RAPID DETECTION AND IDENTIFICATION OF BACTERIAL PATHOGENS IN DRINKING WATER SOURCES USING DNA-BASED METHODS AND IMMUNOIMMOBILIZATION TECHNOLOGY

by

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ABSTRACT

Clean water is a basic requirement for the survival of the planet and is essential for human health. Water-borne pathogens and the diseases they cause have been a serious threat to public health. Drinking water is a major route for spreading of such pathogens. Monitoring of microbial community is one of the most significant practices for prevention of water-related diseases and potential outbreaks. Comprehensive analysis of chemical composition and microbiological structure of drinking water reservoirs is essential for understanding the levels of contamination and pathogenic threats. Rapid pathogen detection methods are critical for on-site experiments.

This study involves two parts. The first part covers investigation of physicochemical and bacteriological composition of drinking water reservoirs in İstanbul. Physicochemical quality was monitored in terms of pH, conductivity, total dissolved solids, salinity, dissolved organic carbon, heavy metals, anions and cations. Bacteriological characterization was performed using culture-independent, DNA-based methods such as DGGE, sequencing and qPCR. The second part includes development of a rapid and sensitive detection method based on immunoimmobilization for selected *E. coli* strains. A recently developed BiyoTrap® technology was used in combination with immunoimmobilization method for analyzing field samples taken from water reservoirs.

Water samples were taken from water intake chambers of drinking water treatment plants. Chemical quality parameters were mostly in agreement with regulation limits. Bacterial diversity was investigated with a combination of DGGE, band excising and sequencing of excised bands. DGGE revealed the presence of 66 OTUs in samples. Similarity research of DGGE band sequences, bacteria available in reservoir samples belong to the members of *Bacteria, Cyanobacteria, Proteobacteria, Actinobacteria*, and *Bacteroidetes* with abundances of 53%, 24%, 16%, 5%, 3% respectively. According to principal component and correlation analysis, Cr, Zn, Co, F^-, and NO_3^- were found to have a significant impact on bacterial community. qPCR was used for quantification of ETEC, EHEC, Legionella and Salmonella targeting specific genes namely LT, *stx*, *mip* and *hilA* respectively. Maximum gene copy numbers for ETEC, EHEC, *Legionella* and *Salmonella*

were quantified as $6.74x10^{11}$, $1.63x10^{4}$, $1.48x10^{6}$ and $1.33x10^{6}$ per 25 ng DNA/μl respectively.

Pathogenic *E. coli* strains were efficiently immobilized on gold surfaces activated with antibodies specific to their fimbrial and LPS antigens. The strains were selectively sorted on antibody microarrays. Minimum antibody requirement for effective immobilization of bacteria was determined as 4 μg/ml. Minimum detection limit of immunoimmobilization method was 10^{4} cfu/ml.

Pre-concentration of environmental samples using BiyoTrap® considerably enhanced the immobilization efficiency of target bacteria on antibody modified chips. Minimum detection limit was improved to 10^{2} cfu/ml using BiyoTrap®.

ÖZET

Temiz su, insan sağlığı ve yaşamın sürdürülmesi için temel bir ihtiyaçtır. Su kaynaklı patojenler ve sebep oldukları hastalıklar, toplum sağlığı için önemli tehdit oluşturmaktadır. İçme suyu, bu patojenlerin yayılmasındaki esas yollardan biridir. Su kaynaklı hastalıkların ve olası salgınların önlenebilmesi için mikrobiyal komünitenin izlenmesi en önemli uygulamalardan biridir. İçme su kaynaklarındaki kirlilik seviyesinin ve patojenik tehditlerin anlaşılabilmesi için kimyasal parametrelerin ve mikrobiyolojik çeşitliliğin kapsamlı olarak analiz edilmesi esastır. Hızlı patojen tayin yöntemlerinin geliştirilmesi, sahada ölçüm yapılabilmesi açısından önemlidir.

