 to 8.2 while acidogens prefer between 4 and 6.5 (Speece 1996). In a well-operating anaerobic digester the pH of the system is around neutral pH.

Aerobic Digestion

One of the other biological stabilization techniques which have a broad range of application is the aerobic digestion process. According to U.S. EPA, aerobic digestion is a process where sewage sludge is agitated with air or oxygen to maintanin aerobic conditions for a specific mean cell residence time at a specific temperature (U.S. EPA, 2003). The working principle of aerobic digestion relies on the direct oxidation of biodegredable matter. In the absence of food supply, aerating the sludge itself results in the production of end by-products such as carbon dioxide, water and nitrogen (Stentiford, 2001; Epstein, 2003). The aerobic digestion process has a great similarity with the activated sludge process. Owing to the interrupted substrate supplementation, the microorganisms involved are forced to consume their own energy reserves to survive which is called as endogenous phase (Andreoli et al., 2007).

The simplified reaction of the aerobic sludge stabilization consists of aerobic oxidation of biodegradable organics (mainly cell mass) to carbon dioxide, ammonia and water.

$$C_5H_7No_2 + 7o_2 + \text{bacteria} \longrightarrow 5Co_2 + No_3 + 3H_2O + H$$

Currently, three types of aerobic digestion processes are used in sludge stabilization; conventional aerobic digestion, aerobic digestion with pure oxygen and thermophilic aerobic digestion. Since heat is the main by-product of organic matter degradation under aerobic conditions, temperature of aerobic digesters can reach to 60°C by insulation and mixing. This temperature is sufficient for the permanence of microbial activity (Andreoli et al., 2007). Typical aerobic microorganisms found in aerobic sludge are able to grow at temperatures of 40-75°C. Therefore, it is preferable to operate aerobic digesters at elevated temperatures (Kepp et al., 2000). Thermophilic aerobic digesters have several advantages such as the reduction of hydraulic retention time and production of

disinfected sludge meeting the regulations requirements. However, high capital costs, operational complexity and foam building are the major drawbacks of the thermophilic aerobic digester process. Therefore, the majority of aerobic digesters currently in use are operated under ambient temperature conditions.

Composting

Composting is another aerobic biological method used for sludge stabilization. In this process, biological degradation of organic material in sludge is accomplished by mesophilic and thermophilic microorganisms under controlled conditions of temperature, moisture, oxygen and nutrients.

Typical aerobic decay equation is;

Complex inorganics + O_2 $\longrightarrow$ $CO_2 + H_2O + NO^-_3 + SO_4$ + less complex organics + heat

The key feature of composting is the utilization of bulking agents such as wood chips, tree leaves,bark, sawdust which are called as green wastes. Mixing the dewatered sludge with those sort of amendments keeps the ingredients in solid form and helps to maintain the moisture content, increase porosity and balance of the carbon-nitrogen ratio (Krogmann, 2001; Warman and Termeer, 2005).

Owing to the exothermic characteristics of the process, when the maximum microorganism activity is achieved, temperature at the inner core of the composting pile increases up to 70 °C approximately. Those elevated temperatures lead to the inactivation of pathogenic microorganisms found in the sludge (Mena et al., 2003). Main current composting technologies are open windows (piles), open aerated static piles and reactor systems. Compost obtained from sewage sludges vary in quality and applicability for beneficial usage. As long as the quality of

composted sludge provide the limitations given in relevant regulations, they can be used as soil conditioners for agricultural or horticultural purposes.

2.2.1.2. Chemical Stabilization

Lime Stabilization

Lime has long been used for sludge stabilization for the purpose of reducing the number of pathogenic and odor-producing organisms as well as to suppress the availability of heavy metals in sludges. In the lime stabilization process, sufficient quantities of lime is added to untreated sludge in order to raise the pH to 12 or higher. Provided that an adequate contact time is implemented, alteration of pH creates an environment that arrest or slow down the microbial reactions that can otherwise lead to odor production and vector (birds, mice, flies, etc.) attraction (Epstein, 2003; Metcalf & Eddy, 2003). According to EPA, when the pH of the mixture of wastewater solids and alkaline material is maintained at or above 12, pathogen reduction requirements can be achieved after 2 hours of contact (U.S. EPA,2000).

Lime stabilization can be a part of a sludge conditioning process since it is a reliable and cost efficient method. However, when compared with the digestion processes, lime stabilization have several drawbacks such as potential for the regrowth of pathogens in case of the pH drops below 9.5 specifically during storage, potential for odor generation at the end use site and increase in the amount of total solids due to chemical precipitation reactions (Haug et al., 1995; U.S. EPA, 2000).

2.2.1.3. Thermal Stabilization

Heat Treatment

Heat treatment is another process utilized for conditioning and stabilization of sludge. It involves heating the sludge for short periods of time under high pressure. The sludge is sterilized and dewatered by the heat treatment since solids are coagulated, gel structure of the sludge is broken down and water affinity of sludge

solids is reduced. (Metcalf & Eddy, 2003). Basic advantages of the process are the microbial reduction, hygienization and the improvement of dewaterability properties. The major disadvantages on the other hand, are associated with high capital cost and the production of side sterams with high concentrations of organics, ammonia nitrogen, odours.

2.2.2. Sewage Sludge Thickening and Dewatering

The removal of the water content is a fundamental operation for the reduction of sludge volume to be treated or disposed of. Since mechanical behavior of sludge depends upon its solids content it should be considered during the design and operation of sludge processing units (Andreoli et al., 2007). In a typical wastewater treatment plant, water removal occurs in two stages of the sludge processing; thickening and water removal. The fundamentals of thickening and dewatering differ in terms of the degree of dewatering. Solid concentration of thickened sludge is typically less than 15% whereas in dewatered sludge it is greater than 15% (Sanin et al., 2011).

The purpose of thickening operation is to reduce the volume of sludge. It is basically implemented on primary or secondary sludge to concentrate sludge in order to improve the effectiveness of further treatment. Common types of thickeners operated in treatment plants are gravity thickeners, flotation thickeners and gravity belt thickeners.

In dewatering process on the other hand, it is aimed to remove as much of water as possible from the suspended solids. For this purpose mechanical dewatering systems such as belt filter presses, centrifuges or vacuum filters are commonly used. Those operations lead to have a final product, known as cake, in which solid content is as high as 30% (Sanin et al., 2011).

Sludge is dewatered in order to reduce the volume for landfill disposal or land application purposes, to minimize the transportation costs, to enhance the sludge

heating capacity prior to incineration and to lower the leachate production when landfill disposal is practiced (Andreoli et al., 2007).

Since the treatment plants investigated in the scope of this study, implement either centrifuge or belt filter press dewatering, only those dewatering processes are described in the following sections.

2.2.2.1. Centrifuge Dewatering

The principle of centrifuge dewatering basically depends on the difference of density between solids and surrounding liquid. Centrifuge applies a centrifugal force as it rotated which is typically several thousand times of gravitional force to separate the solids from liquid. Three sequential operations occur in a typical centrifuge: clarification or removal of solids from the liquids, consolidation of the solids and convey of solids to discharge point (Spinosa and Vesilind, 2001). Vertical and horizontal shaft centrifuges are both used in sludge dewatering but horizontal shaft centrifuges are most widely applied for thickening and dewatering of sludge. The two types of centrifuges are used for municipal sludge dewatering, basket and solid bowl which both operate on the same basic principles. Bowl type horizontal centrifuge, also known as decantor, is the type of centrifuge which is mainly adapted in wastewater treatment plants. After the sludge slurry is introduced to the centrifuge, it is forced against the bowl's interior walls and a basin of liquid is generated. The sludge solid "cake" and the liquid "centrate" are then separately discharged.

The major advantage of centifuge system is the considerably small area required for a large centrfiuge having a feeding capacity of 10-40 L/s (Andreoli et al., 2007).
It can dewater sludge to much higher solids contents. However, high consumption of electricity along with the maintanence costs may limit their use for wastewater treatment plants particularly with flows higher than 100L/s (Andreoli et al., 2007).

2.2.2.2. Belt Filter Press Dewatering

Belt filter presses, also named as belt presses are dewatering units which force water through a fabric by applying a positive pressure. A single or double moving belt continuously presses the incoming sludge via processing three basic operational stages: chemical conditioning, gravity drainage and compaction in a pressure and shear zone.

Belt press dewatering process start with the drainage of free water from the sludge in the gravity drainage stage of the press. Then, sludge is routed to a two-belt contact zone where a second, upper belt gently compresses the sludge and make it to release remaining free water in the sludge. Finally at the high pressure zone, continuously increasing pressures and shear forces are applied to sludge in order to trigger the release of water which is more highly bound.

As in centrifuge dewatering systems, belt pressess require careful maintenance and attention through cleaning at the end of every operating shift. As a major drawback, belt presses are not able to achieve high cake solids concentration as in centrifuges. In additon, since they are open mechanics, they have several disadvantages such as aerosol emission, high noise level and odor problem. However, the low initial costs and reduced electic power consumption can be listed as the most outstanding advantages of belt filter pressing and distinguishing it as one of the most prevailing dewatering options preferred in wastewater treatment plants (McFarland, 2000)

2.2.3. Sewage Sludge Disposal

With the increasing number of treatment plants, sludge quantities have been gradually increasing all over the world. Due to both large quantities and the undesirable characteristics of sludge, handling and disposal of sludge is one of the most significant challenges of the environmental engineering field (Sanin et al., 2011).

After treated by a variety of methods in wastewater treatment plants, invariably a residual sludge is generated and this odiferous, semi solid material should be managed properly with the same attentiveness as in wastewater treatment. The evaluation of final disposal alternatives should be considered as a part of the treatment plant itself (Andreoli et al., 2007). Sludge disposal options aim to incorporate useful characteristics of sludge back into soil as well as managing undesirable properties in an environmentally sustainable manner. Hence, disposal alternatives should be evaluated considering the typical characteristics before the final decision.

Sewage sludge has been disposed by several methods including disposal into sanitary landfills, incineration, agricultural use and ocean dumping. Since the most common disposal alternatives consist of landfilling, incineration and land application, a brief summary of these disposal methods are given below.

2.2.3.1. Landfilling

Landfilling is a disposal technology which aims to confine the disposed waste within the least possible area. More broadly, it can be regarded as any form of deposition on or into land aiming the disposal of the material instead of reuse or improvement (Spinosa and Vesilind, 2001). In terms of the proportion of the sludge obtained in wastewater treatment plants, landfilling is still one of the main disposal options, for instance 40% of sludge generated in Europe is landfilled (Fytili and Zabaniotou, 2008).

During the landfilling operations, the addition of sludge could have a beneficial effect on anaerobic microbial degradation. Owing to the accelerated decomposition of organic material, landfill areas would be used more rapidly which provides oppurtinity for the enhancement of production of methane (Sanin et al., 2011). However, when improperly designed or operated, pollution may affect air through odour and toxic gases, surface water bodies through drainage and soil and also groundwater by infiltration of percolated liquids (Andreoli et al., 2007).

The major benefits of landfill technology can be regarded as low investment costs, large capacity and quick disposal method but regarding the environmental management, landfiling is the least favaourable option for the disposal of sludge. Sludge landfill technology does not ultimately prevent environmental pollution, but it only delay the time of generating pollution. Present trends in management of sewage sludges show that landfill is the least prefereed option and should be used when no alternative exists because of increasing sludge quantities and limited landfilling areas (Wong et al., 2001).

2.2.3.2. Incineration

Incineration refers to the process of transferring dehydrated or dried sludge into a burning furnace for a heat treatment process. During the incineration process, organic solid material in sludge is totally converted to oxidized end products, primarily carbon dioxide, water and ash. The advantage of incineration technology lays in the thoroughness to achieve the purpose of sterility and minimization of sludge with high speed and effectiveness in terms of energy recovery potential as well (U.S. EPA, 1985a).

Incineration may be an expensive alternative owing to the higher transport costs for the sewage treatment plants settled in large cities. Present trend favors the fluidized bed incinerator owing to the smaller operational costs and lower release of air pollutants (Andreoli et al., 2007). However, the potential presence of metals and organic micropollutants in the gaseous effluent and residual ashes restrict the application of incineration process (Mininni, 2001; Khiari et al., 2006). Therefore, during the assessment of an incineration operation for a specific sludge, preliminary characterization of sludge should be carried out in order to determine the presence of pollutants which might pose an environmental risk (Spinosa and Vesilind 2001).

2.2.3.3. Land Application

In the perspective of sludge management, the order of preference is to minimize, utilize and dispose the generated sludge. However, this is a dilemma since better wastewater treatment systems produce more quantities of sludge. Until recently, incineration and landfilling were common practices for sludge disposal. Owing to the limited number of landfilling areas along with the increasing cost of the landfill disposal and prohibition of other disposal options such as ocean dumping with EU law as well, the agricultural use of sewage sludge or biosolids were promoted. Similarly, this option was accepted as one of the best ways to solve the sludge production-disposal paradox in short-term (Werther & Ogada 1999; National Research Council, 2002; Oliver et al., 2005).

United States has become one of the earliest supporters of agricultural reuse through promulgation of regulations that primarily aim is to protect human health. When Environmental Protection Agency (EPA) has promulgated 40 CFR Part 503 in 1993, it was the first time the term of "biosolid" is recommended in order to define sewage sludge treated according to the relevant regulations and which might be used beneficially as a soil conditioner and fertilizer. Even though the designation of biosolids currently appears in technical reports and relevant literature, the terms biosolids, sewage sludge or sludge have been used as synonymous in several publications and by different environmental communities. In fact, the term of biosolid is intended to refer treated sludge leaving the wastewater treatment plant destined for beneficial usage (U.S. EPA, 1993).

Sewage sludge contains large quanities of nitrogen and phosphorous (Metcalf&Eddy, 1991). This content gives sludge unique fertilizing properties, since those elements are essential in terms of plant growth. Hence, the application of sludge on both agricultural and nonagricultural land is a common practice around the world as a disposal option. Sludge can improve soil conditions as well after lime and other alkali addition (Andreoli et al., 2007). Moreover, sludge has beneficial effects for improving poor soils such as strip mine soils (Sanin et al., 2011).

However, sludge may contain various other elements as well, which may be harmful if gets into the food chain. Ground and surface water contamination by phosphorus and nitrogen should also be considered. Those properties inherently cause social and technical problems (Davis, 1996). Since the public concern about the sludge is originating from the odour and the knowledge of where it comes from, it is also a challange to overcome public perception (Sanin et al., 2011). Besides, technical problems arise from the fact that sludge is being produced during the whole year whereas land application is needed for once or twice a year. Consequently, the problem is not still concretely come up with a solution since the sludge should be stored until the land application is needed (Fytili and Zabaniotou, 2008).

The main limitations of biosolids land application arise from heavy metals, toxic organic pollutants along with the pathogenic organisms found in sewage sludge all of which might cause serious threats for envrionment and public health. (Andreoli, et al., 2007). Since pathogenic microorganisms constitute the core subject of this study, more detailed explanation is given below in terms of human pathogens and indicators along with the enumeration techniques.

2.3. Pathogens in Sludge

Several disease causing organisms are found in the sludge since wastewater treatment processes do not completely remove or inactivate pathogenic and parasitic organisms (Andreoli et al., 2007). Instead, organisms attach to the settling solid particles in the separation stages of treatment process and they are known to survive better compared to their suspended state (Scheuerman et al., 1991; Straub et al., 1992). Since microorganisms are inherently transferred to wastewater sludge at the end of the treatment process, concentrations of pathogens in biosolids can be higher than the incoming wastewater (Nell et al., 1983; Pike E.B. 1986).

Municipal wastes collected from large metropolitan area of the world are known to contain a wide variety of pathogens (Lewis and Gattie 2002). The pathogenic

microorganisms mainly arise from human sources and reflect the health status of the population along with the hygenic quality of the region (Andreoli et al., 2007). Similarly, the diversity and concentration of pathogens depend on the occurence of enteric infections within a community and the presence of hospitals and abattoirs in the area (Gerba and Smith 2005; Sidhu and Toze 2009). On the other hand, the amount of pathogens exist in a wastewater from a specific community is greatly influenced by socioeconomic level of the population, geographic region, presence of agro industries and type of sludge treatment process. Apart from human sources, other potential source of pathogens might be the animal sources or vectors such as birds, insects and rodents found in sewers (Andreoli et al., 2007).

Four major types of human pathogens can be found in biosolids: bacteria, viruses, protozoa, and helminths. Some references also include fungi (Gattie and Lewis 2004). Owing to their obligatory parasitic nature, enteric virus, protozoa and helminths are unable to multiply in biosolids, however bacteria found in the sludge may multiply under favorable conditions (Skanavis and Yanko, 1994; Sidhu et al., 2001). Major pathogens found in wastewater sludges and resulting diseases are listed in Table 2.1.

Table 2.1. Principle Pathogens of Concern Found in Domestic Sewage and Sewage Sludge (U.S. EPA, 2003)

Organism	Disease/Symptoms
Bacteria	
Salmonella sp.	Salmonellosis (food poisoning) typhoid fever
Shigella sp.	Bacillary dysentery
Yersinia sp.	Acute gastroenteritis (including diarrhera, abdominal pain)
Vibrio cholerae	Cholera
Compylobacter jejuni	Gastroenteritis
Escherichia coli (pathogenic strains)	Gastroenteritis
Enteric Viruses	
Hepatitis A virus	Infectious hepatitis
Rotaviruses	Acute gastroenteritis with severe diarrhea
Enteroviruses	
Polioviruses	Poliomyelitis
Coxsacklieviruses	Meningitis, pnuemonia, hepatitis, fever,cold-like symptoms etc.
Echoviruses	Meningitis, paralysis, encephalitis, fever, cold-like symptoms,diarherra
Reovirus	Respiratory infections, gastroenteritis
Astroviruses and Calciviruses	Epidemic gastroenteritis
Protozoa	
Cryptosporidium	Gastroenteritis
Entamoeba histolytica	Acute enteritis
Giardia lamblia	Giardiasis (including diarrhea, abdominal cramps, wieght loss)
Blantidium	Diarherra and dysentery
Toxoplasma gondii	Toxoplasmosis
Helmint Worms	
Ascaris lumbricoides	Diegstive and nutritional disturbances, abdominal pain, vomiting, restlessness
Ascaris suum	May produce symptoms such as coughing, chest pain, diarrhea, anemia, weight loss
Trichuris trichura	Abdominal pain, diarrhera anemia weight loss
Toxocara canis	Fever, abdominal discomfort, muscle aches, neurogical symptoms
Taenia solium and T. saginata	Nervousness, insomnia, anorixia, abdominal pain, digestive disturbances
Necator amercanus	Hookworm disease Taeniasis

Bacteria existing in the sludge originated from different sources such as human and animal intestinal flora, soil, air and water. Even though reported incidents are low in terms of diseases transmitted by sewage sludge, increases in the land application of sludge may raise the risk (Andreoli et al., 2007). Among the pathogenic bacteria found in the wastewater and sewage sludge, the principal pathogens considered by EPA in establishing the Part 503 rule are *Salmonella, Shigella*, *Campylobacter jejuni, Yersinia* and *E.coli.* Other newly emerging bacterial pathogens such as *H. pylori* which have been found to cause gastric ulsers and cancer as well as *Listeria* which is found in raw and treated sewage sludge are also causes of concern in terms of land application of biosolids (Gerba et al., 2002b).

Salmonella sp. and *Shigella sp.* are the major infecting agents as being noted the most common pathogenic bacteria found in domestic sewage (Sahlstrom et al 2004). *Salmonella* is frequently isolated from raw and treated sludge but fortunately it is known to be inactivated easily by the treatment processes (Sahlstrom et al 2004). The typical numbers in anaerobically digested solids are relatively low compared to indicator bacteria (Gantzer et al., 2001). However, it is known to survive up to 3 months during storage as well as being capable of regrowth under certain conditions (Sidhu et al., 2001). *Salmonella typhi* and *Salmonella paratyphi* are the main threatening species (Lucero-Ramirez, 2000).

According to EPA, majority of the worldwide undefined outbreaks were caused by enteric viruses (U.S. EPA, 2006). Water and wastewater may become contaminated by approximately 140 types of possible enteric viruses. Existence of enteric viruses in biosolids have been reported in several studies as well (Gallagher and Margolin 2007; Monpoeho et al., 2001; Viau and Peccia 2009). Particularly for adenoviruses, high concentrations have been adressed in several studies for sewage and primary sludge (He and Jiang, 2005; Albinana-Gimenez et al., 2006) probably due to their resistance to heat and UV applications (Gerba et al.,2002a).

In most cases, pathogenic viruses and bacteria in wastewater die after 1–3 months. Protozoan oo(cysts) and helminth ova on the other hand can survive for up to a

year in wastewater and probably much longer in biosolids (Sidhu and Toze 2009). The decay and survival of pathogens depend upon several factors including pH,temperature, competition from other microorganisms, sunlight, soil texture, proper nutrients, and moisture level (Martin et al., 1990; U.S. EPA, 1999; Sidhu et al., 2001; Pietronave et al., 2004). The reaction of pathogens regarding these factors can vary throughout the pathogens as well as sludge treatment process applied (Sidhu and Toze 2009).

2.3.1 Traditional Bacterial Indicators and New Alternatives

In order to validate a treatment process and/or assure about the microbiological quality of wastewater sludge, ideally the direct detection of pathogenic bacteria and viruses along with the protozoan parasites should be conducted. However, it is impractical to enumerate all pathogenic microorganisms in biosolids primarily due to the lack of specific detection techniques which can be applied for all the organisms of concern (Moce-Llivina et al., 2003). In addition, existing procedures are not only time consuming but also expensive and requires well trained labor (Bitton, 2005). These requirements eventually leads to the utilization of indicator organisms which serve as surrogates for the fecal contamination (Sidhu and Toze, 2009).

The ultimate objective with the indicator bacteria concept was to ascertain the risk of gastrointestinal disease from a contaminated site (Yanko, 1988). In 1914, coliforms were adopted as the indicators of fecal contamination in drinking water by the US Public Health Center. Consequently, various microorganisms have been proposed and utilized as indicators of fecal contamination in potable water as well as for the assesment of treatment efficiencies in water and wastewater treatment plants (Bitton, 2005).

The ideal fecal indicator should satisfy several criteria. First of all it should be a non-pathogenic intestinal organism found in all warm blooded animals. In addition, an ideal indicator should de easily and rapidly detectable. Besides, it must be at least

equally resistant to environmental conditions and disinfection processes applied in treatment plants (Hurst et al., 2002; Gabriel Bitton 2005; Sidhu and Toze 2009). Even though there is no single group of microorganisms meeting all of these criteria, Total Coliforms (TC), Fecal Coliforms (FC) and Fecal Streptococci (FS) are known to satisfy most of these requirements, therefore have been used to assess hygenic quality of waterbodies for years.

Total Coliforms include *E.coli*, *Enterobacter*, *Klebsiella*, and *Citrobacter* but they are not all of fecal origin. Hence, their utility as indicators for fecal contamination has been questioned (Charriere et al.,1994). In wastewater treatment plants Total Coliforms are mainly used for determination of the treatment efficiency. Fecal Coliforms or Thermotolerant Coliforms comprise aerobic and facultatively anaerobic gram negative, non-spore forming, rod-shaped bacteria which fermentate lactose at 44.5 C° (Santamaria and Toranzos, 2002). However, the usefulness of Fecal Coliforms is also limited since their presence does not indicate protozoan or viral contamination. More recently, the abundance of *Escherichia coli* has been shown to be a better indicator of the sanitary risk than total coliforms (Edberg et al 2000; Fewtrell and Bartram, 2001).

E.coli is considered to be superior to previous indicators owing to various properties. First of all, it is the only Fecal Coliform bacteria of true fecal origin and high numbers of *E.coli* exist in the feces of warm blooded animals. In addition, it survives longer than some other bacterial pathogens (Hamilton et. al, 2005). Due to these advantages, *E.coli* has been monitored in drinking water regulations in USA (U.S. EPA 1986, U.S. EPA 2000; U.S. EPA 2003) and in Europe (European Union, 1998). In addition, *E.coli* have been utilized in various research concerning drinking water (Edberg et al., 2000); wastewater (Kramer 2002); agricultural soil (Lang et al., 2007); sewage sludge (Eccles et al., 2004) and biosolids (Iranpour et al., 2004). Nevertheless, utilization of *E.coli* as an indicator bacteria has several limitations. First of all, as in most of the intestinal bacteria, *E.coli* has been also shown to enter a VBNC state (Oliver, 2010). However, classical culture methods are unable to

determine VBNC state of bacteria. Moreoever, the conventional *E.coli* analysis is not practical since it requires a hard work and time-consuming methodology (Armisen, 2004). However, those limitations can be overcomed by the development of new detection technologies that rely on enzymatic methods (George et al., 2000; Van Poucke and Nelis, 2000), immunological methods (Pyle et al., 1999) and several molecular tools such as Real Time Polymerase Chain Reaction (RT-PCR) (Juck et al., 1996) or fluorescent in situ hybridization (FISH) (Regnault et al., 2000a).

Fecal Streptococci are used to detect fecal contamination in water. Members of this group commonly inhabit the intestinal track of humans and other warm-blooded animals. They are also reported to be useful for the indication of viruses particularly in biosolids (Bitton, 2005). Fecal Coliform to Fecal Streptococcus ratio has also been utilized for many years for the prediction of the origin of pollution.

Although *Salmonella* is not considered as an indicator bacteria, owing to its broad existence in biosolids and compost, it has been a pathogen of interest mainly for land application of biosolids (Gibbs et al. 1997). It has been also utilized in numerous research as the densities of Fecal Coliform and Salmonella correlate in compost and stored biosolids (Yanko 1988; Gibbs et al. 1994). Similarly, Tiquia et al., (1998) reported that the removal of *Salmonella* corresponds to the decrease of fecal originated bacteria. Quantification of *Salmonella* is also suggested by EPA as to determine pathogenic survival in the analysed material. Since their typical concentrations are higher than other bacterial pathogens, presence of *Salmonella* is believed to be a good determinant for the reduction of other pathogenic microorganisms (U.S. EPA, 2003).

The alternative indicator bacteria which have been progressively used instead of conventional indicators are the anaerobic bacteria such as *Clostridium perfingens* and *Bifidobacteria*, F-specific RNA phages and phages that infect *Bacteroides fragilis.* Those organisms have been proposed as potential indicators of water quality since they possess greater resistance to inactivation compared to Fecal

Coliforms during thermophilic anaerobic digestion and composting (Bagge et al., 2005; Gantzer et al., 2001; Pourcher et al., 2005; Wery et al., 2006).

2.4. Evaluation of Regulations Concerning Sewage Sludge Pathogens in Terms of Reactivation and Regrowth Phenomena

Landspreading or use of sludges in agriculture are the most promising sludge disposal options for utilizing useful characteristics of the material (nutrients, soil-building properties). However, since wastewater sludge also possess undesirable characteristics, government agencies have issued regulations on the land application of biosolids considering the risks from the pathogens and pollutants (Iranpour et al., 2004).

In United States the use and disposal of biosolids are regulated under 40 CFR Part 503. The regulation involves subparts containing general requirements, pollutant limits, management practices and operational standards along with the frequency of monitoring, recordkeeping and reporting requirements for sewage sludge in terms of land application or disposal (Iranpour et al., 2004, U.S. EPA, 1999). In the context of Part 503 Biosolids rule, the land application of biosolids is restricted related to three main considerations; pollutant concentrations, pathogen densities and vector attraction potential of the biosolids (U.S. EPA, 1999).

Based on the indicator microorganism content, sewage sludge is divided into two classes as Class A and Class B in Subpart D of the 40 CFR Part 503 which comprise Pathogens and Vector Attraction Reduction (U.S. EPA, 1994). Class A requirements is enforced for all sewage sludges that are sold or given away for application to the land, or applied to lawns or house gardens (U.S. EPA 1999). The main purpose of Class A requirements is to reduce the level of pathogens below detectable levels at the time the sewage sludge is used or disposed. It requires densities less than 1,000 MPN per gram total solids for Fecal Coliforms and less than 3 MPN per 4 grams of

total solids (dry weight basis) for *Salmonella spp*. Additionally, one of several treatment alternatives must be implemented which are designated as processes to further reduce pathogens (PFRP). Class B requirements on the other hand which is much less strict compared to Class A, enforces that pathogens to be reduced to levels <2 x 10^6 MPN or CFU Fecal Coliforms per gram total dry solids. For Class B biosolids, no analysis is required at the time of use as long as treatment known as processes to significantly reduce pathogens (PSRP) is applied. Since higher densitieas of microorganisms are allowed, several site restriction requirements should be met for land applied sewage sludge that meets Class B criteria. There is no obligation for monitoring of possible regrowth or activation of pathogens or indicator organisms particularly for Class B biosolids. Since time and temperature and the indicator organism options are both crictical criteria for pathogen reduction, it is assumed that biosolids produced with the approved processes meet the indicator organism which is proven as indecisive by several researches that report regrowth of indicator bacteria after dewatering. Even though the regulation requires the Fecal Coliform densities to be met 'in the sewage sludge that is used or disposed' particularly for Class A biosolids, this density is commonly determined after the stabilization process since dewatering process is assumed to decrease the densities of bacteria (Dentel et al., 2008). In the case of Class B biosolids, all these levels are expected to be met right after the stabilization processes. Another sampling is not required after dewatering or prioır to land application in US regulations.

