# QUANTIFICATION OF *SALMONELLA* IN TREATED BIOSOLIDS FROM MUNICIPAL WASTEWATER TREATMENT PLANTS IN FOUR CITIES IN TURKEY USING CULTURE-BASED TECHNIQUES

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# ABSTRACT

**QUANTIFICATION OF *SALMONELLA* IN TREATED BIOSOLIDS FROM MUNICIPAL WASTEWATER TREATMENT PLANTS IN FOUR CITIES IN TURKEY USING CULTURE-BASED TECHNIQUES**

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Large quantities of biosolids are produced every day from wastewater treatment plants. In Turkey, because there is no legally permissible use for this sludge, it is mostly sent to landfills for disposal. Land application of biosolids is a useful and valid alternative for making use of this huge amount of sewage sludge. However, it must be assured that it is adequately safe for human contact and use. In this study, biosolid samples from four Turkish treatment plants (Ankara, Eskişehir, Kayseri and Yozgat) were analyzed for presence and concentration of *Salmonella,* one of the key pathogens present in biosolids. Quantifications were carried out using a culture-based method established by the U.S. EPA (Method 1682). Additionally, a novel molecular method (propidium monoazide assisted Taqman qPCR) was employed to explore more

accurate and rapid quantification. Based on the culture-based method, Yozgat biosolids were found to contain highly variable quantities, reaching up to 693 MPN/4g, higher than would be expected for such a treatment system. *Salmonella* in the biosolid samples from Ankara, Eskişehir, and Kayseri fell mostly in the range of 0-50 MPN/ 4 g dry weight, values consistent with both the published literature values from similar treatment plants around the world, indicating that usage of the biosolids from these treatment plants for agricultural purposes is reasonable in terms of this pathogen. These quantities of *Salmonella* proved too low to be efficiently detected by molecular methods, indicating that the current EPA method is the most applicable for this rare but virulent pathogen.

**Keywords**: *Salmonella*, biosolids, quantification, Method 1682, qPCR

# ÖZ

## *SALMONELLA* BAKTERİLERİNİN TÜRKİYE'NİN DÖRT İLİNDEKİ EVSEL ATIKSU ARITMA TESİSLERİNDEN GELEN ARITILMIŞ BİYOKATILARDA KÜLTÜRE DAYALI YÖNTEMLERLE KANİTATİF OLARAK BELİRLENMESİ

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Tez Yöneticisi: Yrd. Doç. Dr. Robert W. Murdoch

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Atıksu arıtma tesislerinde her gün yüksek miktarlarda biyokatı üretilmektedir. Üretilen bu atıksu çamurlarının Türkiye'de yasal olarak kullanım izni olmadığı için çoğunlukla katı atık sahalarına tasfiye edilmektedirler. Biyokatıların toprakta kullanımı, bu fazla miktarlardaki atıksu çamurlarının işe yarar kullanımının sağlanabilmesi açısından yararlı ve geçerli bir alternatiftir. Ancak çamurların insan teması ve kullanımı bakımından yeterli düzeyde güvenli hale getirilmiş olmaları sağlanmak zorundadır. Bu çalışmada, Türkiye'nin dört arıtma tesisinden (Ankara, Eskişehir, Kayseri, Yozgat) alınan biyokatı örneklerinde, biyokatılarda bulunan en önemli patojenlerden olan *Salmonella* bakterilerinin varlığı ve konsantrasyonu analiz edilmiştir. Miktar belirleme ABD Çevre Koruma Ajansı (U.S. EPA) tarafından belirlenmiş ve kültüre dayalı bir yöntem olan 1682 Metodu ile gerçekleştirilmiştir. Buna ek olarak, daha kesin ve hızlı

miktar belirleme işlemi için yeni geliştirilmiş olan bir yöntem kullanılmıştır (propidyum monoazid ile desteklenmiş qPCR). Kültüre dayalı yöntemler sonunda Yozgat biyokatılarının arıtma sistemi göze alındığında beklenin dışında olarak yüksek oranda değişkenlik gösteren ve 693 MPN/4 g kuru ağırlıka ulaşan sonuçlar verdiği gözlenmiştir. Ankara, Eskişehir ve Kayseri illerinden alınan biyokatı örneklerinin sonuçları, benzer arıtma sistemine sahip dünya üzerindeki başka ülkelerden de elde edilen ve yayınlanmış literatür değerleriyle uyumluluk göstererek çoğunlukla 0-50 MPN/4 g kuru ağırlık olarak elde edilmiştir. Bu sonuçlar göstermektedir ki bu arıtma tesislerinden elde edilen biyokatıların tarım amaçlı faaliyetlerde kullanımı Salmonella patojeni göz önüne alındığında makul görünmektedir. Bu Salmonella miktarları moleküler metotlarla belirlenebilmek için fazla düşük kaldığından güncel EPA metodu bu az rastlanır ancak virulan patojen için en uygulanabilir yöntemdir.

**Keywords:** *Salmonella*, biyokatı, 1682 Metodu, kanitatif miktar belirleme, qPCR

*To my loving and self-sacrificing*
*grandparents Melahat and Selahattin Gölönü,*
*May you both rest in peace…*

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## LIST OF ABBREVIATIONS

EPA: Environmental Protection Agency

U.S.: United States

EU: European Union

MPN: Most Probable Number

DNA: Deoxyribonucleic acid

PMA: Propidium Monoazide

APHA: American Public Health Association

# CHAPTER 1

# INTRODUCTION

## 1.1. What is *Salmonella*?

*Salmonella* is a rod-shaped, gram-negative, facultative intracellular parasitic bacterial genus known to cause more than approximately 1.3 billion cases of disease each year, generally referred to as Salmonellosis (Coburn et al., 2006). *Salmonella* is one of the major causes of foodborne diseases worldwide and is a major public health problem in Turkey (Cetinkaya et al., 2008).

*Salmonella* is a durable organism that can live a non-pathogenic lifestyle. However, it is very virulent, requiring only few cells to cause infection (Varma et al., 2005).

It was in the late $19^{th}$century when *Salmonella* was first identified by the US Bureau of Animal Industry (Feasey et al., 2012).

Throughout time, *Salmonella* has been a very important human pathogen especially as the cause of typhoidal infections, which can have a severe pathology involving multiple organ systems and high death rates (Coburn et al., 2006).

## 1.2. Salmonellosis, the disease

*Salmonella* bacteria that cause human diseases are traditionally divided into two major groups; typhoidal and non-typhoidal (Feasey et al., 2012). Typhoidal Salmonellosis, which can also be referred as the Typhoid fever or Enteric fever (Ohl & Miller, 2001), occurs after *S.enterica* serovar Typhi bacteria is ingested. This mostly comes from contaminated water, animal products, or close contact with infected individuals. (Coburn et al., 2006). The presence of the microorganism in the aquatic environment is mostly due to its direct entrance from infected human or animal feces, or by some indirect routes such as sewage discharge or agricultural land surface runoff (Levantesi et al., 2012).

Non-typhoidal Salmonellosis is a type of gastroenteritis, generally less severe than typhoid (Ohl & Miller, 2001). The incidence of intestinal diseases caused by Salmonella gastroenteritis is as high as typhoid incidence in the developing world, and is substantially important in developed countries as well (Coburn et al., 2006). There are number of different routes by which *Salmonella* can be transmitted, but most of the infections originate from animals (Cetinkaya et al., 2008). Since there exists a very wide range of vertebrate hosts for *Salmonella*, the disease is mostly considered as zoonotic (Feasey et al., 2012). Zoonotic Salmonellae are usually considered foodborne pathogens; the most commonly encountered animals which can host the bacteria are calves, cattles, poultry, pigs, sheep, and even pets (Levantesi et al., 2012). In the developed world, infective Salmonellae are most often transmitted to humans through contaminated food, such as raw eggs or undercooked meat. More recently infection through fresh vegetables like lettuce or sprouts has also gained attention; while the route by which the *Salmonella* reaches these vegetables is not entirely clear, irrigation with contaminated water is a likely possibility (Barrow & Methner, 2013).

Antibiotic resistance in *Salmonella* is an increasing problem. The major reason for this antibiotic resistance is thought to be the overuse of antibiotics for medical purposes in humans (Pang et al., 1995), as well as overuse in livestock veterinary activities (Rabsch et al., 2001).

It has been stated that approximately 94 million gastroenteritis cases and 155,000 deaths occur around the world because of *Salmonella* infections (Majowicz et al., 2010). In the U.S., 1.4 million human cases, 15,000 hospitalizations and more than 400 deaths resulting from *Salmonella* are seen every year (Voetsch et al., 2004) (Mead et al., 1999). The actual number of outbreaks or the frequencies of *Salmonella*-caused diseases is quite uncertain because most of the time, patients suffering from diarrhea do not seek medical attention. The amount of unreported cases is thought to increase the actual number of occurrences by 10 to 15 times (Barrow & Methner, 2013). Additionally, lack of adequate microbiological diagnostics in patients with diarrhea complaints also leads to under-reporting of *Salmonella* cases. When these cases are not reported it becomes harder to recognize the pathogen’s transition or transformation processes earlier (Barrow & Methner, 2013). It is frequently seen that when

diagnostics or report of a disease are inadequate, the burden of the disease will be inevitably underappreciated (Feasey et al., 2012).

In 1999, 10% of foodborne human illnesses, 26% of hospitalizations and 31% of deaths resulting from foodborne pathogen infections were known to be caused by Salmonella in the U.S., making it the first place among other bacterial foodborne pathogens in hospitalizations and deaths. Also, it comes second in the number of overall infectious diseases reported (Mead et al., 1999). In 2009, it became the most reported bacteriological agent of foodborne diseases affecting humans (CDC, 2009).

In conclusion, it should be stated that Salmonellosis is a common seen public health threat and that it will continue to have negative effects on health worldwide when the various transmission routes, the variety of the host organisms and increasing antibiotic resistance are considered as a whole.

### 1.3. *Salmonella* in Biosolids

Generally speaking, the treatment of domestic and urban wastewaters leads to two products, the liquid and the solid portions. The solid portion is the sewage sludge, also called as biosolid (Spicer, 2002). Unimaginably huge amounts of biosolids are produced worldwide every year. Therefore their disposal, safety and possible reuse are important issues that are being discussed all over the world. There are number of areas in which biosolids can be used, such as parks, forests or agricultural lands (Zaleski et al., 2005). Biosolids can be very valuable resources in land applications for agriculture due to their nutrient contents, but prior to use, pathogens and other harmful components should be reduced to safe levels.

Currently, 37% of the biosolids produced in Europe are used in land applications. However, this is an average percentage; there are actually very wide differences in the usage rates of different member states (Evans, 2012). When it comes to the U.S., nearly 60% of biosolids produced are land-applied; even this high rate is only 1/40 of the amount of animal manure used for the same purpose (Spicer, 2002).

Gibbs et. al (1994) reported *Salmonella* concentrations in wastewater sludges to be in the range of $1.1x10^{1}$-$5.9x10^{3}$ $g^{-1}$dry weight, with a mean value of $2.9x10^{3}$ (Sidhu & Toze, 2009). However, during and after many types of wastewater treatment processes,

inactivation of *Salmonella* is generally high (Sahlström et al., 2004). After applying mesophilic digestion to sludge, the amount of *Salmonella* is generally in the range of 1.8-30 MPN/4 g. Further decreases are observed when thermophilic digestion is applied instead. Therefore, it can be said that thermophilic digestion is more effective for inactivating Salmonella (Watanabe et al., 1997).

In some studies carried out in 18 cities of the U.S., amounts of *Salmonella* in biosolids digested under mesophilic conditions were found to be between < 3 and 13.4 MPN/4g of dry solids with a median value of 6.1 (Pepper et al., 2010). After long term supervision of a facility in Arizona *Salmonella* counts were found to vary from 20-45 MPN/4 g of dry solids; this concentration stayed fairly constant over a period of 18 years (Pepper et al., 2010). Wong et al. (2010) also reported somewhat similar numbers in a separate study. They reported that mesophilic digestion reduced the *Salmonella* counts from 194 MPN/4 g of dry solids to below detection limit. Therefore, it can be concluded that mesophilic digestion generally reduces the number of *Salmonella* as measured by traditional methods to approximately between 0-50 MPN/4 g dry solids in urban wastewater treatment plants, although viable but not countable (VBNC) state of the bacteria and possible inaccuracies in the counting methods are not taken into account.

Biosolids are sometimes stored before land application. When this is the case, pathogens may continue to grow, which is a major cause for concern (Zaleski et al., 2005). In a study conducted by Gibbs et al. (1997), it was found that bacterial pathogens show a tendency to regrow. Two sets of experiments were conducted within the context of the study. One set lasted for 15 months, from one autumn to other winter, and the other for 6 months, from summer to winter. In the first experiment series, *Salmonella* became undetectable only after 16 weeks, but following the start of the winter rains, 2.2 MPN/gram of *Salmonella* were detected. In the second set of experiments, an increase in the amounts of both fecal coliform and *Salmonella* were seen in correspondence with rain events. On the contrary, moisture content alone did not explain the levels of increase in the bacterial amounts. However, rain may play a role in increasing the amount or availability of growth factors (Zaleski et al., 2005). In another study by Yeager et al. (1981) it was reported that moisture content directly

affected regrowth of bacteria during long term storage, since the bacteria needed water to survive and grow. They also stated that if the moisture content of the sludge is between 10-50% it may provide effective inactivation of the pathogens, but if the moisture content is less than 10% then it may lead to problematic results, such as favoring long term survival of *Salmonella* (Zaleski et al., 2005).

## 1.4. Detection and Quantification of *Salmonell*a in Biosolids

There are many pathogens in biosolids from different domains, classes, species etc. The presence of these pathogens should be detected with an acceptable level of confidence. Current techniques for the determination and quantification of *Salmonella* are originally derived from applications with food samples or from medical applications. Detection of *Salmonella* species in environmental samples can be challenging due to the high solids content in the biosolids and soils. The high amounts and diversity of other types of bacteria also increase the difficulty of *Salmonella* detection because they are present at higher densities than in clinical samples (Yanko et al., 1995). Currently, the methods that are applied to detect pathogens in samples are divided into three main categories; culture based methods, molecular methods, which are mostly nucleic acids based, and microscopy (Sidhu & Toze, 2009). Within the context of the present study, emphasis will be given on the former two.

### 1.4.1. Culture Methods

There are many culture based methods available which can be used for detection of pathogenic bacteria, *Salmonella* species being only one of them. There are U.S. EPA approved and stated methods in the regulation as well as the methods developed by some researchers, such as Hussong et al. (1984), and Walker and Yanko (1987) (Yanko et al., 1995). These culture based methods most commonly apply “most probable number (MPN)” techniques. When detecting pathogens that are present in low numbers, enrichment and selective enrichment are usually used (Sidhu & Toze, 2009).

Gorski et al. (2011) conducted several studies for detecting different serovars of *Salmonella* in soil/sediment samples. They made use of different techniques and made comparisons of these techniques in the end. The culture based enrichment methods they were using in their study were variations of standardized methods approved by

U.S. FDA, USDA FSIS and also the 1682 Method of U.S. EPA. The isolates obtained at the end of the culturing methods were subjected to polymerase chain reactions (PCR) targeting the *Salmonella*-specific invA gene to eliminate false positives. After comparing this set of different quantification methods, the researchers decided that EPA Method 1682 was superior because it gave fewer false positive results in their study. However, they added that since the purpose of the Modified Semisolid Rappaport-Vassiliadis (MSRV) medium used in the method is to exclude non-motile bacteria, non-motile *Salmonella* serovars will be missed (Gorski et al., 2011). Nevertheless, when it is used as an additional enrichment medium but not as an agar, the Rappaport-Vassiliadis broth is found to be more efficient than other enrichment media that can be used for detecting *Salmonella* (Hu et al., 1997).