Bu çalışma iki bölümden oluşmaktadır. Birinci bölüm, İstanbul'a su temin edilen içme suyu kaynaklarındaki fizikokimyasal ve bakteriyolojik bileşenlerin incelenmesini kapsamaktadır. Fizikokimyasal kalite izlemesi kapsamında pH, iletkenlik, çözünmüş madde, tuzluluk, çözünmüş organik karbon, ağır metaller anyonlar ve katyonlar ölçülmüştür. Bakteriyolojik karakterizasyon için DGGE, dizi analizi ve qPCR gibi kültürden bağımsız, DNA'ya dayalı yöntemler kullanılmıştır. İkinci bölümde farklı *E. coli* suşlarının tespiti için immunoimmobilizasyona dayalı hızlı ve hassas bir tespit yöntemi geliştirilmiştir. Saha numunelerinin ölçümü için immunoimmobilizasyon yöntemi ile birlikte yeni geliştirilmiş olan BiyoTrap® teknolojisi kullanılmıştır.

Su numunleri, içme suyu arıtma tesislerindeki su alma yapılarından alınmıştır. Kimyasal parametreler çoğunlukla yönetmelikte verilen değerlerle uyumludur. Bakteriyel çeşitlilik, DGGE ve kesilen bantların dizi analizi yapılarak incelenmiştir. DGGE, su örneklerinde toplam 66 farklı türün bulunduğunu ortaya çıkarmıştır. Dizileme sonuçları, mevcut veritabanları ile karşılaştırılmış ve su örneklerindeki bakterilerin 53%, 24%, 16%, 5% ve 3% oranlarında, sırasıyla, *Bacteria, Cyanobacteria, Proteobacteria, Actinobacteria,* and *Bacteroidetes* taksonomik gruplarına ait olduğu tespit edilmiştir. İstatistiksel analiz sonuçlarına göre Cr, Zn, Co, F^-, ve NO_3^- miktarının bakteriyel çeşitlilik üzerinde önemli etkileri bulunmaktadır. ETEC, EHEC, Legionella ve Salmonella türlerine özgü sırasıyla, LT, *stx, mip* ve *hilA* genleri, qPCR kullanılarak sayılmıştır. ETEC, EHEC, *Legionella* ve

Salmonella için 25 ng DNA/µl'de bulunan maksimum gen kopya sayısı sırasıyla $6.74x10^{11}$, $1.63x10^{4}$, $1.48x10^{6}$ ve $1.33x10^{6}$ olarak bulunmuştur.

Patojenik *E. coli* suşları, fimbrial ve LPS antijenlerine özgü antikorlar kullanılarak active edilmiş altın kaplı yüzeylere etkin olarak immobilize edilmiştir. *E. coli* suşları antikor dizileri üzerinde seçici olarak ayrılmıştır. Etkin bir immobilizasyon sağlanabilmesi için gereken en düşük antikor miktarı 4 µg/ml olarak belirlenmiştir. İmmunoimmobilizasyon yöntemi için minimum ölçüm sınırı 10^{4} cfu/ml olarak hesaplanmıştır.

Çevre numunelerinin BiyoTrap® kullanılarak konsantre edilmesi immobilizasyon verimini oldukça arttırmıştır. Ayrıca, minimum ölçüm sınırı BiyoTrap® kullanılarak 10^{2} cfu/ml olarak geliştirilmiştir.

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Abbreviation	Explanation	Units used
AFM	Atomic force microscopy	
APTES	Aminopropyltriethoxylsilane	
BMPS	N-(3-maleimidopropionyloxy) succinimide	
BSA	Bovine serum albumin	
cDNA	Complementary deoxyribonucleic acid	
DAPI	4′,6-diamidino-2-phenylindole, dihydrochloride	
DGGE	Denaturing gradient gel electrophoresis	
DNA	Deoxyribonucleic acid	
dNTP	Deoxyribonucleoside triphosphate	
DOC	Dissolved organic carbon	mg L^{-1}
EDC	1-Ethyl-3-[3-dimethyl aminopropyl] carbodiimide hydrochloride	
EDTA	Ethylenediaminetetraacetic acid	
EHEC	Enterohemorrhagic *Escherichia coli*	
ETEC	Enterotoxigenic *Escherichia coli*	
FESEM	Field emission scanning electron microscope	
FITC	Fluorescein isothiocyanate	
GFP	Green fluorescent protein	
LB	Lysogeny broth	
LPS	Lipopolysaccharide	
LT	Heat labile enterotoxin	
MES	2-(*N*-morpholino) ethanesulfonic acid	
MHA	16-mercaptohexadecanoic acid	
MUA	11-mercapto-undecanoic acid	
NHS	*N*-hydroxysuccinimide	
OTU	Operational taxonomic unit	
PCR	Polymerase chain reactions	
qPCR	Quantitative real time polymerase chain reactions	