In Europe, land application of wastewater sludge is regulated by the directive 86/278/EEC. The EU 1986 Directive does not specify limits for pathogen densities, but requires treatment of biosolids prior to land application in order to reduce pathogen densities unless the biosolids are injected or incorporated into the soil. However, all member states have a chance to adapt more stringent standard values according to the 86/278/EEC directive. Therefore, some of the member states such as France, Italy and Luxemburg implement pathogen restrictions by their own legislations. The proposed regulations in the 2000 working document developed

by the EU are more specific towards pathogen reductions, treatment processes and site restrictions in land application which is comparable to regulation implement in USA. However, this proposed regulation do not cover any issues regarding pathogen regrowth as well (Iranpour, 2004).

A new regulation concerning the use of domestic and municipal sewage sludge came into effect on August 3, 2010 by the Ministry of Environment and Forests in Turkey. By the implementation of this regulation, the Soil Pollution Control Regulation (SPCR) which was formerly regulated the use of stabilized sludge in soil is abolished. The regulation limits the values for several parameters including heavy metals, organic chemicals and dioxins for the land application of sewage sludge. In addition, this new regulation obligates 2log10 removal of *Escherichia coli* in the sludge by the stabilization processes applied. However, any issue related with the pathogenic regrowth is not covered in the scope of this regulation.

One critical point about the regulation applied in Turkey as well as other countries is that the indicator bacteria content of sewage sludge is expected to be determined not immediately before the application of sewage sludge to soil, but right after the stabilization processes. However, regarding the reports disseminated about the pathogen regrowth after centrifuge dewatering and further storage all over the world, it is clear that sampling point for pathogens of current regulations is inappropriate. Therefore, it is of great importance to examine and modify these regulations if necessary by considering pathogen regrowth. In addition, mechanisms behind the pathogen regrowth phenomena should be investigated and fully understood in order to take necessary precautions.

2.5. Emerging Molecular Techniques for the Assesment of Indicator Bacteria: Traditional Methods vs. Recent Molecular Tools

Until recently, detection and enumeration approches for the organism of interest was only based on laboratory cultivation of indicator microorganism from environmental samples (Prosser, 2002). However, it is a well known and proven fact

that conventional cultivation methodologies have significant limitations (Rousselon et al., 2004). First of all, assessment of the number of indicator bacteria lead indirect estimation of pathogen concentrations in sample material (Ulrich et al., 2005). In addition, microorganism specific infectious doses are not taken into account during the conventional enumeration techniques. Moreover, elongated incubation time along with the difficult and labour-intensive methodologies are some of the other major drawbacks of culture dependent techniques (Lemarchand et al., 2005).

One of the main debate regarding the standart culture methods is the ability of microorganisms to appear in a VBNC state (Alexandrino et al., 2004). Several reasons such as deficiency of nutrients (Cook and Bolster, 2007), altered temperatures (Besnard et al., 2002), oxidative and osmotic stresses (Asakura et al., 2008), and inhibitory substances might cause bacteria to enter the VBNC state (Grey and Steck, 2001;). However, a critical point about the VBNC concept is that, even though the cells enter the VBNC state, they are still considered viable and carry their infectious properties in vivo (Cappelier et al., 2007). In addition, those bacteria do not persist in the VBNC state indefinitely. Provided that growth promoters and enrichments are available, cells typically continue to the growth on media which is called as ''resuscitation'' or ''reactivation'' (Lleo` et al., 2001; Reissbrodt et al., 2002).

A significant portion of human bacterial pathogens involve viable bacteria such as the injured forms and the VBNC forms that are not cultivable in standard culture media (Oliver, 2010). E. coli, Salmonella sp., Enterococcus faecalis, Shigella dysenteriae, Vibrio cholerae, and Helicobacter pylori are some of the bacteria which have been proven to enter a VBNC state (Reissbrodt et al., 2002; Adams et al., 2005; Mizunoe et al., 1999; Gupte et al., 2003). Even though the determined cultivability of gastrointestinal bacteria is relatively higher than most of the microbial ecosystems, the culturable fraction is still known to be narrow (Ranjard et al., 2000).

The VBNC state may be a survival strategy of bacteria to persist in several conditions since they are known to reactivate under desired conditions. Owing to the potential danger for human health rising from VBNC bacteria, methods have to be designed for the routine analysis of nonculturable bacteria as in viable counts of bacteria (Lleo et al., 2005).

Owing to these negative features of culture dependent techniques, rapid and accurate alternatives were investigated for the assesment of pathogens in water and wastewater which do not rely on cultivability (Straub and Chandler, 2003). Following the recent advances in the field of molecular biology such as extraction of nucleic acids, development of polymerase chain reaction (PCR) amplification, along with the cloning and sequencing of DNA, isolation and culture of bacteria is no longer seemed as the only available technology. Thus, flexibility of choosing the proper enumeration technique also helped to reduce the bias against the obligatory applications of culture methods (Rousselon et al., 2004).

The molecular microbial ecology can be defined as the application of molecular technologies to environmental processes. It is an invaluable tool for the identification or enumeration of microorganisms in a particular environment. Moreover, functional role of a specific group of organisms is also assesed by means of comparative nucleic acid sequence information.The basic mechanisms behind molecular microbial investigations rely on three major steps: direct lysis of bacterial cells, extraction of genetic material from the matrix and the analysis of targeted sequences or whole genetic information (Ranjard et al., 2000). Although several molecular methods have been found widely applicable in the field of environmental microbiology; fundamental approches basically consist of PCR and modifications, denaturing and temperature gradient gel electrophoresis (DGGE and TGGE), single-strand conformation polymorphism (SSCP), 16S rRNA gene clone libraries and FISH. A summary of current techniques used is given in Table 2.2.

Table 2.2. Summary of current techniques used in microbial ecosystems (Zoetendal et al., 2004)

Methods	Uses	Limitations
Cultivation	Isolation; "the ideal"	Not representative; slow & laborious
16S rDNA sequencing	Phylogenetic Identification	Laborious; subject to PCR biases
DGGE/TGGE/TTGE	Monitoring of community/population shifts; rapid comparative analysis	Subject to PCR biases; Semi-quantitative; identification requires clone library
T-RFLP	Monitoring of community shifts; rapid comparative analysis; very sensitive; potential for high throughput	Subject to PCR biases; semi-quantitative; identification requires clone library
SSCP	Monitoring of community/population shifts; rapid comparative analysis	Subject to PCR biases; semi-quantitative; identification requires clone library
FISH	Detection; enumeration; comparative analysis possible with automation	Requires sequence information; laborious at species level
Dot-blot hybridization	Detection; estimates relative abundance	Requires sequence information; laborious at species level
Quantitative PCR	Detection; estimates relative abundance	Laborious
Diversity microarrays	Detection; estimates relative abundance	In early stages of development; expensive
Non-16S rRNA profiling	Monitoring of community shifts; rapid comparative analysis	Identification requires additional 16S rRNA-based approaches

Among those molecular techniques; PCR has proved to be essential in terms of detecting low amounts of specific DNA from an excessive number of procaryotic and eucaryotic cells and organic material present in environmental samples (Brauns et al. 1991; Leser et al. 1995; Lleo et al. 1999). Moreover modification of PCR methodology allows DNA quantification through competitive PCR (cPCR) and quantitative PCR (qPCR) along with the evaluation of cell viability by mRNA detection in Reverse Transcriptase PCR (Lleo et al., 2005). Analysis of 16S rRNA genes also have extensive usage for the analysis of bacterial populations; such as determination of genetic diversity in a specific area (Giovannoni et al.,1990) or revealing uncultured microorganisms in a natural community (Ward et al., 1990). Ribosomal rRNA genes as possessing conserved regions among all bacteria or fungi, enable distinction between different groups which provide the basis for the establishment of phylogenetic relations between different groups. One of the other rapid analysis is achieved by means of fingerprinting techniques. A variety of microbial systems have been analyzed using Denaturating Gradient Gel Electrophoresis (DGGE) or similar techniques, such as Temperature Gradient Gel Electrophoresis (TGGE) (Muyzer et al., 1998). The other microbial community fingerprinting techniques consist of Single Strand Conformation Polymorphism (SSCP) and Terminal-Restriction Fragment Length Polymorphism (TRFLP) analyses. All of the fingerprinting technologies rely on PCR methodologies and even though the mechanism and technical procedures vary, the basic principle of

the all techniques is to generate profiles representing the sequence diversity within the selected ecosystem (Zoetendal et al., 2004). Sequence information of target organism can also be used to design and construct fluorescently labelled oligonucleotide probes specific for particular microbial groups. These probes can then be used to detect cells in situ by using fluorescent in situ hybridization (FISH) technique (Amann et al., 1995).

Following the more frequent application of molecular tools in microbial ecology, Quantitative PCR has became one of the most widely used molecular technique for determination of the gene or transcript numbers in environmental samples. The technique has been used previously to study pathogenic bacteria in manure (Lebuhn et al., 2005) or soil (Marsh et al., 1998) and has recently been applied to detect bacterial pathogens during municipal wastewater treatment (Lee et al., 2006; Wery et al., 2008).

Quantitative PCR method is based on the combination of the detection of the target template with the resulting product during the repeated cycles in PCR. This is provided by corresponding fluoroscent signal with the amplified PCR product. Quantitative PCR follows the same steps as end-point PCR essentially. Denaturation of the template DNA is initialized, annealing of selected primers for the target sequences takes place, the extension of a complemantary strand across the annealed primers with DNA polymerase is followed and resulted with an exponential increase of the target sequence during the cycles of PCR. Contrary to end-point PCR, the increase in the number of amplicons is dispatched simultaneously by a fluoroscent reporter through each cycle. Two widely known reporter systems are the intercalating SYBR green assay (Wittwer et al., 1997) and the TaqMan probe system (Holland et al., 1991; Livak et al., 1995). Schematic represantation of the two reporter system is given in Figure 2.2.

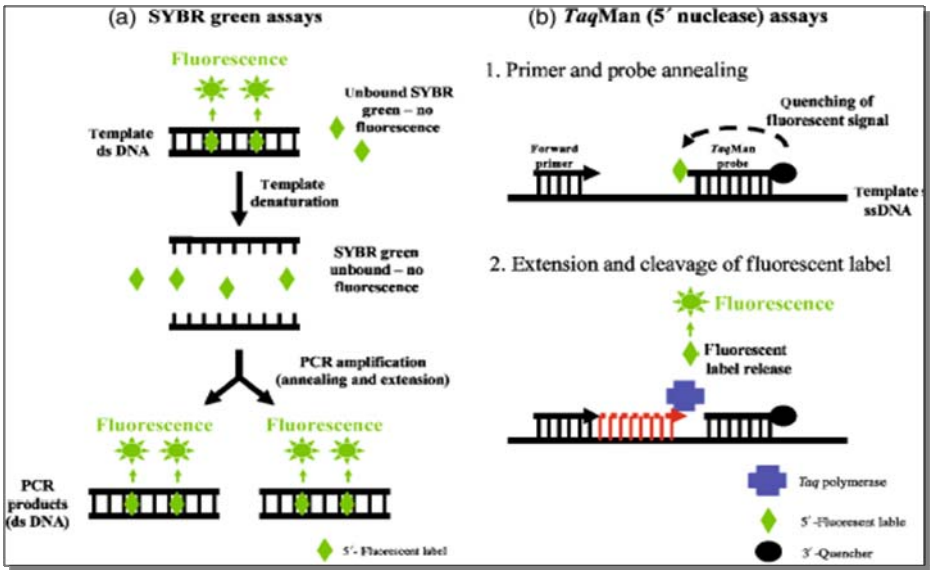

Figure 2.2. Quantitative PCR Chemistries (Smith and Osborn, 2008)

Regardless of the fluoroscence applied, quantification of the target DNA is carried out in the same pattern. In the SYBR Green based assay, SYBR Green which is a double-stranded DNA intercalating dye, fluoresces once bound to the DNA. The amount of dye applied is proportional to the amount of generated target. The dye emits at 520 nm and fluorescence emitted can be detected and related to the amount of target. The main drawback of this technique is that the SYBR Green I binds to any amplified dsDNA. Therefore, primer dimers or unspecific products may cause inaccurate quantification. However, this drawback of SYBR Green system is overcomed by the validation of the specificity of the system through running a melt curve at the final stage of each PCR run.

The TaqMan probe method utilizes a fluorescently labelled probe which contains a quencher molecule at the 3'end and a fluorophore at the 5'end (Heid et al.,1996). During the annealing step of the repeated cycles of PCR, primers and the intact probe bind to the target sequences. During the subsequent template extension, the exonuclease activity of the Taq polymerase enzyme cleaves the fluorophore from the TaqMan probe and a fluorescent signal is detected. Amplification of the template is thus measured by the release and accumulation of the fluorophore

during the extension stage of each PCR cycle. One significant advantage of TagMan probe is the ensured specifity of the generated fluorescence.

Concentrations of the analyzed material and/or a gene are determined by either Relative or Absolute Quantification. The net change in the target sequence is compared to a co-amplified gene in Relative Quantification assay. In Absolute Quantification procedure, the amount of the target transcript is calculated with the help of a standard curve obtained from an already known concentrations of the gene amplified. This standard can be a genomic or plasmid DNA which varies regarding to the analyzed material.

2.6. Motivation of the Study: Reactivation Potential of Indicator Bacteria in Anaerobically Digested Sludge after Dewatering Process

As mentioned before, the classification of biosolids for disposal purposes are based on Fecal Coliform or E.coli densities determined with relevant regulations in many countries. A common aspect for the stabilization alternatives involved in these regulations regarding the use of sewage sludge is the elimination of pathogenic microorganisms with different chemical or thermal mechanisms which assure the hygienic quality of sludge. Since it has long been known to succesfully reduce the number of pathogens and indicator organisms, anaerobic digestion process is one of the most common alternatives to meet pathogen requirements for particular classes of sludge. Therefore, anaerobic digestion process is widely adopted by many wastewater treatment facilities and sludges obtained after digestion has been used for a number of beneficial purposes including agricultural applications all over the world.

Although it is stated in the relevant regulations that Fecal Coliform or E.coli densities must accomplish the given criteria for sewage sludge that is used or disposed, the indicator bacteria content of sewage sludge is expected typically to be determined not immediately before the application of sewage sludge to soil but right after the stabilization processes. However, it has recently been reported in the literature that significantly higher densities of Fecal Coliforms and/or Escherichia

coli have been measured in dewatered cake samples compared to samples collected after anaerobic digestion. In other words, the reduced densities of Fecal Coliform and/or E. coli resulting from anaerobic digestion seemed to increase particularly after centrifuge dewatering (Iranpour et al., 2003; Cheung et al., 2003; Qi et al., 2004; Hendrickson et al., 2004). Moreover, densities of indicator bacteria have been observed to further increase in case of storage of the cake samples (Erdal et al., 2003; Qi et al., 2007; Higgins et al., 2007).

In 2001, when the largest known two phased anaerobic digester was taken into operation, the plant was assessed whether to perform all of the time and temperature requirements of the U.S. EPA's regulations for the production of particular classes of biosolids. The Fecal Coliform analysis of the anaerobic digester effluent was met the expactations and indicated any detectable level of Fecal Coliforms. However, subsequent analysis of centrifugally dewatered biosolids revealed high levels of Fecal Coliforms. This occurence was tested for several times through the analysis of numerous samples and one of the first reports related with the incerases in Fecal Coliform densities following centrifuge dewatering was published (Hendrickson et al., 2001).

Iranpour et al., (2002 and 2003) also reported that typical concentrations of Fecal Coliforms met the criteria for Class A biosolids after a thermophilic digestion process applied. Yet after dewatering, significant increaeses were assessed in the Fecal Coliform densities. Iranpour et al., (2002) also reported that centrifugation of digested solids in a lab scale centrifuge did not result in the high densities of Fecal Coliforms, suggesting that g-force of the centrifugal dewatering is not the only cause of increases in the number of Fecal Coliforms.

In their study Erdal et al., (2003) compared centrifuge and belt filter press dewatering processes and they reported similar increases in the mesophically digested biosolids only after centrifuge dewatering. Additionally, moderate increases were assessed following the storage of biosolids. Similarly, in their investigation concerning a mesophilic anaerobic digester, Cheung et al., (2003)

found that there were approximately 2,17 log units of increases in the *E.coli* densities after dewatering process.

In another investigation conducted by Monteleona et al., (2004), five treatment plants in U.K. which operate mesophilic anaerobic digesters were examined. In four of the plants dewatering process was accomplished by centrifuge dewatering whereas the other one operates belt filter press dewatering. In the context of the study, samples obtained from digester effluent and dewatered sludge were examined for their *E.coli* content. In the same manner with the other reports, increased densities of *E.coli* were reported in all of the centrifugally dewatered samples. The increases were recorded as 63%, 74%, 394% and 3452% for each of the treatment plants. On the other hand, in the treatment plant operating belt filter press dewatering, 44% of decrease in the number of *E.coli* was observed.

In fact, sludge dewatering process is not expected to have such a significant impact on indicator bacteria concentrations of stabilized sludge. However, it is clearly mentioned in the relevant literature that, anaerobic digestion followed by centrifuge dewatering process leads to the increases in the number of indicator bacteria regardless of the differences in the anaerobic digestion processes and centrifuges utilized. Two terms were used to explain observed types of increases in the indicator bacteria content of anaerobically digested sludges in the course of dewatering or storage. Increases assessed immediately after centrifugation was named as *reactivation* whereas *regrowth* was referred to the increases occur during storage (Higgins et al., 2007; Qi et al., 2008).

The term of regrowth confers on increase in the indicator bacteria content during the storage of biosolids which has been stabilized and centrifugally dewatered. In this case, indicator bacteria levels of the biosolids increase in a manner which is equal to doubling time of *E.coli* which is approximately 20 minutes or shorter. Reactivation on the other hand, refers to the multiplication of indicator bacteria more rapidly than their normal duplication period which is also termed as Sudden

Increase (SI). These two terms can be simply distinguished by means of the representation available in Figure 2.4

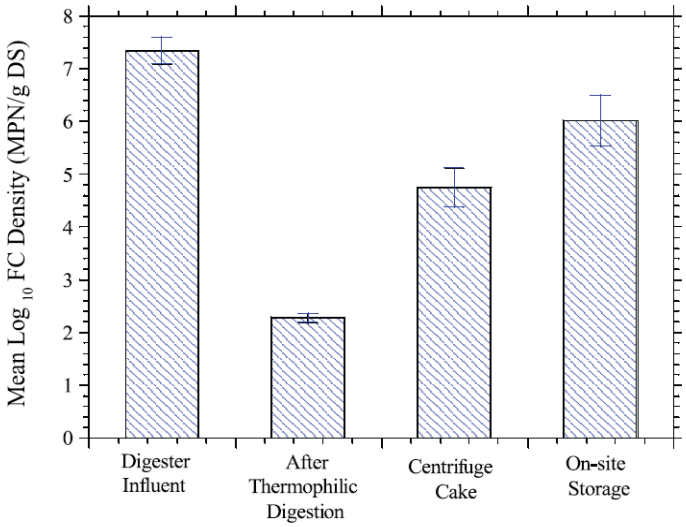

Figure 2.3. Sudden increase in FC density after thermophilic digestion (reactivation), followed by additional increases during long-term storage (regrowth) (Higgins et al., 2007)

Fecal Coliform concentrations of anaerobic digester influent, effluent and dewatered sludge samples taken from a thermophilic digester are given in the Figure 2.3. It is obvious that Fecal Coliforms are removed substantially during thermophilic anaerobic digestion process. However, after centrifuge dewatering, there is a significant increase in the number of Fecal Coliforms. In addition, a further increase was determined following the storage of the samples.

2.6.1. Hypothesis Emerged Regarding Reactivation and/or Regrowth of Indicator Bacteria

It is vital to determine possible reasons for the reported increases in order to assess the risk associated with this phenomenon (Dentel et al., 2008). Several predictions

have been postulated to explain this occurrence. Theories which have been emerged by several researches are explained briefly in the following sections.

False Positive Results Associated with Enumeration Techniques

In their study Qi et al., (2007) assessed increases in the Fecal Coliform content of samples taken from five treatment plants which operate mesophilic and thermophilic anaerobic digesters. In the context of the study, they hypothesized different mechanisms to explain possible reasons for measured increases. One of the hypotheses was based on the false positive results associated with the enumeration technique. Since growth of *Bacillus spp*. was formerly cited by Baker et al., (2004) as the cause of the false positive results in enumeration of Fecal Coliforms by A-1 method (Standard Methods 9221E.2), digested biosolids spiked with *Bacillus spp*., unspiked biosolids, and pure *Bacillus spp.* culture were investigated for their Fecal Coliform content with both of the enumeration methods. However, findings of the study indicated an agreement between spiked and unspiked biosolids which reveals that *Bacillus spp*. does not constitute an effect on the enumeration of Fecal Coliform bacteria.

Contamination from the centrifuge

One possible mechanism to explain the sudden increase is believed to be due to a layer of colonized bacteria developed in a centrifuge which lead to the contamination of digested solids during dewatering. In order to test this hypothesis Hendrickson et al., (2004) evaluated a centrifugation process with a cake sample which has been reported to have a significant increase in the content of indicator bacteria after the dewatering operation. The centrifuge was disinfected by using sodium hypochlorite solution and right after sterilization, digester effluent samples were taken to centrifuge dewatering process. However, immediate analysis of dewatered cake samples indicated similar increases in the Fecal Coliform content of the sludge.

Chen et al., (2007) performed a similar test with a lab. scale centrifuge which is known to generate cake samples similar to full scale centrifuges. Then, samples were taken from a plant that utilizes pre-pasteurization followed by mesophilic anaerobic digestion which has been reported a sudden increase in FC and *E. coli* after centrifuge dewatering. All of the equipment were sterilized before the processing of samples by centrifuge. The cake samples were collected after processing and analyzed for their Fecal Coliform and *E.coli* content. Determined *E. coli* densities were similar to field data which display approximately four orders of magnitude increases within 24 hours. Obtained results signify that the increase can occur without centrifuge contamination.

Effect of floc break up on improved culturability

Since large numbers of bacteria are embedded in the floc structure, prior to enumeration of target bacteria, floc needs to be broken up to prevent the enumeration of multiple bacteria as one bacteria.The theory behind the regrowth point of view is related with the shear applied to the sample during dewatering process which leads to a better dissemination and increased quantity of indicator bacteria.

In this context, several researchers have suspected whether dispersing of flocs during dewatering affect the enumeration procedure (Cheung et al., 2003, Monteleone et al., 2004, Qi et al., 2007). In their study, Qi et al. (2007) experimented the use of a kitchen blender, a sonic dispersion unit and a helical extrusion device to shear sludge but none of the homogenization methods output a statistically significant increase in the number of Fecal Coliforms. Likewise, Cheung et al. (2003) evaluated the effect of using a stomacher or an ultrasonic homogenizer in order to enhance the floc dispersion in the disgested sludges. Moreover, addition of a surfactant to enhance the floc dispersion is experienced. However, none of these treatments improved the detected quantities of indicator bacteria in the liquid sludge samples. Researchers concluded that, floc dispersal is

not the basic mechanism for the increased quantities of Fecal Coliforms after centrifuge dewatering.

Removal of growth inhibitors during dewatering

Owing to the complex biochemical pathways involved in anaerobic digestion process, many inhibitors are produced during the anaerobic reactions such as long chain fatty acids, ammonia, sulfide which might also be found in the incoming wastewater (Hwu et al., 1996; Visser et al., 1993). Since the role of those inhibitory substances on the survival of bacteria has not been studied so far, it is hypothesized that those substances might be released into the centrate during dewatering allowing Fecal Coliforms and other bacteria grow in the cake (Gardner et al., 2010). In their study, Higgins et al., (2007) designed an experiment to reveal the effect of centrate additon to the indicator bacteria concentrations in anaerobically digested sludge. Their results surprisingly led to an increase in the number of indicator bacteria (Higgins et al., 2007) which is not expected if inhibitors were preventing growth. However, repeated experiments did not indicate the same reactivation and it is concluded in the study that additional research is necessary to understand the factors affecting reactivation and regrowth. Besides, in their study which examines several factors including inhibitory substances on reactivation of indicator bacteria, Gardner et al., (2010) concluded that sulfid generated during anaerobic digestion proces, had a toxic effect on the growth of Fecal Coliforms. However, since many other factors were evaluated in the scope of this study, it is not clear that if the only reason for the reactivation of indicator bacteria is the removal of growth inhibitors or not (Gardner at al., 2010).

Effect of Total Solid content

In a study conducted by Qi et al., (2008) quantitiy of Fecal Coliforms assesed in centifugally dewatered anaerobic sludge is suggested to be related with the Total Solid content of the biosolids. Findings of the relevant investigation revealed that, increases observed after dewatering process is proportional to the Total Solid content of the final sludge. One of the conclusions obtained in the study indicated

that the concentration of solids or liquid to solid ratio governs regrowth. Nevertheless, it is also stated that the effect of shear, polymer and oxygen is still needed to be differentiated in order to validate the role of Total Solids on regrowth of Fecal Coliforms.

Effect of day-light throughout the storage of biosolids

Gozen et al., (2010) evaluated the effect of daylight on regrowth of total coliforms, *Salmonella* and *Clostridium perfringens* in centrifugally dewatered anerobic sludge. In the scope of the study, half of the sludge cake and centrate samples were taken from a treatment plant stored in daylight whereas the other half was kept at dark for three weeks. Although, it is found that, presence of daylight increased the regrowth of *Salmonella spp.* both in sludge cake and centrate, nonetheless it concluded that, more research is required in order to understand the regrowth mechanism related with the daylight.

Effect of Viable but Not Culturable Bacteria

Another possible theory which is supported by many of the researchers is related with the the ability of indicator bacteria persisting in VBNC state during anaerobic digestion process. As mentioned before, bacteria have long been known to enter the VBNC state owing to the environmental stress such as deficiency of nutrients low temperatures or some other factors including metals, chlorine and high salinity (Mizunoe et al., 1999; Grey and Steck, 2001). Therefore, it is suggested in the relevant literature that, anaerobic digestion process may be the reason for indicator bacteria to enter a VBNC state owing to stressful conditions applied during the process. Bacteria in VBNC state can not be cultured by Standart Culture Methods even though they are regarded as viable. Consequently, these bacteria can not be enumerated by means of conventional Standart Culture Methods. On the other hand, during the dewatering process, the bacteria are reactivated or regrow owing to the ceasing of stress related conditions. It is also believed that centrifugation has a resuscitation effect for the indicator bacteria allowing them to grow and be enumerated using SCMs. In the relevant literature, the term of

reactivation is more commonly used instead of resuscitation (Qi et al. 2004; Higgins et al. 2007; WERF 2007).

Along with the common use of molecular tools for environmental samples which provide an approach to enumerate bacteria without relying on standard culturing techniques, research all over the world intensified in order to investigate those phenomena of VBNC state for the purpose of elucidating the observed increases. In order to validate existence of VBNC bacteria, Higgins et al., (2007) compared the *E.coli* densities of a sludge sample determined by standard culture method to a Real Time polymerase chain reaction (RT-PCR) method. Figure 2.4. represents summary of the findings obtained in this study during the investigation of a themophilic anaerobic digetser operating plant.

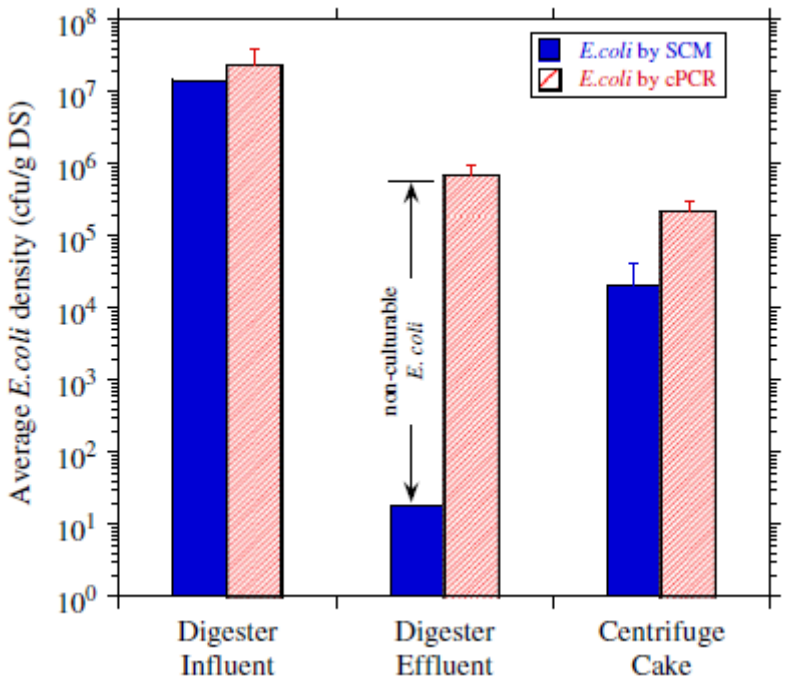

Figure 2.4. *E.coli* densities obtained by SCM and competitive PCR in digestion and dewatering processes (Higgins et al., 2007)

The evidence for the VBNC state of bacteria was because the acquired concentrations of *E.coli* are almost the same in the digester influent however a

significantly higher number of *E. coli* was measured by Real Time PCR in the digester effluent. The differences of two enumeration method is believed to indicate the VBNC portion of bacteria found in the anaerobic digester effluent. Since the detected levels of *E.coli* were similar again in the dewatered cake samples, increased cultivability was verified (Higgins et al., 2007).