The EPA 1682 method, which was published in 2006, is the current gold-standard method for quantifying *Salmonella* in biosolids. The precise and well-researched technique aims to analyze the safety of Class A biosolids via accurate quantification, and is also the accepted method for the quality analysis of Class B biosolids (Wong et al., 2010). This method is also the culture based method that was used throughout the present study.

Method 1682 is most probable number (MPN) method involving enrichment, selection and biochemical conformation phases in order to overcome the various challenges faced when trying to isolate *Salmonella* from biosolids. The method also includes strict guidelines for sample collection, which is quite significant when the capacity of *Salmonella* to increase in number is taken into consideration.

The enrichment phase aims to create an ideal metabolic environment for the target organism in particular, also allowing any injured organisms to metabolically recover before being placed on more challenging selective media. Selection represents more specific isolation strategies such as the use of selective antibiotics. The last stage of the experiments, biochemical confirmation, refers to three additional specific metabolic tests for ultimate confidence in identifying the presumptive *Salmonella* specimens which reach the final stage.

Although the steps of Method 1682 are widely accepted, there exists some major disadvantages. There is the question about the potential presence of non-culturable portion of bacteria. Also, the method requires a lot of time, labor, equipment and reagents. For MPN counting, hundreds of culture tubes and petri dishes and several days of sequential culturing are necessary.

### 1.4.2. Molecular Methods

Polymerase Chain Reactions (PCR) and quantitative real-time PCR (qPCR, q RT-PCR) analyses are commonly applied techniques for molecular detection (Wong et al., 2010). PCR is a tool by which the detection of organisms can be achieved at the species level by targeting and amplifying species-specific nucleic acid sequences (Burtscher & Wuertz, 2003). Use of PCR in the detection of various pathogenic bacteria can be considered as a common practice which has been applied to many bacteria including *Salmonella* (Taskin et al., 2011). The various practices in which PCR techniques are used include food studies, medical or veterinarian practices. (Daum et al., 2002) (Mocellin et al., 2003).

If the cell density of the bacteria is too low to be detected by PCR, standard two-step enrichment (one general enrichment medium+ MSRV medium) can be performed before applying the PCR procedure (Burtscher & Wuertz, 2003), although such a method will not be quantitative.

One of the major drawbacks of using PCR-based methods in the detection of pathogens is that they tend to give false positive results because DNA is a stable molecule; even if the target organism has died, its DNA continues to exist in the sample for quite a long time (Drahovska et al., 2001). To eliminate this issue, Gonzalez-Escalona et al. (2009) targeted mRNA instead of DNA to amplify only living organisms during PCR. As long as an organism is alive, it continues to produce mRNA, but mRNA is a very unstable molecule that does not persist long after death. Therefore by targeting the mRNA only the viable bacteria will be detected. The assay used throughout the study was a TaqMan qRT-PCR targeting invA mRNA (González-Escalona et al., 2009), very similar to that of ours except that DNA was made use of during our experiments, not

RNA. Again however, such a method is not strictly quantitative since the number of mRNA molecules cannot be used to reliably deduce the number of organisms present.

Another option that can be applied for reducing overestimation of the amount of bacteria present in biosolids is the use of selective nucleic acid intercalating agents such as ethidium monoazide (EMA) or propidium monoazide (PMA). These compounds interact with free, extra-cellular DNA and also can penetrate into dead cells which have lost their cell membrane integrity to form a more complex compound by binding with DNA, inhibiting DNA amplification during PCR (Taskin et al., 2011) (Nocker et al., 2007). DNA in viable, intact cells will be left unaffected by this procedure however.

In the selection of which target to be used as the indicator sequence, in PCR applications, non-specific amplification should be addressed. In the case of *Salmonella* in particular a primer set for the metabolic gene invA is a well-accepted target for the primer/probe system since the gene is unique to *Salmonella* and does not yield significant signals from other bacteria, and is present in known *Salmonella* strains (Daum et al., 2002). Since PCR primers may have partial sequence analogy to non-target DNA sequences, non-specific amplification is a universal challenge in all PCR applications. In order to prevent possible non-specific amplifications more specific targets can be used in the system. This was achieved in the present study by using an internal probe, the most popular probe system currently, the Taqman system. A Taqman probe is a third nucleic acid oligomer with high specificity to the target region internal to the primer hybridization sites.

An invA Taqman probe system has been successfully applied in a number of studies aiming to quantify Salmonella and achieving detection sensitivities down to 5.8 copies per qPCR reaction (Novinscak et al., 2007), (Shannon et al., 2007), (González-Escalona et al., 2009), (van Frankenhuyzen et al., 2011).

### 1.4.3. The Viable But Not Culturable (VBNC) State of Bacteria

In order to be able to estimate the number of living bacteria in an environmental sample, the most commonly applied method is plate counting. However, plate count numbers are usually much lower than the actual amount of living bacteria present in

the sample partially because of the presence of VBNC state bacteria (Su et al., 2013). The viability of a bacterial cell is traditionally equated with whether it is culturable or not, and its ability to grow in a laboratory environment when an appropriate growth medium is provided (Kell et al., 1998) (Bogosian & Bourneuf, 2001). However, beginning especially in the 1990s, it was realized that the question of whether a bacterium is alive or dead is more complicated.

It is quite clear that if bacteria are subjected to adverse conditions some portion of the population will not grow which will lead to them being considered as "dead" by traditional definitions. However, some cells which do not grow may show signs of biological activity or signs of general metabolic activity indicating that non-culturable cells might, in fact, still be alive (Barer & Harwood, 1999) (Kell et al., 1998).

A host provides bacteria an environment with a constant warm temperature and high concentrations of amino acids and sugars, both having important roles in growth of bacteria. Some pathogens have the ability to live outside of a host, although they face hostile environmental conditions such as starvation, non-ideal temperatures or salinity, competition and predation. Under such conditions the bacteria might favor a VBNC state from which cells might only emerge under certain conditions consistent with access and entry to a host (Winfield & Groisman, 2003). The VBNC state is a survival strategy mainly employed by gram negative bacteria (Gupte et al., 2003), such as Salmonella.

A VBNC bacterium is not simply a cell which is badly damaged, yet may or may not recover if suitable conditions or nutrients are given. Rather, VBNC is currently defined as an intentional biological strategy taken for maximizing the chances of continuing life and growth at a later time (Bogosian & Bourneuf, 2001). This is very much different than the case in which cells are damaged and might not be able to grow unless they can repair themselves.

Still, it is quite impossible to distinguish between cells resuscitated from the VBNC state and the cells which simply regrow after they were injured but otherwise were viable (Bogosian & Bourneuf, 2001).

The VBNC state is sometimes called as “dormant” by some, however the dormancy is defined as cells having negligible metabolic activity, but ultimately being culturable. On the other hand, VBNC cells are claimed to have opposing properties; they show metabolic activities but they are non-culturable (Kell et al., 1998).

Whether these non-culturable cells pose potential health threats to humans or not is a very important question that needs to be answered. The role that non-culturable cells have in the transmission of diseases still remains unclear (Barer et al., 2000). However, in a study done by Smith et al. (2002) it was found that the non-culturable cells of *Salmonella typhimurium* were not able to infect mice. Besides, the potential infective hazards that can be posed are managed to be detected by some indirect tests of cell integrity, which can be molecular, biochemical, or staining procedures (Barer et al., 2000).

### 1.5. Current State of *Salmonella* in Turkey

The amount of *Salmonella* in Turkish biosolids has not been reported in any study yet. Presence of pathogens, including *Salmonella*, in biosolids at a certain level even after treatment is suspected, but the actual numbers have not been established or reported. Yet, the occurrence of the bacteria in other environmental samples and especially in food products has been demonstrated many times. In this section, examples of *Salmonella* detection in Turkey will be presented. As previously mentioned, Salmonellosis is a disease commonly accepted as originating from food. However, increasing outbreaks of Salmonellosis in EU countries resulting from non-animal origin have been encountered, leading to the examination of fresh produce since animal or human waste contaminated irrigation water can be a very important route of transmission. A study was conducted with many different kinds of produce from tomato, parsley, lettuce to varieties of peppers all obtained within the province of Ankara. In the end *Salmonella* species were found in low percentages of the fresh produce samples. These percentages were even lower when they were compared to that of the published data of other countries, yet the results are solid proof that fresh produce can be contaminated with *Salmonella* and thus pose a threat to public health (Gunel et al., 2015).

Moreover, it is likely to encounter *Salmonella* species in various dairy products. Occurrence of *Salmonella* in Van oltu cheese has also been demonstrated. This is a public health concern since many foodborne disease outbreaks are known to result from cheese made from unpasteurized or improperly pasteurized milk (Tekinşen & Özdemir, 2006).

Not only dairy products but also poultry meat can be infected with *Salmonella*. Examinations were done on chicken doner kebab from 72 establishments for the prevalence of many pathogens including *Salmonella*. In this study, no *Salmonella* species were detected in the samples, however it was noted that *Salmonella* tends to attach to the skin of the poultry and is very difficult to remove. Therefore, there exists a high risk of disease when the kebab, or any other food for that matter, is being prepared with the chicken skin added (Vazgecer et al., 2004).

As the last but not the least example of *Salmonella* contaminated food, Turkish sausage (sucuk) can be given. *Salmonella* was detected in 7% of the samples coming from shops and markets of Afyon province (Sırıken et al., 2006). In all of these cases, amounts of the pathogen may seem relatively low at first sight, but it should be kept in mind that the samples that have been mentioned are all foods that will directly be ingested, leading to the very most important route of transmission.

Lastly, it is necessary to mention that *Salmonella* is also encountered in environmental media. It is suggested by current data that Salmonellae are reaching surface waters and food supply chains in Turkey. However, available data on this issue are very few. It was demonstrated by Aytac et al. (2010) that a remarkable number of leafy green vegetables which were irrigated by stream water taken from downstream of Ankara turned out to be positive for the presence of *Salmonella*. This suggests that released urban wastewater and biosolids that are used for land application purposes are significant sources. The frequency of detection was much higher than the results typically obtained in other developing countries. Over 10% of Turkish samples were positive, whereas in similar studies less than 1% of specimens in Spain were reported as contaminated (Levantesi et al., 2012).

## 1.6. Regulations on Pathogen Limits in Biosolids for Land Application

Even before there were modern wastewater treatment techniques, human feces was considered a valuable resource for agriculture in that it could be used as fertilizers. In this sense, the use of biosolids for agricultural purposes is an attractive and an advantageous option because biosolids provide some necessary nutrients, such as nitrogen and phosphorus, organic matter and minerals to the soil (Spicer, 2002). However, since biosolids originate from human and animal excreta, they are expected to contain a high variety of pathogens (Evans, 2012). The fact that even treated sludge may contain many bacterial or viral pathogens still raises some public concerns about the use of biosolids for land applications. These concerns make it difficult for the biosolid producers, wastewater treatment facilities mainly, to market their biosolids since the amount of sludge generated has significantly increased over the recent years (Iranpour et al., 2004).

Therefore, it is an obligation in many parts of the world to process the sludge so as to reduce the amount of pathogens present to a level that can be considered as “safe”, and also to monitor the sludge further to check for the presence of these pathogens (Evans, 2012). Government agencies from both the U.S. and the European Union (EU) have set some regulations on the land application of biosolids with the purpose of reducing the risks that may come from pathogens present (Iranpour et al., 2004).

The regulations set in the U.S., EU and in Turkey are explained in the following sections. It should be noted that the regulations are examined mainly for pathogen reduction and their limit amounts for the sake of the context. They contain other criteria regarding sewage sludge land applications, but not much emphasis will be given to them.

### 1.6.1. The U.S. Regulations on Biosolids

The regulations in the U.S. regarding with the fate of sewage sludge is called “The Standards for the Use or Disposal of Sewage Sludge”. It was developed and published by the U.S. EPA as the 40 CFR Part 503 Biosolids Rule on February 19, 1993 and it is came into force on March 22, 1993. The law is commonly known as the 503 Rule (Lu et al., 2012).

The 503 Rule is the most extensive legislation made on sludge and it sets the quality requirements for biosolids land application. Among these requirements pathogen and vector reduction, metal loading and concentrations and nutrient limits are stated (Lu et al., 2012).

When it comes to pathogen reduction two main classifications are set in the 503 Rule: Class A and Class B. Reducing the pathogen amounts until Class A requirements are met is necessary when the biosolid will be applied to lawns or gardens or will be used for any other land application purposes which might require direct human contact (U.S.EPA, 1994). According to Class A requirements, pathogen levels should be reduced below detection limits. These limits are less than 1000 MPN/ g total solids for fecal coliform density, less than 3 MPN/4 g total solids (dry weight) for *Salmonella* species, less than 1 PFU/4 g total solids (dry weight) for enteric viruses, and less than 1 viable helminth ova/4 g total solids (dry weight) for helminth (U.S. EPA, 1994, U.S. EPA, 2003).

Class B pathogen reduction requirements are valid for any use other than the ones mentioned for Class A requirements. It is stated that a fecal coliform density of 2 million MPN or CFU/ g of total solids (dry weight) in the treated sewage sludge is required (U.S. EPA, 2003).

For Class B biosolids, there does not exist an obligation to monitor the biosolids for possible regrowth or activation of pathogens or indicator organisms because it is thought that biosolids obtained at the end of an approved process would meet the most important time, temperature and indicator organism criteria.

Class A requirements, unlike Class B, are not based on average values. Each sample taken for analysis must comply with the exact given numerical limit. However, for achieving Class B requirements measuring the geometric mean fecal coliform density of 7 treated samples is one of the options that can be used (U.S. EPA, 2003).

For biosolids that meet the Class A requirements it is not necessary to restrict public access, but biosolids meeting the Class B requirements still contain considerable amounts of pathogens, therefore public access is restricted and there are stated site

restrictions in the regulation that limit crop harvesting, animal grazing and public access for a certain amount of time (Lu et al., 2012).

### 1.6.2. EU Regulations on Biosolids

Regulations regarding the use of biosolids for agricultural purposes in the EU is described in the 1986 Directive which contains 18 articles. The regulatory directive, 86/278/EEC has the purpose of preventing hazardous effects on soil, vegetation, animals and humans.

It is an obligation for each member state to adopt this directive to their national legislations (Evans, 2012). In addition, the member states are allowed to adopt the standards more strictly. There are no specified limits for pathogen densities in 86/278/EEC, but the necessity of treating the biosolids before land application is stated. The member states decide the required treatment level and technology for themselves (Iranpour et al., 2004). France, Italy, Luxemburg and Poland are some of the countries that set some limitations on pathogens even though no requirements are presented in the main directive (National Research Council, 2002). The selected pathogen limitations of these member states are as given in Table 1.1.

There are more specific limitations about pathogen reductions, treatment processes and site restrictions in the 2000 working document of EU (Iranpour et al., 2004).

### 1.6.3. Turkey's Regulations on Biosolids

The standards and limitations about land application of biosolids is handled in a very specific regulation, "Regulation on the Land Application of Domestic and Urban Sewage Sludge". The regulation was issued on August 3, 2010 in Official Gazette with number 27661. The regulation includes technical and administrative issues on the controlled application of the sludge resulting from the treatment of domestic and urban wastewater on land without causing any harm to soil, plants, animals or humans.

In the regulation there are some limit values given for heavy metals and for some organic compounds that might be present in the treated sludge. However, the only limit given regarding pathogen reduction is that at the end of the stabilization method

applied to sludge, at least 2 log10 (99%) reduction in the amount of *E. coli* should be obtained.

**Table 1.1.** European Limit Concentrations for Pathogens (National Research Council, 2002)

| Country | Salmonella | Other Pathogens |
|---|---|---|
| **France** | 8 MPN/10 g dry matter | Enterovirus: 3 MPCN/10 g of dry matter |
| **Italy**<br>**Luxemburg** | 1000 MPN/g dry matter | Enterobacter: 100/g<br>No egg of worm likely to be contagious |
| **Poland** | Biosolids cannot be used for agriculture if they contain Salmonella | “Parasites”: 10/kg dry matter |

### 1.7. Aim of the Study

The overall objectives of this study are as follows. The first objective was to accurately measure the amount of *Salmonella* in Turkish biosolids by applying the internationally accepted best available methods. There is an increasing need to know the pathogen amounts in Turkish biosolids so as to be able to be sure about their safety. This is necessary for further use of the potential use of biosolids in land applications. Since there is no published data on this specific issue, this study is expected to be useful in that matter. Additionally, by obtaining data from treatment plants of different cities comparison between the technologies and city conditions can be achieved.