Abbreviation	Explanation	Units used
RFP	Red fluorescent protein	
RNA	Ribonucleic acid	
RT PCR	Reverse transcriptase polymerase chain reactions	
SAM	Self assembled monolayer	
TAE	Tris-acetate-EDTA	
TDS	Total dissolved solid	$mg\ L^{-1}$
TR QSWDS	Turkish Regulation on Quality of Surface Waters used as Drinking Water Supply	
Tris	Tris(hydroxymethyl)aminomethane	
TRITC	Tetramethyl rhodamine isocyanate	
TSA	Tryptic soy agar	
TSB	Tryptic soy broth	
stx	Shiga toxin	
WPCR	Water Pollution Control Regulation	
XPS	X-ray photoelectron spectroscopy	

1. INTRODUCTION

Domestic water supply, hygiene of water and water resources management are priority concerns to protect human health, reduce child mortality and protect natural resources. However, industrialization, increasing population growth, uncontrolled urbanization in the vicinity of drinking water reservoirs, and politics that disregard the environment are serious threats to water resources in terms of drought and contamination. Industrial activities significantly increased consumption of water. In addition, uncontrolled and untreated discharge of effluents to receiving water bodies is another stress of industrialization on clean water. Excess growth rate raises the demand for clean water. In case sanitation of water cannot be provided, high population means more people susceptible to infections and more people transmitting the pathogens (Theron et al., 2008). Uncontrolled urbanization is a result of overpopulation, which also leads to inadequacy, and failures of sanitation of clean water supply and wastewater treatment. Water environments with poor hygiene are provides favorable conditions for spread and growth of pathogens. Untreated and contaminated water bodies promote evolution of microbes into more virulent, toxic and antibiotic-resistant forms through adaptation and exchange of genetic material which may end up in more deadly strains (Theron et al., 2008). Last but not least, actions such as deforestation for urbanization and highway construction are not only a massacre of wild life, but also an unrecoverable damage to hydrological cycle. Considered on the worldwide base, scarcity of clean water is an inevitable fact that humanity will be facing. For drawing a global solution to clean water supply, one must keep in mind that ecosystem borders are not limited to national borders.

Every threat on water resources ends up with physical, chemical, microbiological and even radiological pollution. Chemicals and microbial agents are generally reported as the causes of waterborne outbreaks. Pollutants can be spread in the environment through point and non-point pollution sources. Point sources are comparatively easier to track and analyze since they generally come from industrial or domestic wastewater discharges. Agricultural runoff is an important source of non-point pollution, which carries both chemical pollutants such as fertilizers, herbicides and insecticides and microbiological pollutants such as pathogenic bacteria. They might be easily diffused into groundwater and

contaminate surface waters. Therefore, it is important to carefully monitor water quality from source to end-use in terms of these pollutants.

In many cases, the abundance of coliform bacteria is analyzed as the indicator of fecal contamination. Nevertheless, coliform index represents only 13% of the pathogenic microorganisms. Traditional methods based on cultivation and colony counting were standardized and they have been a common practice for decades. However, since the conventional methods are time consuming and labor intensive, they are not appropriate for modern water and food quality assurance. On the other hand, polymerase chain reactions (PCR) is the most common culture-independent method to identify pathogenic bacteria. Polymerase chain reaction (PCR) has reduced the assay time to 4–6 h. Quantitative real-time-PCR enables obtaining results in a few hours. Other approaches that have been studied or are currently being studied for rapid pathogen detection include miniaturized biochemical tests, physicochemical methods, nucleic acid-based tests, antibody-based methods, and some fully automated instrumental diagnostic systems. Besides these, collaborations among physics, chemistry, biology, engineering, and microelectronics in the field of nanotechnology have yielded the improvement of devices such as sensors and biosensors by filling the gaps between micro- and macro range.