Similarly, Wery et al., (2008) quantified selected indicator bacteria including *E.coli* and *Salmonella spp.* at different stages of a municipal wastewater treatment plant by both Real Time PCR and standart culture methods to compare their survival throughout the process. Particularly for *Salmonella spp.* and *E.coli*, the obtained values were higher when compared to results of standart culture methods in each phase of the wastewater treatment process. However, a crucial point with the study is that; the difference between culture methods and Real Time PCR of *E.coli* were closer after centrifuge dewatering than determined after anaerobic digestion. This conclusion is in accordence with non-culturable bacteria concept which is believed to be induced during anaerobic digestion. The reduced differences after centrifuge dewatering is proposed to be due to the reactivation of those non-culturable bacteria.

Following these findings, Water Environment Research Foundation (WERF) initiated a research at 2008. Throughout the study, a number of treatment plants were examined which operate either mesophilic or thermophilic anaerobic digestion process with centrifuge or belt filter presses used for dewatering. Samples were analysed for their indicator bacteria content by means of both Real Time PCR and conventional standart culture methods. For, all of the treatment plants evaluated which operate centrifuge dewatering, a sudden increase and/or regrowth except for one of the plants (which operates thermophilic reactors in series) were reported. Plants with belt filter press dewatering on the other hand, did not have significant increases after dewatering. It is also revealed that, increases in the number of indicator bacteria found in sewage sludges processed with thermophilic digesters are 4-5 logs whereas 0-1 log in mesophilic anaerobic digesters (WERF, 2008). A number of factors were investigated throughout the

study including almost all of the aforementioned hypotheses. Yet, none of them was found the be the main reason for the determined increases even though they have some complementary impacts. However, since *E.coli* levels obtained by Real Time PCR was higher than those measured by standart culture methods whereas after the digestion process both of the methods give similar results, principle investigators of the study concluded that sudden increase after dewatering is largely due to the recativation of non-culturable bacteria. Moreover, it is also reported that, in addition to Fecal Coliforms, pathogens such as *Salmonella* also possibly regrow in biosolids (Higgins et al., 2007).

2.6.2. Recent Findings Concerning Reactivation and Regrowth of Bacteria

Regarding the findings that indicate substantial increases after dewatering of the biosolids, the effectiveness of thermophilic digestion is questioned. Since digestion process occurs under elevated temperatures, in order to evaluate the bacterial response to heat over time a research was conducted by Boczek et al., (2010). Pure *E.coli* cultures containing sterile buffer and buffer amended with 1% nutrient broth were subjected to heat at 55°C for 4, 6, and 24 hours and their survival strategies were examined for ten days including day zero. It is reported that, none of the samples gave positive results immediately after heat stress. Yet, samples which have buffer with nutrient broth heated for 4 and 6 hours gave positive *E.coli* results after 24 hours. Recovery of *E.coli* inoculated buffer sample was observed after 72 hours. In addition, a similar setup was designed for biosolid sample from a full-scale treatment plant which operates pre-pasteurization followed by mesophilic digestion. No measurable densities of *E. coli* were assessed after digestion, but, following the storage of the samples, perceptable densities of *E. coli* were measured over a period of several days. It is concluded in the research that, owing to the elevated temperatures achieved in the thermophilic digesters, bacteria may not grow on standard media but recover from thermal stress over time by supporting the VBNC phenomena.

Similarly, Yankey et al., (2010) investigated the potential of formation of VBNC bacteria in their study. Experiments were conducted by pure cultures of *E.coli*

which were thermally treated *at* 55°C. Quantities of *E.coli* were observed over a prolonged time by using both standart culturing method (SCM) and Live/Dead test methodology which rely on a two-color DNA staining procedure that allows rapid total counts of live bacteria regarding to their impermeability to the staining agents. The results obtained by Live/Dead test showed that *E. coli* remained alive after prolonged thermal treatment at 55°C, although no culturable *E. coli* is observed after 16 hours of heat treatment and it is suggested as the reason for increases assessed in thermophilically digested sludges after dewatering .

Cooper et al., (2009) investigated different types of dewatering process in terms of their effect on *E. coli* concentrations. They conducted their study on conventionally treated sludge, anaerobically digested sludge (primary and secondary liquid digestion) and raw sludge from eight wastewater treatment plants operated in U.K. The dewatering methods examined was consisting of centrifuge conditioning, belt filter and filter-plate pressing. Their findings revealed an increase in the number of *E.coli,* up to 0.8 log10 *E.coli*/gTS for centrifugally dewatered sludge. More importantly, since dewatering of raw sludge by either centfiuge, filter belt or filter plate methods did not result in any increase in the number of *E.coli*, it is demonstrated in this study that the regrowth of the bacteria is not solely related with the dewatering process.

In one of the recent and detailed studies related with the bacterial regrowth after dewatering, the correlation between odor production and bacterial regrowth was tested. The hypothesis was established on the idea that both odor production and regrowth of pathogens are signs of microbial activity. For this reason, the mechanisms of biosolids odor production and pathogen regrowth were evaluated by additionally considering several factors such as shear, oxygen and substrate which might contribute to the microbial growth at the stabilized sludge. For this reason samples taken from five municipal wastewater treatment plants of which anerobic digesters are operated in various time and temperature combinations were examined. Results of the related experiments revealed that, high shear applied during centrifuge dewatering lead to release of substrates which are the

food source for Fecal Coliforms. Similarly, proteins released can be degraded and eventually form odor. It is also believed that, increased shearing provide oxygen to the system which negatively effect methanogenic activity and consequently lead to accumulation of odor precursors. In addition, it is also possible that provided oxygen might enhance the growth of Fecal Coliforms. Obtained results suggest that, both odor and indicator bacteria decrease after the storage of samples since both oxygen and substrate are consumed over time (Chen et al., 2011).

CHAPTER 3

MATERIALS AND METHODS

3.1. Determination of the Wastewater Treatment Plants Suitable for the Scope of the Study

At the early stages of the study, various wastewater treatment plants which have different operating configurations from all over Turkey were examined. Six wastewater treatment plants were determined to be evaluated during the study. Those plants were chosen among treatment plants which utilize mesophilic anaerobic digester as the stabilization process. In order to designate the effect of different dewatering processes, 3 treatment plants using centrifuge and 3 treatment plants using belt filter press as dewatering processes were selected.

3.2. Wastewater Treatment Plants Investigated within the Scope of the Study

Throughout the study, Ankara, Adana and Kayseri wastewater treatment plants were monitored in order to examine the possible microbial increases during belt filter press dewatering. Konya, Eskişehir and Mersin wastewater treatment plants were chosen since they implement centrifugal dewatering to the anaerobically digested sludge; so their performance for the microbial quality was examined. Main process characteristics of the wastewater treatment plants evaluated in the scope of the study are described in the following sections. Additionally, some properties of interest concerning selected treatment plants are summarized in Table 3.1.

Table 3.1. Relevant Properties of Selected Treatment Plants

Wastewater Treatment Plants	Inflow Rate (m^3/d)	Digester Conditions	Dewatering Process	Number of Samplings Done
Ankara	765,000	T:35°C, SRT: 24 d	Belt Filter	5
Adana	174,000	T:35°C, SRT: 20 d	Belt Filter	2
Kayseri	140,000	T:37°C, SRT: 20 d	Belt Filter	1
Konya	245,000	T:35,5 °C, SRT: 11.2 d	Centrifuge	4
Mersin	130,000	T:35°C, SRT: 20 d	Centrifuge	2
Eskişehir	105,000	T:37°C, SRT: 20.5 d	Centrifuge	2

SRT:Solids Retention Time

3.2.1. Ankara Wastewater Treatment Plant

The wastewater treatment plant of Ankara treats daily 765,000 m^3 of municipal wastewater generated from a population of 4 million residents. Apart from domestic wastewater input, treatment plant receives industrial inputs from Organized Industrial Zones. The treatment process consists of pretreatment station, grit and scum removal, primary sedimentation and conventional activated sludge with sludge retention time of 3 days. Sludge generated from primary and secondary clarifier are thickened together and stabilized in a mesophilic anaerobic digester with the operation temperature of 35°C. Sludge retention time is approximately 24 days in anaerobic digesters. Anaerobic sludge is dewatered in belt filter presss and then landfilled. Total sludge production is about 200 ton/day. Flow chart of Ankara wastewater treatment plant is shown in Figure 3.2.

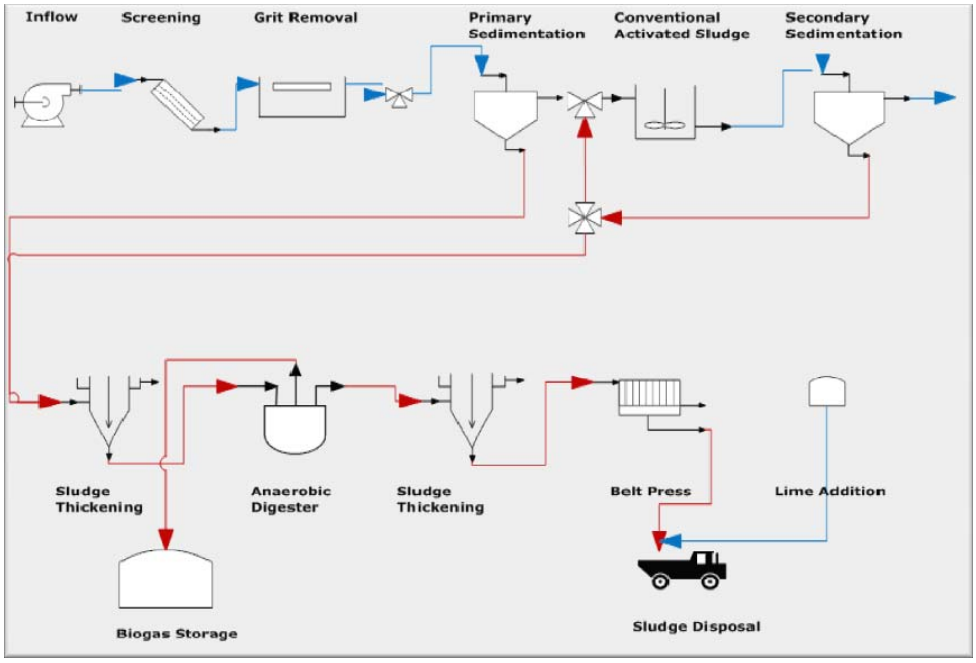

Figure 3.2. Flow Chart of Ankara Wastewater Treatment Plant

3.2.2. Adana-Seyhan Wastewater Treatment Plant

Adana Seyhan wastewater treatment plant serves 1,151,000 residents by treating 174,303 m^3 of wastewater per day. In addition to municipal wastewater, treatment plant receives industrial wastewater generated from food industry along with a minor input generated from textile industry. A conservative approach is employed in the treatment process by using screening, primary settlement, trickling filters and conventional activated sludge methods. Subsequent sludge stabilization is processed by anaerobic digesters operating between 36°C to 37°C with retention time of 30 days. Sludge generated after the digestion process is thickened and dewatered by belt filters. After the dewatering process, the resultant 90 ton/day sludge-cake is landfilled.

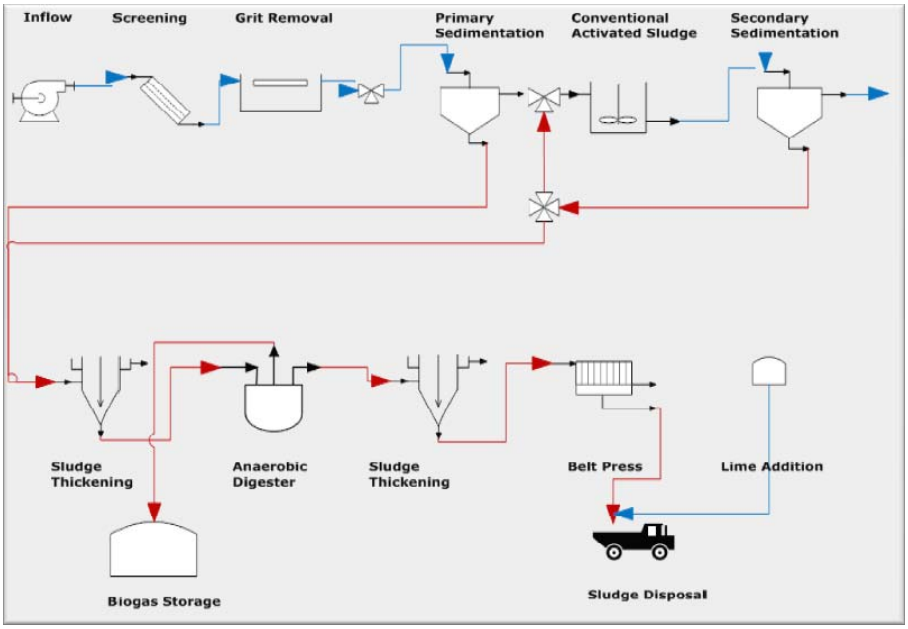

Figure 3.2. Flow Chart of Adana Wastewater Treatment Plant

3.2.3. Kayseri Wastewater Treatment Plant

The wastewater treatment plant of Kayseri treats 140,000 m^3/day of wastewater generated from approximately 912,000 residents. The treatment plant receives additional industrial input from food, metal, paper, textile chemical and wood industries. Wastewater treatment process consists of screening and grit and grease chambers, primary sedimentation, biological nutrient removal with anoxic anaerobic oxic process (A^2O), and secondary sedimentation. There are two different sludge-producing units in the stream which are primary sedimentation and secondary sedimentation. Sludge obtained from primary sedimentation is transported to pre-thickening unit. The solid content of primary sludge is increased by pre-thickening unit. Primary thickened sludge is digested in a mesophilic anaerobic digester operated at 37 °C with the SRT of 20 days and transported to the secondary thickening unit. A major difference in the flow scheme of Kayseri wastewater treatment plant from other plants investigated is the mixing of

biological sludge with secondary thickened sludge before the dewatering process. This way the secondary sludge is not digested and directly taken into dewatering operation. Total sludge production is about 64 ton/day. Flow chart of wastewater treatment plant of Kayseri is shown in Figure 3.3.

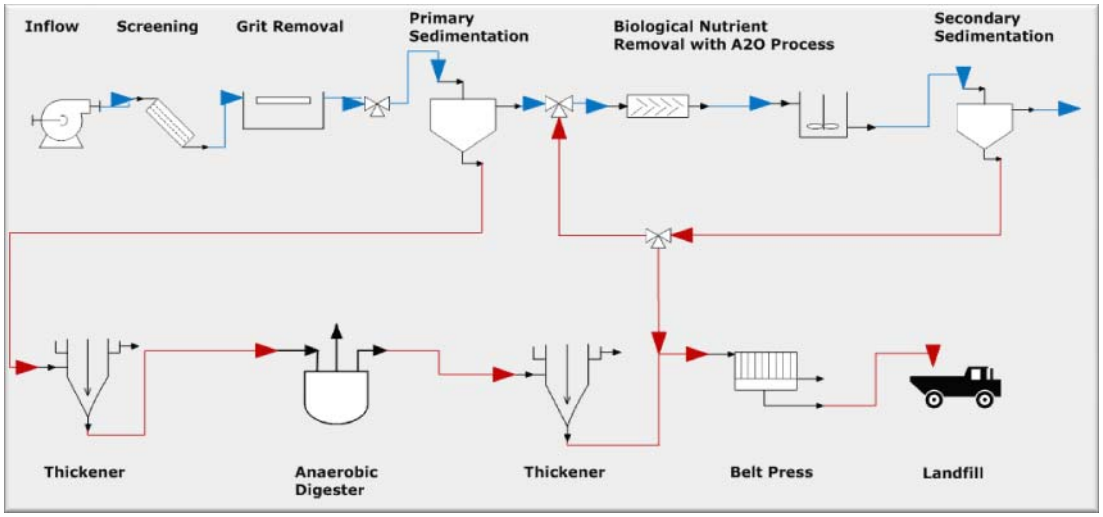

Figure 3.3. Flow Chart of Kayseri Wastewater Treatment Plant

3.2.4. Konya Wastewater Treatment Plant

The wastewater treatment plant of Konya treats 145,000 m^3 of municipal wastewater per day. The population served by the plant is approximately 1,000,000. Treatment plant receives industrial inputs generated from dairy and bakery industries. The treatment processes include pretreatment station, grit and scum removal, primary sedimentation and biological treatment utilizing Bardenpho Process in Biological Nutient Removal. Total sludge production is about 163 ton/day. Primary and secondary sludges are transferred to sludge thickening tank from which the thickened sludge is transferred to anaerobic sludge digestion with sludge retention time of 11.2 days for stabilization. Stabilized sludge is transferred to a second thickener and then is dewatered in belt filter press

dewatering unit. Flow Chart of Konya wastewater treatment plant is given below in Figure 3.4.

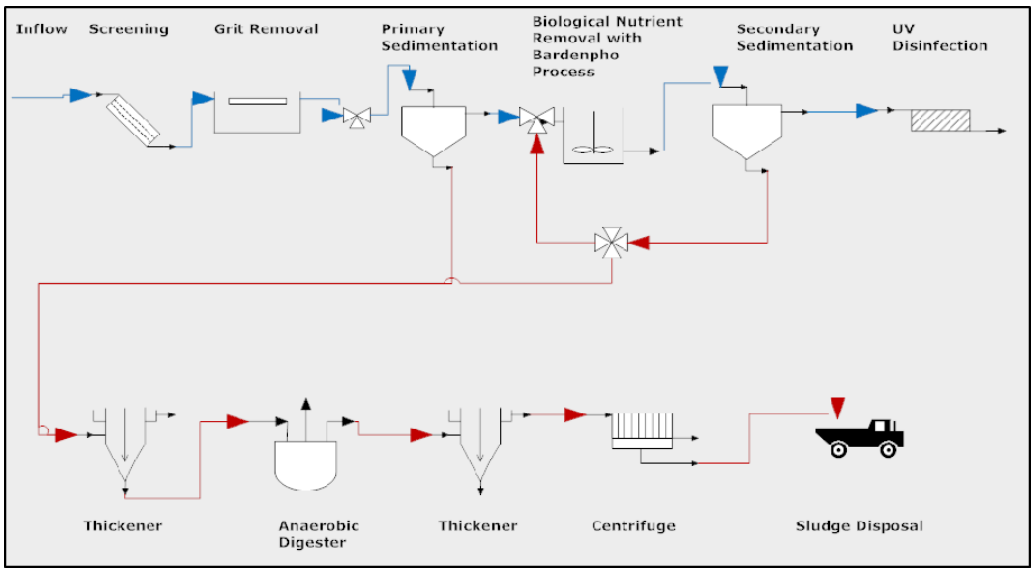

Figure 3.4. Flow Chart of Konya Wastewater Treatment Plant

3.2.5. Mersin Wastewater Treatment Plant

Mersin wastewater treatment plant serves to 1,050,000 residents by treating 130,000 m^3/day of municipal wastewater. The industrial input arises from cement, food and olive oil industries. Preliminary treatment process consists of screenings and grit and grease chambers. Primary sedimentation process is followed by Bardenpho Process with sludge retention time of 7-9 days. Resulting primary and secondary sludge is stabilized in mesophilic anaerobic digesters operated at 35°C with solids retention time of 20 days. Stabilized sludge is transferred to a second thickening tank, and then is sent to centrifuge for dewatering. Dewatered sludge is solar dried in a pilot plant. Total quantity of the generated sludge is about 70 ton/day. Flow Chart of Mersin wastewater treatment plant is given below in Figure 3.5.

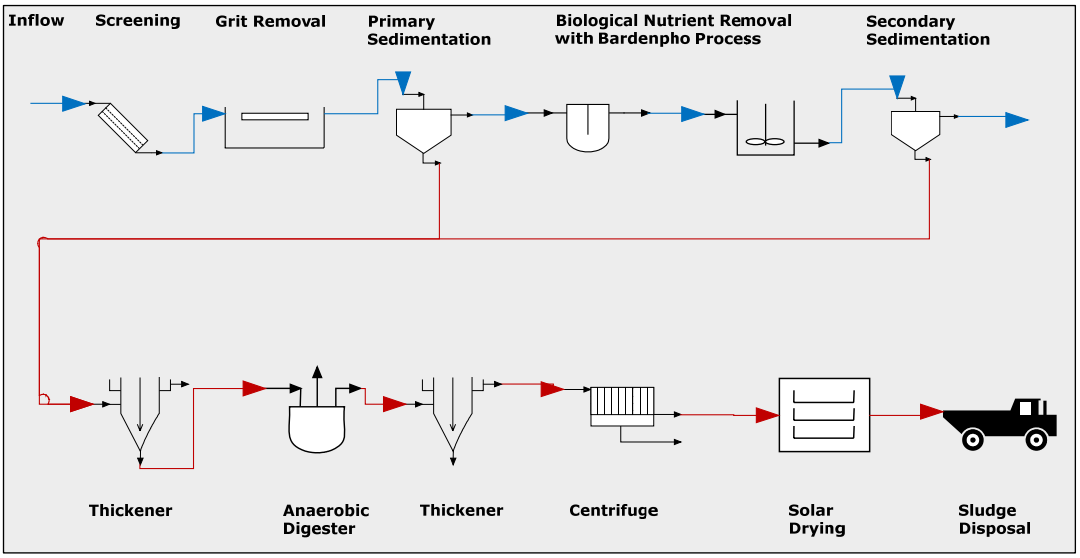

Figure 3.5. Flow Chart of Mersin Wastewater Treatment Plant

3.2.6. Eskişehir Wastewater Treatment Plant

The wastewater treatment plant of Eskişehir treats 105,000 m^3 of municipal wastewater per day. The population serrved is approximately 650,000. The treatment plant receives additional wastewater input from sugar mill industry, textile industry and aircraft industry. Treatment process applied includes pretreatment such as, grit and scum removal, primary sedimentation followed by biological treatment using University of CapeTown (UCT) processes with a sludge retention time of 9.2 days. Stabilization process consists of anaerobic digesters operated at 37 °C with a retention time of 20.5 days. Afterwards, the obtained sludge is directly routed to dewatering unit which applies centrifuge dewatering. Total sludge production is about 40 ton/day. Flow chart of Eskişehir wastewater treatment plant is given in Figure 3.6.

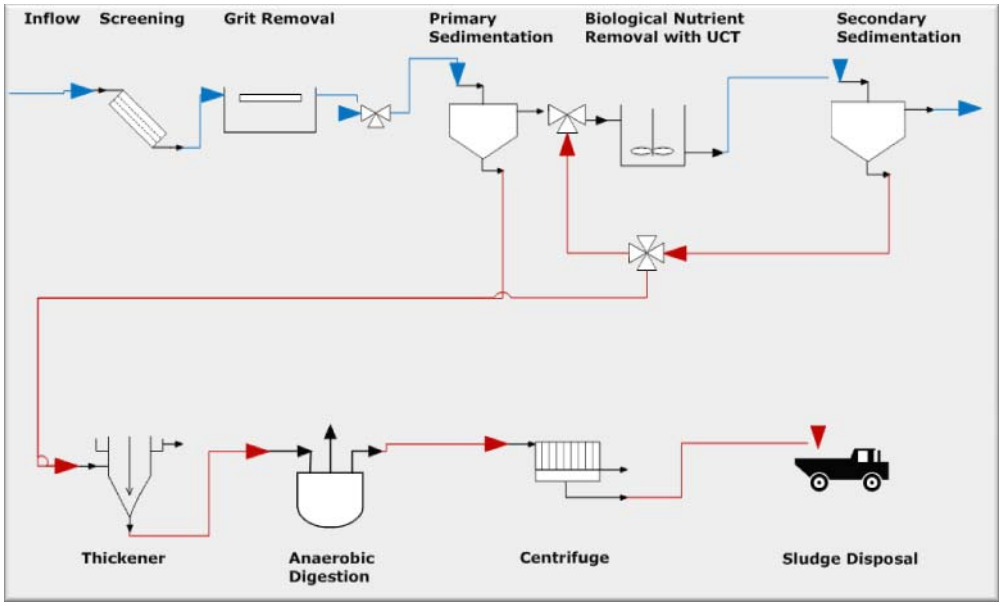

Figure 3.6. Flow Chart of Eskişehir Wastewater Treatment Plant

3.3. Collection and Pretreatment of the Samples

3.3.1. Collection of Samples from Selected Treatment Plants

Following the determination of the treatment plants, samples including anaerobic digester influent, anaerobic digester effluent and dewatered sludge were collected from the selected treatment plants.

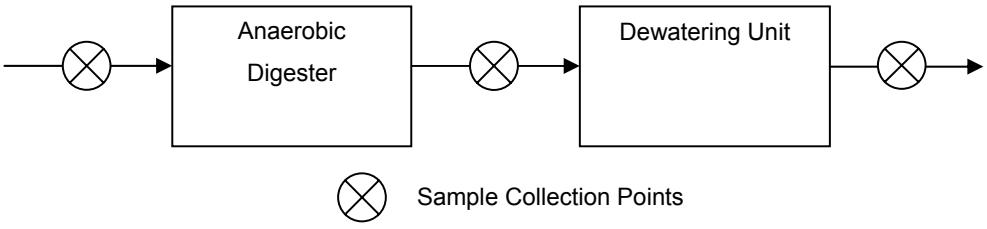

Figure 3.7. Sample Collection Points

Samples were taken in plastic containers and immediately transferred to the labarotory in a cooler. Standart culturing methods and DNA isolations were conducted within 24 hours of sample collection as recommended by U.S. EPA (1999).

3.3.2. Pretreatment of Samples for Standard Culture Methods

As a part of the standart culturing methods, initially samples were processed according to EPA Method 1680 (U.S. EPA, 2003). Owing to the heterogeneous nature they possess, first samples were homogenized with a blender. Approximately 300 mL of the liquid samples (digester influent and effluent) were blended for two minutes and for the dewatered samples, 70 g of solid samples were blended with 230 mL of phosphate buffer dilution water. Then the pH of those homogenized samples was brought to 7.0-7.5 wtih 1N HCl or 1N NaOH. Since Fecal Coliform and *E.coli* concentrations of undiluted samples could exceed the determination range of methods applied, samples were diluted with phosphate buffer dilution water. This dilution procedure applied before inoculation of the samples to the culture media are recommended in Control of Pathogens and Vector Attraction in Sewage Sludge (EPA/625/R-92/013), Standard Methods, Method 2540 D (Standard Methods, 2005) and the standart culturing method used in this study (U.S. EPA, 2006).

3.3.3. Preparation of the Phosphate Buffer Dilution Water

Phosphate buffered dilution water consists of stock phosphate buffer solution and stock magnesium chloride solution. Those stock solutions used for the preparation of phosphate buffer dilution water were prepared monthly and stored in a refrigerator until used. Preparations of the stock solutions are as follows:

For the preparation of stock phosphate buffer solution: 3,4 g KH_2PO_4 were dissolved in 50 mL reagent-grade water. The pH of the solution was adjusted to 7.2

with 1 N NaOH and after the volume was brought to 100 mL with reagent-grade water, the solution was autoclaved at 121°C (15 PSI) for 15 minutes.

Stock Magnesium Chloride Solution was prepared by dissolving 8,1 g $MgCl_2 \bullet 6H_2O$ in 100 mL reagent-grade water. The solution was autoclaved at 121°C (15 PSI) for 15 minutes.

Combining 1.25 mL of the stock phosphate buffer and 5 mL of the $MgCl_2$ stock per liter of reagent-grade water, working phosphate buffer dilution water was prepared freshly for each analysis. It was autoclaved at 121°C (15 PSI) for 15 minutes. In order to provide a neutral environment to the bacteria, after diluting the samples, the final pH of phosphate dilution water was set to 7.0 ± 0.2.

3.4. Enumeration of Indicator Bacteria by Standard Culture Methods

Standard Culturing Methods (SCMs) have been utilized for years to identify and isolate organisms, store cultures, develop antigens for serological diagnosis and enumerate organism of interest as well. In the case of this study, SCMs were utilized in order to analyze samples in terms of their indicator bacteria content by Membrane Filter Method; SMWW 9222D Fecal Coliform Procedure (Standard Methods, 2005) and Multiple Tube Fermentation Technique of U.S. EPA Method 1680; Fecal Coliforms in Sewage Sludge by using Lauryl Tryptose Broth and EC Medium (U.S.EPA, 2006). For this reason, samples in which the microorganism concentrations were to be determined, were inoculated to culture specific media and incubated at elevated temperatures. All of the samples collected were analyzed for their total solid content according to SMWW 2540B (Standard Methods, 2005). Afterwards, a serial of dilution was applied to the homogenized samples with the phosphate buffer dilution water. The dilution method was applied in a distinctive manner for liquid and solid samples.