Second objective was to evaluate the effectiveness of PMA assisted Taqman qPCR on *Salmonella* quantification, and finally to compare the data obtained by the two different quantification methods; culture based and molecular. Each of these objectives are expected to offer new data and perspective on the issue.

# CHAPTER 2

# MATERIALS AND METHODS

## 2.1. Sample Collection

The biosolid samples used throughout the experiments are taken from the municipal wastewater treatment plants of 4 different cities of Turkey. The cities from which the biosolid samples are taken are; Ankara, Eskişehir, Kayseri and Yozgat. The biosolid samples are taken right after the dewatering units of the treatment plants.

Samples were collected in clean plastic containers and transported in insulated carriage bags in which ice blocks are put to retard bacterial growth and activity.

The samples are collected monthly starting from September, 2015 till May, 2016. The treatment plant configurations of these cities are mentioned in section 2.3.

## 2.2. Total Solids Determination

Total solids determination is necessary to determine the dry weight percentage of the biosolid samples. This is done for expressing the results in "per dry weight" basis. After weighing the portion of biosolids for use in microbiological analysis, another portion of 30 g was weighed and placed in a crucible. This sample was dried overnight at 105°C and cooled in a desiccator prior to weighing. Then, the dry weight percentage was calculated.

## 2.3. Culture Based Experiments

U.S. EPA's "Salmonella in Sewage Sludge (biosolids) by Modified Semi-Solid Rappaport-Vassiliadis Medium" method was used to conduct culture-based quantifications for nine months of biosolid samples. The method is commonly known as Method 1682.

### 2.3.1. Materials of Method 1682

**Phosphate Buffered Dilution Water**

Before preparing the phosphate buffered dilution water there are two stock solutions that needs to be prepared: phosphate buffer and magnesium chloride ($MgCl_2$) solutions. For phosphate buffer solution, 34 g of monopotassium phosphate ($KH_2PO_4$) was dissolved in 500 mL of reagent-grade water. The pH of the solution was brought to 7.2 by adding 1 N NaOH, and then the volume was brought to 1 L with reagent-grade water. The solution was then autoclaved at 121 °C for 15 minutes. For $MgCl_2$ solution, either 38 g anhydrous $MgCl_2$ or 81.1 g magnesium chloride hexaydrate ($MgCl_2 \cdot 6H_2O$) was added to 1 L reagent-grade water. The solution was then autoclaved at 121 °C for 15 minutes. The stock solutions were stored at 4°C.

Phosphate buffered dilution water was prepared by mixing 1.25 mL of stock phosphate buffer and 5 mL of $MgCl_2$ stock per liter of reagent-grade water. The dilution water was also autoclaved at 121 °C for 15 minutes. Phosphate buffered dilution solution was stored at room temperature.

**Tryptic Soy Broth (TSB)**

Tryptic Soy Broth was prepared as in the forms of single strength (1X) and triple strength (3X) solutions. For 1X TSB solution, 6 g TSB (VWR Chemicals/Merck) was added into 200 mL of reagent-grade water. It was then divided to tubes in volumes of 10 mL. These 10 mL 1X TSB tubes were prepared to be used further for the inoculation of samples in 1 mL volumes. 3X TSB was obtained by adding 12 g TSB (VWR Chemicals, Merck) in 133.4 mL reagent-grade water. The broth was then divided into tubes in 10 and 5 mL of volumes.

During sample dilution, the 3X TSB tubes containing 10 mL of media were inoculated with 20 mL of sample, and the tubes with 5 mL of media were inoculated with 10 mL of sample. The tubes were all autoclaved at 121 °C for 15 minutes.

TSB was stored at 4 °C or freshly prepared on the day of use, but in either case was acclimated to room temperature prior to analysis.

**Modified Semi-Solid Rappaport-Vassiliadis (MSRV) Medium**

12 g of MSRV (Merck) and approximately 2.66 g of agar powder (VWR Chemicals) were added into 400 mL reagent-grade water. The mixture was heated to boiling until complete dissolution, yielding a clear solution with a blue-green color. Dissolution was aided by magnetic stirring. The glassware was then placed in a hot water bath with stirring. MSRV medium should not be autoclaved. After dissolution, the mixture was cooled down to 50 °C and 0.4 mL of a 2% stock solution of novobiocin was added into 400 mL medium. The medium was mixed thoroughly following the addition of novobiocin. It should be noted that after pouring the medium into petri plates, the plates were not inverted since MSRV is a semi-solid medium. Plates were stored at room temperature prior to use. The novobiocin in the MSRV medium selects against non-*Salmonella* species.

Novobiocin stock was obtained by dissolving 0.2 g sodium novobiocin in 10 mL distilled water. It was sterilized by passing through a sterile 0.22 µm filter into a sterile container. The stock solution was then divided into 2 mL Eppendorf tubes and stored at -20 °C.

**Xylose-Lysine Desoxycholate (XLD) Agar**

5.5 g of XLD powder (VWR Chemicals) and 1.5 g agar (VWR Chemicals) were added per 100 mL for the preparation of XLD medium. Just like MSRV, XLD was heated to boiling with constant stirring until complete dissolution, yielding a bright red color. The glassware container was placed in a hot water bath and the chosen temperature of the stirrer is 135 °C by experience. After dissolution was completed, the media was cooled to 50 °C, poured into petri dishes and allowed to cool.

**Triple Sugar Iron (TSI) Agar**

19.5 g TSI powder (Merck) and 3 g agar (VWR Chemicals) were added into 300 mL of distilled water for preparing the TSI agar. The media was heated and stirred until complete dissolution and then divided into test tubes in volumes of 7 mL. The tubes were then autoclaved at 121 °C for 15 minutes. After autoclaving, the test tubes were

left to cool and solidify in a rack which is tilted such that the media would form a slant in the tubes.

**Lysine Iron Agar (LIA)**

For the preparation of LIA, 9.6 g LIA powder (Merck) and 3 g (VWR Chemicals) were added into 300 mL distilled water. The media was heated and stirred to dissolution, and then divided into test tubes in volumes of 7 mL. The tubes were then autoclaved at 121 °C for 15 minutes. After autoclaving, the test tubes were left to cool and solidify in a rack which was tilted such that the media would form a slant in the tubes.

**Urea Broth or Urease**

3.85 g of urea broth powder was added into 100 mL distilled water while preparing urease. The solution was mixed thoroughly with mild heating to dissolution. For sterilization, the solution was passed through a sterile 0.22 μm filter into a sterile flask. The sterile solution was then divided into test tubes in volumes of 3 mL.

**Heart Infusion Agar (HIA)**

For preparing HIA, 7.5 g Brain Heart Infusion Broth powder (Fluka) and 5.25 g BactoAgar (BD) were added into 300 mL distilled water. The solution was mixed and heated to dissolution and then autoclaved at 121 °C for 15 minutes. After cooling to 50 °C, it was poured into petri dishes and allowed to cool and solidify.

### 2.3.2. Culture-Based Quantification Procedure

#### 2.3.2.1. Sample Preparation

Sample preparation was aimed primarily at homogenization. 30 g of the biosolid sample was weighed. Pieces of wood, plant stems or leaves were removed and discarded if present. The sample was transferred into a sterile blender and 270 mL of sterile phosphate buffered dilution water was added. The mixture was then blended at high speed for 1-2 minutes. Each sample was quantified in triplicate and the results were taken as the average. Additionally, each sample was analyzed alongside one negative control with a soil sample which is *Salmonella*-free was analyzed. The soil sample in question is simply garden soil purchased from a random local store. Method 1682 was previously applied to the soil to confirm *Salmonella* absence.

#### 2.3.2.2. Enrichment Step

The enrichment medium that was used in this step was tryptic soy broth (TSB). Inoculation of the samples started with the enrichment step. This step encouraged the growth of *Salmonella*, but not exclusively for *Salmonella*. Therefore, several other steps were required for confirmation.

20 mL of homogenized sample was inoculated into test tubes containing 10 mL of 3X TSB; to test tubes with 5 mL of 3X TSB, 10 mL of homogenized sample was inoculated; and finally to the test tubes that contained 10 mL of 1X TSB, 1 mL of homogenized sample was inoculated. The test tubes were incubated for 24 hours at 36.5 °C. At the end of the incubation time, all tubes were highly turbid in most instances because of the rich and non-inhibitory nature of the enrichment medium; therefore, they were all taken as positives at this first step. However, it should be noted that the true positive results are not obtained until the last step of the procedure. Appearance of no growth in the tubes would have indicated the presence of a toxic substance or that the tubes were not inoculated in the first place (EPA Method 1682 Guideline, 2006). The dilutions made during the enrichment step is seen on Figure 2.1.

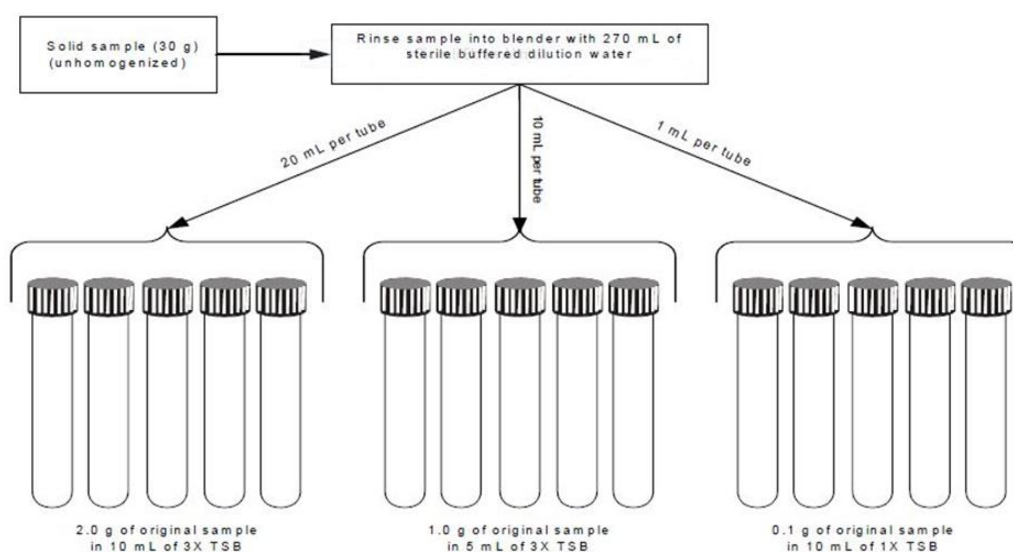


**Figure 2.1.** Schematic representation of the dilutions during enrichment step (EPA Method 1682 Guideline, 2006)

#### 2.3.2.3. Selection Step

From each TSB tube, six separate 30 µL drops were applied onto one MSRV plate. The drops were placed evenly over the area of the plate. MSRV plates were incubated at 42.5 °C for 18 hours. After the incubation period, the plates were examined for the signs that indicate the motility of present bacteria, indicated by a whitish halo around the point of inoculation. An MSRV plate with the six even inoculation points and with the white halos after incubation is illustrated in Figure 2.2.

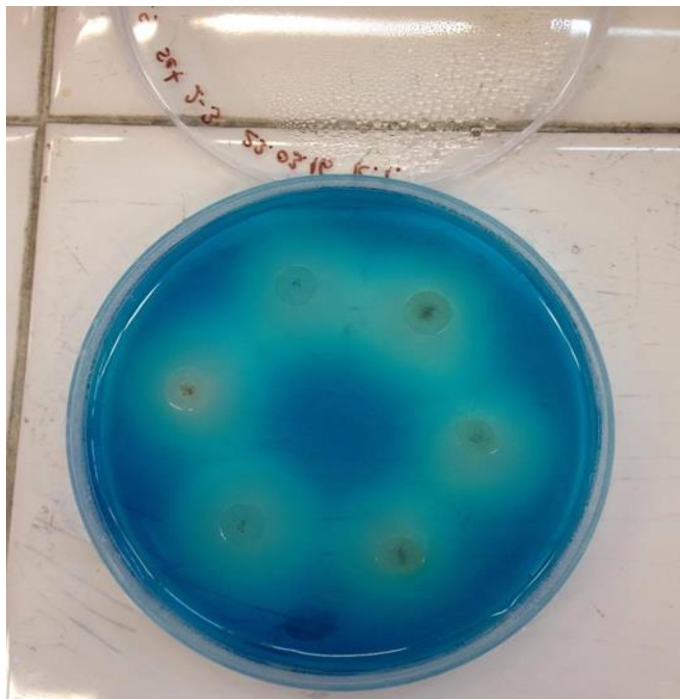

**Figure 2.2.** View of a positive MSRV plate

With a sterile inoculating loop, a representative halo is stabbed from the outer edge of a target colony and streaked onto a XLD plate. The loop should penetrate the MSRV medium at least half-way because *Salmonella* predominantly locate within the media. XLD plates are incubated for 24 hours at 36.5 °C. Black or pink/red colonies with black centers were considered *Salmonella* and taken as positive (Figure 2.3 and Figure 2.4). The positive XLD plates were later submitted to biochemical confirmation.

This part of the selection step, obtaining single *Salmonella* colonies on XLD plates, is called isolation.

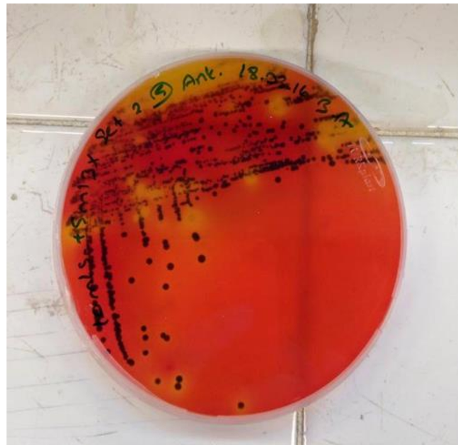


**Figure 2.3.** View of a positive XLD plate, black colonies representing Salmonella species

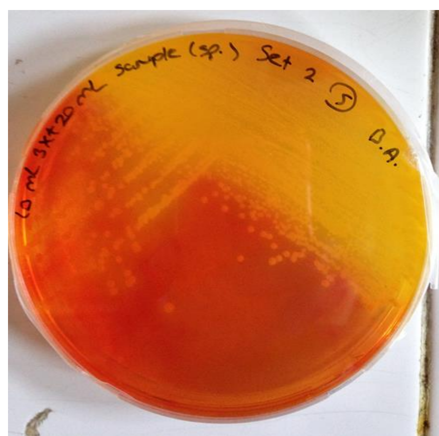


**Figure 2.4.** View of a negative XLD plate having no black colonies to indicate the presence of Salmonella

#### 2.3.2.4. Biochemical Confirmation Step

From the colonies isolated on XLD agars, a representative one was chosen and inoculated on triple sugar iron (TSI) agar slants, lysine iron agar slants and urea broth. Inoculation on slant agars were done by stabbing the butt and streaking the slant. Since the urea broth is a liquid media, it is enough to just immerse the loop in the tube and mix. It is more preferable to use the same colony for all three media. The slants and urea broth were incubated for 24 hours at 36.5 °C.

The blackening in the color of the butts of both TSI and LIA slants is considered a positive reaction for *Salmonella.* The urea broth is originally an orange-pink medium and its color changes to deep pink or purplish-red if the result is positive. However, in our case we looked for a negative result with the urea broth because *Salmonella* are negative with urease, which means there needs to be no color change after inoculation, and then incubation.