1.1. Problem Definition and Aim

Istanbul is one of the most crowded metropolises of the world, with a population of 13.6 million. Domestic water is supplied from seven drinking water reservoirs located in European and Asian sides. In addition, Melen River, located outside the boundaries of Istanbul has been used as domestic water resource to meet the demands. Due to unplanned urbanization and inadequate infrastructure, most clean water resources are surrounded by residential areas and are under threat of contamination. Determination of the microbial diversity in these resources and rapid detection of pathogenic bacteria will help to understand the level of bacteriological threats and prevent any possible outbreaks.

There is a lack of research in the field of investigating microbiological quality of water resources in Turkey. In addition, available studies were performed using cultivation based methods and targeted a limited number of species (Özler and Aydın, 2007; Tonguc

Yayintas, et al., 2007; Koksal et al., 2007). Thus, a majority of microbial diversity was underestimated. In addition, the procedure of such a method usually includes microbiological culturing and isolation of the pathogen, followed by confirmation by biochemical or serological tests, taking up to 5-7 days to get a confirmed result for a particular pathogen. For a comprehensive investigation of the microbial community, water resources need to be analyzed using culture independent methods. In addition, more rapid and practical methods are required for precise detection of pathogens on-site so that the contamination risk until analysis is minimized and immediate action can be taken. Based on these requirements, the objectives of this research are outlined as:

- Screening the bacterial diversity of drinking water resources of İstanbul via Denaturing Gradient Gel Electrophoresis (DGGE).
- Excising and sequencing distinct DGGE bands for revealing the bacteria available in drinking water basins.
- Quantification of selected pathogens, i.e. enterohaemorragic *E. coli* (EHEC), enterotoxigenic *E. coli* (ETEC), *Legionella* and *Salmonella*, using quantitative real time PCR.
- Correlation of microbial diversity and physicochemical water quality parameters such as pH, conductivity, salinity, total dissolved solids, heavy metals, anions, cations and dissolved organic carbon.
- Development of a sensitive immunoimmobilization method for rapid detection of pathogens by optimizing the chemical and microbiological parameters that will enhance the binding of bacterial pathogens using surface-bound antibodies.
- Application of immunoimmobilization method in field samples pre-concentrated via BiyoTrap® technology.

1.2. Main Findings

The scientific merit of this thesis lies in (i) identification of bacterial species present in drinking water reservoirs of Istanbul using DNA-based methods, (ii) determination of the chemical and microbiological parameters that will enhance the capturing of bacterial pathogens using antibodies and (iii) application of novel bacteria capture and concentration

technologies to determine rapidly the presence of pathogens in contaminated water sources.

This is a pioneering study that investigates the microbial community of drinking water reservoirs in İstanbul using culture-independent methods in correlation with physicochemical characterization. Therefore, this study provides a basic reference for future studies involved in microbiological investigation of water quality. In addition, a rapid detection method based on specific antigen-antibody interactions was optimized for selected *E. coli* strains and used for determination of *E. coli* in field samples within a short period of time (~ one hour). Combination of immunoimmobilization with BiyoTrap® technology provided significant improvement of the limit of detection in field samples. Microarrays constructed using antibodies targeting different *E. coli* strains proved that the method is capable of discriminating between different strains of the same species.

Microbial characterization and quantification of pathogens will be a useful guide for further investigations and detection of pathogens in water reservoirs. Moreover, there is a wide selection of surface antigens against which antibodies are commercially available or can be easily produced for immunoimmobilization experiments. The practical impact of this method lies in its promise as an immunoassay sensor.

2. LITERATURE REVIEW

2.1. Water Quality Parameters

Domestic water is mostly provided from ground water and surface water. In Turkey, water for domestic and beneficial use is provided mostly from surface waters coming from rains by a ratio of 85%, from groundwater by 12.5% and from water resources of neighboring countries by 2.5%.

According to Water Pollution Control Regulation (WPCR) of Turkey, water bodies are evaluated in four classes according to their beneficial uses:

Class I: High quality water

- Water supply after disinfection
- Recreational purposes including contact sports
- Trout production
- Animal breeding and farm use
- Other purposes

Class II: Slightly polluted water

- Water supply after adequate treatment
- Recreational purposes
- Fish production other than trout
- Irrigation, provided that the standards given in the Technical Methods Circular are complied

Class III: Polluted water

- Industrial uses after adequate treatment, except for those like food and textile industries, which need high quality water

Class IV: Highly polluted water

- Surface water of lower quality, by means of specifications given for Class I, II and III.