3.4.1. Dilution Procedure for Liquid Samples

Three hundred mL of liquid samples which consisted of samples taken from digester influent and digester effluent units were blended and their pH was adjusted to the interval of 7.0-7.5. A serial dilution procedure which was composed of five different concentrations of homogenized liquid samples was applied as described below.

In Dilution A; 11 mL of the homogenized sample was transferred to a screw cap bottle containing 99 mL phosphate buffer dilution water. A 1 mL of dilution A contained 10^{-1} g of the original sample.

In Dilution B; 11 mL of dilution A was transferred to a second bottle containing 99 mL of dilution water using a sterile pipette and mixed carefully. A 1 mL of dilution B contained 10^{-2} g of the original sample.

In Dilution C; 11 mL of dilution B was transfered to another bottle containing 99 mL of sterile buffer dilution water and mixed carefully. A 1 mL of dilution C contained 10^{-4} g of the original sample

In Dilution D; 11 mL of dilution C was transferred to another bottle containing 99 mL of sterile buffer dilution water and mixed carefully. A 1 mL of dilution D contained 10^{-5} g of the original sample.

In Dilution E; 11 mL of dilution D was transferred to another bottle containing 99 mL of sterile buffered dilution water and mixed carefully. A 1 mL of dilution E contained 10^{-6} g of the original sample.

3.4.2. Dilution Procedure for Solid Samples

As for solid samples, 30 g of the dewatered sludge samples, which constituted the only solid samples in our study, were blended with 270 mL of phosphate buffer dilution water. The pH was set to 7.0-.7.5. A 1 mL of this homogenized sample

contained 10^{-1} g of the original sample. The details of the applied procedure are given below:

For solid samples, in Dilution A; 11 mL of homogenized solid sample was transferred to a screw cap bottle containing 99 mL of phosphate buffer dilution water and mixed. A 1 mL of this sample contained 10^{-2} g of the original sample. As with liquid samples, the same series of dilution procedure was conducted for the rest of the dilution procedure. In fact, owing to the different initial dilution ratios applied to original samples which were 1/10 for liquid samples and 1/100 for solid samples, concentrations obtained through the dilution procedure were different for liquid and solid samples. In other words, in case of solid sample dilution, 1 mL of dilution A, B, C, D, E and F contained 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6} and 10^{-7} g of the original solid sample, respectively which were tenfolds more diluted compared to the liquid samples.

Following the dilution procedures for both liquid and solid samples, five samples which were tenfold diluted compared to the former one were prepared in order to assess their indicator bacteria content by culture based methods. *E.coli* content of sludge samples were estimated by Multiple Tube Fermentation Technique. For the determination of the Fecal Coliforms, on the other hand, both Membrane Filter Method and Multiple Tube Fermentation Technique were applied. The details of implemented methods are given below in the following sections.

3.4.3. Membrane Filter Procedure

Fecal Coliform analyses were performed according to the Membrane Filter procedure at the early stages of the study. In accordance with SM 9222D (Standard Methods, 2005) analyses were conducted by filtering 100 mL of diluted samples through a 0.45 µm pore sized sterile membrane filter. After the filtration process, membrane filters were placed into sterile petri dishes containing commercially

available M-FC medium (Millipore) consisting of selective growth media for Fecal Coliforms. Then, petri dishes were incubated at 44.5±0.5° C for 24 hours. After the incubation period, blueish colonies were considered as Fecal Coliform bacteria as mentioned in the data sheet of the Millipore M-FC medium and the densities were recorded per 100 mL (Standard Methods, 2005). Colony numbers of Fecal Coliforms per gram of dry weight of samples were calculated for each of the dilution applied and the average number was assessed considering the colony numbers for all of the dilutions. However, if the colonies were nested or high in number, results were reported as "too numerous to count" and number of dilutions were increased. Numbers of Fecal Coliform colonies per gram of dry weight of sewage sludge are calculated using Equation 1.

$$\text{Fecal Coliforms/g dry weight} = \frac{\text{coliform colonies counted} \times 100}{\text{dilution chosen} \times \text{dry solids}} \quad \text{..... (Equation 1)}$$

3.4.4. Multiple-Tube Fermentation Technique

Multiple Tube Fermentation technique is one of the oldest but most reliable tests among other methods applied for the enumeration of coliforms in water and wastewater. This method which is also called as the Most Probable Number (MPN) method is mainly based on lactose fermentation with production of acid and gas within 48 hours. This technique consists of three steps; the presumptive, confirmed, and completed tests. In the presumptive test, a selective lactose broth medium (Lauryl Tryptose Broth) is inoculated with the sample material to induce the recovery and growth of potentially stressed coliforms in the sample. In presumptive phase, a tube containing both growth and gas is recorded as a positive result. Since it is possible for non-coliforms to give false positive results by growing in this medium, all positive tubes are then inoculated into a more selective medium (Brilliant Green Lactose Broth or EC Broth). Eventually in the completed test, Eosin Methylene Blue (EMB) agar is inoculated by streaking a loopful of the growth from a positive completed phase tube in order to provide more precise results.

In the scope of the study, enumeration of Fecal Coliforms was conducted by Multiple Tube Fermentation Technique. The same method was utilized for the enumeration of *E.coli* regarding the broad range of application in relevant literature. Owing to the high level of Fecal Coliform and *E.coli* concentrations of undiluted samples, both enumeration methods applied require homogenization and dilution of the samples. Therefore, the dilution procedure which is given both for liquid and solid samples is applied to the homogenized samples before the enumeration process. The details of the applied procedures for the enumeration of Fecal Coliforms and *E.coli* are given below in the following sections

3.4.4.1. Enumeration of Fecal Coliforms by Multiple Tube Fermentation Technique

Fecal Coliform analyses were conducted according to the EPA Method 1680: Fecal Coliforms in Sewage Sludge (Biosolids) by Multiple Tube Fermentation using Lauryl Tryptose Broth (LTB) and EC Medium (U.S. EPA, 2006). Briefly, Fecal Coliforms are identified based on their growth at the culture specific media and gas production resulting from fermentation of lactose after incubation at elevated temperature.

Since prior enrichment in presumptive medium is required for optimum recovery of Fecal Coliforms, LTB is used at the initial phase of the Method 1680. During the experimental study, sufficient amount of LTB was prepared freshly the day before the analyses. Ten mL of medium were dispensed into tubes containing inverted fermentation vials and tubes were sterilized by autoclaving at 121°C for 15 minutes.

Each series represent the dilutions expressed as dilution C, D and E and F (EPA Method 1680). Each of the Dilution C, D, E and F is inoculated into five test tubes, containing sterile LTB and an inverted vial. Inoculated tubes were incubated at 35°C ± 0.5°C. After 24 ± 2 hours, each tube was mixed gently and examined for growth and gas production. If no gas has been formed, tubes were reincubated for an additional 24 ± 2 hours since final assessment should be done within a total of 48

± 3 hours. After 48 ± 3 hours period the tubes were reexamined for growth and gas production. Failure to produce gas in LTB medium within 48 ± 3 hours is a negative presumptive test. For tubes with growth, the presence of gas in inverted vials within 48 ± 3 hours signifies a positive presumptive reaction. Then, the presumptive phase was followed by the confirmation phase.

EC medium is utilized as the confirmative medium for Fecal Coliforms in the scope of the Method 1680. After 24 hours of incubation at the presumptive phase, tubes were observed for growth and gas production. An average number for positive tubes were assesed roughly and tubes containing EC medium was freshly prepared and autoclaved at 121°C for 15 minutes. Each of the positive LTB tube was shaked gently to homogenize the bacterial content of the tube. For each of the positive LTB tube, one EC tube was inoculated while transferring a loop of growth from LTB to corresponding tubes. All EC tubes were placed in an 44.5°C ± 0.2°C incubator and incubated for 24 ± 2 hours. After incubation, each tube was examined for growth and gas production. Gas production with growth in EC broth after 24 ± 2 hours was considered as positive Fecal Coliform. Failure to produce gas indicates a negative reaction. All positive and negative results were recorded for EC tubes. Utilizing the MPN table given in EPA Method 1680, MPN / g of total solids (dry weight) was calculated considering the number of positive EC tubes.

3.4.4.2. Enumeration of Escherichia coli by Multiple Tube Fermentation Technique

The enumeration of *E. coli* in the samples was accomplished by SM 9221F-*Escherichia coli* Procedure which is performed as a multiple tube procedure (Standard Methods, 2005). After conducting the same presumptive phase with Fecal Coliform detection method which utilizes LTB, in the case of *E.coli* enumeration, this method differs at the confirmative phase which requires inoculation of EC-MUG media. In this method, *E.coli* are defined as coliform bacteria that process the enzyme ß-glucuronidase which hydrolyzes substrate of 4-

methylumbelliferyl-ß-D-glucuronide (MUG) to yield a fluorogenic product that is detectable under long-wave (366 nm) UV light. After the incubation period, tubes were examined for growth and gas production as in Fecal Coliform enumeration. Additionally, exhibition of fluorescence were investigated by using a long-wavelength UV lamp (Merck) and tubes displaying blueish fluorescence under UV light were considered as positive *E.coli* test. The same MPN index was utilized for the assessment of *E.coli* content of the samples. The summary of the procedure applied for the enumeration of both Fecal Coliforms and *E.coli* are provided in Figure in A.1 in Appendix A.

The estimated densities of Fecal Coliform bacteria and *E.coli* which are based on the confirmation test using EC and EC-MUG, respectively, was calculated in terms of most probable number (MPN). In the scope of the applied enumeration methods, Fecal Coliform and *E.coli* content of samples were determined by using the MPN Index which is given in EPA Method 1680. The MPN index utilized and the sample calculation for the assessment of bacterial content of the sludge samples is provided in Appendix B.

3.5. Enumeration of Indicator Bacteria by Quantitative PCR Analyses

In addition to culture based enumeration methods, Quantitative PCR analyses were also conducted paralelly in order to investigate if the indicator bacteria are potentially being underestimated by conventional culture methods. *E. coli* was chosen as the target indicator bacteria to be enumerated by Quantitative PCR since a significant portion of Fecal Coliform bacteria consists of *E.coli*. DNA extractions from sludge samples were performed in our laboratory according to the extraction protocol given in the relevant section. Extracted DNAs were labelled and stored at - 20 °C until Quantitative PCR analysis. On the other hand, Quantitative PCR analyses were carried out at the Central Laboratory of Ankara University, Biotechnology Institute by expert researchers. The details of the DNA extraction

procedure along with the applied Quantitative PCR program and conditions are provided below.

3.5.1. Extraction of DNAs from Sludge Samples

Due to the heterogeneous nature of the sample material analyzed throughout the study, it is essential to assess the most efficient DNA extraction method. However, there is no standard and/or recommended method for materials with high solid content. Therefore, DNA extraction methods addressed for environmental samples in relevant literature were investigated. Since the lysis protocol combines chemical and mechanical methods at the cell lysis step, Mo-Bio Power Soil Extraction Kit is reported to provide high DNA yield among other DNA extraction kits. Hence, throughout the study, DNA extractions were accomplished with Mo-Bio Power Soil Extraction Kit. Particularly for the liquid samples, a specific pretreatment process was applied shortly before the DNA extraction procedure.

3.5.1.1. Pretreatment of Samples for DNA Extraction Procedure

In order to concentrate samples and remove naked DNA found in the suspension, digester influent and digester effluent samples were centrifuged for 15 min at 4000 *g* before initiating the DNA extraction procedure. Specifically for the preliminary experiments, Volatile Solid (VS) content of samples were controlled before and after centrifugation. Since the organic portion of the samples consists of live or dead bacteria, a reduction in the VS content indicates the removal of bacteria during centrifugation which might affect the calculated number of bacteria. In fact, according to the results, there was no VS reduction after centrifugation, indicating that the organic portion is not removed during the centrifugation step with the removal of centrate.

3.5.1.2. DNA Extraction Procedure

DNA extractions from the sample materials were conducted in our laboratory according to the given protocol in Mo-Bio Power Soil Extraction Kit. The content of the solutions included in Mo-Bio Power Soil Extraction Kit are listed in Appendix C.

DNA isolation procedure given in Mo-Bio Power Soil Extraction Kit Manuel is as follows:

1. To the PowerBead Tubes provide 0.25 grams of soil sample.

2. Gently vortex to mix.

3. Check Solution C1. If Solution C1 is precipitated, heat solution to 60°C until dissolved before use.

4. Add 60 µL of Solution C1 and invert several times or vortex briefly.

5. Secure PowerBead Tubes horizontally using a vortex adapter tube holder for the vortex. Vortex at maximum speed for 10 minutes.

6. Centrifuge tubes at 10,000 x *g* for 30 seconds at room temperature.

7. Transfer the supernatant to a clean 2 ml Collection Tube (provided).

8. Add 250 µL of Solution C2 and vortex for 5 seconds. Incubate at 4°C for 5 minutes.

9. Centrifuge the tubes at room temperature for 1 minute at 10,000 x *g.*

10. Avoiding the pellet, transfer up to, but no more than, 600 µl of supernatant to a clean 2 ml Collection Tube.

11. Add 200 µL of Solution C3 and vortex briefly. Incubate at 4°C for 5 minutes.

12. Centrifuge the tubes at room temperature for 1 minute at 10,000 x *g.*

13. Avoiding the pellet, transfer up to, but no more than, 750 µL of supernatant into a clean 2 mL Collection Tube.

14. Shake to mix Solution C4 before use. Add 1200 µL of Solution C4 to the supernatant and vortex for 5 seconds.

15. Load approximately 675 µL onto a Spin Filter and centrifuge at 10,000 x *g* for 1 minute at room temperature. Discard the flow through and add an additional 675 µL of supernatant to the Spin Filter and centrifuge at 10,000 x *g* for 1 minute at room temperature. Load the remaining supernatant onto the Spin Filter and centrifuge at 10,000 x *g* for 1 minute at room temperature.

16. Add 500 µL of Solution C5 and centrifuge at room temperature for 30 seconds at 10,000 x *g*.

17. Discard the flow through.

18. Centrifuge again at room temperature for 1 minute at 10,000 x *g*.

19. Carefully place spin filter in a clean 2 ml Collection Tube (provided). Avoid splashing any Solution C5 onto the Spin Filter.

20. Add 100 µL of Solution C6 to the center of the white filter membrane.

21. Centrifuge at room temperature for 30 seconds at 10,000 x *g*.

22. Discard the Spin Filter. The DNA in the tube is now ready for any downstream application. No further steps are required.

Extracted DNAs were labeled and stored at -20°C until the Quantitative PCR Analyses. Particularly for initial extraction experiments, the concentrations and purity of isolated DNAs were questioned in order to have a general idea concerning the suitability of the extraction method chosen. In addition to that, since the quality and purity of the DNA are critical factors for further analysis, isolated DNAs

were examined by a Nanodrop Spectrophotometer. The ratio of absorbances at 260 and 280 nm, indicate high purity of DNA when found to be between 1.8 and 2 (Bonot et al., 2010). Even though absorbations obtained for DNAs isolated from digester influent and effluent samples indicate a high quality, a lower ratio was assed for dewatered sludge samples. Nevertheless, it is acceptable owing to the heterogenous nature of the sample and variety of protein inpurites included.

3.5.2. Primer Design

In the relevant literature gad AB, uid A, rRNA and dxs genes are reported to be used as target for the detection and enumeration of *E.coli*. Primers amplifying these genes were evaluated to be utilized in the Quantitative PCR analysis. Since it is recommended to use a single copy gene for enumeration purposes with Quantitative PCR analysis, the dxs gene; a single copy gene in *E.coli* genome which yields a final 305 bp PCR product was selected as the target gene for quantification of *E.coli. dxs* gene encodes D-1-deoxyxylulose-5-phosphate which is a common precursor for isoprenoid, thiamin, and pyridoxol biosynthesis (Lois et al., 1998). The primer oligonucleotides were obtained in the lyophilized form through BM Labosis (Ankara,Turkey) from Metabion (Germany). The specificity of the primer pair was validated in Lee et al. (2008) and checked further through Primer BLAST (http://www.ncbi.nlm.nih.gov/tools/primer-blast accession number: AF035440.1). The sequence information found in the NCBI GenBank database of the gene is listed in Appendix D. Sequences and main properties of the primer pair selected are given in Table 3.2.

Table 3.2. Primers used for the amplification of dxs gene

	Sequence (5'- 3')	Length (bp)	Start	Stop	Tm	GC%
Forward Primer	CGAGAAACTGGCGATCCTTA	20	1509	1528	51.70	50.00%
Reverse Primer	CTTCATCAAGCGGTTTCACA	20	1621	1602	50.96	45.00%
Product Length		113 bp				

3.5.3. Quantitative PCR Analysis

In the scope of the study Quantitative PCR analyses were conducted at the Central Laboratory of Ankara University Biotechnology Institute for the enumeration of the indicator bacteria. As mentioned earlier, *E.coli* was chosen as the target bacteria to be enumerated by Quantitative PCR analysis. Basically, the number of *E.coli* was assessed by the Absolute Quantification of the dxs gene by Quantitative PCR analysis. In the context of Absolute Quantification, a standard curve was constructed to correlate the number of dxs gene determined by q PCR analysis with the quantity of bacteria found in the sample material. Since dxs gene is a single copy gene found in the *E.coli* genome, quantification of dxs gene with Quantitative PCR analysis eventually leads to enumeration of *E.coli* present in the sample material. The SYBR Green based amplification analyses were executed on Roche-LightCycler 480 System. Each reaction was run in triplicate to assure the accuracy of the analysis. In order to assess the specificity of the amplified PCR product melting curve analyses were also performed for the discrimination of primer dimmers and specific products. The components used in the reaction are given in Table 3.3.

Table 3.3. Components of the reaction mixture for Quantitative PCR Analysis

Component	Volume	Final Concentration
2x SybrGreen Mix	5 µL	1x
Forward Primer	0.5 µL	10 pmol/ul
Reverse Primer	0.5 µL	10 pmol/ul
Nuclease free H_2O	2 µL	-
DNA	2 µL	50 ng/ul
Total Volume	**10 µL**	

The cycling parameters for the dxs gene detection system can be observed in Table 3.4 which outlines the conditions applied during the denaturation, amplification, melting curve and cooling steps.

Table 3.4. q PCR Program for SYBR Green Method for LightCycler 480 System

Analysis Mode	Cycles	Segment	Target Temperature	Hold Time	Acquisition Mode
Denaturation					
none	1		95	5 min	none
Amplification					
Quantification	45	Denaturation	95	30 s	none
		Annealing	60	30 s	none
		Extention	72	30 s	single
Melting Curve					
Melting Curves	1		95 °C	10 s	none
			60 °C	1 min	none
			72 °C		continuous
Cooling					
Cooling			40 °C	10 s	none

3.5.4. Construction of the Standard Curve

In this study, Applied Biosystems; the method suggested in "Creating Standard Curves with Genomic DNA or Plasmid DNA Templates for Use in Quantitative PCR" manuel is followed for the construction of the Standard Curve. Standard Curve was generated using serially diluted *E.coli* (ATCC 25922) DNA. Isolated DNAs were procured from Ankara University; Department of Biology, Molecular Biology Laboratory. Concentrations of the isolated genomic DNAs which were used in the preparation of Standard Curve were calculated.

In order to construct a Standard Curve genomic size, mass of the haploid genome and copy number of the target gene of interest in the genome of investigated organism is considered. The procedure applied for the generation of standart curve is as follows;

First of all the mass of the genome was identified according to the formula given below,

$$m = (n)\, x \left(1,096 e^{-21} \frac{g}{bp}\right)$$

where n = plasmid size (bp)

m = mass (m)

After calculation of the mass of a single genome, mass of the genome needed for a certain copy number was calculated (Eq.2).

Copy # of interest × mass of haploid genome=mass of genomic DNA needed (Eq.2)

Since the volume pipetted into each reaction was known, the concentration of genomic DNA needed to achieve the copy number of interest was easily calculated. The concentration of *E.coli* genomic DNA was determined spectrophotometrically and serial dilutions were conducted. Regarding the copy number of the analysed gene, Standard Curve was generated by the amplification of prepared dilutions in predetermined Quantitative PCR conditions. The applied dilution scheme and resulting copy numbers are given in Table 3.5.

Table 3.5. Preparation of Standards and Dilution Scheme Applied

Source	Initial concentration (pg/µL)	Volume of gDNA (µL)	Volume of diluent (µL)	Final Volume (µL) V_2	Final concentration of dilution (pg/µl) C_2	Resulting copy # gene
Stock	2.000.000	8	92	100	160.000	64.000.000
Dilution 1	160.000	10	90	100	16.000	6.400.000
Dilution 2	16.000	10	90	100	1600	640.000
Dilution 3	1600	10	90	100	160	64.000
Dilution 4	160	10	90	100	16	6400

The detailed computation procedure followed during the construction of Standard Curve with *E.coli* genomic DNA is given in Appendix E. Standard Curve and sample Amplification and Melting Curves obtained during Quantitative PCR analysis are given in Appendix F. The sample computation for the conversion of obtained Cp values to the bacterial numbers are provided in Appendix G.

CHAPTER 4

RESULTS AND DISCUSSION

4.1. Preliminary Results Obtained by Membrane Filtration Method

At the early stages of the study, Fecal Coliform analyses were conducted by Membrane Filtration method following the above mentioned dilution and application procedure. However, early results obtained by the method were found improper since they did not indicate growth in any of the dilutions even for a sample which was expected to have at least 10^6 Fecal Coliform bacteria. In the same manner, it is revealed that the original MF method did not only underestimate the bacterial concentrations of the samples but also gave non-replicable results.

Therefore, a number of experiments were conducted by changing some parameters with the purpose of determining possible reasons for problematic results obtained. For a period of approximately 2 months, the effect of several parameters was evaluated by altering pH, dilution water utilized, medium and membrane filters. Results obtained by the applied procedures are presented in Table H1 given in Appendix H.

Although a variety of parameters have been changed, the reason for the inaccurate results obtained could not be assessed by the experimentation. Nevertheless, one of the possible reasons is believed to be clogging of membrane filters by colloidal structures and solid particles found in the sample material. Even though a number of different laboratories use MF method for wastewater and wastewater sludge analysis due to its very easy to apply nature, MF method is mainly recommended

for the analysis of drinking water and a variety of natural waters. Although this method is mentioned to be applicable for groundwater, wastewater and even for sludge samples, major disadvantages of the MF technique are already stated both in Standard Methods (2005) and EPA Method 1103.1 (U.S EPA, 2002)

According to Standard Method 9222D (Fecal Coliform by Membrane Filtration Using m-FC) and EPA Method 1103.1 (Escherichia coli in Water by Membrane Filtration Using membrane-Thermotolerant Escherichia coli Agar) this technique is inappropriate for the analysis of waters which contain high amount of particulate matter and high numbers of non-coliform background bacteria. It is believed that, this inconvenience might be the reason for the inaccurate results obtained. Consequently, an alternative method was sought for Fecal Coliform analyses. After reviewing the related research in the literature, EPA Method 1680: Fecal Coliforms in Sewage Sludge by Multiple Tube Fermentation using LTB and EC Medium (U.S EPA, 2006) was chosen and immediately implemented for the Fecal Coliform analyses. Results obtained by Multiple Tube Fermentation Technique are given in the following section.

4.2. Examination of the Indicator Bacteria Content of Selected Treatment Plants

Since the main purpose of the study is to figure out the impact of dewatering processes on indicator bacteria content of anaerobically digested sludge, six different treatment plants which apply either belt filter press or centrifuge dewatering to anaerobically digested sludges were examined throughout the study. Samples taken from three main phases of the sludge treatment process were investigated for their Fecal Coliform and *E.coli* densities by means of both Standard Culture Methods (SCM) and Quantitative PCR analysis.

As mentioned before, findings of the relevant literature regarding reactivation and regrowth of indicator bacteria are based on the results obtained by cultivation methods. Moreover, pathogen removal standards implemented in the regulations

rely on results obtained by standard culture methods all around the world including Turkey. Thus, in the scope of our study, the initial evaluation of treatment plants investigated for reactivation and/or regrowth potential of indicator bacteria depend on the results obtained by SCM.

In the scope of this study Quantitative PCR analyses were also conducted as to determine the indicator bacteria concentrations of analysed samples by an additional culture independent method. In order to provide a clear comprehension for the outcomes of the study, results obtained during the investigation period are divided into two major sections which consist of Enumeration of Indicator Bacteria by Standard Culture Methods and Quantitative PCR Analyses and Comparision of the Results obtained by those two methods.

4.2.1. Enumeration of Indicator Bacteria by Standard Culture Methods

This part of the study involves determination of Fecal Coliform and *E.coli* content of samples by means of standard culture methods. Anaerobic digester influent, effluent and dewatered sludge cake samples taken from all of the six different treatment plants were analysed for their Fecal Coliform and *E.coli* content in accordance with the dilution and enumeration procedures given in the Material and Methods section of the study. Results are expressed as Most Probable Number per gram Total Solids (MPN/gTS).

Determination of the indicator bacteria content of the samples taken from anaerobic digester influent and effluent provides an additional information regarding the indicator bacteria removal rates obtained in anaerobic digesters of the investigated treatment plants. These removal efficiencies are given in the following sections for each treatment plants. In addition, acquired removal rates of

E.coli were evaluated in order to examine the land applicability of stabilized sludge generated in treatment plants in terms of the regulation promugulated in Turkey in August 2010.

4.2.1.1. Ankara Wastewater Treatment Plant

Among the treatment plants investigated in the scope of the study, Ankara wastewater treatment plant is one of three treatment plants operating belt filter press dewatering unit after anaerobic digestion process. Throughout the study, five samples taken from Ankara wastewater treatment plant were analyzed for their Fecal Coliform and *E.coli* content by means of cultivation techniques.

Fecal Coliform and *E.coli* densities of samples obtained from anaerobic digester influent, anaerobic digester effluent and dewatered cake samples along with the average densities calculated are given in Table 4.1 and Table 4.2, respectively.

Table 4.1. Fecal Coliform densities in Ankara Wastewater Treatment Plant revealed by Standard Culture Methods

Sampling Date	Fecal Coliform Densities (MPN/gDS)		
	Digester Influent	**Digester Effluent**	**Dewatered Cake**
04.08.2010	2,51E+05	3,09E+04	8,00E+02
24.11.2010	4,46E+07	2,20E+06	1,80E+06
01.12.2010	3,86E+07	1,13E+06	8,00E+05
02.02.2011	4,45E+07	1,70E+06	1,90E+05
14.03.2011	1,42E+07	6,00E+05	1,30E+05
ave	**2,84E+07**	**1,13E+06**	**5,84E+05**

Table 4.2. *E.coli* Densities in Ankara Wastewater Treatment Plant revealed by Standard Culture Methods

Sampling Date	*E.coli* Densities (MPN/gDS)		
	Digester Influent	**Digester Effluent**	**Dewatered Cake**
04.08.2010	7,98E+04	1,92E+04	6,00E+02
24.11.2010	7,70E+06	5,00E+05	4,80E+05
01.12.2010	2,20E+07	7,10E+05	4,40E+05
02.02.2011	1,99E+07	1,13E+06	8,00E+04
14.03.2011	8,40E+06	5,37E+05	1,01E+05
ave	**1,16E+07**	**5,79E+05**	**2,20E+05**

As provided in Tables 4.1 and 4.2, the number of indicator bacteria in digester influent is determined to be in the range of 10^6 and 10^7 whereas after the digestion operations, concentrations are reduced to levels of 10^4 to 10^5. Even though several samplings were conducted over a seven months period, significant variations occur in the number of indicator bacteria. However, it is quite acceptable since the number of indicator bacteria are known to change owing to a number of operational and seasonal variations (Saleem et al.,2001). For instance, in the sample taken on 04.08.10, a different trend which is not detected later on was determined for the number of indicator bacteria. However, it is understood in a short time that, acquired low concentrations of indicator bacteria result from a breakdown occured in the pumps of anaerobic digester. Since the sludge was stored for a long time before taken into anaerobic digester, it was already degraded in the storage tank owing to the anaerobic conditions occur gradually. For these reasons, the following sampling were done after approximately 3.5 months.

Results obtained from Ankara wastewater treatment plant indicate that anaerobic digester achieve a clear reduction in the number of both Fecal Coliforms and *E.coli*. For each sample obtained, removal rates for both Fecal Coliforms and *E.coli* were calculated and provided in the Table 4.3.

Table 4.3. Indicator Bacteria Removal Efficiencies of Ankara Wastewater Treatment Plant

Sampling Date	Removal Efficieny of Anaerobic Digester	
	Fecal Coliforms (%)	***E.coli* (%)**
04.08.2010	87,7	75,9
24.11.2010	95,1	93,5
01.12.2010	97,1	96,8
02.02.2011	96,2	94,3
14.03.2011	95,8	93,6

The removal rates are determined based on the influent and effluent concentrations of Fecal Coliforms and *E.coli*. Apart from the first sampling which

corresponds to an operationally problematic time for the treatment plant, the overall removal of indicator bacteria is higher than 93 % which is an expected ratio for a properly operated mesophilic anaerobic digestion process (Sanin et al., 2011). However, obtained removal ratio for *E.coli* do not meet the removal criteria of the regulation implemented in Turkey concerning the land application of stabilized sludge which requires 99% removal of E.coli .