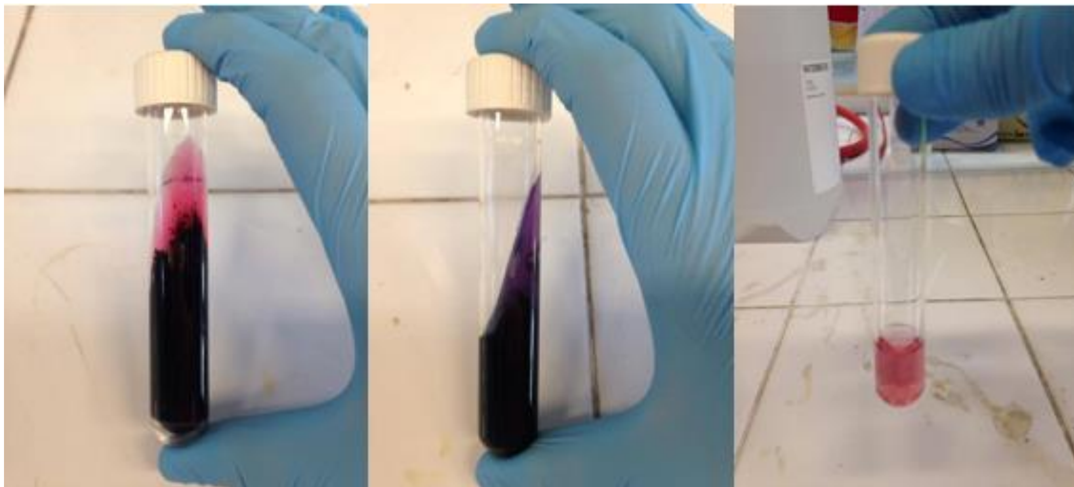

**Figure 2.5.** Views of positive TSI and LIA slants, respectively. Urea broth does not give positive results with Salmonella (photo on the right)

At the end of the biochemical confirmation step, in order for the original TSB tube to be taken as positive for *Salmonella,* all the corresponding inoculations must be MSRV,

XLD, TSI, LIA positive and urease negative. From the final results, which are actually a series of numbers indicating the number of positive tubes, the MPN value is determined from the MPN index table relevant for Method 1682 provided with the method.

If, for example, there exists 3 positive tubes among the 20 mL sample containing tubes, 4 among 10 mL sample containing tubes, and 2 among 1 mL sample containing ones; the combination will be 3-4-2. The corresponding MPN index result is read from the relevant tables for this combination, which is 0.0984.

Figure 2.6 shows these mentioned inoculation steps of Method 1682 on a scheme.

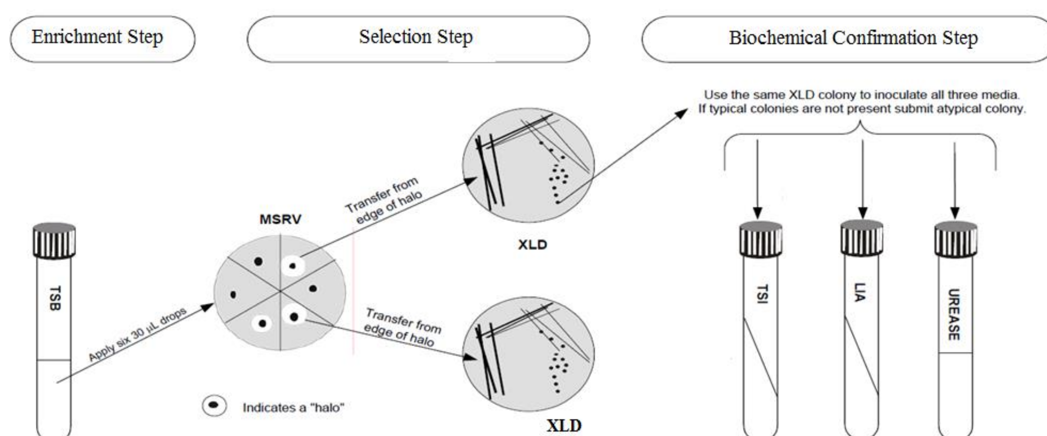


**Figure 2.6.** Schematic representation of the steps of Method 1682 (EPA Method 1682 Guideline, 2006)

### 2.3.3. Spiking Procedure

The spiking procedure consisted of adding *Salmonella* from pure culture to a soil sample known to be *Salmonella*-free throughout the steps of Method 1682 instead of the biosolid samples in order to calculate and obtain the recovery percentage of pure *Salmonella* in the end. In addition to calculating the recovery percentage, the spiking procedure was used for initial precision determination. This is called as initial

precision and recovery (IPR). The IPR analyses are used to achieve an acceptable method performance, both for recovery and precision, and should be performed before the method is used for the analysis of actual biosolid samples (EPA Method 1682 Guideline, 2006). In the first few months of experimentation, 16 experimental set-ups were conducted in total from May, 2015 until August, 2015 to stabilize the recovery percentages and also to be able to perform the method effectively in an established order.

#### 2.3.3.1. Preliminary Preparation to Spiking

In advance to the spiking procedure, an "undiluted spiking suspension" was prepared. First, a stock culture was prepared by inoculating *Salmonella typhimurium* onto a heart infusion agar (HIA). The plate was incubated at 36.5 °C for 24 hours. Then, a 1% TSB solution was prepared, meaning that 1 mL of 1X TSB was mixed with 99 mL of phosphate buffered dilution water. The solution was mixed very well to obtain a homogeneous solution. From the *Salmonella typhimurium* stock culture, a loopful of colony was transferred and inoculated in the 1% TSB solution and vigorously shaken at least 25 times. The solution was incubated at 36.5 °C for 24 hours. This undiluted spiking suspension contained approximately $10^7$to $10^8$ *Salmonella typhimurium* colony forming units (CFU) per mL (EPA Method 1682 Guideline, 2006).

#### 2.3.3.2. Sample Spiking

The first step of the sample spiking procedure was to dilute the spiking suspension. After the undiluted spiking suspension was prepared and mixed intensely, 1 mL was transferred into 99 mL sterile phosphate buffered dilution water. It was also capped tightly and mixed by shaking the bottle at least 25 times. The bottle containing this spiking suspension dilution was labeled as "A". Bottle "A" was a 1/100 dilution of the original undiluted spiking suspension. Then 1 mL of diluted spiking suspension "A" is added to 99 mL of phosphate buffered dilution water. The bottle was also capped tightly and vigorously shaken at least 25 times. This was spiking suspension dilution "B", which was a 1/10,000 dilution of the original undiluted spiking suspension. To obtain dilution "C", 10 mL of "B" was transferred into 90 mL of buffered solution, yielding a 1/100,000 dilution of the original undiluted spiking suspension. In the same

way, 10 mL of “C” was transferred into buffered solution to obtain a 1/1,000,000 dilution, sample “D”.

The soil sample which needed to be confirmed not to contain *Salmonella* species prior to analysis was first homogenized. To the homogenized sample, 0.5 mL of spiking suspension “D” was added and then blended on high speed for 1-2 minutes. This was called as the “spiked” sample. Once the spiked sample was obtained the procedure proceeded exactly the same way as it did with actual biosolids.

#### 2.3.3.3. Enumeration of Undiluted Spiking Suspension

Once the diluted spiking suspensions were obtained, they were inoculated onto HIA plates to estimate the amount of *Salmonella* which was originally present in the undiluted spiking suspension. For that the following steps were conducted in three replicates:

- 0.1 mL of dilution “B” was spread on the surface of a HIA plate, obtaining $10^{-5}$ of the original spiking suspension
- 0.1 mL of dilution “C” was spread on the surface of a HIA plate, obtaining $10^{-6}$ of the original spiking suspension
- 0.1 mL of dilution “D” was spread on the surface of a HIA plate, obtaining $10^{-7}$ of the original spiking suspension

Since the procedure was repeated three times, in the end nine spread plates are obtained. The inoculum on each plate was distributed evenly over the surface of the plate with the help of a glass rod or a spreader of any kind while rotating the dish by hand. The plates were left for a few minutes to allow for the inoculum to be absorbed into the medium completely. The plates were inverted and incubated at 36.5 °C for 24 hours. The number of colonies were counted and recorded for each plate.

The recovery percentage calculated from this spiking procedure was 88% with a standard deviation of 26.18%. Both these values are within the range and in compliance with the values stated in the guideline of U.S. EPA Method 1682, presented in Table 2 of Section 9.3. The given range for the recovery percentage is 0%-254%, and the maximum relative standard deviation value is given as 92%.

## 2.4. Molecular Experiments

### 2.4.1. DNA extraction

A pure culture of *Salmonella enterica* Typhimurium was grown overnight in rich media to an $OD_{600}$ of approximately 1 and subjected DNA extraction using the E.Z.N.A.Bacterial DNA Kit (Omega Bio-Tek, GA, USA). For biosolids samples, total DNA was extracted from 500mg of sample using the E.Z.N.A. Soil DNA Kit (Omega Bio-Tek, GA, USA) and eluted twice with 25 ul of sterile water for a total of into 50 ul elution. DNA samples were quantified and checked for quality via NanoDrop (Thermo Fisher Scientific).

### 2.4.2. PMA Treatment

Propidium monoazide (PMA) was purchased from Biotium (CA, USA) and prepared as a 10 mM stock solution in 20% dimethyl sulfoxide (DMSO). 50 ul of biosolid sample diluted in 450 ul of phosphate buffer was mixed with 5 ul of the PMA stock solution, placed in an Epindorf tube, and incubated in the dark at room temperature for 10 minutes. The tube was then placed on a sheet of aluminum foil on top of ice. The sample was then placed under a 400W halogen light at a distance of 15 cm for 10 minutes with vigorous mixing every minute. Following PMA treatment, biosolid samples were subjected to DNA extraction and quantification as described in the previous sections.

### 2.4.3. PCR Conditions and t-cloning

PCR using the *Salmonella invA* primers SalF/SalR was used to confirm the presence of quality DNA in all extracts. The reaction mixture consisted of 10 ul Hotstart PCR Mastermix, 1ul 1/100X diluted template DNA, 0.2 uM of each primer, and water to 20 ul. The thermocycling conditions consisted of an initial activation at 95°C for 5 minutes followed by 40 cycles consisting of 95°C for 15 seconds, 52°C for 20 seconds, and 72°C for 40 seconds, followed by a final extension of two minutes at 72°C. Products were visualized by gel electrophoresis.

#### 2.4.3.1. Creation of Template Standard

*invA* PCR product using *S. enterica* as template was t-cloned into the plasmid pGEMt-easy using the manufacturer's instructions and transformed into *E. coli* JM109. The

successful construct was confirmed by PCR. This construct, pGEMt:*invA*, was then be grown, extracted by standard alkaline lysis procedure (Sambrook&Russel), and used as a quantification standard for all subsequent assays. DNA was quantified and its quality checked (A260/280 and A260/230) by NanoDrop.

#### 2.4.3.2. qPCR Methods

Primers and TaqMan® probes were added to TaqMan® Universal PCR Mastermix (Applied Biosystems, Foster City, CA, USA) to a final concentration of 250 nM TaqMan® probes and 1 µM for each primer. Real-time PCR reactions were executed in an ABI7900 qPCR Thermocycler amplification conditions of 50 °C for 2 min, 95 °C for 10 min, followed by 45 cycles at 95 °C for 15 s and 60 °C for 60 s (Daum et al., 2002).

**Table 2.1.** Primers and probe used in the Taqman qPCR experiment

| **Primers** | |
|---|---|
| Sal-F | GCGTTCTGAACCTTTGGTAATAA |
| Sal-R | CGTTCGGGCAATTCGTTA |
| **TaqMan Probes** | |
| Sal-TM | TGGCGGTGGGTTTTGTTGTCTTCT |

## 2.5. Wastewater Treatment Plants

Within the context of the study biosolid samples are taken from 4 different municipal treatment plants of Turkey. The samples are collected monthly, for 9 months starting from September, 2015. The descriptions of the features of these treatment plants are as stated below.

### 2.5.1. Ankara Wastewater Treatment Plant

Ankara Tatlar Central Wastewater Treatment Plant is Turkey's biggest wastewater treatment plant, and it is also the 4$^{th}$ biggest treatment plant in the world. It is designed to work with the capacity of 765,000 $m^3$ wastewater/day to treat. The approximate

number of people that the plant serves is 4 million. The sludge produced at the end of the treatment processes is disposed by storing at storage areas.

The first stage of the treatment process is preliminary treatment and it consists of coarse/fine screens and grit chambers. These units are followed by primary sedimentation, aeration and secondary sedimentation units. The sludge coming from primary and secondary sedimentation tanks is mixed in sludge thickening unit. The retention time of the thickening process is 2 days. Thickened sludge is then stabilized in mesophilic anaerobic digestion tanks in 35°C. Generated biogas is used for electricity production, obtaining hot water, and for the mixing process occurring in the digestion tanks. Digested and thickened sludge is then dewatered with the use of centrifugal decanters. Total sludge production in the treatment plant is about 200 tons/days.

The process flow chart of Ankara Tatlar Wastewater Treatment Plant is shown in Figure 2.7 given below.

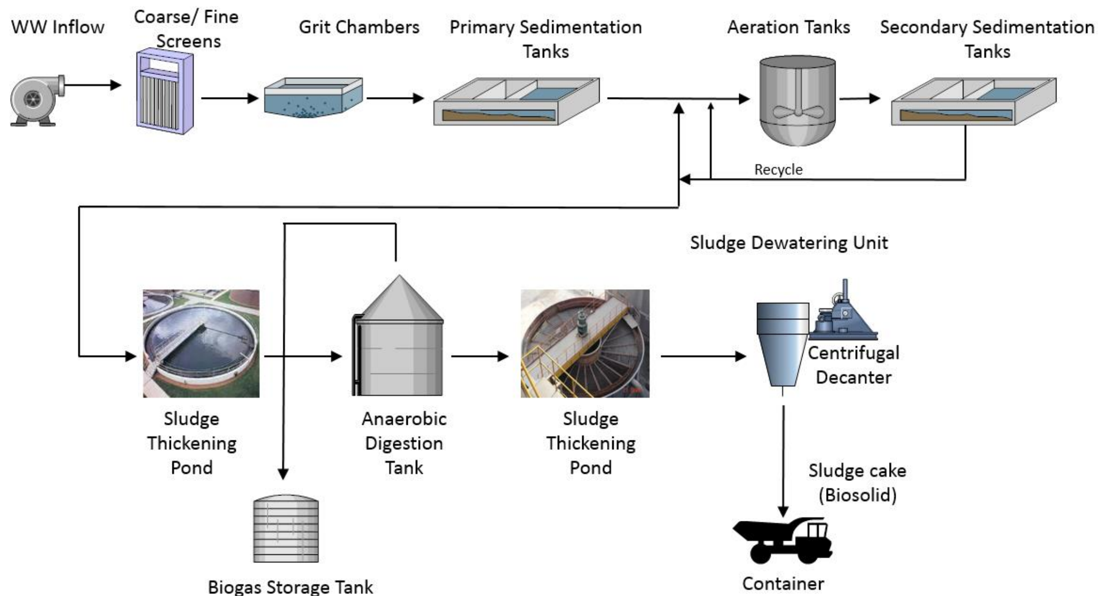


**Figure 2.7.** Flow scheme of Ankara Wastewater Treatment Plant

### 2.5.2. Eskişehir Wastewater Treatment Plant

Eskişehir Biological Wastewater Treatment Plant has started its operation with its current, existing plant (in 2006) and the recent additional plant in 2010. The plant has the capacity of treating 105,000 $m^3$ wastewater/day, and the approximate number of people that the plant gives service is 650,000.

The existing plant uses primary sedimentation tanks and biological treatment units (aeration tanks) actively. During preliminary treatment wastewater is passed through coarse and fine screens, and grit chambers. After this stage the wastewater is transferred to primary sedimentation tanks and sent to aeration tanks afterwards. The existing plant is operated with an activated sludge system that will provide the removal of carbon.