Water resources were categorized based on the criteria for classification of Inland Surface Waters as given by WPCR. Parameters for the classification are grouped under four headings: (1) physical and inorganic chemical parameters (mainly to identify the constituents of the water), (2) organic parameters, (3) inorganic pollution parameters (as direct indicators of pollution), and (4) bacteriological parameters.

Physical parameters are basically temperature and pH. *Inorganic chemical* parameters that define water quality are given as dissolved oxygen, oxygen saturation, chloride, sulfate, ammonium, nitrite, nitrate, total phosphorus, total dissolved solids, sodium and color. *Organic* parameters are chemical oxygen demand, biochemical oxygen demand, organic carbon, total Kjeldahl nitrogen, emulsified oil and grease, methylene blue active substances, phenolic compounds mineral oils and derivatives and total pesticides. *Inorganic pollution* parameters are most heavy metals such as mercury, cadmium, lead, arsenic, copper, chromium (total Cr and Cr^{6+}), cobalt, nickel, zinc, iron, manganese, boron, selenium, barium, aluminum; fluoride, sulfur, free chlorine, cyanide and radioactivity (alpha- and beta-activity). *Bacteriological* parameters are given as total and fecal coliforms. Although these two parameters give an estimate of fecal contamination, they underestimate a vast majority of other microbiological threats.

2.2. Water and Wastewater-Related Pathogens

Water and wastewater related pathogens could be mainly classified as viruses, bacteria and parasites (Toze, 1997; USEPA, 2009). They mostly occur as enteric pathogens whereas a minority of them is non-enteric and opportunistic pathogens. (Toze, 1997). Selected pathogenic bacteria, viruses and parasites are summarized in Table 2.1.

More than 250 diseases that cover urinary system and wound infections, pneumonia, poisoning, respiratory system infections, nervous system disorders, gastrointestinal diseases, dermatological illnesses are caused by water-borne pathogenic microorganisms,

most commonly by bacteria. Amongst the pathogenic bacteria, *Escherichia coli, Listeria monocytogenes, Legionella pneumophilia, Campylobacter jejuni, Salmonella enterica* serovar Typhimurium were reported as the most frequently studied microorganisms (Lazcka et al., 2007).

Table 2.1. Pathogenic agents in the environment.

Bacteria	**Viruses**	**Parasites**
Acinetobacter	Adenoviruses	*Acanthamoeba*
Aeromonas	Astroviruses	*Ascaris lumbricoides*
Campylobacter	Parvoviruses	*Balamuthia mandrillaris*
Cyanobacteria	Coronaviruses	*Balantidium coli*
Enterohemorragic *E. coli*	Polyomaviruses	*Blastocystis hominis*
Escherichia coli	Picobirnaviruses	*Cryptosporidium parvum* *C. hominis*
Flavobacterium	Circoviruses	*Cyclospora cayetanensis*
Klebsiella	Enteroviruses	*Entamoeba histolytica*
Legionella	Parechoviruses	*Giardia lamblia*
Pseudomonas	Hepatitis A virus	*Isospora belli*
Salmonella	Hepatitis E virus	Microsporidia
Serratia	Human Caliciviruses	*Naegleria fowleri*
Shigella	Reoviruses	Schistosomatidae
Staphylococcus	Rotaviruses	*Toxoplasma gondii*
Vibrio chlorae		*Trichus triciura*
Yersinia		
Mycobacterium avium Complex		

Escherichia coli is a natural inhabitant in the intestinal tracts of humans and warm-blooded animals (Geng et al., 2008). However, more than 700 antigenic types (serotypes) of *E. coli* are available some of which are severe pathogens to human. The serotypes recognized based on O, H, and K antigens (O refers to somatic antigen; H refers to flagellar antigen; K refers to capsular polysaccharides).

Different *E. coli* strains cause diverse intestinal and extraintestinal diseases by means of virulence factors. Pathogenic strains of *E. coli* are responsible for three types of infections in humans: urinary tract infections, neonatal meningitis, and intestinal diseases. Whether the diseases caused (or not caused) by a particular strain of *E. coli* depend on distribution and expression of an array of virulence determinants, including adhesins, invasins, toxins, and abilities to withstand host defenses. These enteric *E. coli* can cause

several intestinal and extra-intestinal infections such as urinary tract infection and menengitis.