In order to visualize reductions occur in the whole process, average concentrations for Fecal Coliforms and *E.coli* in each step are calculated and placed in the following graph given in Figure 4.1.

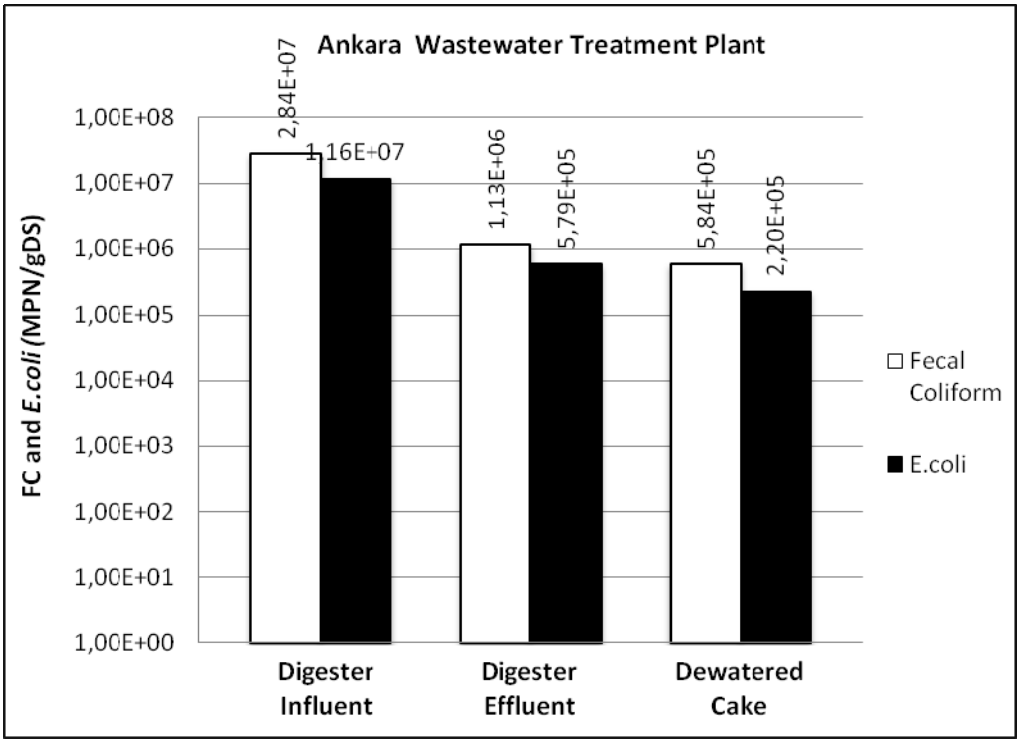

Figure 4.1. Average Fecal Coliform and *E.coli* content of samples taken from Ankara Wastewater Treatment Plant

On average, *E.coli* to Fecal Coliform ratios are determined to be 42%, 60% and 55% for digester influent, digester effluent and dewatered sludge, respectively. As can be gathered from Figure 4.1, besides the reduction occur during the anaerobic digestio process, a slight decline in the number of indicator bacteria also takes place after the dewatering process. It is not suprising since it has been reported in the literature for several times that belt filter press process does not cause an increase in the number of indicator bacteria (Erdal et al., 2003, Monteleona et al., 2004). This tendency is valid for both Fecal Coliforms and *E.coli*. Since Fecal Coliforms comprise a number of coliform bacteria including *E.coli*, the initial and final concentrations of Fecal Coliforms are higher than *E.coli.*

4.2.1.2. Adana Wastewater Treatment Plant

Adana wastewater treatment plant was investigated as the second treatment plant operating belt filter press dewatering unit in the scope of the study. Since results obtained from Ankara wastewater treatment plant clearly indicate that, belt filter press dewatering does not result in any increases in the indicator bacteria content, taking two samples from Adana wastewater treatment plant was found to be sufficient to reveal if the same trend occur in an analogous wastewater treatment operation. Obtained densities for Fecal Coliforms and *E.coli* along with the reduction rates computed are given in the Table 4.4, Table 4.5 and Table 4.6, respectively.

Table 4.4. Fecal Coliform Densities in Adana Wastewater Treatment Plant revealed by Standard Culture Methods

Sampling Date	Fecal Coliform Densities (MPN/gDS)		
	Digester Influent	Digester Effluent	Dewatered Cake
23.05.2011	3,49E+07	1,01E+05	6,90E+04
13.07.2011	1,34E+06	1,28E+05	1,10E+04
ave	**1,81+07**	**1,15E+05**	**4,00E+04**

Table 4.5. *E.coli* Densities in Adana Wastewater Treatment Plant revealed by Standard Culture Methods

Sampling Date	*E.coli* Densities (MPN/gDS)		
	Digester Influent	Digester Effluent	Dewatered Cake
23.05.2011	7,55E+06	7,30E+04	3,40E+04
13.07.2011	9,25E+05	6,50E+04	9,00E+03
ave	**4,24E+06**	**6,90E+04**	**2,15E+04**

Table 4.6. Indicator Bacteria Removal Efficiencies of Adana Wastewater Treatment Plant

Sampling Date	Removal Efficieny of Anaerobic Digester	
	Fecal Coliforms (%)	***E.coli* (%)**
23.05.2011	99,71	99,03
13.07.2011	99,04	92,97

Results obtained from Adana wastewater treatment plant show that, a significant reduction is achieved in the Fecal Coliform and *E.coli* densities through the anaerobic digestion process. The reduction ratios are determined to be 95,1 % for Fecal Coliforms and 96% for *E.coli* on the average. Although a relatively higher reduction is achieved in the concentrations of *E.coli*, the removal ratio is still insufficient in terms of the regulation which requires a 99% removal in the *E.coli* concentrations by the stabilization process applied for the land application purposes.

The average densities of Fecal Coliforms and *E.coli* found in samples taken from digester influent, digester effluent and dewatered cake are provided in Figure 4.2 to provide a visual comparison.

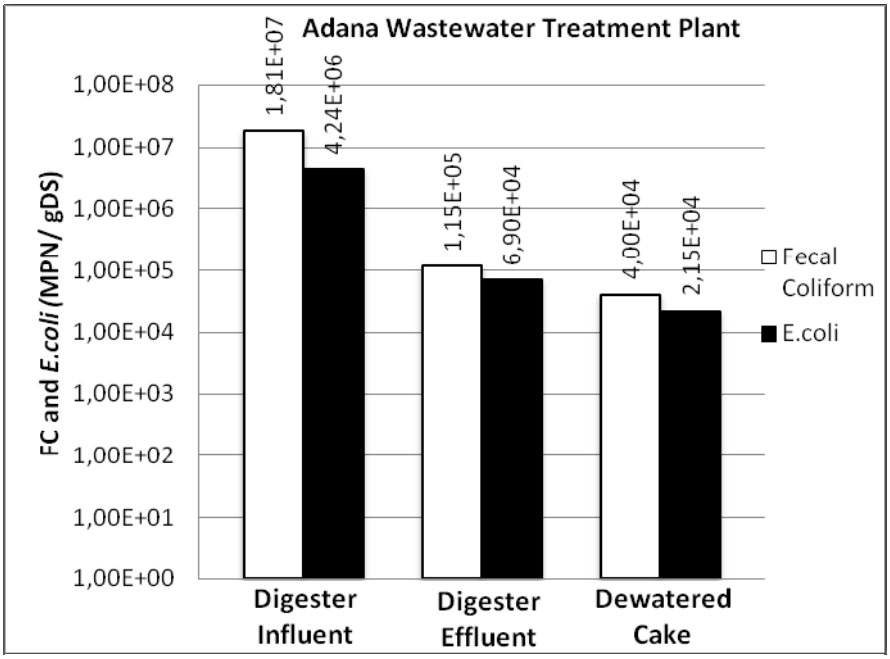

Figure 4.2. Average Fecal Coliform and *E.coli* content of samples taken from Adana Wastewater Treatment Plant

Our results show that, on average Fecal Coliforms to *E.coli* ratios are 45%, 61% and 65% for anaerobic digester influent, digester effluent and dewatered sludge, respectively. As can be revealed from Figure 4.2., similar to the results obtained from Ankara wastewater treatment plant, the decreasing trend of the number of indicator bacteria proceeds following the belt filter press dewatering process. Therefore, in Adana wastewater treatment plant etiher, the regrowth of microorganisms during dewatering process was not observed.

4.2.1.3. Kayseri Wastewater Treatment Plant

In addition to Ankara and Adana wastewater treatment plants, Kayseri Wastewater treatment plant was investigated as the other model treatment plant for evaluating the effect of belt filter press dewatering on indicator bacteria content of sludge. However, the treatment process applied for wastewater sludge differs from the other treatment plants investigated that operate belt filter press dewatering process. Although, anaerobic digestion is utilized as the stabilization process, it is used only for primary sludge. Immediately after the digestion process, waste

activated sludge (unstabilized sludge) is combined with the stabilized primary sludge and both sludges dewatered alltogether. In order to designate the effect of this different type of sewage sludge treatment, samples were taken from pre-determined phases of stabilization and dewatering processes. However, due to this distinctive feature of Kayseri wastewater treatment plant, results obtained indicate a different tendency than the other investigated treatment plants. Therefore, only a single sample was taken and analysed during the study. Obtained results are provided in Table 4.7, Table 4.8 and Table 4.9.

Table 4.7. Fecal Coliform Densities in Kayseri Wastewater Treatment Plant revealed by Standard Culture Methods

Sampling Date	Fecal Coliform Densities (MPN/gDS)		
	Digester Influent	Digester Effluent	Dewatered Cake
22.12.2010	1,45E+07	5,83E+05	1,87E+07

Table 4.8. *E.coli* Densities in Kayseri Wastewater Treatment Plant revealed by Standard Culture Methods

Sampling Date	*E.coli* Densities (MPN/gDS)		
	Digester Influent	Digester Effluent	Dewatered Cake
22.12.2010	1,70E+05	1,30E+05	3,00E+05

Table 4.9. Indicator Bacteria Removal Efficiencies of Kayseri Wastewater Treatment Plant

Removal Efficieny of Anaerobic Digester (%)	
Fecal Coliforms	*E.coli*
95,98	94,83

Even though a good reduction efficiency is achieved after the anaerobic digestion process, significantly different results were obtained after dewatering process in

Kayseri, compared to the results obtained from Ankara and Adana wastewater treatment plants. As can be seen in the relevant tables given above, concentrations of the indicator bacteria were determined to increase subsequent to belt filter press dewatering.

Regarding the land application of sludge that is obtained from Kayseri wastewater treatment plant is not suitable to apply on land owing to two main reasons. First of all, 99% removal of *E.coli* which is required by the current regulation is not met by the stabilization process applied. In addition, even though it is not considered in the scope of the regulation, stabilized sludge is combined with waste activated sludge after the stabilization process which results a sudden increase in the number of indicator bacteria. Figure 4.3 represents the general tendency of indicator bacteria after the stabilization and dewatering processes applied in Kayseri wastewater treatment plant.

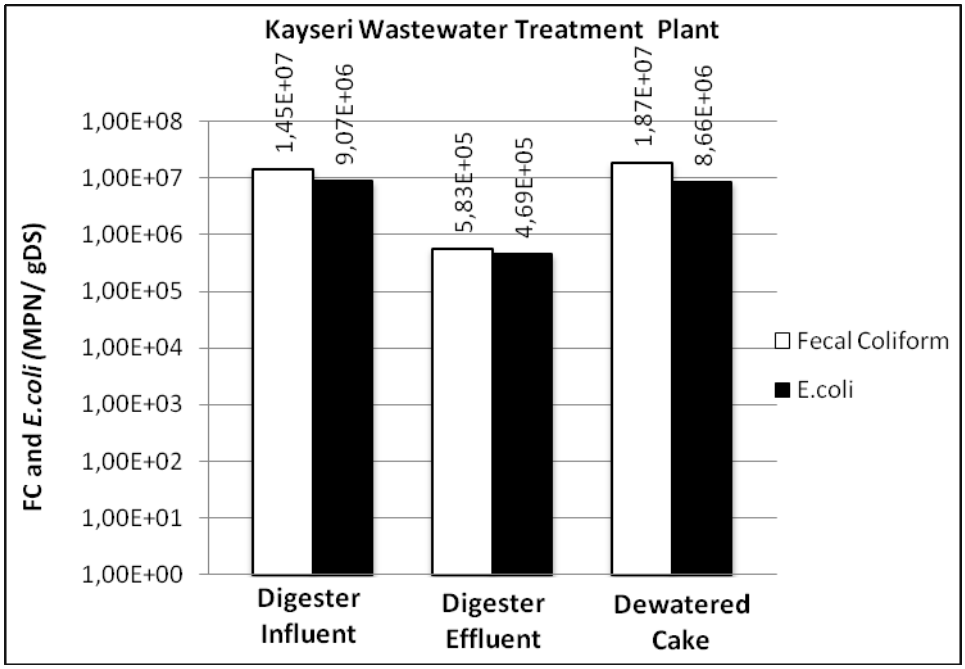

Figure 4.3. Fecal Coliform and E.coli content of sample taken from Kayseri Wastewater Treatment Plant

E.coli is determined to comprise 62%, 80%, 46% of Fecal Coliforms for anaerobic digester influent, anaerobic digester effluent and dewatered sludge, respectively. As represented in Figure 4.3, both of the indicator bacteria contents of the stabilized sludge increase remarkably following the dewatering process.

The reason for this is realized to be due to the aforementioned difference exist in the wastewater treatment system operated in Kayseri wastewater treatment plant. Owing to the high levels of Fecal Coliforms and *E.coli* found in the unstabilized secondary sludge, the analysis of dewatered sludge show higher concentrations of indicator bacteria compared to stabilized sludge. The details of the treatment process can be seen from the flow chart of Kayseri wastewater treatment plant available in Materials and Methods part of the study.

4.2.1.4. Konya Wastewater Treatment Plant

Konya wastewater treatment plant is one of the treatment plants surveyed in the scope of the study which dewater the digested sludge using centrifugal dewatering proccess.

Throughout the study, 4 samples were taken at different times from the predetermined stages of the sludge treatment process and the samples were investigated for their Fecal Coliform and E.coli content. The Fecal Coliform and *E.coli* contents of the samples taken from digester influent, digester effluent and dewatered cake samples are shown in Table 4.10 and Table 4.11, respectively.

Table 4.10. Fecal Coliform Densities in Konya Wastewater Treatment Plant revealed by Standard Culture Methods

Sampling Date	Fecal Coliform Densities (MPN/gDS)		
	Digester Influent	Digester Effluent	Dewatered Cake
17.08.2010	3,70E+05	1,50E+05	8,80E+05
21.09.2010	8,40E+05	1,90E+04	1,33E+06
28.09.2010	1,08E+06	4,90E+04	2,33E+06
18.03.2011	8,04E+06	2,50E+05	7,34E+07
ave	**2,58E+06**	**1,17E+05**	**1,95E+07**

Table 4.11. *E.coli* Densities in Konya Wastewater Treatment Plant revealed by Standard Culture Methods

Sampling Date	*E.coli* Densities (MPN/gDS)		
	Digester Influent	Digester Effluent	Dewatered Cake
17.08.2010	2,90E+05	7,00E+04	4,80E+05
21.09.2010	6,13E+05	8,00E+03	4,90E+05
28.09.2010	9,27E+05	3,30E+04	1,49E+06
18.03.2011	3,94E+06	1,67E+05	4,18E+07
ave	**1,44E+06**	**6,95+04**	**1,11E+07**

As presented in the Table 4.10 and Table 4.11, following the anaerobic digestion process, a decrease was observed in the number of Fecal Coliforms and *E.coli.* Removal efficiencies obtained for the anaerobic digestion process operated in Konya wastewater treatment plant is given in Table 4.12.

Table 4.12. Indicator Bacteria Removal Efficiencies of Konya Wastewater Treatment Plant

Sampling Date	Removal Efficieny of Anaerobic Digester	
	Fecal Coliforms (%)	***E.coli* (%)**
17.08.2010	59,46	75,86
21.09.2010	97,74	98,69
28.09.2010	95,46	96,44
18.03.2011	96,89	95,76

The removal efficiencies calculated for Fecal Coliforms and E.coli generally vary between 95 % to 96 %. As mentioned before, the typical removal ratios reported in the literature for a mesophilic anaerobic digester is similar with the obtained removal rates. Relatively low rate of removal obtained for 17.08.2010 might be caused by an operational problem existed in the anaerobic digester of the treatment plant. In fact, the plant was taken into operation in June 2010. Therefore, rather low values of removal is acceptable during the early phases of plant operation. Nevertheless, the results indicate that biosolids obtained from Konya wastewater treatment plants do not satisfy the *E.coli* removal rates required by the implemented regulation.

When the results of the Fecal Coliform and *E.coli* analyses conducted on dewatered cake samples are evaluated, it is easily seen that a significant increase occurs in the number of both group of indicator bacteria. The results expressed as bar graphs in Figure 4.4 to provide a quick view of situation.

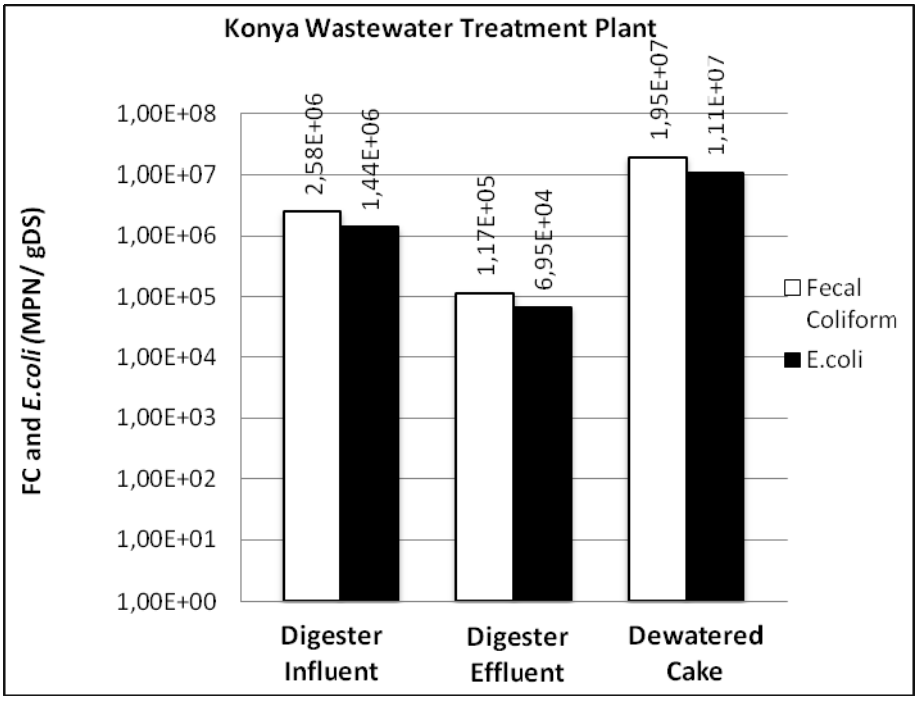

Figure 4.4. Average Fecal Coliform and *E.coli* content of samples taken from Konya WWTP

E.coli to Fecal Coliform ratio is determined to be 71%, 55%, 53% for anaerobic digester influent, anaerobic digester effluent and dewatered sludge, respectively. As can be gathered from Figure 4.4, a certain reduction in the number of indicator bacteria is achieved after the anaerobic digestion process. However, significant increases occur upon the centrifugal dewatering process in such a ratio that lead indicator bacteria to exceed the initial concentrations found in the anaerobic digester influent. These results are parallel with the relevant literature which assert the effect of centrifuge dewatering process on the increased number of indicator bacteria compared to the corresponding anaerobically stabilized sludge (Higgins et al., 2007; WERF 2008; Dentel et al., 2008; Qi et al., 2008). Regarding the land application of biosolids obtained from Konya wastewater treatment plant, increases occur after the dewatering process should be considered even though the *E.coli* removal requirement could be met by the stabilization process applied.

4.2.1.5. Mersin Wastewater Treatment Plant

Mersin wastewater treatment plant is another treatment plant sampled to investigate the effect of centrifuge dewatering on the indicator bacteria content of stabilized sludge. Since the results acquired from Konya wastewater treatment plant showed the occurence of significant increases in the number of both Fecal Coliforms and *E.coli* after dewatering, two different samplings were conducted on samples obtained from Mersin wastewater treatment plant to reveal whether the same trend is avaliable in this treatment plant.

Indicator bacteria concentrations and removal ratios of the samples taken from Mersin wastewater treatment plant are provided below in Table 4.13 through Table 4.15.

Table 4.13. Fecal Coliform Densities in Mersin Wastewater Treatment Plant revealed by Standard Culture Methods

Sampling Date	Fecal Coliform Densities (MPN/gDS)		
	Digester Influent	**Digester Effluent**	**Dewatered Cake**
26.04.2011	2,55E+06	9,00E+04	1,10E+06
08.06.2011	1,07E+07	5,72E+05	2,90E+06
ave	**6,63E+06**	**3,31E+05**	**2,00E+06**

Table 4.14. *E.coli* Densities in Mersin Wastewater Treatment Plant revealed by Standard Culture Methods

Sampling Date	*E.coli* Densities (MPN/gDS)		
	Digester Influent	**Digester Effluent**	**Dewatered Cake**
26.04.2011	1,27E+06	6,20E+04	7,60E+05
08.06.2011	7,40E+06	3,60E+05	1,71E+06
ave	**4,34E+06**	**2,11E+05**	**1,24E+06**

Table 4.15. Indicator Bacteria Removal Efficiencies of Mersin Wastewater Treatment Plant

Sampling Date	Removal Efficieny of Anaerobic Digester	
	Fecal Coliforms (%)	***E.coli* (%)**
26.04.2011	96,47	95,12
08.06.2011	94,65	95,14

As shown in given tables above, a significant reduction in the concentrations of indicator bacteria is achived by anaerobic digestion process applied. Computed removal efficiencies for Fecal Coliforms and *E.coli* indicate approximately 95% reduction in concentrations which is an expected ratio for a typical mesophilic anaerobic digester. However, biosolids obtained from Mersin wastewater treatment plant do not provide a 99% removal in the *E.coli* concentrations. In addition, as also can be gathered from the concentrations of indicator bacteria found in Table 4.13 and 4.14, a certain increase takes place after the centrifuge dewatering process. Therefore, as in Konya wastewater treatment plant, an

additional attention should be paid to the increases occur after dewatering process. Figure 4.5 provides a visual demonstration of the obtained increases.

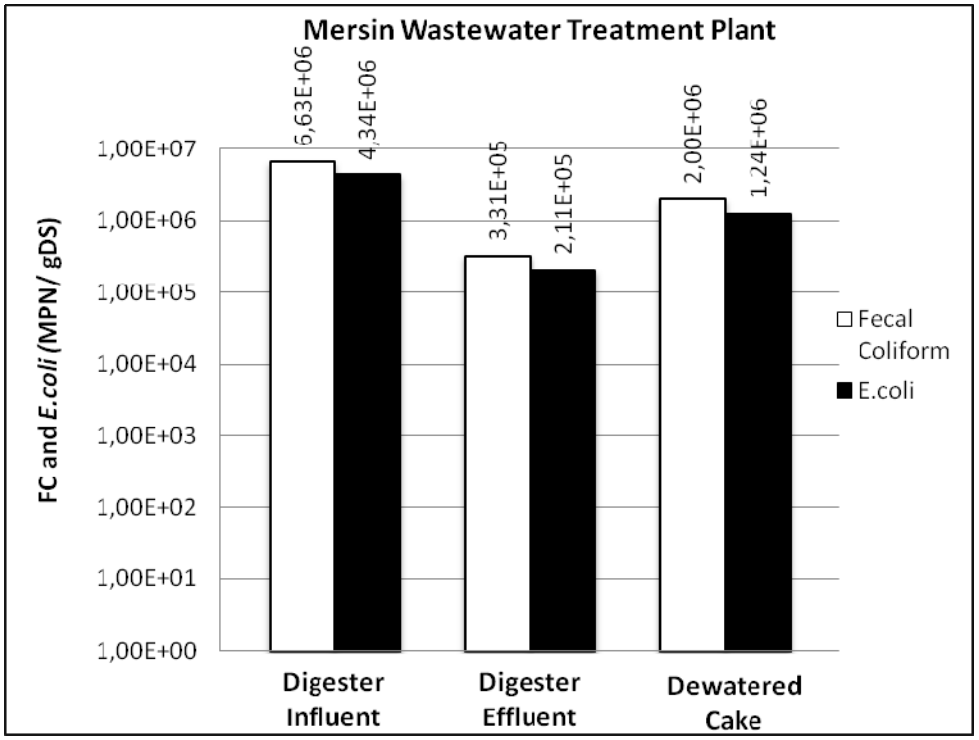

Figure 4.5. Average Fecal Coliform and *E.coli* content of samples taken from Mersin WWTP

E.coli to Fecal Coliform ratios are computed to be 65%, 64% and 67% for anaerobic digester influent, anaerobic digester effluent and dewatered sludge, respectively. As shown in Figure 4.5, increases after dewatering is determined to be valid for Mersin wastewater treatment plant which operates a centrifuge for the dewatering of stabilized sludge.

4.2.1.6. Eskişehir Wastewater Treatment Plant

As being one of the treatment plants operating centrifuge dewatering subsequent to anaerobic digestion process, Eskişehir wastewater treatment plant was investigated in terms of changes occur in the number of indicator bacteria after stabilization and dewatering process. Two samples were taken from the treatment

plant. Results comprising the Fecal Coliform and *E.coli* concentrations are given in Table 4.16 and Table 4.17, respectively.

Table 4.16. Fecal Coliform Densities in Eskişehir Wastewater Treatment Plant revealed by Standard Culture Methods

Sampling Date	Fecal Coliform Densities (MPN/gDS)		
	Digester Influent	**Digester Effluent**	**Dewatered Cake**
08.04.2010	3,34E+07	2,03E+05	9,60E+05
13.05.2010	3,77E+06	2,41E+04	4,57E+05
ave	**1,86E+07**	**1,14E+05**	**7,09E+05**

Table 4.17. *E.coli* Densities in Eskişehir Wastewater Treatment Plant revealed by Standard Culture Methods

Sampling Date	*E.coli* Densities (MPN/gDS)		
	Digester Influent	**Digester Effluent**	**Dewatered Cake**
08.04.2010	1,90E+07	1,35E+05	5,20E+05
13.05.2010	2,15E+06	1,13E+04	2,95E+05
ave	**1,06E+07**	**7,32E+04**	**4,08E+05**

Table 4.16 and Table 4.17 show the indicator bacteria concentrations obtained from digestion and dewatering processes. As can be seen from the influent concentrations, levels of indicator bacteria are relatively high compared to the other treatment plants investigated. However, these densities are similar to the several results reported in literature (Higgins et al., 2007). Nevertheless, the indicator bacteria content of a wastewater is known to be influenced by several factors including geographic region, presence of agro industries and type of sludge treatment process (Andreoli et al., 2007).

The indicator bacteria removal ratios of the Eskişehir wastewater treatment plant are provided below in Table 4.18.

Table 4.18. Indicator Bacteria Removal Efficiencies of Eskişehir Wastewater Treatment Plant

Sampling Date	**Removal Efficieny of Anaerobic Digester**	
	Fecal Coliforms (%)	***E.coli* (%)**
08.04.2010	99,39	99,29
13.05.2010	99,36	99,47

The average removal rate calculated for Eskişehir wastewater treatment plant is determined to be 99 % which is the highest ratio assessed among all the treatment plants investigated in the scope of the study. Even though the concentrations of indicator bacteria reach the levels of 10^7 per gram of unstabilized sludge, the anaerobic digestion process applied provides a major reduction in the concentrations of indicator bacteria.

Eskişehir wastewater treatment plant is the only treatment plant which meet the 99% *E.coli* removal requirement of the implemented regulation for the use of biosolids obtained on the soil. This success of reduction is believed to be related with the long retention times applied in the anaerobic digestion process which is 20 days. Since, SRT values higher than 15 days enhance the growth of slowly growing methanogens, the performance of the system improves correspondingly (Gerardi 2003).

Unfortunately, reduced numbers of indicator bacteria do not persist after the dewatering process as can be gathered from in Figure 4.6. Even though the pathogen removal requirement of the regulation is stasfied by the stabilization process applied, *E.coli* densities increase approximately 1 log after the centrifuge

dewatering operation. Since the implemented regulation does not consider the post stabilization stages, utilization of these sludges might still pose a threat to human health and environment.