On the other hand, the additional plant consists of an activated sludge system that will accomplish the removal of carbon, nitrogen and phosphorus (anaerobic-anoxic-aerobic). Anaerobic zone is the first part of these types of activated sludge systems to provide the biological removal of phosphorus. While at anoxic zone denitrification part of nitrogen removal process occurs, at aerobic zone nitrification occurs. After these processes wastewater is sent to secondary sedimentation tanks.

Sludge coming from the primary sedimentation tanks of both of the plants are sent to gravity sludge thickener, while polyelectrolyte is being added to the excess sludge from the secondary sedimentation tanks and sent to mechanical sludge thickener. All thickened sludge is then mixed and transferred to mesophilic anaerobic digestion tanks. The gas produced in the digesters is used for electricity production and for the heating of the digesters and the buildings within the treatment plant. Digested sludge is then dewatered by centrifugal decanters. Total sludge production of the plant is 40 tons/day.

The flow chart of Eskişehir Wastewater Treatment Plant is given in Figure 2.8 below.

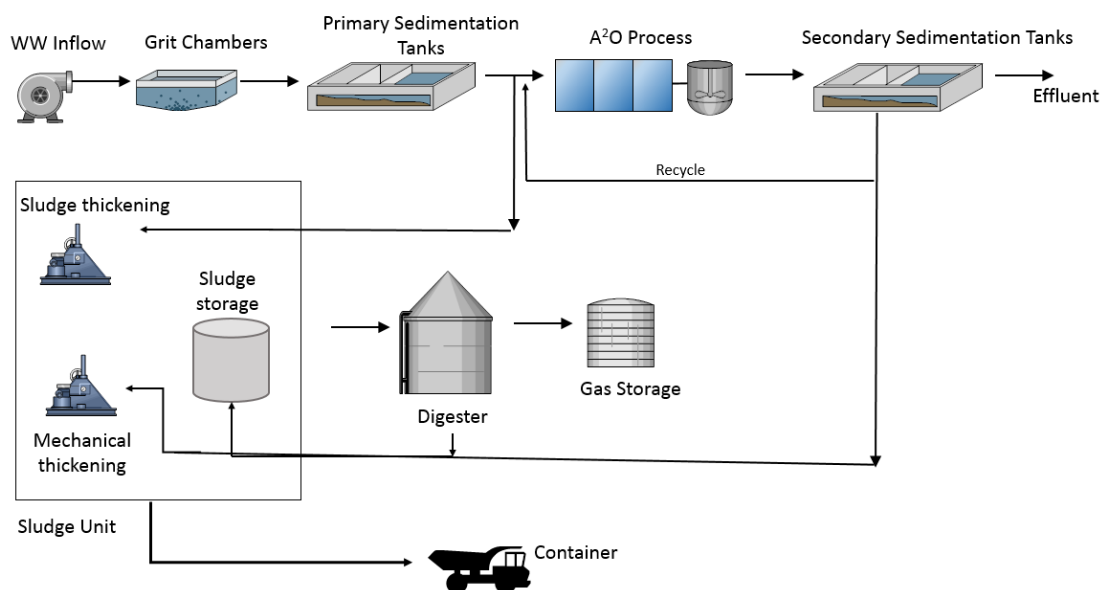


**Figure 2.8.** Flow scheme of Eskişehir Wastewater Treatment Plant

### 2.5.3. Kayseri Wastewater Treatment Plant

Kayseri Advanced Biological Wastewater Treatment Plant is under operation since 2004, and has the capacity to treat 140,000 $m^3$wastewater/day. The approximate number of people that the treatment plant serves is 912,000. The sludge produced is disposed by pouring it at storage areas.

The first stage of the treatment process is comprised of coarse/fine screens and grit chambers. Wastewater then enters to primary sedimentation tanks and then the overflow is sent to selector/bio-phosphorus tank. The anaerobic tank is used for the purpose of removing phosphorus from the wastewater with the help of microorganisms, and then the wastewater is transferred to anoxic/oxic aeration tanks for the occurrence of denitrification and nitrification processes. After this stage wastewater is sent to secondary sedimentation tanks.

In Kayseri Advanced Wastewater Treatment Plant sludge is obtained after two different process stages. Sludge coming from primary sedimentation tank is sent to primary thickener. Thickened sludge is then digested in mesophilic anaerobic digesters at 37 °C with a solids retention time (sludge age) of 20 days. Digested sludge is taken

to secondary thickening tank and dewatered from there with the use of a belt press. Another source of this dewatered sludge is the part which settled down in the secondary clarifier. This portion of sludge is transferred directly to the belt press without coming over to the digesters.

One of the major differences of this treatment plant, when compared to others, is that the sludge digested and thickened before the dewatering process is mixed with the activated sludge coming from the secondary sedimentation tank and sent to belt presses in this way.

The total sludge production in this treatment plant is 64 tons/day.

The flow chart of Kayseri Wastewater Treatment Plant is presented in Figure 2.9 below.

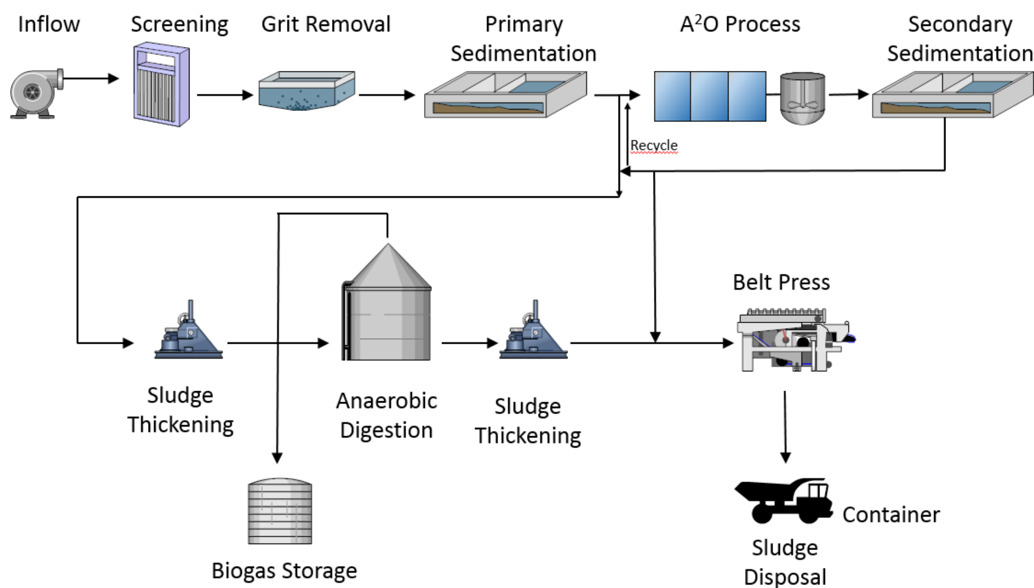


**Figure 2.9.** Flow scheme of Kayseri Wastewater Treatment Plant

### 2.5.4. Yozgat Wastewater Treatment Plant

Yozgat Municipal Wastewater Biological Treatment plant has started its operation in 2006 and has the capacity to treat 24,000 $m^3$ wastewater/day. The number of people that the treatment plant serves is around 76,250. The disposal of the produced sludge is ensured by pouring it to Yozgat Municipality Solid Waste Landfill.

The first stage in the treatment processes is preliminary treatment which consists of coarse/fine screens and grit chambers. Primary sedimentation, aeration and secondary sedimentation units proceed this stage. Sludge coming from primary and secondary sedimentation tanks is mixed in sludge thickening ponds, and thickened there. Thickened sludge is then stabilized in aerobic sludge digestion units with the help of aerators. The digested sludge is then dewatered by belt press. Total sludge production of the treatment plant is around 1.2 tons/day.

The flow chart of Yozgat Wastewater Treatment Plant is in Figure 2.10.

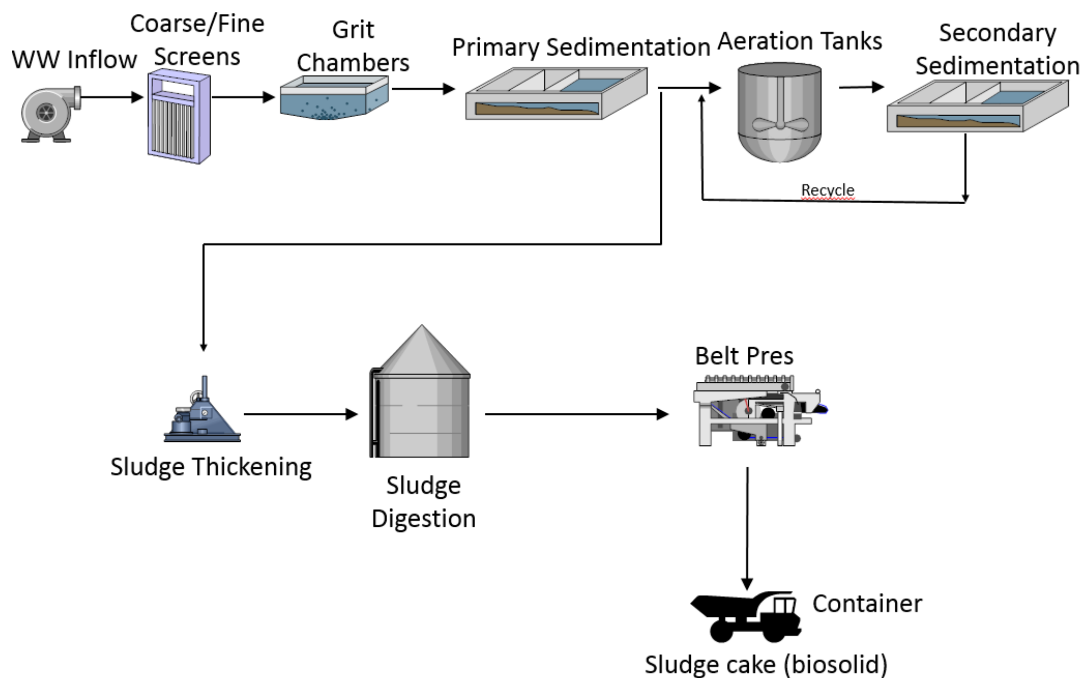


**Figure 2.10**. Flow scheme of Yozgat Wastewater Treatment Plant

## 2.6. Temperature and Rainfall Data

Temperature and Rainfall information for the cities in which the treatment plants are located were gathered from *accuweather.com*.

## 2.7. Statistical Methods

The experimental methods are conducted in 3 replicates in order to be able to obtain an average result in the end. However, in the data set there had been some data points that seemed to be unlikely to occur when compared to other results. In determining these outliers in the data sets, Grubbs' test is used.

# CHAPTER 3

# RESULTS AND DISCUSSION

## 3.1. MPN Results

The first samples arrived in September, 2015 and the last ones arrived in May, 2016; the results given are cover nine months. The average MPN/4 g dry weight values of three sets data are taken.

In the following sections the quantification results obtained from the wastewater treatment plants of 4 cities of Turkey by using the culture based Method 1682 are presented.

### 3.1.1. Ankara

The MPN Results for the biosolid samples coming from Ankara's wastewater treatment plant are given in Table 3.1. The results obtained from Ankara biosolid samples were fairly consistent month-to-month, ranging from 10-27 MPN/4g. The outlier test used for the detection of outliers within the data set detected no outliers.

To be able to explain the distribution of the results, the correlation of the results with some climactic factors were examined.

**Table 3.1.** Ankara MPN Results

| | Sampling Date | *Salmonella* quantity (*Salmonella spp.*/4 g-dry weight) |
|---|---|---|
| **Ankara Tatlar Wastewater Treatment Plant** | 30.09.2015 | 14,7±2,5 |
| | 31.10.2015 | 15,4±3,6 |
| | 24.11.2015 | 15,3±1,4 |
| | 24.12.2015 | 10,4±1,8 |
| | 30.01.2016 | 27,9±5,9 |
| | 26.02.2016 | 15,2±2,7 |
| | 23.03.2016 | 9,7±3,5 |
| | 30.04.2016 | 5,2±0,7 |
| | 31.05.2016 | 7,6±2,9 |

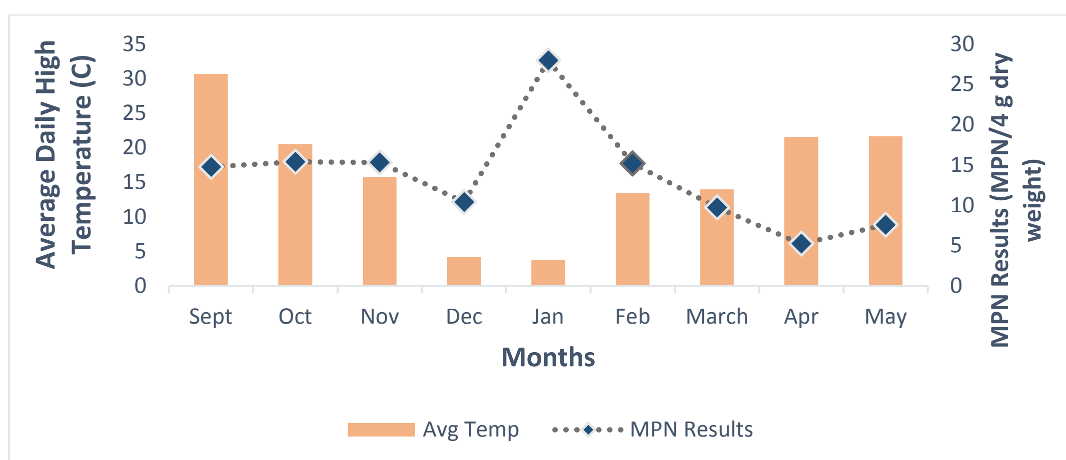


**Figure 3.1.** Ankara MPN results and temperature

Figure 3.1 represents the MPN results and the monthly average temperatures of Ankara. The MPN results showed an inverse trend with temperature. Whenever the average temperature value decreased, the MPN results tend to increase, and there was an increase in the temperature, the results decreased. This pattern can most clearly be seen at January, the month in which the MPN results have their peak, and at the same time the month in which the average temperature was the lowest. Additionally, through

the 4 months following January, the same inverse pattern seems to be present. As the temperature increased approaching summer, MPN results dropped.

Figure 3.2 shows the MPN results along with the monthly precipitation. Precipitation and the amount of pathogens in biosolids seemed to show a correlation with each other, especially in some months. Through November till April, the amount of precipitation and the quantification results increase and decrease together. However, the lack of this pattern throughout the whole sampling period makes an exact conclusion impossible.

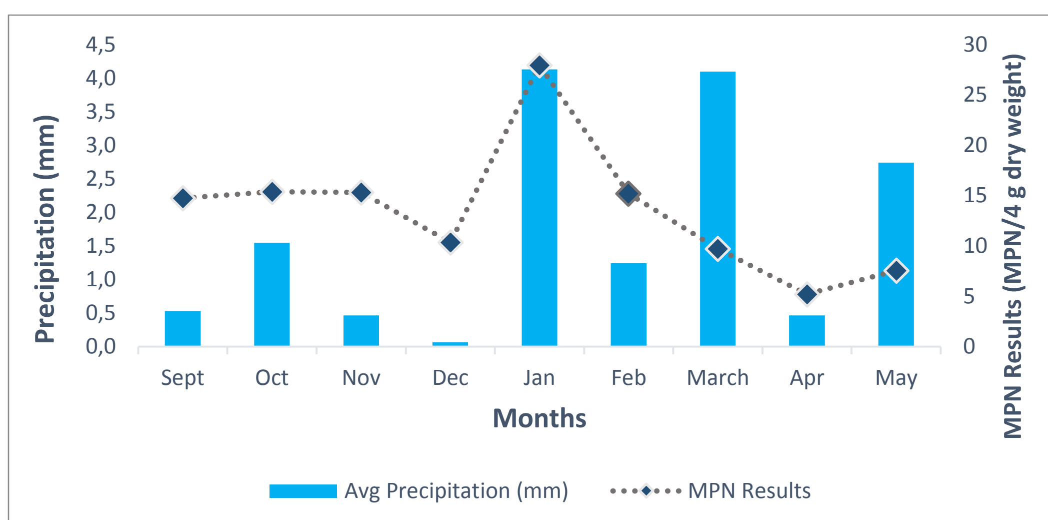


**Figure 3.2.** Ankara MPN results and precipitation

The TSS amount of Ankara biosolid samples was 0.27, and this value stayed constant through the whole sampling period.

### 3.1.2. Eskişehir

The MPN results for biosolid samples from Eskişehir Wastewater Treatment Plant are given in Table 3.2. The data of Eskişehir city were also consistent month-to-month, ranging from 8 to 39 MPN/4g. Overall, these results are consistent with previously analyzed Class B biosolids.

Figure 3.3 shows MPN results and the monthly average temperatures of Eskişehir. There seems to be no clear correlation between the two data sets. Figure 3.4 shows the

MPN results distribution along with the monthly average precipitation amounts. Again, during some month intervals MPN results seemed to increase with increasing rainfall amounts, whereas during some other month intervals the results decrease as the amount of rainfall increases. However, it seemed like the effects of the precipitation were observed in the MPN results in the following month.

**Table 3.2.** Eskişehir MPN results

| | **Sampling Date** | ***Salmonella* quantity (*Salmonella spp.*/4 g-dry weight)** |
|---|---|---|
| **Eskişehir Wastewater Treatment Plant** | 28.09.2015 | 10.2±1.8 |
| | 28.10.2015 | 9.6±4.4 |
| | 20.11.2015 | 17.9±2.3 |
| | 24.12.2015 | 11.2±1.4 |
| | 20.01.2016 | 23.5±1.2 |
| | 26.02.2016 | 39.3±7.2 |
| | 21.03.2016 | 27.6±8.2 |
| | 25.04.2016 | 13.9±1.2 |
| | 27.05.2016 | 23.4±0.7 |

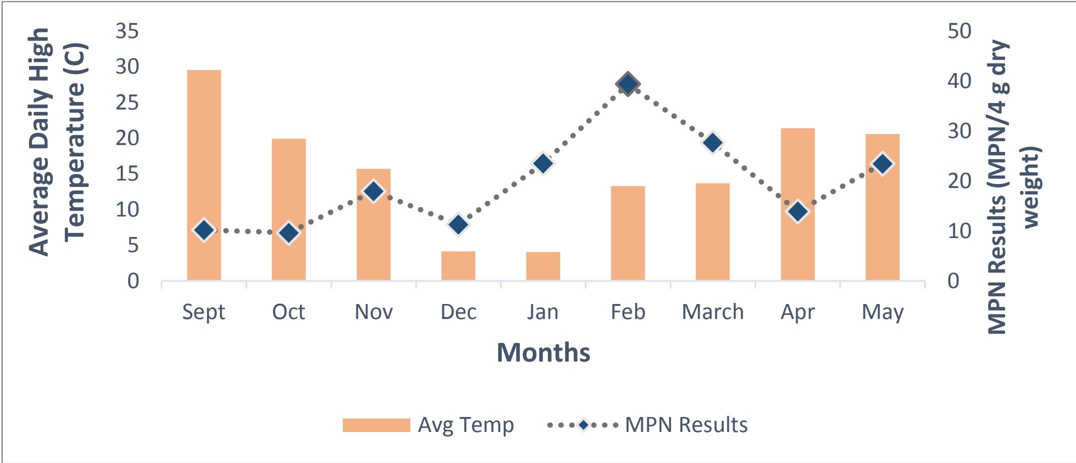


**Figure 3.3.** Eskişehir MPN results and temperature

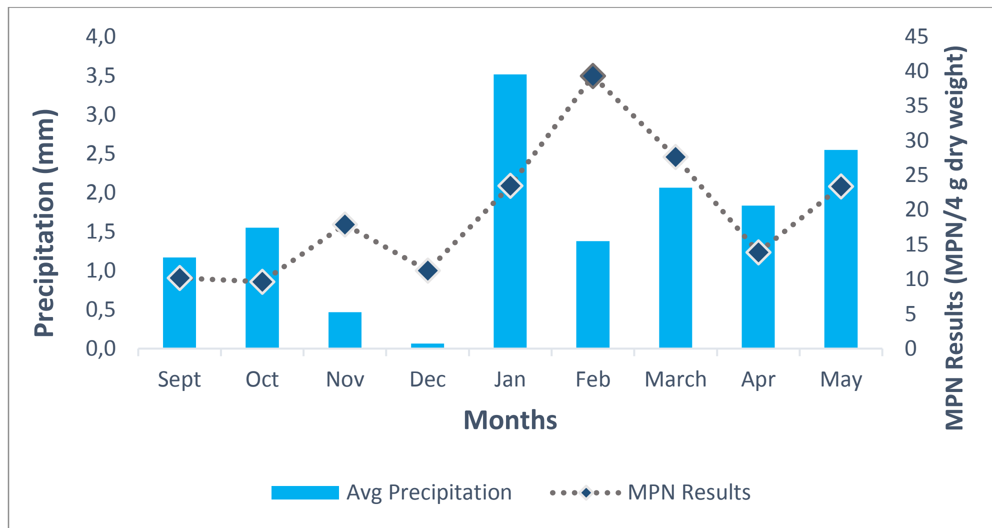


**Figure 3.4.** Eskişehir MPN results and precipitation

The TSS values of Eskişehir samples were constant at 0.2 throughout the sampling period.

### 3.1.3. Kayseri

The MPN results of biosolids from Kayseri Wastewater Treatment Plant are shown in Table 3.3. Kayseri data was consistent month-to-month (10-49 MPN/4g).

The correlation of the MPN results with monthly average temperature and the average precipitation the city receives can be seen in Figure 3.5 and Figure 3.6.

For Kayseri samples, there seems to be an inverse relationship between MPN and both temperature and rainfall. The average temperature values dropped drastically between September and December, but the MPN results increased and reached a peak at the month of December. After that for two months a direct relationship occurred between the temperature and the MPN results, however it stopped immediately and the results fluctuated in small amounts while the temperature continued to increase.

**Table 3.3.** Kayseri MPN results

| | Sampling Date | *Salmonella* quantity (*Salmonella spp.*/4 g-dry weight) |
|---|---|---|
| **Kayseri Wastewater Treatment Plant** | 29.09.2015 | 9.5±0.0 |
| | 30.10.2015 | 12.7±0.9 |
| | 20.11.2015 | 30.4±4.4 |
| | 29.12.2015 | 44.4±8.8 |
| | 22.01.2016 | 36.7±4.0 |
| | 18.02.2016 | 45.7±1.4 |
| | 19.03.2016 | 14.2±4.3 |
| | 14.04.2016 | 48.8±1.7 |
| | 19.05.2016 | 31.1±3.0 |

When it comes to the amount of rainfall, again the results seemed to have an inverse correlation with precipitations. In fact, we see the highest result at the month in which the lowest amount of precipitation was observed.

The TSS values of Kayseri biosolids were 0.2 and stayed constant throughout the sampling period.

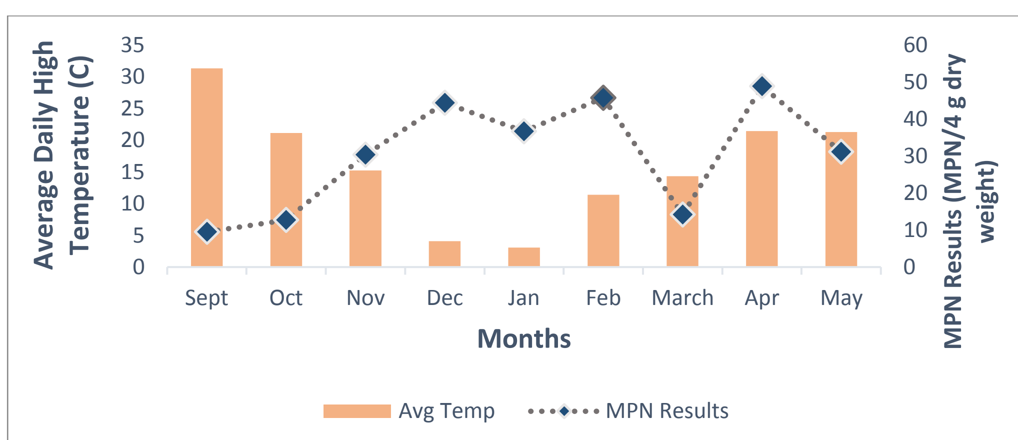


**Figure 3.5.** Kayseri MPN results and temperature

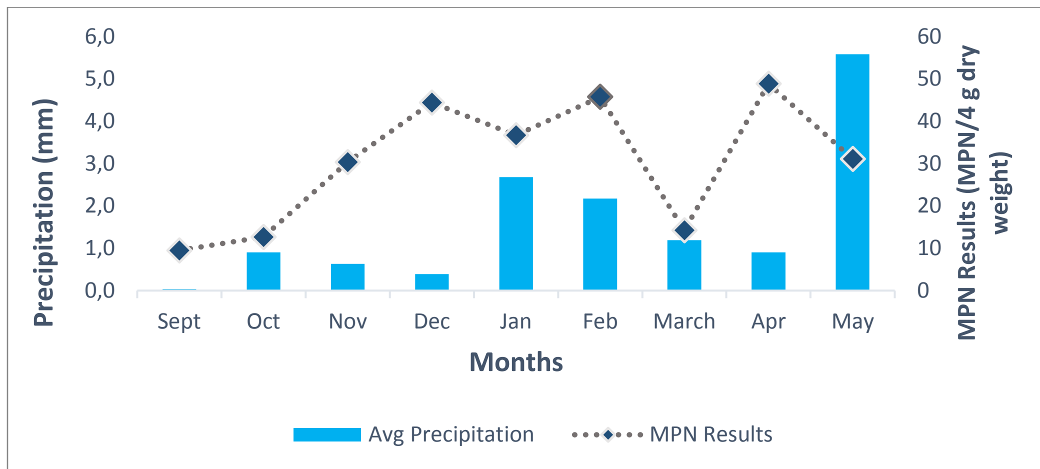


**Figure 3.6.** Kayseri MPN results and precipitation

### 3.1.4. Yozgat

With the biosolid samples of Yozgat city, the results obtained at the end of the first months of sample collection (October and November) were higher than the quantifiable limits given in the MPN tables of Method 1682. Therefore, the methodology regarding the dilution of the homogenized samples used for the enrichment step of the experiments had to be altered to account for the high *Salmonella* levels Yozgat biosolid samples. Since the enrichment dilutions stated in Method 1682 appeared to be too high for Yozgat biosolid samples, another set of dilutions were used for Yozgat. For that, the MPN calculations given in the Standards Methods for the Examination of Water and Wastewater, under section Estimation of Bacterial Density (APHA, 1992). The enrichment dilutions, which are normally 20 mL, 10 mL and 1 mL, were changed to 10 mL, 1 mL, 100 µL and 10 µL to account for higher *Salmonella* concentrations. The MPN results obtained at the end of the experiments for Yozgat biosolids are given in Table 3.4.

**Table 3.4.** Yozgat MPN results

| | **Sampling Date** | ***Salmonella* quantity (*Salmonella spp.*/4 g-dry weight)** |
|---|---|---|
| **Yozgat Wastewater Treatment Plant** | 30.09.2015 | 17.0±4.0 |
| | 31.10.2015 | 154.7±10.1 |
| | 24.11.2015 | 265.1±19.7 |
| | 24.12.2015 | 693.3±62.6 |
| | 30.01.2016 | 175.0±9.8 |
| | 26.02.2016 | 100.0±7.2 |
| | 23.03.2016 | 6.0±0.6 |
| | 30.04.2016 | 32.0±2.5 |
| | 31.05.2016 | 63.7±4.4 |

Results of the samples of Yozgat city were highly variable, ranging from 6 to 693 MPN/4g. During first few months of experimentation the results seemed to have a drastic increase, however that pattern changed with February and the results began to be comparatively small except for May.

Although the process technologies present in Yozgat Wastewater Treatment Plant are among the approved and accepted processes necessary for the exiting sludge to be classified as a Class B biosolid based on U.S. EPA classifications, the MPN results obtained with Method 1682 were high enough to call their safety into question. There are serious doubts about the authenticity of the samples that were provided by treatment plant personnel; in some cases, the samples contained fresh plant material, suggesting that samples long-term storage areas were provided or simply soil from the surrounding area.

The graphs showing the correlation between monthly average temperatures and precipitation amounts to MPN results are as follows.

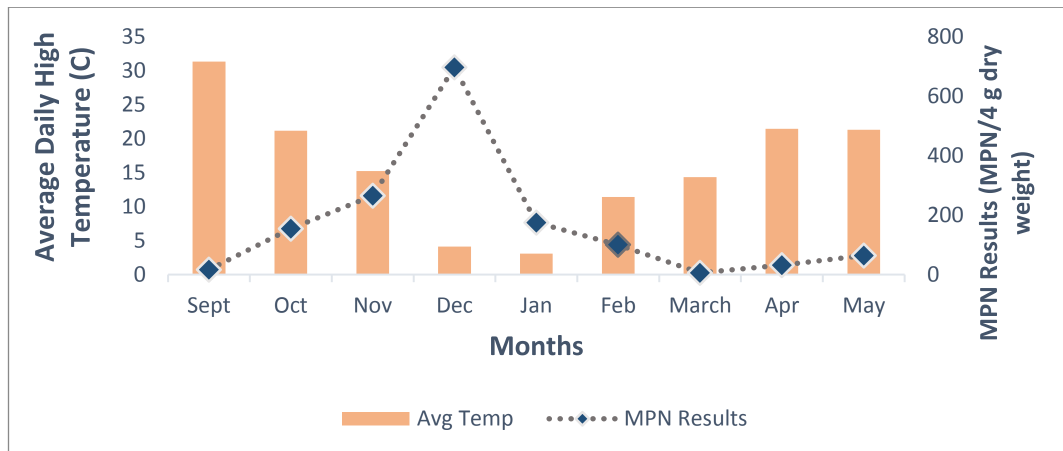


**Figure 3.7.** Yozgat MPN results and temperature

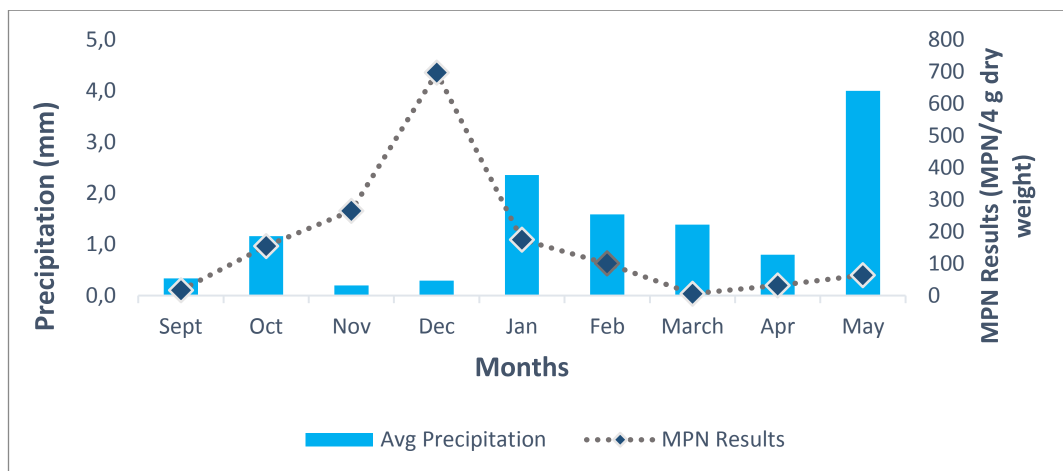


**Figure 3.8.** Yozgat MPN results and precipitation

Given the wide variation in results and the consequent doubts about the authenticity of the samples, it seems improper to speculate about the effects of temperature and rainfall on the MPN results.