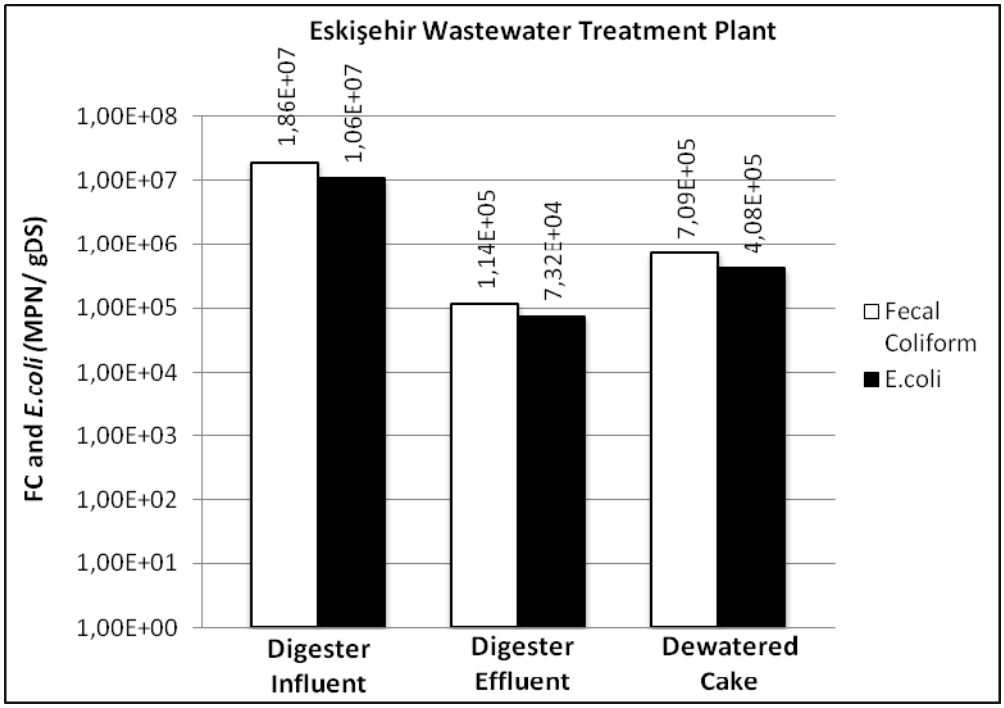

Figure 4.6. Average Fecal Coliform and *E.coli* content of samples taken from Eskişehir WWTP

E.coli is assessed to be 57%, 56% and 59% of Fecal Coliforms in anaerobic digester influent, anaerobic digester effluent and dewatered sludge, respectively. As revealed in Figure 4.6, reduced number of indicator bacteria resulting from anaerobic digestion process applied, increase after the centrifuge dewatering process. This occurence is totally same with the trend of indicator bacteria assessed for both Konya and Mersin wastewater treatment plants which implement centrifuge dewatering process.

Besides revealing an increase in the concentrations of indicator bacteria similar to the other centrifuge applying treatment plants, prominent results obtained from Eskişehir wastewater treatment plant mostly indicate that, high levels of removal

achieved during anaerobic digestion process do not prevent the increases obtained after centrifuge dewatering.

In summary, results obtained by Standard Culture Methods for the treatment plants invesitgated in the scope of the first phase of the study are parallel with the outputs of the relevant literature. In treatment plants which utilize belt filter press after anaerobic digestion process, no increase was observed after dewatering. However, an increase which is either slight or more major, is observed for all of the treatment plants which utilize centrifuge dewatering.

4.2.1.7. Evaluation of the Increases Obtained After Centrifuge Dewatering Process

As revealed by our results on the three of the treatment plants, it is clear that when centrifuge dewatering is coupled with anaerobic digestion process during the sludge treatment, the reduced numbers of indicator bacteria immediately increase after the dewatering processes.

In order to assess whether the amount of increases occuring after centrifuge dewatering was similar to the reported levels in relevant literature, a computation was conducted from the two data points including Fecal Coliform and *E.coli* densities of anaerobically digested sludge and centrifugally dewatered sludge. Thus, the exact amount of increases was determined and evaluated correspondingly. Since the reported increases are broadly expressed as log base, it is preferred here to illustrate these increases as log based numbers to provide an easier comparison.

Table 4.19 represents the increases determined after centrifuge dewatering for treatment plants operating centrifuge dewatering which are Konya, Mersin and Eskişehir Treatment Plants. For the sake of a broader comparison, the results from two other treatment plants (Ankara and Adana) that operate belt filter press dewatering are also included in this table. One should note that a negative number indicates a decrease in concentration (no regrowth) after dewatering whereas a

positive number indicates an increase in concentration (regrowth) after dewatering.

Table 4.19. SCM Based Log Changes Obtained After Dewatering Process Compared to the End of Digestion Concentrations

Sampling Date	Fecal Coliforms	*E.coli*
Ankara WWTP		
04.08.2010	-1,59	-1,51
24.11.2010	-0,09	-0,02
01.12.2010	-0,15	-0,21
02.02.2011	-0,95	-1,15
14.03.2011	-0,66	-0,73
ave*	-0,69	-0,72
Adana WWTP		
23.05.2011	-0,17	-0,33
13.07.2011	-1,07	-0,86
ave*	-0,62	-0,60
Konya WWTP		
17.08.2010	0,77	0,84
21.09.2010	1,85	1,79
28.09.2010	1,68	1,65
18.03.2011	2,47	2,40
ave	1,69	1,67
Mersin WWTP		
26.04.2011	1,09	1,09
08.06.2011	0,71	0,68
ave	0,90	0,88
Eskişehir WWTP		
08.04.2010	0,67	0,59
13.05.2010	1,28	1,42
ave	0,98	1,00

SCM based log changes= SCM (dewatered sludge) - SCM(digested sludge)

*Negative values represent decreases occur in the number of indicator bacteria after belt filter press dewatering process

As gathered in Table 4.19, our results indicated 0,6 to 0,72 log decreases in the number of indicator bacteria after belt filter press dewatering. As discussed earlier, belt filter press dewatering is reported to have no increasing effect on indicator bacteria concentrations of sludge. However, after centrifugal dewatering, concentrations of indicator bacteria are determined to increase by 0.5 to 2,2 logs. Considering the average reactivation rates obtained for Mersin and Eskişehir wastewater treatment plants, it is revealed that the increases obtained are in the range of values reported in the relevant literature. For instance, Qi et al., (2007) reported 0.4-1.6 log increases for six mesophilic anaerobic process investigated whereas 0-1 log increases were stated in the report prepared by WERF (2008).

The level of increase occuring in Konya wastewater treatment plant on the other hand, is determined to be higher than 2 logs on the average which is higher than the reported values in literature for mesophilic anaerobic digesters. One possible reason for higher increases assessed, might be the shorter retention time (11 days) applied in the anaerobic digestion process of Konya wastewater treatment plant. When the obtained increases are evaluated, it is comprehended that more than two folds increases are observed in Konya wastewater treatment plant relative to the other two treatment plants which operate anerobic digesters with SRTs of 20,5 and 20 days. In addition, reported increases which remain 1 to 1.6 logs maximum in the relevant literature are valid for treatment plants applying long SRTs during anaerobic digestion process which is 29-30 days in WERF, whereas 30-35 days in the investigated treatment plants by Qi et al.(2007). Therefore, shorter digestion process of Konya wastewater treatment plant seems to be one of the reasons for relatively high levels of increases obtained after dewatering.

The examination of obtained results lead us to relate the increases with the reactivation of bacteria which is inactivated during anaerobic digestion process as proposed formerly by Higgins et al., (2007). As revealed in relevant literature,

indicator bacteria are not completely removed but partially reduced or inactivated during the anaerobic digestion process (Smith et al., 2005). This inactivation possibly render the bacteria to be non-culturable after anaerobic digestion. As mentioned before, this attitude of bacteria is known as Viable but Not Culturable state. In this state, bacteria have been reported to change their physiology to survive under stressful conditions (Signoretto 2000; Heim 2002). Environmental stressess that cause Viable but Not Culturable State include extreme conditions of temperature, pH, UV irridation, toxic chemicals and oxygen concentrations (Trevors ,2011). As can be understood from the statement of viable but not culturable, those bacteria are considered as viable even though they can not be cultivated by standard culture techniques. However, when the appropriate conditions that favor their growth are provided, they become culturable again.

Since increases are specific to centrifuge dewatering process, it is clear that one of the specific aspects of centrifugal systems promote the growth of indicator bacteria. One of the main differences found between two-types of dewatering system is the enforcement of shear to the sludge during the centrifuge dewatering. As mentioned before, the effect of shear stress was investigated in several researches but no relationship have been found between the shear and improved culturability (Cheung et al., 2003; Qi et al., 2007). However, one more particular distinctiveness found between two methods is the generation of heat in the centrifuges owing to the rotational motion of the centrifugal systems. The centrifuge is a closed system and keep this heat inside. The heat generated during the centrifugation process can raise the temperature.

Therefore, it could be speculated that, this temperature might be essential to support the growth of microorganisms which might be formerly entered in a viable but not culturable state during anaerobic digestion. Considering the typical temperatures of mesophilic anaerobic digestion process which is in the interval of 35°C and 37°C, the heat generated in the centrifuges during dewatering process can lead temperature to rise up to a value which might provide a resuscitation chance for indicator bacteria. In order to check whether this could be correct,

temperature of the dewatered biosolid samples were examined for once in Konya wastewater treatment plant. The temperature of the sludge collected after centrifuge dewatering unit was determined to be 32°C. However, since it was not possible to check temperature during the dewatering process, temperature was determined right after collection of sludges in the collection truck for a period of time. Therefore, this temperature determined is possible to be lower than the exact temperature levels achieved in the centrifuge. As can be seen in Figure 3.4, Konya wastewater treatment plant operates an anaerobic digester which is followed by a thickener and then the sludge enters the dewatering unit. Even though the temperature is around 35°C in the digester, it falls down to ambient temperature values in the thickener. Having this temperature (possibly around 15-20°C on the average year round) sludge enters the dewatering unit. If the sludge temperature can be measured as 32°C (even after some time of waiting in the collection truck) this means that, there is significantly high temperatures are experienced in the centrfiuge. It can be speculated that, this rise in temperature, along with the exposure to more favorable conditions may lead to the increases in microorganism concentration. On the other hand, the belt filter which is a low energy equipment working under ambient temperature does not cause this kind of temperature rises and hence does not cause increases in indicator microorganism concentration.

Such an effect of temperature has not been investigated before in the relevant literature. However, outcomes of the several studies support the hypothesized effect of temperature. For instance, it is reported by Jolis (2006) that, storage of digested samples at 35°C lead reactivation of indicator bacteria without implementing dewatering process to sludge which is an evidence of the effect of temperature in the obtained increases. Moreover, as mentioned before high speed centrifuges are reported to lead greater increases compared to the low speed ones (Qi et al., 2007). It might be speculated that, higher speeds of rotation might result in higher increases in the temperature which lead to higher increases in the number of indicator bacteria as well.

However, since a typical centrifuge dewatering process lasts as short as few minutes, the doubling time of *E.coli* which is 20 minutes is not observed during the centrifugation operation. Therefore, it might be questioned that, even though conditions favor the growth during centrifuge operation, the time interval is not sufficient for the growth of coliforms. However, one should keep in mind that, those bacteria are speculated to be found in a viable but not culturable state. Hence, the case is not a simple multiplication of microorgasims from low concentrations. In the present case, a resuscitation event occurs which does not require long periods of time typically required for doubling of bacteria (Gupte et al., 2003).

In addition, resuscitation of several organisms after temperature changes was already reported. For instance, *Vibrio vulnifucus* (Nilsson et al., 1991), *V. parahaemolyticus* (Bates&Oliver, 2004) were reported to resuscitate after a temperature upshift. In addition, *Salmonella enterica* was resuscitated from the VBNC state after a temperature upshift and nutrient addition (Gupte et al., 2003). However, there is a limited number of studies regarding resuscitation of *E.coli* and results are debated (Arana et al., 2010). Altough, several conditions were investigated in terms of the resuscitation of *E.coli*, the only positive result was observed recently by Pinto et.al., (2010). It is found that, VBNC cells produced at 4°C which were confirmed to be in this state by both culture techniques and microscopic observations, resuscitated after a temperature uphift to 20°C, 30°C and 37°C. Moreover, it is reported that unlike regrowth of culturable cells, resuscitation is possible during a limited time interval and the number of resuscitation event declines gradually (Pinto el at., 2010).

In this context, increases observed after dewatering might be related to the favorable conditions developed during centrifuge dewatering operation. Since both reported results and our inital results obtained by SCMs indicate increases only after the centrifuge dewatering process, it might be speculated that centrifuge dewatering might be providing proper conditions for the reactivation of viable but not culturable bacteria. Since same quantification methodology is applied by an

additional culture independent technique, outcomes of the Quantitative PCR are also expected to give an idea about this occurence by revealing whether the Standard Culture Methods underestimate the number of bacteria due to the uncultivable state they persist.

4.2.2. Enumeration of Indicator Bacteria by Quantitative PCR Technique

As mentioned before, Quantitative PCR technique has been used in several researches in order to provide an insight to the changes occuring after dewatering processes by means of a a culture independent technique. Owing to its wide application in relevant literature, in addition to Standard Culture Methods, samples were analysed with Quantitative PCR analyses as well. Since comparison of results obtained by culture based (SCM) and DNA based (Quantitative PCR) methods is expected to provide an additional understanding about the reactivation of indicator bacteria, results of two methods were evaluated comparatively. In this context, the second part of the results consist of the number of indicator bacteria obtained by Quantitative PCR analysis and evaluation of results obtained by SCM and Quantitative PCR analysis.

E.coli which constitute a major group of indicator bacteria is selected as the indicator bacteria to be monitored by Quantitative PCR. As performed in the Standard Culture Method analyses, samples taken form anaerobic digester influent and effluent and dewatering units of the treatment plants were evaluated for their *E.coli* content by means of Quantitative PCR technique. Analyses were performed as explained in the Materials and Methods part of the study. Determined *E.coli* densities of investigated treatment plants are provided in Table 4.20.

Table 4.20. *E.coli* densities of investigated treatment plants revealed by Quantitative PCR analysis

Sampling Date	*E.coli* Densities (*E.coli*/g TS)		
	Digester Influent	Digester Effluent	Dewatered Cake
Ankara			
04.08.2010	4,94E+07	1,28E+07	1,75E+06
24.11.2010	2,74E+08	1,14E+07	6,82E+06
01.12.2010	2,93E+08	2,04E+07	1,12E+07
02.02.2011	2,50E+07	8,02E+06	5,97E+05
14.03.2011	1,70E+07	6,71E+06	1,18E+06
ave	**1,32E+08**	**1,19E+07**	**4,31E+06**
Adana			
23.05.2011	7,73E+06	2,14E+06	9,23E+05
13.07.2011	1,73E+06	4,92E+06	5,31E+05
ave	**4,73E+06**	**3,53E+06**	**7,27E+05**
Kayseri			
22.12.2010	3,00E+08	9,75E+07	8,73E+06
Konya			
17.08.2010	1,66E+08	5,67E+06	5,80E+07
21.09.2010	1,27E+07	5,00E+06	1,03E+07
28.09.2010	1,72E+07	6,01E+06	8,35E+06
18.03.2011	5,17E+07	4,98E+06	1,54E+06
ave	**6,19E+07**	**5,41E+06**	**1,95E+07**
Mersin			
26.04.2011	4,82E+06	2,56E+06	1,19E+06
08.06.2011	4,85E+07	1,47E+07	1,81E+07
ave	**2,67E+07**	**8,63E+06**	**9,65E+06**
Eskişehir			
08.04.2010	2,11E+07	1,75E+07	5,59E+06
13.05.2010	1,03E+07	3,37E+06	2,29E+06
ave	**1,57E+07**	**1,04E+07**	**3,94E+06**

As can be gathered from Table 4.20, results obtained by Quantitative PCR analyses validate the reduction of microorganisms achieved through anaerobic digestion process impelemented in all of the treatment plants investigated. In Ankara and

Adana wastewater treatment plants similar sludge treatment processes are used and no increases were detected after belt filter dewatering in the number of indicator bacteria by Quantitative PCR analysis. However, results obtained from Kayseri wastewater treatment plant which is the other belt filter press operating plant, differ from the results obtained for Ankara and Adana Wastwater Treatment Plants. As explained before, a completely different sludge treatment system is applied in Kayseri wastewater treatment plant where waste activated sludge is mixed with the digested sludge before dewatering process. However, a reduced number of *E.coli* determined by Quantitative PCR analysis of dewatered sludge sample taken from Kayseri wastewater treatment plant. It is believed to occur due to the reduced amount of DNAs isolated during the extraction procedure. During the DNA extraction from dewatered sludge sample, membrane of the spin column was congested probably due to the high number of microorganisms added with the waste activated sludge. Therefore, a portion of the available DNAs could not be captured by the column and a portion of the available DNA could not be isolated. A similar decrease in the DNA extraction efficiency is speculated by Higgins et al. (2007) as one of the reasons for the low levels of *E.coli* determined by competitive PCR (cPCR) analysis in a centrifugally dewatered sludge sample taken from a mesophilic anaerobic digestion process.

On the other hand, for samples obtained from Konya, Mersin and Eskişehir wastewater treatment plants, Quantitative PCR analyses do not show a common pattern after centrifuge dewatering. For instance, Quantitative PCR results of samples taken from Eskişehir wastewater treatment plant indicate a reduction in the number of *E.coli* after centrifuge dewatering process. However, results obtained for Konya and Mersin wastewater treatment plants vary for different samples.

In Konya wastewater treatment plant, three of the samples indicate occurence of increases whereas the other one indicate a reduction in the number of *E.coli* for dewatered cake sample. Similarly, for Mersin wastewater treatment plant, one of the samples analysed indicate a reduction in the number of *E.coli* after dewatering

process. On the other hand, for the other sample analysed, a decreased concentration of *E.coli* was determined for dewatered cake sample. This variation is observed for the treatment plants investigated in relevant studies in literature. For instance, two different treatment plants which operate mesophilic anaerobic digestion and centrifuge dewatering systems were investigated by Higgins et al., (2007). According to the Quantitative PCR results, one of the treatment plants was determined to exhibit an increase in the number of *E.coli* whereas the other one was determined to show decreases after centrifuge dewatering. Similarly in pathogen report prepared for WERF, for different thermophilic anaerobic digestion processes surveyed, both decreases and increases were determined for particular plants by Real Time PCR analyses. In addition, Higgins et al., (2007) proposed the low yield of DNA extraction procedure and high levels of PCR inhibitors as the possible reasons for the decreases revealed by Competitive PCR (cPCR) after the centrifugal dewatering process.

In order to summarize the changes observed in the *E.coli* concentrations, log based differences exist between Quantitative PCR results of anaerobic digester effluent and dewatered cake samples are provided Table 4.21. Here again, a positive number means an increased concentration, whereas a negative number means a decreased concentration of microorganisms after dewatering.

Table 4.21. Q-PCR based Log Changes Obtained by After Dewatering Process Compared to the End of Digestion Concentrations

Treatment Plants	Ankara WWTP	Adana WWTP	Konya WWTP	Mersin WWTP	Eskişehir WWTP
Log Changes in *E.coli* (ave)	-0,65	-0,67	0,24	-0,12	-0,33

As can be seen from the computed changes in *E.coli* concentrations after dewatering processes, results obtained by Quantitative PCR analysis do not follow a typical trend particularly for centrifuge dewatering systems. Both literature and our results indicate that, variations can be related to a number of different factors

including operational differences exist in the treatment plants, efficieny of the DNA extraction protocols and existence of high levels of inhibitor substances.

It is clear that both the bacterial concentrations and the level of increases obtained vary between the results by standard culture methods and Quantitative PCR. In order to gain a better understanding about the differences and explain the possible reasons, results obtained by two enumeration methods are evaluated comparatively in the following section.

4.2.2.1. Evaluation of the Results Obtained by Standard Culture Methods and Quantitative PCR

The examination of results obtained by a culture based and non-culture based method first of all allows us to reveal whether cultivation techniques applied underestimate the bacteria available in the sample materials owing to the Viable but Not Culturable portion of bacteria which might be induced during anaerobic digestion process. In addition, comparison of results obtained for cake samples dewatered by either belt filter press or centrifuge dewatering processes provide an insight to the increases observed by both of the enumeration techniques. Therefore, results obtained by Standard Culture Methods for both Fecal Coliforms and *E.coli* along with the *E.coli* concentrations revealed by Q-PCR for all of the treatment plants investigated are given in Table 4.22 for comparison purposes.

Table 4.22. Results obtained by SCM and Quantitative PCR for treatment plants investigated

SAMPLING DATE	DIGESTER INFLUENT			DIGESTER EFFLUENT			DEWATERED CAKE		
ANKARA WWTP									
	FC SCM	EC SCM	EC Q-PCR	FC SCM	EC SCM	EC Q-PCR	FC SCM	EC SCM	EC Q-PCR
04.08.2010	2,51E+05	7,98E+04	4,94E+07	3,09E+04	1,92E+04	1,28E+07	8,00E+02	6,00E+02	1,75E+06
24.11.2010	4,46E+07	7,70E+06	2,74E+08	2,20E+06	5,00E+05	1,14E+07	1,80E+06	4,80E+05	6,82E+06
01.12.2010	3,86E+07	2,20E+07	2,93E+08	1,13E+06	7,10E+05	2,04E+07	8,00E+05	4,40E+05	1,12E+07
02.02.2011	4,45E+07	1,99E+07	2,50E+07	1,70E+06	1,13E+06	8,02E+06	1,90E+05	8,00E+04	5,97E+05
14.03.2011	1,42E+07	8,40E+06	1,70E+07	6,00E+05	5,37E+05	6,71E+06	1,30E+05	1,01E+05	1,18E+06
ave	2,84E+07	1,16E+07	1,32E+08	1,13E+06	5,79E+05	1,19E+07	5,84E+05	2,20E+05	4,31E+06
ADANA WWTP									
23.05.2011	3,49E+07	7,55E+06	7,73E+06	1,01E+05	7,30E+04	2,14E+06	6,90E+04	3,40E+04	9,23E+05
13.07.2011	1,34E+06	9,25E+05	1,73E+06	1,28E+05	6,50E+04	4,92E+06	1,10E+04	9,00E+03	5,31E+05
	1,81+07	4,24E+06	4,73E+06	1,15E+05	6,90E+04	3,53E+06	4,00E+04	2,15E+04	7,27E+05
KAYSERİ WWTP									
22.12.2010	1,45E+07	1,70E+05	3,00E+08	5,83E+05	1,30E+05	9,75E+07	1,87E+07	3,00E+05	8,73E+06
KONYA WWTP									
17.08.2010	3,70E+05	2,90E+05	1,66E+08	1,50E+05	7,00E+04	5,67E+06	8,80E+05	4,80E+05	5,80E+07
21.09.2010	8,40E+05	6,13E+05	1,27E+07	1,90E+04	8,00E+03	5,00E+06	1,33E+06	4,90E+05	1,03E+07
28.09.2010	1,08E+06	9,27E+05	1,72E+07	4,90E+04	3,30E+04	6,01E+06	2,33E+06	1,49E+06	8,35E+06
18.03.2011	8,04E+06	3,94E+06	5,17E+07	2,50E+05	1,67E+05	4,98E+06	7,34E+07	4,18E+07	1,54E+06
ave	2,58E+06	1,44E+06	6,19E+07	1,17E+05	6,95+04	5,41E+06	1,95E+07	1,11E+07	1,95E+07
MERSİN WWTP									
26.04.2011	2,55E+06	1,27E+06	4,82E+06	9,00E+04	6,20E+04	2,56E+06	1,10E+06	7,60E+05	1,19E+06
08.06.2011	1,07E+07	7,40E+06	4,85E+07	5,72E+05	3,60E+05	1,47E+07	2,90E+06	1,71E+06	1,81E+07
ave	6,63E+06	4,34E+06	2,67E+07	3,31E+05	2,11E+05	8,63E+06	2,00E+06	1,24E+06	9,65E+06
ESKİŞEHİR WWTP									
08.04.2010	3,34E+07	1,90E+07	2,11E+07	2,03E+05	1,35E+05	1,75E+07	9,60E+05	5,20E+05	5,59E+06
13.05.2010	3,77E+06	2,15E+06	1,03E+07	2,41E+04	1,13E+04	3,37E+06	4,57E+05	2,95E+05	2,29E+06
ave	1,86E+07	1,06E+07	1,57E+07	1,14E+05	7,32E+04	1,04E+07	7,09E+05	4,08E+05	3,94E+06

The comparative results obtained for treatment plants operating belt filter press process are given in Figure 4.7 and Figure 4.8 for treatment plants of Ankara and Adana, respectively. Since the treatment operation applied in Kayseri wastewater treatment plant completely differs, it was not investigated for the comparison purposes.

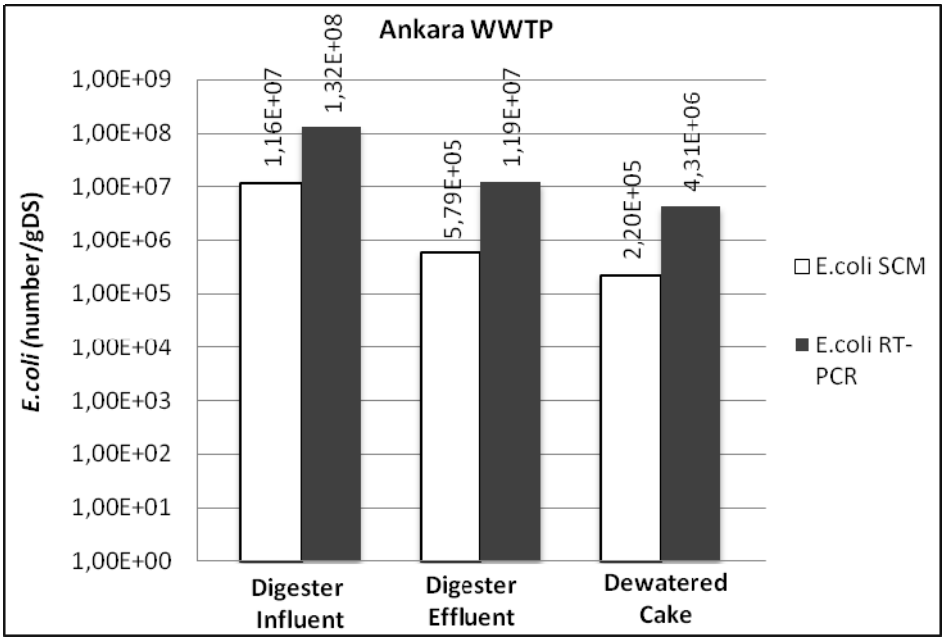

Figure 4.7. SCM and Q-PCR Results for Ankara WWTP

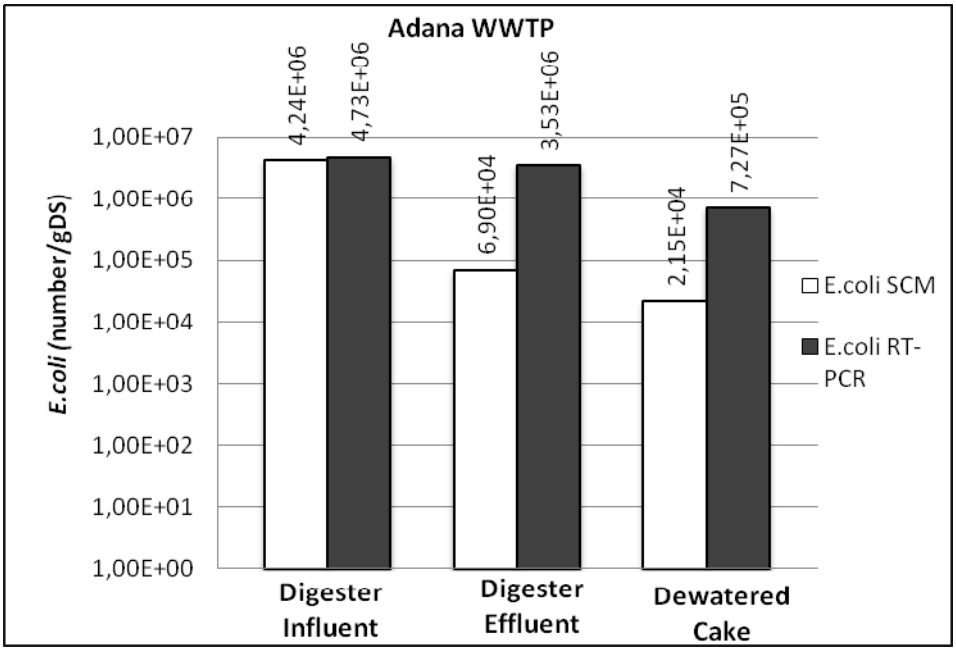

Figure 4.8. SCM and Q-PCR Results for Adana WWTP

As can be gathered from the bar graphs representing different sampling stages, results obtained by Quantitative PCR are one to two logs higher than SCM in all of the three sludge samples of Ankara treatment plant. Although, the first thing coming to mind is the existence of bacteria in VBNC state, since the differences are valid for all three of the stages of the treatment and the concentrations are not getting particularly higher after dewatering, it is clear that the reason behind the observed variations is not only the existence of VBNC state of bacteria. Instead, the observed differences are believed to be caused partially by the high numbers of dead bacteria present in the sludge samples used. The difference between the SCM and Quantitative PCR results are comparatively smaller in digester influent; whereas this difference increases in the digester effluent. The digestion process reduces the number of bacteria by either killing them or by inactivating them. If bacteria are killed; it will be impossible to quantifiy them by SCM. In this situation, there is not necessarily a problem about the SCM. The Quantitative PCR would still quantify these bacteria since it is based on DNA and does not differentiate between dead and live cells. The higher difference in the digester effluent indicates that, bacteria got either killed and therefore can not be cultured during the SCM or the

bacteria entered a VBNC state and became non-culturable even though they are alive.