The TSS values of Yozgat biosolid samples were highly variable and changed in the range of 0.15-0.43 through the sampling period.

Maximum, minimum, and average MPN results for each of the plants are provided in Table 3.5. With the exception of Yozgat, CFU/4g values were consistent with previous reports from the USA regarding the levels normally present in Class B biosolids.

**Table 3.5.** Maximum, minimum and average values of the MPN/4 g dry weight and the related months for each city

| **Cities** | **ANKARA** | | **ESKİŞEHİR** | | **KAYSERİ** | | **YOZGAT** | |
|---|---|---|---|---|---|---|---|---|
| | ***value*** | ***month*** | ***value*** | ***month*** | ***value*** | ***month*** | ***value*** | ***month*** |
| **Max** | 27.94 | January | 39.3 | February | 48.8 | April | 696.4 | December |
| **Min** | 5.2 | April | 9.6 | October | 9.5 | September | 6 | March |
| **Avg** | 13.48 | - | 19.63 | - | 30.39 | - | 167.77 | - |

### 3.1.5. Comparison and Discussion over MPN Results

It might provide a better understanding of the results if they are considered together as a whole. For that purpose, for The MPN results of all 4 cities are given in Figure 3.9 for better and easier comprehension of the results.

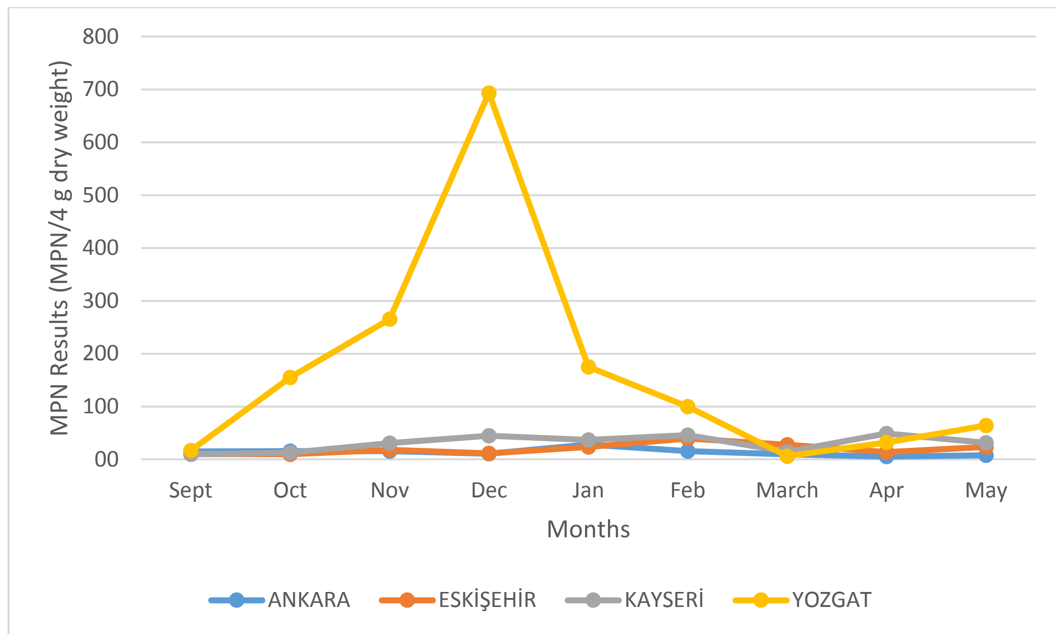


**Figure 3.9.** MPN results of the 4 cities

As it can clearly be seen from the graph, at Figure 3.9 at some months the MPN results of Yozgat city are considerably higher than the other cities, therefore it becomes harder to make a comparison between the rather smaller results belonging to other cities. To clarify the scene a little bit more a graph excluding the Yozgat results is also provided in Figure 3.10.

The MPN results of the cities Ankara, Eskişehir and Kayseri have a rather neat distribution when they are all examined as a whole. The results are not too high to prevent them from being classified as Class B biosolids, and prevent the potential land applications of the biosolids in the upcoming future.

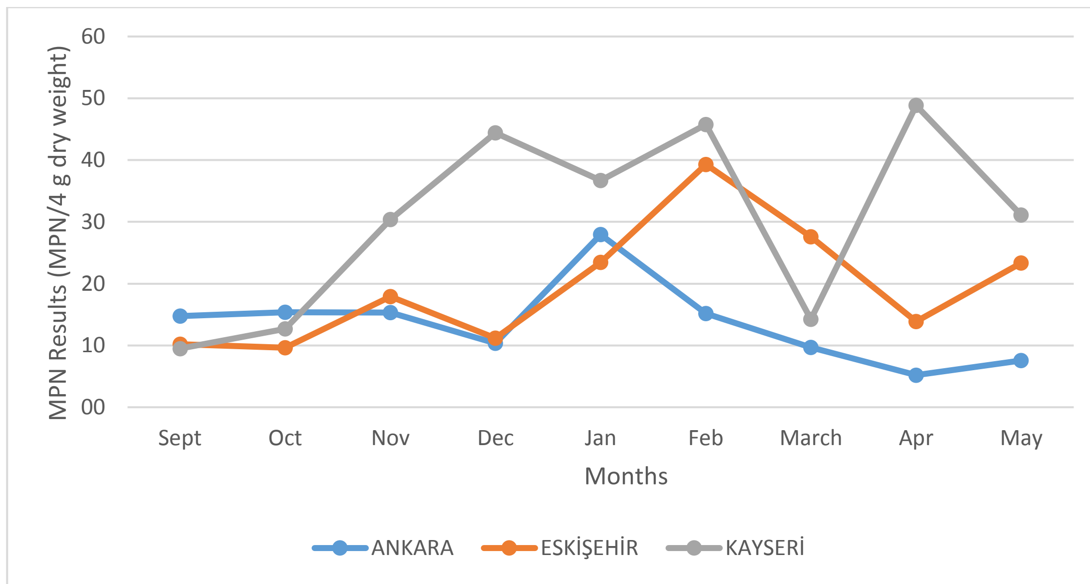


**Figure 3.10.** MPN results of cities Ankara, Eskişehir and Kayseri

At first thought, the reasons behind the single highest data points seen among both Eskişehir and Kayseri data sets which could not be accepted as outliers and eliminated, and nearly half of Yozgat data were thought to be explained by the potential correlations between climatic variables and the pathogen amounts in the biosolids. However, though there are some relations between those, there were no significant relationship that could have explained the furthest results.

Other reasons that should be considered include disease outbreaks that might be seen in the associated cities, and operational state of the treatment plants. Since there are not any public database systems through which we can learn about Salmonellosis disease outbreaks, the required information can be tried to be gathered through personal communication with medical workers, preferably authorities, in the cities. However, due to the difficulties in reaching out to the authorities, not much information could be obtained. We were able to learn only that there were no reported outbreaks in Eskişehir city in February, the month in which the highest MPN result was recorded, through information from medical workers. Also, there had been attempts to make contacts with the responsible engineers working in the treatment plants in question about the operational states of the plants during the months showing

high results. Unfortunately, there weren't any replies from them. It is possible that employees might be unwilling to report if there had been any complications with the operation of the treatment plants.

Considering that the treatment plant configurations of the 4 cities from which the biosolid samples are coming are not significantly different from each other except for their dewatering units, consideration of the processing techniques may not be of help to discuss the differences observed in the results. On the other hand, it has been found that during digestion processes, indicator bacteria can enter a non-culturable state but are reactivated during centrifuge dewatering, thereby increasing the amount of quantifiable bacteria present in the sludge sample (Higgins et al., 2007). In this study, we did not have the opportunity to compare the bacterial concentrations of sludges entering and leaving the dewatering units since we only received sludge cake samples coming out of dewatering. Nevertheless, this might be an explanation for the relatively high results observed with the samples. Moreover, the increase seen after dewatering units is most commonly observed after centrifugal dewatering but not after belt press type dewatering units (Erkan & Sanin, 2013). The reason proposed for this difference is the heat generated to centrifugal dewatering units during operation. The conditions that the indicator bacteria meet once they enter to centrifugation after anaerobic digestion conditions are favorable to reactivate and become culturable again (Erkan & Sanin, 2013).

One other reason that might be causing the increase in the pathogen amounts in biosolids could be the livestock activities and facilities going on in the cities, unregulated or unsupervised use of animal wastes for agricultural purposes. What is more, as far as we can assume, the biosolids obtained at the end of the treatment procedure might already being used for agricultural purposes, although it is unregulated. Therefore, after the agricultural activities take place, the pathogens present in the biosolids in the first place will have contaminated the crops, which will then lead to infections and end up increasing the amount of pathogens in the sewage, and consequently in the biosolids at the end. This is suspected to be the case that might have been going on in Yozgat.

Another point that might have an effect in the high and varying pathogen amounts and highly variable dry weight percentages observed in Yozgat is the duration and place of the storage of biosolids. The place at which the treated sludge coming from the belt press unit is stored is unknown along with the amount of time that it has being stored. Therefore, the possibility of the biosolids staying in storage for a long time before being collected as our samples remains. It is mentioned before that storage of biosolids prior to further use might result in regrowth of pathogenic bacteria (Zaleski et al., 2005). It is reported that digested biosolids stored at 35 °C leads to the reactivation of indicator bacteria (Jolis, 2006). Also, we suspect that there is the chance of the sludge being mixed with regular animal waste at the place of storage for Yozgat city.

Occasionally, when the weather is heavily rainy or snowy, treatment plants shut down operations and bypass the wastewater. This practice does not proceed for more than a few days, ideally. However, this might be a reason for the fluctuations seen on the results.

Seasonality is a known and a very important factor affecting pathogen growth; the occurrences and the survival rates of pathogens vary with different times of the year. Just like many other pathogenic diseases, Salmonellosis is seen most in warmer months (Ravel et al., 2010) (Naumova et al., 2007), leading us to the conclusion that the survival rate of *Salmonella* is higher in warm temperatures. However, it seems this fact is contradictory to what we observed with our experiments given that we mostly found that with increasing temperature the quantified *Salmonella* in the biosolid samples declined. At this point, the operational states of the treatment plants may come back into questioning. Most of the treatment plants use $A^2O$ process for biological removal and anaerobic digestion for stabilization. $A^2O$ process requires different operational conditions in the winter time than in summer. If the plants are not being operated under required conditions this might ultimately affect the pathogen amounts in the biosolids.

Altogether, there are several possible reasons that might affect the amount of residual pathogens in sludge even after treatment and the variations of these amounts in time.

However, when all these factors had to be considered as a whole each one having a role in the outcome, it becomes quite complicated to draw a single conclusion.

## 3.2. DNA Extraction Results

Tables from Table 3.6 to Table 3.13 list the DNA quantities and quality results for each of the biosolid extractions. Each table includes also the quantity and quality of PMA-treated DNA. Values were generally acceptable, although qualities were occasionally poor (above 2.0 or below 1.8).

**Table 3.6.** Biosolid DNA extraction concentrations for Ankara samples

| | Sampling Month | DNA concentration (ng/µl) | A260/A280 | A260/A230 |
|---|---|---|---|---|
| Ankara Wastewater Treatment Plant | September | 57,70 | 1,82 | 1,98 |
| | October | 197,75 | 1,80 | 2,01 |
| | November | 118,18 | 1,84 | 2,08 |
| | December | 59,14 | 1,76 | 1,96 |
| | January | 51,12 | 1,88 | 2,12 |
| | February | 220,30 | 1,79 | 1,96 |
| | March | 125,44 | 2,04 | 2,16 |
| | April | 110,65 | 1,87 | 2,10 |
| | May | 114,38 | 1,82 | 1,98 |

**Table 3.7.** PMA-treated biosolid DNA extraction concentrations for Ankara samples

| | Sampling Month | DNA concentration (ng/µl) | A260/A280 | A260/A230 |
|---|---|---|---|---|
| **Ankara Wastewater Treatment Plant** | September | 116,48 | 1,88 | 2,16 |
| | October | 161,70 | 1,79 | 2,22 |
| | November | 120,21 | 1,82 | 2,18 |
| | December | 131,92 | 1,85 | 2,12 |
| | January | 121,90 | 1,92 | 2,08 |
| | February | 96,42 | 1,79 | 2,00 |
| | March | 75,83 | 1,81 | 2,12 |
| | April | 88,64 | 1,78 | 1,98 |
| | May | 76,12 | 1,86 | 2,22 |

**Table 3.8.** Biosolid DNA extract concentrations for Eskişehir samples

| | Sampling Month | DNA concentration (ng/µl) | A260/A280 | A260/A230 |
|---|---|---|---|---|
| **Eskişehir Wastewater Treatment Plant** | September | 96,40 | 1,90 | 2,12 |
| | October | 337,72 | 1,81 | 2,08 |
| | November | 311,60 | 1,82 | 2,02 |
| | December | 58,76 | 1,79 | 1,98 |
| | January | 56,48 | 1,85 | 2,21 |
| | February | 190,21 | 1,86 | 2,14 |
| | March | 136,52 | 1,81 | 2,10 |
| | April | 117,30 | 1,92 | 2,18 |
| | May | 86,42 | 1,96 | 2,12 |

**Table 3.9.**PMA-treated biosolid DNA extraction concentrations for Eskişehir samples

| | Sampling Month | DNA concentration (ng/µl) | A260/A280 | A260/A230 |
|---|---|---|---|---|
| Eskişehir Wastewater Treatment Plant | September | 96,40 | 1,91 | 2,10 |
| | October | 87,80 | 1,82 | 2,18 |
| | November | 81,36 | 1,98 | 1,98 |
| | December | 52,53 | 1,79 | 2,06 |
| | January | 51,82 | 1,76 | 2,12 |
| | February | 76,46 | 1,78 | 2,12 |
| | March | 96,20 | 2,01 | 2,06 |
| | April | 88,74 | 1,96 | 2,11 |
| | May | 76,42 | 1,85 | 2,14 |

**Table 3.10.** Biosolid DNA extraction concentrations for Kayseri samples

| | Sampling Month | DNA concentration (ng/µl) | A260/A280 | A260/A230 |
|---|---|---|---|---|
| Kayseri Wastewater Treatment Plant | September | 125,86 | 1,89 | 2,00 |
| | October | 387,81 | 1,82 | 1,98 |
| | November | 372,40 | 1,84 | 2,14 |
| | December | 156,47 | 1,81 | 2,00 |
| | January | 112,72 | 1,77 | 1,96 |
| | February | 152,60 | 1,83 | 2,16 |
| | March | 119,63 | 2,05 | 2,12 |
| | April | 121,98 | 1,98 | 1,97 |
| | May | 116,26 | 1,92 | 2,01 |

**Table 3.11.** PMA-treated biosolid DNA extraction concentrations for Kayseri samples

| | Sampling Month | DNA concentration (ng/µl) | A260/A280 | A260/A230 |
|---|---|---|---|---|
| Kayseri Wastewater Treatment Plant | September | 114,00 | 1,90 | 1,98 |
| | October | 152,82 | 1,81 | 2,06 |
| | November | 110,65 | 1,82 | 1,96 |
| | December | 97,32 | 1,79 | 1,97 |
| | January | 66,14 | 1,85 | 2,10 |
| | February | 113,54 | 1,86 | 2,01 |
| | March | 75,90 | 1,81 | 1,98 |
| | April | 58,74 | 1,92 | 2,00 |
| | May | 102,42 | 1,96 | 1,98 |

**Table 3.12.** Biosolid DNA extraction concentrations for Yozgat samples

| | Sampling Month | DNA concentration (ng/µl) | A260/A280 | A260/A230 |
|---|---|---|---|---|
| Yozgat Wastewater Treatment Plant | September | 88,72 | 1,96 | 1,98 |
| | October | 76,43 | 1,86 | 2,05 |
| | November | 113,62 | 1,84 | 2,18 |
| | December | 96,24 | 1,76 | 1,98 |
| | January | 124,86 | 1,91 | 2,14 |
| | February | 172,85 | 1,82 | 2,08 |
| | March | 156,38 | 1,78 | 2,00 |
| | April | 106,65 | 1,92 | 2,00 |
| | May | 110,12 | 2,02 | 2,16 |

**Table 3.13.** PMA-treated biosolid DNA extraction concentrations for Yozgat samples

| | Sampling Month | DNA concentration (ng/µl) | A260/A280 | A260/A230 |
|---|---|---|---|---|
| Yozgat Wastewater Treatment Plant | September | 66,42 | 1,83 | 2,23 |
| | October | 75,53 | 1,80 | 2,09 |
| | November | 138,12 | 1,77 | 2,02 |
| | December | 124,28 | 1,79 | 2,14 |
| | January | 72,46 | 1,84 | 2,00 |
| | February | 100,54 | 1,88 | 1,98 |
| | March | 78,57 | 1,80 | 2,00 |
| | April | 68,92 | 1,78 | 2,18 |
| | May | 56,52 | 1,92 | 2,22 |

## 3.3. Confirming PCR Reactions with Sal Primers

SalF/SalR PCR reactions on *Salmonella enterica* Typhimurium genomic DNA yielded a high concentration reaction product of a size consistent with the expected product, 102 bp (Figure 3.11). Reactions performed with biosolids extracts and PMA-treated biosolids extracts yielded the expected product, but in low concentration, indicating a significant degree of reaction inhibition (Figure 3.12). This is consistent with DNA preparations from complex media, which tends to include inhibitory humic acids. Additionally, the MPN results suggest that the DNA extracts would contain very low concentrations of *Salmonella*, perhaps beneath detection thresholds.

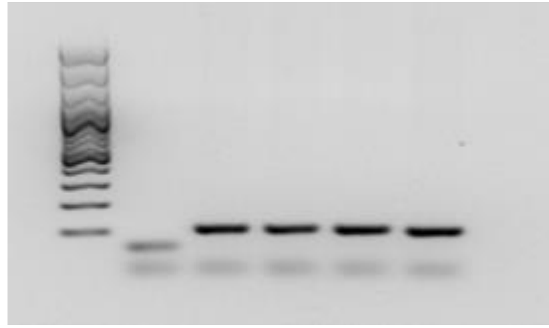

**Figure 3.11**. SalF/SalR PCR reactions run on agarose gel. Lane 1 is no-template control while other four represent different pure *Salmonella* culture extracts

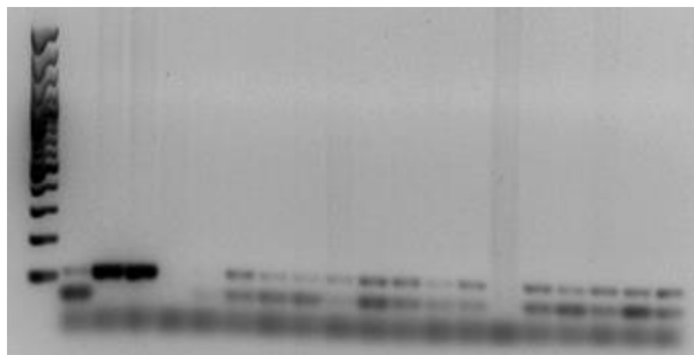

**Figure 3.12.** SalF/SalR PCR reactions run on agarose gel. Negative control is in lane 1, positive controls are in lanes 2 and 3. The remaining lanes represent reactions performed with various biosolids DNA extracts.

### 3.4. qPCR Results

__Standard Curve__

The Taqman qPCR method yielded a reliable standard curve over copy numbers per reaction ranging over five orders of magnitude, from 48 million to 125 copies (Table 3.14. Over this range, the best-fit curve (based on exponential line fit) had an $R^2$ value of 0.9994 Figure 3.14. At the lower copy numbers tested (25, 5, and 1 copy), Ct values became erratic, with wide inter-replicate variation or no detection at all. 125 copies per reaction was accepted as the threshold of detection for this assay. 125 copies yielded an average Ct value of 34.9; consequently, Ct value results of 35 or above were considered to represent copy numbers beneath the threshold of detection.

**Table 3.14.** Standard curve Ct values according to copy number. Values in *italics* indicate that due to the variance in Ct among the replicates, these concentrations were excluded from further analysis

| *invA* copy number | Ct | | | Ct average |
|---|---|---|---|---|
| 4.8E+07 | 15.39 | 15.30 | 15.28 | 15.32 |
| 9.6E+06 | 17.76 | 17.77 | 17.91 | 17.81 |
| 1.9E+06 | 19.67 | 19.96 | 20.21 | 19.95 |
| 3.8E+05 | 22.31 | 22.68 | 22.88 | 22.62 |
| 7.7E+04 | 24.98 | 25.01 | 24.56 | 24.85 |
| 1.5E+04 | 27.48 | 27.30 | 27.17 | 27.31 |
| 3.1E+03 | 29.99 | 30.14 | 29.83 | 29.98 |
| 6.2E+02 | 32.56 | 33.03 | 32.41 | 32.67 |
| 1.3E+02 | 35.07 | 34.42 | 35.20 | 34.90 |
| *2.5E+01* | *37.30* | *40.58* | *36.78* | *38.22* |
| *5.1E+00* | *ND* | *37.31* | *40.34* | *38.82* |
| *1.0E+00* | *ND* | *39.33* | *ND* | *39.33* |

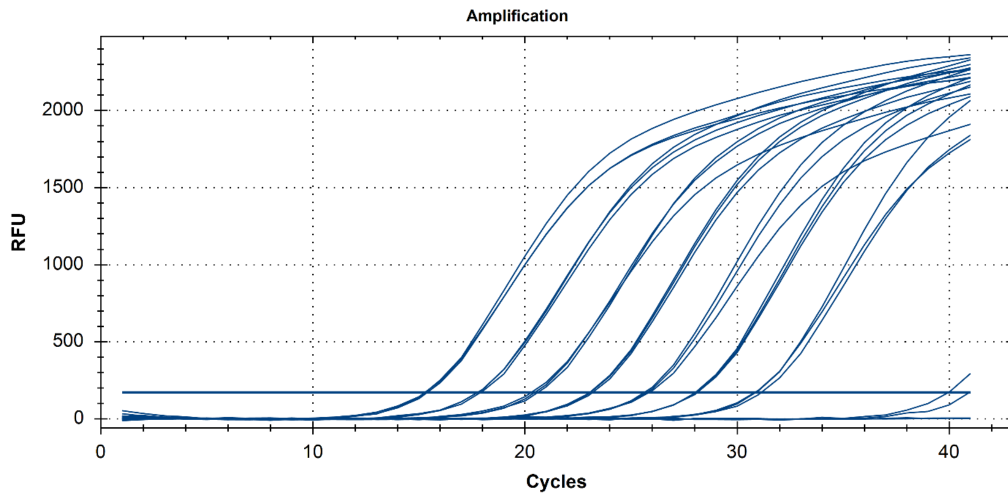


**Figure 3.13.** qPCR amplification curves for standard curve samples. The straight line along the bottom is the threshold used for determination of Ct

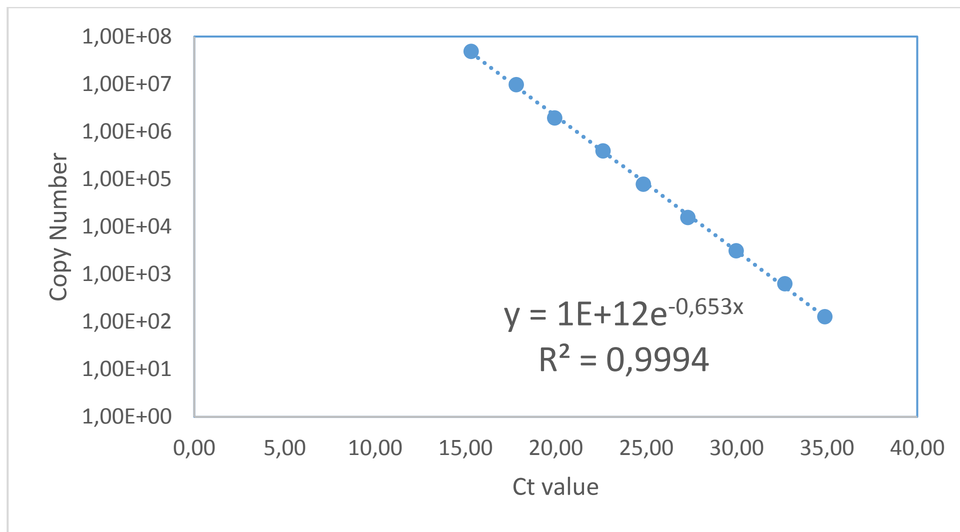


**Figure 3.14.** Determination of best-fit line (exponential equation) for use in calculating copy number based on Ct results

Ct values for each of the qPCR reactions performed using DNA templates derived from biosolids extracts yielded Ct values above the threshold of detection (Ct > 35), indicating that *Salmonella invA* gene copy numbers were beneath 125 copies per reaction (Table 3.15 to Table 3.18). Given the low concentrations of *Salmonella* according to the MPN results and the low amount of DNA that is was possible to extract from the biosolids samples, this lack of qPCR results is not surprising. Due to the high cost and labor of the biosolid DNA reaction and especially to the high cost of the PMA chemical, PMA-treated extracts represented only 50 microliters of the original biosolid, or about 20 mg dry weight. The average MPN/4g of Ankara, according to MPN results, was 16/4g, or 4/g, actually lower than is normally achieved by comparable sewage treatment methods.

Clearly, when considering the small quantity of biosolid which was practical to extract and treat, it becomes clear that this qPCR assay had no chance to succeed. Even the poorest performing plant, Yozgat, showed an average of 195 MPN/4g, or 49 MPN/g. This would be represented by only a handful of cells in the average DNA extract assuming perfect extraction. Accordingly, in the study in which this *invA* Taqman qPCR system was developed and applied to soil system (Novinscak et al., 2007), the concentration of *Salmonella* was many thousands per gram, three orders of magnitude higher than we observed in these biosolids.

**Table 3.15.** Average Ct values resulting from Taqman SalF/R qPCR reactions for monthly samples taken from Ankara Wastewater Treatment Plant

| **Sampling Month** | **Average Ct Value** | |
|---|---|---|
| | **PMA (-)** | **PMA (+)** |
| September | 39,08 | 39,28 |
| October | 37,56 | 36,11 |
| November | 35,61 | 37,37 |
| December | 36,06 | 35,66 |
| January | 38,26 | 35,54 |
| February | 35,89 | 36,05 |
| March | 36,42 | 37,12 |
| April | 35,58 | 36,82 |
| May | 36,18 | 36,56 |

**Table 3.16.** Average Ct values resulting from Taqman SalF/R qPCR reactions for monthly samples taken from Eskişehir Wastewater Treatment Plant

| Sampling Month | Average Ct Value | |
|---|---|---|
| | PMA (-) | PMA (+) |
| September | 36,75 | ND |
| October | 36,16 | 38,03 |
| November | 35,95 | 37,88 |
| December | 36,81 | 35,94 |
| January | 37,56 | 37,27 |
| February | ND | 36,24 |
| March | 35,92 | ND |
| April | 37,61 | 37,48 |
| May | 36,82 | 37,42 |

**Table 3.17.** Average Ct values resulting from Taqman SalF/R qPCR reactions for monthly samples taken from Kayseri Wastewater Treatment Plant

| Sampling Month | Average Ct Value | |
|---|---|---|
| | PMA (-) | PMA (+) |
| September | 38,22 | 37,42 |
| October | 37,06 | 39,47 |
| November | 36,70 | 36,92 |
| December | 35,60 | 35,60 |
| January | 37,18 | 37,74 |
| February | 36,93 | 37,39 |
| March | 36,24 | 37,42 |
| April | 35,82 | 36,59 |
| May | 36,18 | 36,84 |

**Table 3.18.** Average Ct values resulting from Taqman SalF/R qPCR reactions for monthly samples taken from Yozgat Wastewater Treatment Plant

| Sampling Month | Average Ct Value | |
|---|---|---|
| | PMA (-) | PMA (+) |
| September | 37,02 | 37,12 |
| October | 35,85 | 36,64 |
| November | 35,34 | 38,12 |
| December | 35,71 | 36,23 |
| January | 37,17 | 38,26 |
| February | 35,24 | 35,92 |
| March | 38,56 | 37,87 |
| April | 37,43 | 37,45 |
| May | 36,65 | 37,34 |

qPCR reactions performed on the biosolids extracts yielded no Ct values below the limit of detection, indicating low or no templates present. This is most likely due to the low concentrations of *Salmonella* according to the MPN results and the low amount of DNA that is was possible to extract from the biosolids samples. Due to the high cost and labor of the biosolid DNA extraction, each sample represented 500 ml or about 200 mg of solids. Additionally, due to the high cost of the PMA chemical, PMA-treated extracts represented only 50 microliters of the original biosolid, or about 20 mg dry weight. In each case, 1/20 of the total extract was subjected to molecular analysis. The average CFU/4g of Ankara, according to MPN results, was 16/4g, or 4/g; this would lead to 0.04 cells per reaction or 0.004 cells per PMA-treated reaction; clearly, even if the qPCR reaction had shown perfect sensitivity, as in capable of detecting one copy per reaction, it still would have been far from able to detect these low concentrations. In the study in which this *invA* Taqman qPCR system was developed and applied to soil systems (Novinscak et al., 2007) , the concentration of *Salmonella*

was thousands per gram, three orders of magnitude higher than we observed in these biosolids. The only way around this limitation would be to extract exceedingly large amounts of biosolid followed by concentration down to a very small volume, which in addition to being very expensive and labor intensive, would lead to co-concentration of large amounts of reaction-inhibiting humic acids.

# CHAPTER 4

# CONCLUSION

The primary purpose of this study was to enumerate the amount of the pathogenic bacterium *Salmonella* in sewage sludge from Turkey. The information obtained is useful in that there are no published data on the matter. Most of the studies regarding this issue in Turkey have been related with the enumeration of fecal indicators, especially, *E. coli.* For this purpose sludge samples of four treatment plants were collected.

The enumeration techniques included culture-based methods and molecular methods applied together with the purpose of comparing the results to detect possible underestimations due to the presence of VBNC bacteria present in the samples.

Taqman qPCR has been successfully applied especially for detection of pathogens on food surfaces and in water or when present in high concentrations in soil in previous studies. In the case of biosolids, extraction of pure DNA is a very laborious and expensive procedure taking a full day with over thirty steps. Quantification of such low numbers of *Salmonella*, in this case around 20-200 cells per 4g according to the MPN results, would require extraction from a prohibitively large amount of biosolid and accordingly, a very large amount of PMA, an expensive reagent. Such procedures, those based on detection of DNA, are not reasonable for detection of such rare pathogens in biosolids.

U.S. EPA's Standard Method 1682 was the culture based method used, while PMA-assisted qPCR with Taqman probe was the molecular one. What we expected to observe at the end of the experiments was to see that the quantification results obtained

with molecular methods were higher than the culture based method MPN results. However, the amounts of *Salmonella* in the biosolid samples were not high enough to allow for effective quantification by molecular methods.

The MPN results revealed that for the cities Ankara, Eskişehir and Kayseri, amounts of *Salmonella* were at acceptable levels if they are compared with the classification limitations of the U.S. EPA 503 Rule. All the MPN results were between 0-50 MPN/4 g dry weight consist values reported in literature for sewage sludge that has being treated with mesophilic or thermophilic digestion (Pepper et al., 2010) (Wong et al., 2010).

The findings of this study provide significant data since it is a first to be enumerating the amount of *Salmonella* in Turkish biosolid samples. Further studies conducted in the light of this one may further aid the regulatory developments that are currently in question in Turkey. The results may be used as references for the limit criteria of pathogens in biosolids. Once the limits are established and the legislative regulations come into force, then it can also be possible to talk about making use of the sludge in practices such as land applications under regulated and properly supervised condition.

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