In Adana wastewater treatment plant, 2 logs and 1 log differences exist for digester effluent and dewatered cake samples, respectively. However, for the digester influent samples, results obtained by SCM and Quantitative PCR are almost the same. Although it depends on the type of operations applied before the anaerobic digestion process, low numbers of available dead cells are expected to be found in the system before the anaerobic digestion process. Therefore, a contribution resulting from dead cells is not expected to happen for particularly digester influent samples as observed in Adana wastewater treatment plant which indirectly evidence the contribution of dead cells to the results obtained by Quantitative PCR. Since Quantitative PCR method performed did not discriminate DNAs originating from dead and alive bacteria, differences obtained in belt filter press operating plants are believed to arise from the amplification of DNAs resulting from non-viable cells during the Quantitative PCR analysis.

In summary, since Quantitative PCR method performed did not discriminate DNAs originating from dead and alive bacteria, differences obtained in belt filter press operating plants are believed partly to arise from the amplification of DNAs resulting from non-viable cells during the Quantitative PCR analysis. The presence of DNAs originating from non-viable cells along with the contribution of those to the results obtained by DNA based methods were discussed by several authors before. For instance, Wery et al., (2008) reported 1.8-2 log differences between Real Time PCR and SCM results for the assessed number of indicator bacteria found in dewatered cake samples and concluded that besides DNA from viable cells, values obtained by Real Time PCR also include extracellular DNA and DNA originating from non-viable cells.

On the other hand, the comparative results of SCM and Quantitative PCR illustrated for treatment plants which operate anaerobic digestion and centrifuge

dewatering sequentially are given in Figure 4.9, Figure 4.10 and Figure 4.11 for Konya, Mersin and Eskişehir wastewater treatment plants, respectively.

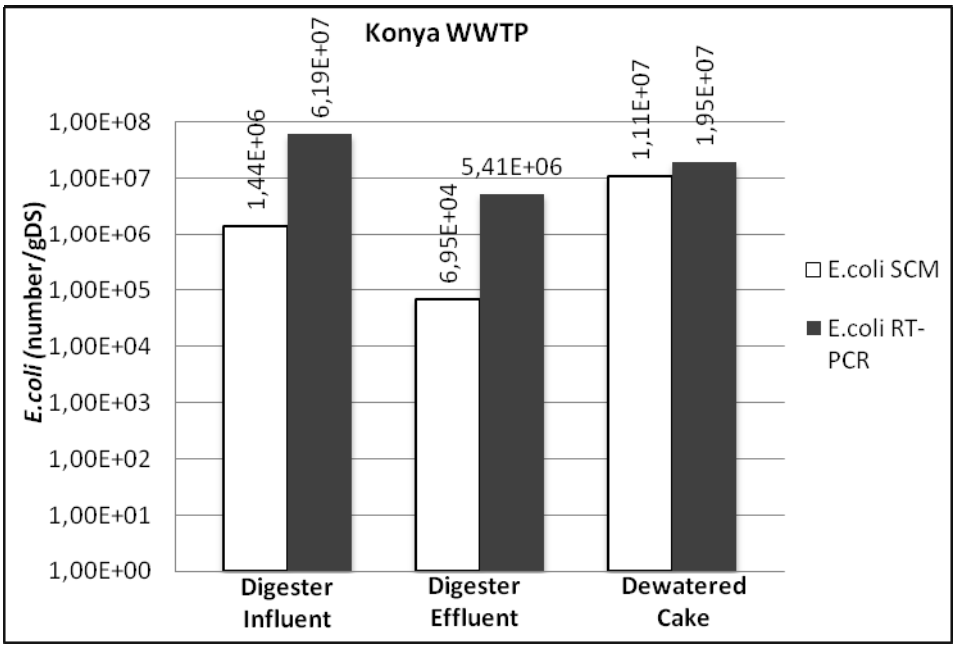

Figure 4.9. SCM and Q-PCR Results for Konya WWTP

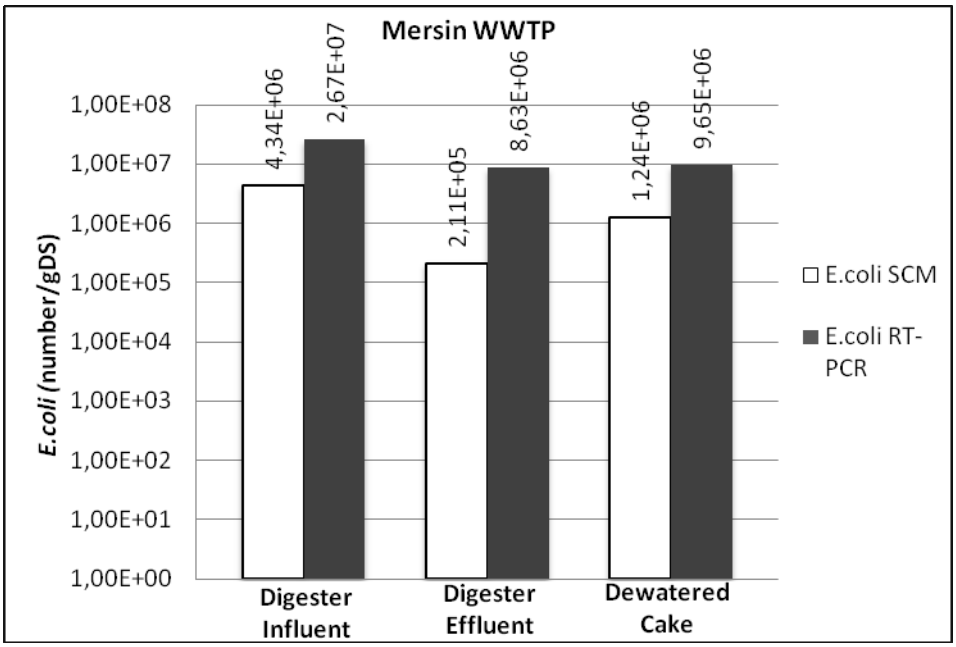

Figure 4.10. SCM and Q-PCR Results for Mersin WWTP

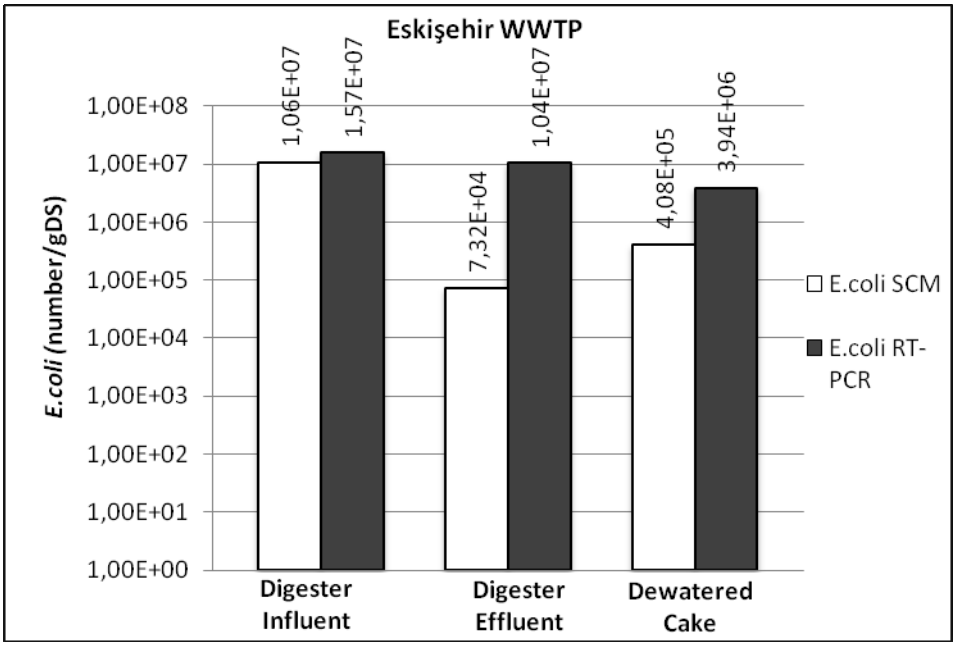

Figure 4.11. SCM and Q-PCR Results for Eskişehir WWTP

When results obtained for centrifuge operating systems are examined, it is easily concluded that differences exist between SCM and Quantitative PCR vary through the anaerobic digestion effluent and dewatered cake samples. Contribution of dead DNAs to the obtained results might be suspected since it is known that the existence of dead cells cause a false positive result on the enumeration of indicator bacteria during Quantitative PCR analyses as explained previously. It was reported in the literature for several times that, even though providing a valuble tool for detecting bacteria, molecular approaches overestimate the number of enumerated organisms since these methods rely on the enumeration of whole available DNA in the environment without distinguishing DNAs originating from live and dead cells (Taskin et al., 2011; WERF 2008; Wery et al., 2008). However, in the case of belt filter press dewatering which is also expected to have high numbers of dead cells available, no major difference exists between the results of SCM and Quantitative PCR for anaerobically digested sludge samples and dewatered sludge samples. In addition, the other reason for the existing differences obtained by SCM and Quantitative PCR could be due to the presence of VBNC bacteria as well.

Therefore, a more detailed examination of the differences is believed to bring an explanation to this inconsistent trend of two enumeration methods for belt filter press and centrifuge dewatering processes. Therefore, results were converted to log bases and the differences between SCM and Quantitative PCR were calculated. The differences between log based results obtained by SCM and Quantitative PCR for each of the sampling from digester influent, digester effluent and dewatered sludge samples are presented in Table 4.23. In this table a positive number indicates that the Quantitative PCR method gives a higher measurement compared to SCM. The higher the positive number is, the higher is the difference between the two methods. On the other hand, the one single negative number that is given for Konya sample shows that the SCM gives a larger measurement compared to Quantitative PCR.

Table 4.23. Log based differences obtained between Standard Culture Methods and Quantitative PCR(*)

Sampling Date	Digester Influent	Digester Effluent	Dewatered Cake
	Ankara WWTP		
04.08.2010	2,79	2,82	3,46
24.11.2010	1,55	1,36	1,15
01.12.2010	1,12	1,46	1,41
02.02.2011	0,10	0,85	0,87
14.03.2011	0,31	1,10	1,07
ave	**1,17**	**1,52**	**1,59**
	Adana WWTP		
23.05.2011	0,01	1,47	1,43
13.07.2011	0,27	1,88	1,77
ave	**0,14**	**1,67**	**1,60**
	Konya WWTP		
17.08.2010	2,76	1,91	1,82
21.09.2010	1,18	2,42	1,32
28.09.2010	1,27	2,26	0,75
18.03.2011	1,12	1,47	-1,43
ave	**1,58**	**2,02**	**1,30**
	Mersin WWTP		
26.04.2011	0,58	1,62	0,20
08.06.2011	0,82	1,61	1,02
ave	**0,79**	**1,61**	**0,61**
	Eskişehir WWTP		
08.04.2010	0,04	2,11	1,03
13.05.2010	0,68	2,47	0,89
ave	**0,36**	**2,29**	**0,96**

(*) Log based differences= log(SCM)-log(Q-PCR)

The log based differences given in the Table 4.23 show the general variation tendency of differences obtained by Quantitative PCR analyses and Standard Culture Methods. Since the indicator bacteria content of digester influent varies

owing to the characteristics of wastewater inflow, even though several sampling has been conducted, the number of bacteria assessed do not follow a consistent trend. Therefore, the assessed differences between two enumeration techniques are also expected to vary. However, evaluating the average differences obtained distinctively for treatment plants operating belt filter press and centrifuge dewatering, a consistent trend can be gathered.

In all of the treatment plants, 0.1 to 1.5 log differences exist between results obtained by SCM and Quantitative PCR in the digester influent samples. In the effluent samples on the other hand, differences get higher due to the inactivation of bacteria which is consistent with the purpose of the digestion. These log based differences are higher than some of the reported differences obtained from the effluent of mesophilic anaerobic digesters. For instance, in their study Higgins et al., (2007) reported 1 log difference whereas, 0 to 1 log differences between Real Time PCR and SCM results were reported by WERF (2008) for six of the investigated treatment plants throughout the study. However, Wery et al., (2008); reported 3.4 log difference between the results obtained by SCM and Real Time PCR. Therefore, it is concluded that, the obtained differences vary from plant to plant depending on the applied wastewater and sludge treatment process.

After the anaerobic digestion process, differences get higher which is an expected result since indicator bacteria are either inactivated and entered a VBNC state or removed (killed) by the anaerobic digestion process. Which one of this two mechanisms is the exact reason for the determined difference can not be identified from the results. DNAs originating from dead cells might be contributing to the results obtained by Quantitative PCR analysis and eventually cause an over-estimated result. On the other hand, the difference might be related with the bacteria which entered the VBNC state during anaerobic digestion process. Since they can not be enumerated by SCM, the higher differences obtained is possible to be correlated with Viable but Not Culturable bacteria.

The differences obtained after dewatering process bring some further explanation to this picture. As revealed in Table 4.23, the differences between SCM and Quantitative PCR remain almost the same after the belt filter press process applied in Ankara and Adana wastewater treatment plants. This is acceptable since in the case of VBNC bacteria, it is hypothesized that, belt filter press process does not favour their reactivation. Therefore, the difference obtained can be accepted as from DNAs originating from dead cells. Although several studies have reported the rapid degredation of DNA in wastewater systems (Ruiz et al., 2010) it is a debate since others suggest that, when DNAs are bound to surfaces, the degredation slows down (Demaneche et al.,2001). If the latter is correct, as stated by pathogen report of WERF (2008) it could be expected for DNAs to remain in the sludge for a long time, due to the high concentrations of solid particles found in the sludge samples.

On the contrary, the differences between Quantitative PCR and SCM gets lower with an interval of 0,7-1 after the centrifuge dewatering operation applied in the Konya, Mersin and Eskişehir wastewater treatment plants. This decrease in the obtained differences is believed to be the evidence for reactivation of bacteria which has been speculated to enter to Viable but Not Culturable State due to several stress imposing conditions generated during anaerobic digestion process as proposed by several researchers (Qi et al., 2004; Higgins et al., 2007; Dunaev et al., 2008; Taskin et al., 2011). By knowing that majority of the dead cells' DNAs are also measured in Quantitative PCR, one can assume that, total DNA measured by Quantitative PCR should not vary too much within a treatment plant. If the difference between SCM and Quantitative PCR gets smaller due to the increase observed in results obtained by SCM, it can be judged that, cultivability of the microorganisms increase. This can be taken as an indication of VBNC state of bacteria. Nevertheless, it is believed that there is a portion of dead cells of which quantity is still an unknown. Therefore, it is speculated that, the reported increases after dewatering by centrifuge dewatering primarly occur due to the reactivation of Viable but Not Culturable Bacteria. As mentioned before, it is speculated that, the

heat generated during centrifuge dewatering process might be inducing indicator bacteria to reactivate and become culturable again. However, the impact of DNAs originating from dead cells has to be considered as well.

CHAPTER 5

CONCLUSION

In this study, it is aimed to examine different wastewater treatment plants to be able to assess the microorganism reactivation potential in stabilized sludge during dewatering process. For this purpose, six treatment plants; three operating belt filter press and three operating centrifuge dewatering are investigated. Samples from influent and effluent of anaerobic digesters along with the dewatered cake samples were analyzed for their indicator bacteria content. The enumerations of Fecal Coliforms and *E.coli* were conducted by means of both Standard Culture Methods and Quantitative PCR. Since Standard Culture Methods are questioned in terms of possible underestimation of bacteria which are hypothesized to enter Viable but Not Culturable state during the anaerobic digestion process, Quantitative PCR was conducted as an alternative tool which is a culture independent method that can bring a new perspective for the reactivation phenomena.

Conclusions of the study are multifaceted since different phases of treatment plants were investigated by means of different enumeration techniques. Regarding the obtained results, conclusions drawn in this study can be summarized as follows.

Preliminary results obtained by Membrane Filter method was assessed to be inappropriate for the analysis of Fecal Coliforms and *E.coli* that exist in sewage sludge owing to the high turbidity and large numbers of non-coliform background bacteria they comprise.

Treatment plants investigated in the scope of the study achieve 95% to 99% removal for Fecal Coliforms and *E.coli* by means of the mesophilic anaerobic digesters operated. The removal amounts vary depending on the operational parameters of the anaerobic digestion processes conducted. The highest removal rate (99,4%) is obtained for Eskişehir wastewater treatment plant which operate the anaerobic digester at 37°C and with an SRT of 20, 5 days.

Relevant regulation regarding the land application of sludges for agricultural purposes requires 2 log (99 %) reduction in E.coli content of sludge with the stabilization process applied. However, none of the treatment plants except for Eskişehir wastewater treatment plant meets that criterion.

Ankara, Adana and Kayseri wastewater treatment plants using belt filter dewatering investigated in this study provide data for indicator bacteria content after dewatering of anaerobically stabilized sludge. In Ankara and Adana wastewater treatment plants, the reduced concentrations of indicator bacteria, does not change any trend after the belt filter press dewatering. Since raw sludge is mixed with stabilized sludge immediately before dewatering operation, regardless of the belt filter press dewatering, in Kayseri wastewater treatment plant, higher numbers of indicator bacteria are determined after belt filter press dewatering. However, in Konya, Mersin and Eskişehir wastewater treatment plants which apply centrifuge dewatering to the stabilized sludge, although a significant reduction is achieved by anaerobic digestion process, the indicator bacteria of sludge increase immediately after centrifuge dewatering operation.

Examination of the log based increases indicates in Konya wastewater treatment plant, the increases are significantly higher compared to other centrifuge operating plants. This is thought to originate from the relatively lower SRT applied in Konya wastewater treatment plant. Eskişehir and Mersin wastewater treatment plants are operated very similar to each other and the results show that indicator bacteria

increases expressed as log differences are pretty much the same for both of the treatment plants.

It is possible that bacteria may enter in a Viable but Not Culturable (VBNC) state during anaerobic digestion. Since one of the main differences that exist between belt filter press and centrifuge dewatering is the heat generated and temperature rise during centrifuge dewatering process, it is speculated in this study that, this temperature increase might lead to the reactivation of indicator bacteria which are formerly found in VBNC state.

In order to provide a better understanding about the increases obtained by culture methods, samples were analysed for their indicator bacteria content by an additional culture independent enumeration method. For this purpose, *E.coli* content of samples was determined by the Quantitative PCR analysis as well. Results obtained by Quantitative PCR did not indicate any particular trend for belt filter press and centrifuge dewatering processes as in SCM. Therefore, results obtained by two enumeration methods were evaluated comparatively. Comparison of results obtained by Quantitative PCR and SCM showed that, Quantitative PCR method gives 1-2 order of magnitude higher numbers of bacteria. However, since these differences is valid for all of the treatment plants investigated regardless of the dewatering process applied, it is believed that DNAs originating from dead cells are also amplified during the Quantitative PCR analysis and lead to the overestimation of indicator bacteria. However, comparative evaluation of the differences obtained for belt filter press and centrifuge dewatering processes indicate that, in Konya, Mersin and Eskişehir wastewater treatment plants which operate centrifuge dewatering system, differences between SCM and Quantitative PCR results are lower which can be considered as an indication of reactivation of VBNC bacteria after centrifuge dewatering.

Obtained increases are critical for potential health risks that may be posed by the land application of sludge. Even if the indicator bacteria limits set by the current

regulation would be met, due to reactivation, the higher indicator and pathogen contents would be possible for sludges coming from centrifuge operating plants. Therefore, the reactivation potential of indicator bacteria should be taken into account in the scope of the regulations concerning the final use and disposal of sludge. In addition, the convenience of the Fecal Coliforms and *E.coli* as indicator bacteria and the accuracy of results rely on those bacteria should be re-examined by considering the Viable but Not Culturable State of these bacteria as well.

CHAPTER 6

RECOMMENDATIONS FOR THE FUTURE STUDIES

The findings of this study reveal that during anaerobic digestion process indicator bacteria might be enter to a Viable but Not Culturable state owing to several stress conditions. Subsequently, centrifuge dewatering of stabilized sludge lead to reactivation of indicator bacteria exist in stabilized sludge. The proposed mechanism for the resuscitation of bacteria is speculated to be owing to the heat generated during centrifuge processing which is determined to be the main reason for reactivation of several group of bacteria before. However, the exact mechanisms regarding the entrance of Fecal Coliforms to Viable but Not Culturable state and resuscitation to culturable phase are still debated. Therefore, understanding of these mechanisms specifically for coliform bacteria is believed to be supportive for the explanation of obtained increases after centrifuge dewatering process.

In addition, since differentiation of viable and non-viable cells is not intended in the scope of our study, the portion of viable and non-viable cells were estimated indirectly. However, the discrimination of dead, alive and VBNC bacteria is pretty significantly vital in terms of the understanding the reactivation mechanism. In recent years, several techniques were proposed to differentiate DNAs originating from viable cells from the non-viable cells including nucleic acid staining with fluorescence-based dyes; LIVE/DEAD test kits and molecular probes (Khan et al.,2010). Most of these techniques, typically rely on the membrane integrity of viable cells (Taskin et al., 2011). However, besides the requirement of a extensive optimization for the procedure, it is also a known fact that these dyes can be

incorporated with viable cells and cause misleading results (Khan et al, 2010). One of the other molecular approaches providing a tool for the discrimination of viable and non-viable cells is the utilization of the Reverse Transcriptase PCR which provides the live-dead differentiation since m-RNA synthesis is one of strongest signals of viability (Conway & Schoolnik, 2003). However, considering the unstable behaviour of RNA and the difficulty of RNA extraction from environmental matrices, the credibility of RNA based techniques is questioned (Gedalanga and Olson, 2009). Therefore, in order to examine the effect of Viable but Not Culturable cells to the reported increases after dewatering, a proper and practical way for discrimation of dead, live and VBNC bacteria has to be developed.

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APPENDIX A

SUMMARY OF FECAL COLIFORM AND *E.COLI* ENUMERATION PROCEDURE

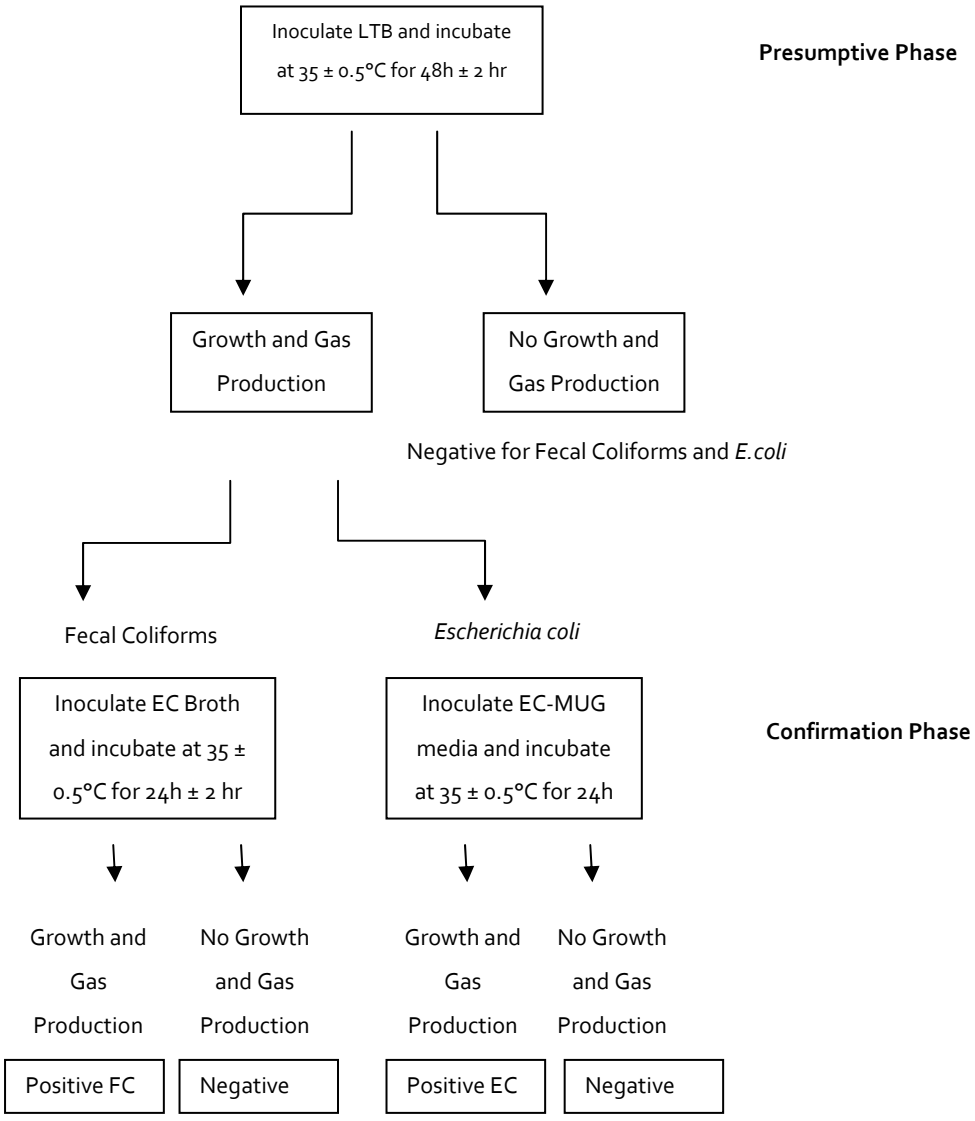

Figure A.1. Summary of Fecal Coliform and *E.coli* Analysis

APPENDIX B

MPN INDEX AND SAMPLE COMPUTATION FOR THE ASSESSMENT OF BACTERIAL CONTENT OF SLUDGE

Table B.1. MPN Index and 95% Confidence Limits for Various Combinations of Positive Results (EPA Method 1680, 2006)

Combination of Positives	MPN Index mL	95% Confidence Limits		Combination of Positives	MPN Index mL	95% Confidence Limits	
		Lower	Upper			Lower	Upper
0-0-0	<0.1803			1-3-0	0.83	0.12	1.96
0-0-1	0.18	0.03	0.63	1-3-1	1.04	0.20	2.43
0-0-2	0.36	0.03	1.01	1-3-2	1.25	0.29	2.96
0-0-3	0.54	0.03	1.37	1-3-3	1.47	0.38	3.64
0-0-4	0.72	0.08	1.74	1-3-4	1.69	0.48	4.60
0-0-5	0.91	0.15	2.12	1-3-5	1.91	0.57	5.66
0-1-0	0.18	0.03	0.63	1-4-0	1.05	0.21	2.45
0-1-1	0.36	0.03	1.01	1-4-1	1.27	0.30	3.00
0-1-2	0.55	0.03	1.38	1-4-2	1.48	0.39	3.70
0-1-3	0.73	0.08	1.75	1-4-3	1.70	0.48	4.68
0-1-4	0.91	0.15	2.14	1-4-4	1.93	0.58	5.75
0-1-5	1.10	0.23	2.56	1-4-5	2.15	0.67	6.57
0-2-0	0.37	0.03	1.02	1-5-0	1.28	0.30	3.03
0-2-1	0.55	0.03	1.39	1-5-1	1.50	0.40	3.75
0-2-2	0.74	0.08	1.76	1-5-2	1.72	0.49	4.77
0-2-3	0.92	0.15	2.15	1-5-3	1.95	0.58	5.83
0-2-4	1.11	0.23	2.58	1-5-4	2.17	0.68	6.64
0-2-5	1.29	0.31	3.07	1-5-5	2.40	0.77	7.31
0-3-0	0.56	0.03	1.40	2-0-0	0.45	0.03	1.19
0-3-1	0.74	0.09	1.77	2-0-1	0.68	0.06	1.64
0-3-2	0.93	0.16	2.17	2-0-2	0.91	0.15	2.13
0-3-3	1.12	0.23	2.60	2-0-3	1.15	0.25	2.69
0-3-4	1.30	0.31	3.10	2-0-4	1.39	0.35	3.38
0-3-5	1.49	0.39	3.72	2-0-5	1.64	0.46	4.37
0-4-0	0.75	0.09	1.79	2-1-0	0.68	0.06	1.66
0-4-1	0.94	0.16	2.19	2-1-1	0.92	0.15	2.16
0-4-2	1.12	0.24	2.63	2-1-2	1.16	0.25	2.72
0-4-3	1.31	0.32	3.13	2-1-3	1.41	0.36	3.43
0-4-4	1.50	0.40	3.77	2-1-4	1.66	0.46	4.47
0-4-5	1.69	0.48	4.62	2-1-5	1.92	0.57	5.71
0-5-0	0.94	0.16	2.21	2-2-0	0.93	0.16	2.18
0-5-1	1.13	0.24	2.65	2-2-1	1.18	0.26	2.76
0-5-2	1.33	0.32	3.17	2-2-2	1.43	0.36	3.49
0-5-3	1.52	0.40	3.82	2-2-3	1.68	0.47	4.56
0-5-4	1.71	0.48	4.70	2-2-4	1.94	0.58	5.81
0-5-5	1.90	0.56	5.63	2-2-5	2.21	0.69	6.75
1-0-0	0.20	0.03	0.68	2-3-0	1.19	0.26	2.79
1-0-1	0.40	0.03	1.08	2-3-1	1.44	0.37	3.55
1-0-2	0.60	0.03	1.49	2-3-2	1.70	0.48	4.67
1-0-3	0.81	0.11	1.91	2-3-3	1.97	0.59	5.91
1-0-4	1.01	0.19	2.36	2-3-4	2.23	0.70	6.83
1-0-5	1.22	0.28	2.87	2-3-5	2.51	0.82	7.59
1-1-0	0.40	0.03	1.09	2-4-0	1.46	0.38	3.61
1-1-1	0.61	0.03	1.50	2-4-1	1.72	0.49	4.77
1-1-2	0.81	0.11	1.92	2-4-2	1.99	0.60	6.00
1-1-3	1.02	0.19	2.38	2-4-3	2.26	0.72	6.92
1-1-4	1.23	0.28	2.90	2-4-4	2.54	0.83	7.68
1-1-5	1.44	0.37	3.54	2-4-5	2.82	0.94	8.36
1-2-0	0.61	0.03	1.51	2-5-0	1.74	0.50	4.88
1-2-1	0.82	0.12	1.94	2-5-1	2.01	0.61	6.10
1-2-2	1.03	0.20	2.40	2-5-2	2.29	0.73	7.00
1-2-3	1.24	0.29	2.93	2-5-3	2.57	0.84	7.76
1-2-4	1.46	0.38	3.59	2-5-4	2.86	0.95	8.45
1-2-5	1.67	0.47	4.51	2-5-5	3.15	1.07	9.10

Table B.1. Continued

3-0-0	0.79	0.10	1.88	4-3-0	2.71	0.90	8.09
3-0-1	1.06	0.21	2.46	4-3-1	3.26	1.11	9.34
3-0-2	1.35	0.33	3.23	4-3-2	3.86	1.32	10.60
3-0-3	1.65	0.46	4.40	4-3-3	4.51	1.54	11.92
3-0-4	1.96	0.59	5.89	4-3-4	5.21	1.76	13.31
3-0-5	2.29	0.73	6.99	4-3-5	5.93	1.96	14.77
3-1-0	1.07	0.22	2.50	4-4-0	3.35	1.14	9.53
3-1-1	1.37	0.34	3.29	4-4-1	3.98	1.37	10.84
3-1-2	1.67	0.47	4.52	4-4-2	4.66	1.59	12.23
3-1-3	1.99	0.60	6.01	4-4-3	5.39	1.81	13.68
3-1-4	2.32	0.74	7.10	4-4-4	6.15	2.02	15.21
3-1-5	2.67	0.88	8.00	4-4-5	6.93	2.23	16.81
3-2-0	1.38	0.35	3.35	4-5-0	4.11	1.41	11.11
3-2-1	1.70	0.48	4.64	4-5-1	4.83	1.64	12.56
3-2-2	2.02	0.62	6.13	4-5-2	5.59	1.87	14.09
3-2-3	2.36	0.76	7.20	4-5-3	6.39	2.09	15.70
3-2-4	2.71	0.90	8.10	4-5-4	7.22	2.30	17.39
3-2-5	3.08	1.04	8.94	4-5-5	8.06	2.50	19.16
3-3-0	1.72	0.49	4.77	5-0-0	2.40	0.76	7.63
3-3-1	2.05	0.63	6.24	5-0-1	3.14	1.06	9.08
3-3-2	2.40	0.77	7.31	5-0-2	4.27	1.46	11.42
3-3-3	2.76	0.92	8.21	5-0-3	5.78	1.92	14.46
3-3-4	3.13	1.06	9.06	5-0-4	7.59	2.39	18.16
3-3-5	3.52	1.20	9.89	5-0-5	9.53	1.65	22.34
3-4-0	2.09	0.64	6.36	5-1-0	3.29	1.12	9.40
3-4-1	2.44	0.79	7.42	5-1-1	4.56	1.56	12.02
3-4-2	2.81	0.93	8.33	5-1-2	6.31	2.07	15.53
3-4-3	3.19	1.08	9.18	5-1-3	8.39	2.57	19.85
3-4-4	3.58	1.23	10.02	5-1-4	10.62	3.04	24.85
3-4-5	3.99	1.37	10.86	5-1-5	12.93	3.04	30.90
3-5-0	2.48	0.80	7.53	5-2-0	4.93	1.67	12.76
3-5-1	2.86	0.95	8.44	5-2-1	7.00	2.24	16.94
3-5-2	3.25	1.10	9.31	5-2-2	9.44	2.80	22.13
3-5-3	3.65	1.25	10.17	5-2-3	12.05	3.31	28.43
3-5-4	4.07	1.40	11.03	5-2-4	14.79	3.81	37.14
3-5-5	4.50	1.54	11.89	5-2-5	17.67	5.03	52.30
4-0-0	1.30	0.31	3.11	5-3-0	7.92	2.47	18.86
4-0-1	1.66	0.46	4.45	5-3-1	10.86	3.08	25.44
4-0-2	2.07	0.64	6.31	5-3-2	14.06	3.68	34.45
4-0-3	2.53	0.82	7.64	5-3-3	17.50	4.34	51.31
4-0-4	3.02	1.02	8.81	5-3-4	21.22	5.29	67.98
4-0-5	3.55	1.21	9.96	5-3-5	25.27	8.14	79.71
4-1-0	1.69	0.48	4.60	5-4-0	12.99	3.48	31.08
4-1-1	2.12	0.66	6.46	5-4-1	17.24	4.29	49.75
4-1-2	2.58	0.85	7.79	5-4-2	22.12	5.63	70.87
4-1-3	3.10	1.05	8.98	5-4-3	27.81	8.82	86.00
4-1-4	3.65	1.25	10.16	5-4-4	34.54	11.59	101.10
4-1-5	4.25	1.45	11.38	5-4-5	42.56	14.37	118.00
4-2-0	2.16	0.67	6.61	5-5-0	23.98	7.62	76.29
4-2-1	2.64	0.87	7.94	5-5-1	34.77	11.72	101.60
4-2-2	3.17	1.08	9.15	5-5-2	54.22	17.91	141.90
4-2-3	3.75	1.29	10.37	5-5-3	91.78	26.72	220.10
4-2-4	4.38	1.50	11.64	5-5-4	160.90	38.37	410.30
4-2-5	5.04	1.71	12.97	5-5-5	>160.90		

Table B.2. Sample Computation for Assessment of Bacterial Content of the Sludge

Fecal coli / *E.coli* Densities										
Dilution	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	Significant Dilutions	MPN Index mL	MPN / mL wet weight	%dry weight	MPN/g (dry weight)
Influent	5/5	5/5	4/5	2/5	-	5-4-2	22,12	$22,12x10^{4}$	2,75	$8,04x10^{6}$
Effluent	5/5	2/5	0	0	-	5-2-0	4,93	$4,93x10^{3}$	1,97	$0,25x10^{6}$
Dewatered Sludge	5/5	5/5	5/5	5/5	4/5	5-5-4	160,9	$160,9x10^{5}$	21,91	$7,34x10^{7}$

APPENDIX C

DNA EXTRACTION KIT COMPONENTS

The PowerBead Tube contains a buffer that will (a) help disperse the soil particles, (b) begin to dissolve humic acids and (c) protect nucleic acids from degradation.

Solution C1 contains SDS and other disruption agents required for complete cell lysis. In addition to aiding in cell lysis, SDS is an anionic detergent that breaks down fatty acids and lipids associated with the cell membrane of several organisms.

Solution C2 is patented Inhibitor Removal Technology (IRT). It contains a reagent to precipitate non-DNA organic and inorganic material including humic substances, cell debris, and proteins.

Solution C3 is patented Inhibitor Removal Technology (IRT) and is a second reagent to precipitate additional non-DNA organic and inorganic material including humic acid, cell debris, and proteins..

Solution C4 is a high concentration salt solution. Since DNA binds tightly to silica at high salt concentrations, this will adjust the DNA solution salt concentrations to allow binding of DNA.

Solution C5 is an ethanol based wash solution used to further clean the DNA that is bound to the silica filter membrane in the Spin Filter. This wash solution removes residual salt, humic acid, and other contaminants while allowing the DNA to stay bound to the silica membrane.

Solution C6 is an elution buffer. DNA that was bound in the presence of high salt is selectively released by Solution C6 (10 mM Tris) which lacks salt.

APPENDIX D

SEQUENCE OF *dxs* GENE AND SPECIFICATIONS OF PRIMER PAIR

Table D.1. Fasta sequence of dxs gene and primers utilized

ATGAGTTTTGATATTGCCAAATACCCGACCCTGGCACTGGTCGACTCCACCCAGGAGTTACGACTGTTGCCGAA
AGAGAGTTTACCGAAACTCTGCGACGAACTGCGCCGCTATTTACTCGACAGCGTGAGCCGTTCCAGCGGGCACT
TCGCCTCCGGGCTGGGCACGGTCGAACTGACCGTGGCGCTGCACTATGTCTACAACACCCCGTTTGACCAATTG
ATTTGGGATGTGGGGCATCAGGCTTATCCGCATAAAATTTTGACCGGACGCCGCGACAAAATCGGCACCATCCG
TCAGAAAGGCGGTCTGCACCCGTTCCCGTGGCGCGGCGAAAGCGAATATGACGTATTAAGCGTCGGGCATTCA
TCAACCTCCATCAGTGCCGGAATTGGTATTGCGGTTGCTGCCGAAAAAGAAGGCAAAAATCGCCGCACCGTCTG
TGTCATTGGCGATGGCGCGATTACCGCAGGCATGGCGTTTGAAGCGATGAATCACGCGGGCGATATCCGTCCTG
ATATGCTGGTGATTCTCAACGACAATGAAATGTCGATTTCCGAAAATGTCGGCGCGCTCAACAACCATCTGGCA
CAGCTGCTTTCCGGTAAGCTTTACTCTTCACTGCGCGAAGGCGGGAAAAAAGTTTTCTCTGGCGTGCCGCCAATT
AAAGAGCTGCTCAAACGCACCGAAGAACATATTAAAGGCATGGTAGTGCCTGGCACGTTGTTTGAAGAGCTGG
GCTTTAACTACATCGGCCCGGTGGACGGTCACGATGTGCTGGGGCTTATCACCACGCTAAAGAACATGCGCGAC
CTGAAAGGCCCGCAGTTCCTGCATATCATGACCAAAAAAGGTCGTGGTTATGAACCGGCAGAAAAAGACCCGA
TCACTTTCCACGCCGTGCCTAAATTTGATCCCTCCAGCGGTTGTTTGCCGAAAAGTAGCGGCGGTTTGCCGAGCT
ATTCAAAAATCTTTGGCGACTGGTTGTGCGAAACGGCAGCGAAAGACAACAAGCTGATGGCGATTACTCCGGC
GATGCGTGAAGGTTCCGGCATGGTCGAGTTTTCACGTAAATTCCCGGATCGCTACTTCGACGTGGCAATTGCCG
AGCAACACGCGGTGACCTTTGCTGCGGGTCTGGCGATTGGTGGGTACAAACCCATTGTCGCGATTTACTCCACT
TTCCTGCAACGCGCCTATGATCAGGTGCTGCATGACGTGGCGATTCAAAAGCTTCCGGTCCTGTTCGCCATCGA
CCGCGCGGGCATTGTTGGTGCTGACGGTCAAACCCATCAGGGTGCTTTTGATCTCTCTTACCTGCGCTGCATACC
GGAAATGGTCATTATGACCCCGAGCGATGAAAACGAATGTCGCCAGATGCTCTATACCGGCTATCACTATAACG
ATGGCCCGTCAGCGGTGCGCTACCCGCGTGGCAACGCGGTCGGCGTGGAACTGACGCCGCTGGAAAAACTACC
AATTGGCAAAGGCATTGTGAAGCGTCGTGGCGAGAAACTGGCGATCCTTAACTTTGGTACGCTGATGCCAGAA
GCGGCGAAAGTCGCCGAATCGCTGAACGCCACGCTGGTCGATATGCGTTTTGTGAAACCGCTTGATGAAGCGTT
AATTCTGGAAATGGCCGCCAGCCATGAAGCGCTGGTCACCGTAGAAGAAAACGCCATTATGGGCGGCGCAGGC
AGCGGCGTGAACGAAGTGCTGATGGCCCATCGTAAACCAGTACCCGTGCTGAACATTGGCCTGCCGGACTTCTT
TATTCCGCAAGGAACTCAGGAAGAAATGCGCGCCGAACTCGGCCTCGATGCCGCTGGTATGGAAGCCAAAATC
AAGGCCTGGCTGGCATAA

(http://www.ncbi.nlm.nih.gov/nuccore/2665585?report=genbank (Accession number: AF035440.1)

Table D.2. Primers used for amplifications of *dxs* gene

	Sequence (5'->3')	Strand on template	Length	Start	Stop	Tm	GC%
Forward primer	CGAGAAACTGGCGATCCTTA	Plus	20	1509	1528	51.70	50.00%
Reverse primer	CTTCATCAAGCGGTTTCACA	Minus	20	1621	1602	50.96	45.00%
Product length	113						

(http://www.ncbi.nlm.nih.gov/tools/primer-blast/primertool.cgi)

APPENDIX E

CREATING A gDNA STANDARD CURVE

Step 1

The genome size of the organism of interest is identified.

E.coli: 4.571E+6 bp

Step 2

Mass of DNA per genome is identified according to the below given formula.

$m = (n)\left(1{,}096 \times 10^{-21} \frac{g}{bp}\right)$ where n: genome size (bp)

m: mass

m=(4.571e6 bp)(1.09e-21 g/bp)

≈ 5e-15 g

The calculation below converts the mass to picogram units.

(5e-15g) x (1+E12 pg/g) = 0.005 pg

Step 3

The mass of the genome is dividing by the copy number of the gene of interest per haploid genome.

The X gene is a target that exists as a single copy gene per haploid genome

0.005 pg/genome ÷ 1 copy X/genome = 0.005 pg / 1 copy X

Therefore, 0.005 pg of *E.coli* gDNA contains one copy of the X gene.

Step 4

The mass of gDNA containing the copy #s of interest is calculated which is 64,000,000 to 6400 copies in our study.

Copy # of interest × mass of haploid genome = mass of gDNA needed

Step 5

The desired concentrations of gDNA correspond to the copy numbers of interest is calculated. Then, the mass needed (calculated in Step 4) is divided by the volume pipetted into each PCR reaction which is 2 µL.

Concentration of gDNA is calculated which is needed to achieve the required masses of gDNA.

Table E.1. Calculation of the final concentration of gDNA

Copy Number	Mass of gDNA needed (pg)		Final concentration (pg/µl) of gDNA
64.000.000	320.000	÷ 2 ul	160.000
6.400.000	32.000		16.000
640.000	3.200		1600
64.000	320		160
6400	32		16

Step 6

A serial dilution of the gDNA is prepared. The following formula was utilized for the praperation of dilutions.

$$C_1V_1 = C_2V_2$$

The stock concentration of E. coli gDNA was determined by spectrophotometric analysis to be 2 µg/µl which corresponds to 2,000,000 pg/µL. Each dilution prepared has a final volume (V_2) of 100µL.

Table E.2. Calculation of the resulting copy number

Source	Initial concentration (pg/µL)	Volume of gDNA (µL)	Volume of diluent (µL)	Final Volume (µL) V_2	Final concentration of dilution (pg/µl) C_2	Resulting copy # gene
Stock	2.000.000	8	92	100	160.000	64.000.000
Dilution 1	160.000	10	90	100	16.000	6.400.000
Dilution 2	16.000	10	90	100	1600	640.000
Dilution 3	1600	10	90	100	160	64.000
Dilution 4	160	10	90	100	16	6400

Table E 3. Standard Copy Number and Cp Values Measured

ID	Standard Copy Number	Cp Values Measured
st1	6,21E+07	15,18
	6,36E+07	15,14
	6,47E+07	15,12
st2	6,45E+06	18,69
	6,45E+06	18,69
	6,36E+06	18,71
st3	6,50E+05	22,24
	6,42E+05	22,26
	6,41E+05	22,26
st4	4,89E+04	26,24
	6,71E+04	25,76
	6,37E+04	25,84
st5	7,11E+03	29,23
	7,16E+03	29,22
	6,90E+03	29,28

APPENDIX F

STANDARD CURVE AND SAMPLE AMPLIFICATION AND MELTING CURVES

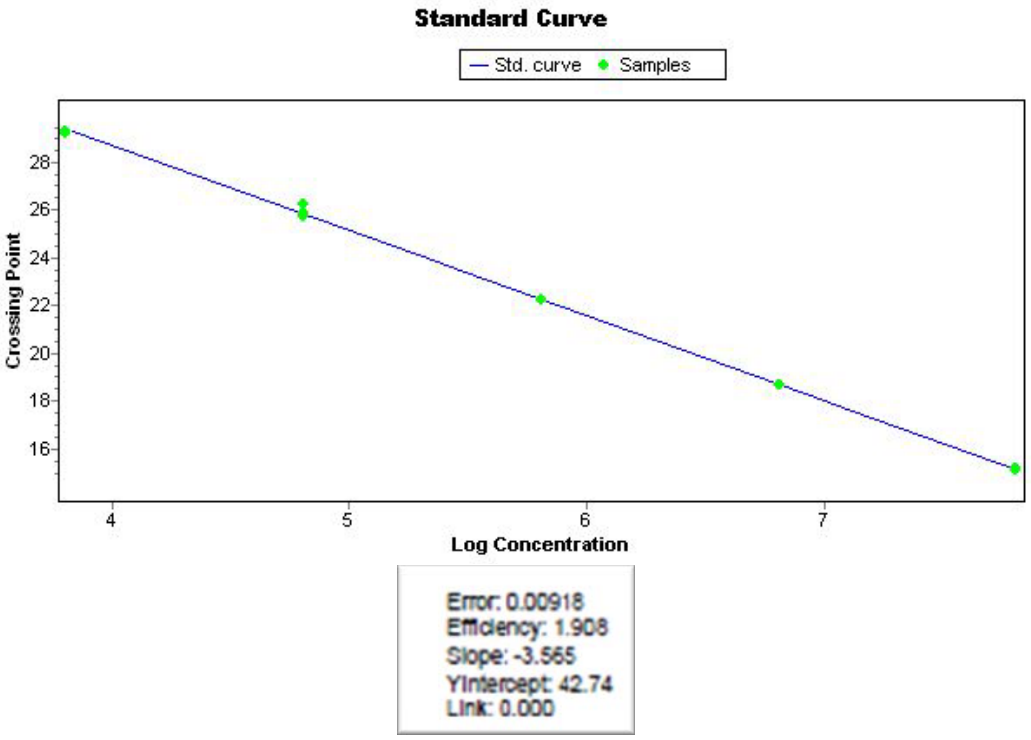

Figure F.1. Standard curve obtained for a dilutional series of genomic DNA standards

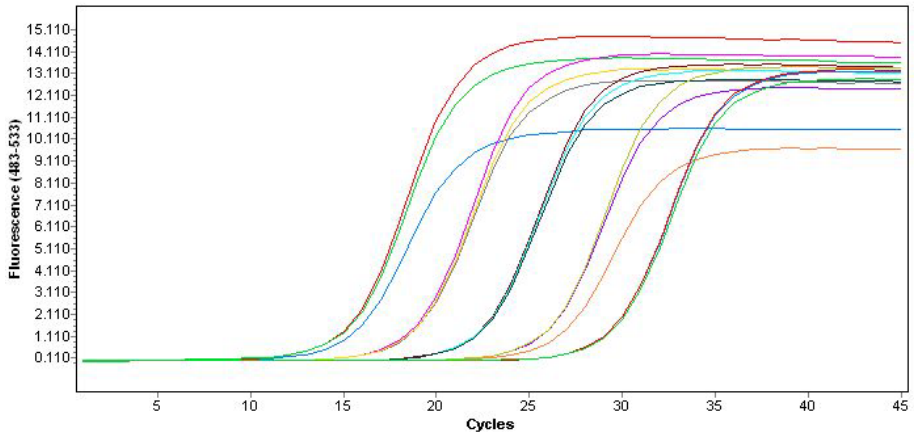

Figure F.2. Amplification Curve of a dilutional series of genomic DNA standard

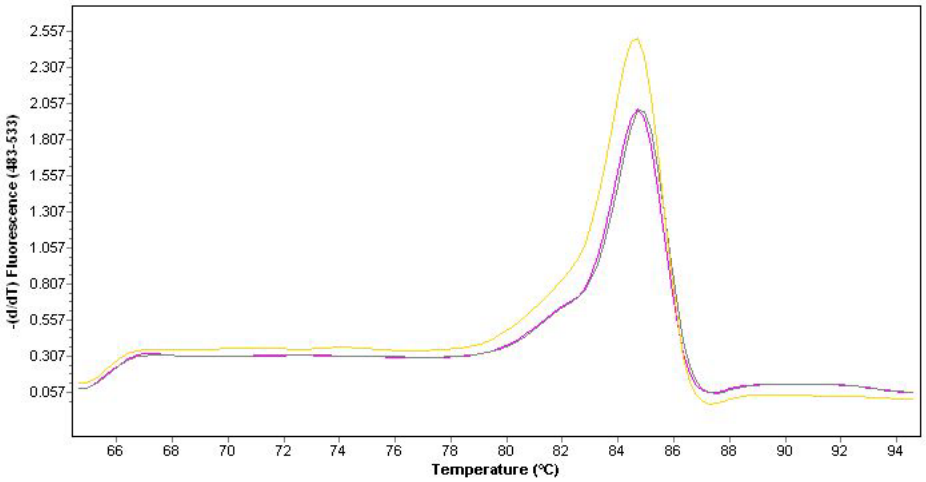

Figure F.3. Melting Curve of a dilutional series of genomic DNA standards

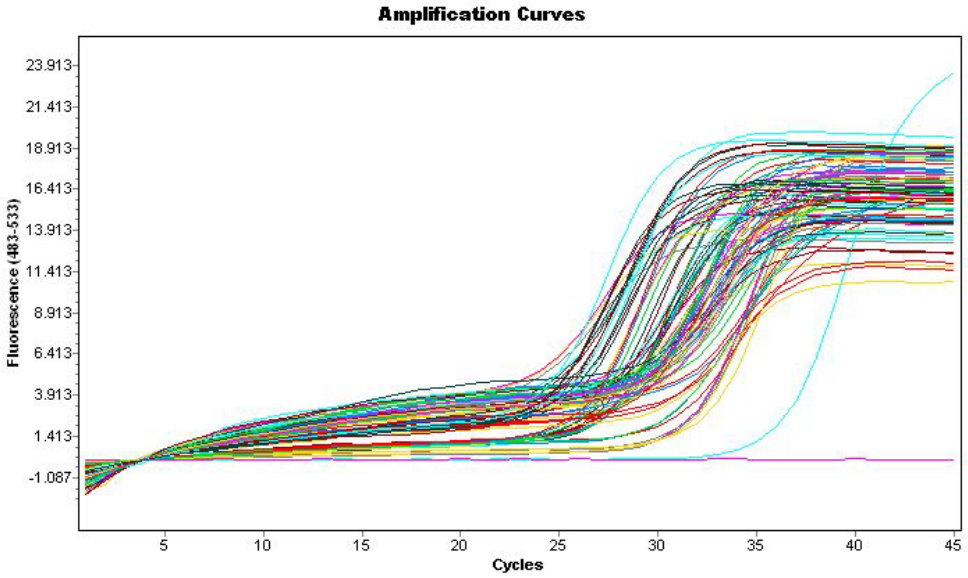

Figure F.4. Amplification Curve Obtained during Quantitative PCR Analysis of Konya (28.09.10), Ankara (24.11.10), Kayseri (22.12.10) and Ankara (01.12.10) Samples

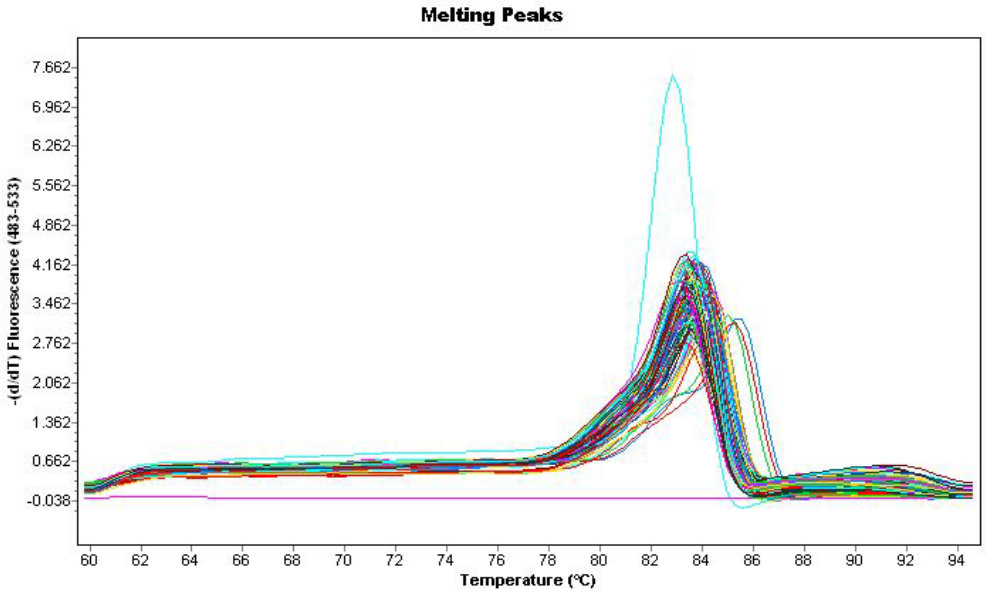

Figure F.5. Melting Curve Obtained during Quantitative PCR Analysis of Konya (28.09.10), Ankara (24.11.10), Kayseri (22.12.10) and Ankara (01.12.10) Samples

APPENDIX G

EVALUATION OF THE QUANTITATIVE PCR RAW DATA

Sample ID: I (influent)

Cp I $_{mean}$: 26.45

Concentration $_{mean}$: 5.40+E4

Copy number of the dxs gene in 2 uL DNA solution is 5.40+E4

So the concentration is 2.7+E4 copy dxs gene/uL

Determination of the mass:

$5x10^{-3}$ pg *E.coli* DNA 1 copy of dxs gene

x $2,7 \times 10^4$ copy

x: 135pg

1 ul of the DNA solution (total DNA) include 135 pg E.coli DNA

So *E.coli* DNA concentration in the solution is: 135pg/uL = 135.000pg/mL = $135x10^9$ g/mL

TS $_{Influent}$:19,25 %

100 mL ınfluent sample 19,25 gTS

1 mL influent sample 0,19 g TS

19 x 10^{-2} g TS 135 x 10^{-9} g *E.coli* DNA

1 g TS x

x= 7,1 x 10^{-7} g *E.coli* DNA

1 g TS contain 7,1x 10^{-7} g E.coli DNA

From the mass formula

$$m = [n][1.096e\text{-}21 \ \frac{g}{bp}]$$

where: n = genome size (bp)
m = mass
e-21 = ×10^{-21}

7,1x 10^{-7} = (n) (1.096x10^{-21}g/bp)

n= 6,47 x 10^{14} bp

The total genome size of 7,1 x 10^{-7} g E.coli DNA is 6,47 x 10^{14} bp

1 *E.coli* 4,57 x 10^{6} bp

x 6,47 x 10^{14} bp

x: 1,41 x 10^{8}

The the number of E.coli find in Sample is 1,41 x 10^{8} E.coli/g TS

APPENDIX H

PRELIMINARY RESULTS OBTAINED BY MEMBRANE FILTER METHOD

Table H.1. Preliminary Results Obtained by Membrane Filter Method

Date of Sampling and Analyses	Conditions/Procedures Applied	Results
17.02.10	Original Procedure	Inaccurate results
24.02.10	Original Procedure	Inaccurate results
26-27. 02.10	Original Procedure	No growth
02.03.10	Different Filtrate Volumes	No growth
04.03.10	Different pH Values and Different Medium Lots	No growth
05.03.10	Different Dilution Procedures with Phosphate Buffer, Sterile Water and Pure water	A few colonies only in dilutions with pure water
06.03.10	Different Medium Lots and Different Dilution Procedures with Phosphate Buffer and Sterile Water	A few colonies observed only in sterile water dilution.
09.03.10	Changing Filtration Equipment	No growth
10.03.10	Checking pH	No growth
16.03.10	Original Procedure	A few colonies observed only in pure water dilution for dilution series 10^{-3}
17.03.10	Changing Medium Pouring Method	No growth
22.03.10	Original Dilution Procedure at Different pH Values	Only dilution series 10^{-3} gave positive results
29.03.10	Different Dilution Procedures with Buffers Prepared at Different Temperature	Inaccurate results
31.03.10	Original Procedure	No growth
01.04.10	Parallel Analyses with Original Membrane Filter Procedure and Multiple Tube Fermentation Technique	No growth at membrane filters. MTF method gave accurate results.