Intestinal pathogens of *E. coli* can be classified into six categories such as enterotoxigenic *E. coli* (ETEC), enteropathogenic *E. coli* (EPEC), enteroinvasive *E. coli* (EIEC), enterohemorrageic *E. coli* (EHEC), enteroaggregative *E. coli* (EAEC), and diffuse adherent *E. coli* (DAEC) (Kaper et al., 2004).

EPEC was the first pathogenic *E. coli* strain reported to cause infant diarrhea in 1945 in United Kingdom (Chen and Frankel, 2005). Virulence factors such as non-fimbrial adhesins (intimin), bundle forming pili (BFP) and EPEC adherence factor (EAF) enable localized adherence of bacteria to intestinal cells (Chen and Frankel 2005; Todar, 2008).

ETEC and EIEC were discovered in 1970s. ETEC differed from EPEC strains by the production of heat-labile enterotoxins (LT) and heat-stable enterotoxins (ST). ETEC strains have a variety of fimbrial antigens for adhering and colonizing the intestinal mucosa (Nataro and Kaper, 1998). Among these fimbrial antigens, K99 has a diverse range of targets such as calves, lams and pigs, whereas K88 and 987P antigens can colonize in only pigs. Human are infected by strains carrying colonization factor antigens (CFAs) (Nataro and Kaper, 1998; Johnson and Nolan, 2009). It is he cause of travelers' diarrhea in individuals from industrialized countries traveling to developing countries.

EIEC stains possess *Shigella*-like invasiveness and cause an inflammatory dysentery-like disease and diarrhea (Chen and Frankel, 2005). EIEC strains are non-motile and invade the intestinal epithelial cells by penetration (Andrade, 2002; Todar, 2008).

EAEC are capable of adhering to intestinal cells mediated by aggregative adherence fimbriae (AAF) and forming aggregates that resemble "stacked-bricks". Once adhered to the surface, the bacteria produce enterotoxins and cytotoxins and induce inflammation (Okhuysen and DuPont 2010). EAEC causes persistent diarrhea in children and adults.

DAEC represent a diffuse pattern of adherence to the intestinal epithelial cells that form long, finger-like cellular projections, which wrap around the bacteria. Fimbrial adhesin, F1835, is responsible for the diffuse cell adherence of DAEC (Kaper et al., 2004).

EHEC produces a potent cytotoxin, shiga toxin (Stx), that expands the spectrum of illness to include non-bloody diarrhea, bloody diarrhea, hemolytic uremic syndrome (HUS), a potentially fatal kidney disease. *E. coli* O157:H7 was the first EHEC recognized as a cause of human disease in 1982 (Kaper et al., 2004). Although *E. coli* O157:H7 is a natural inhabitant of cattle and a wide range of animals such as sheep, goat and deer, it is a severe enteric human pathogen. EHEC differs from other *E.coli* strains by its non-lactose fermenting characteristic, which prevents it from being detected by coliform index.

Listeria monocytogenes is the agent of listeriosis, a serious infection caused by eating food contaminated with the bacteria. Listeriosis has been recognized as an important public health problem in the United States. The disease affects primarily persons of advanced age, pregnant women, newborns, and adults with weakened immune systems. Listeriosis is a serious disease for humans; the overt form of the disease has a mortality greater than 25 percent. The two main clinical manifestations are sepsis and meningitis. Meningitis is often complicated by encephalitis, a pathology that is unusual for bacterial infections (Todar, 2008).

Legionella, a water borne organism of worldwide public health significance, proliferates in thermally altered waters such as those found in cooling water reservoirs of air conditioning plants, hot water systems, spas and fountains. Legionellosis, the infectious disease caused by *Legionella pneumophilia* takes two distinct forms as Legionnaires' disease and Pontiac fever. Legionnaires' disease is the more severe form of the infection and produces pneumonia. Advanced stages of the disease cause problems with the gastrointestinal tract and the nervous system and lead to diarrhea and nausea. However, the disease is generally not a threat to most healthy individuals, and tends to lead to harmful symptoms only in those with a compromised immune system and the elderly (Winn, 1996). Consequently, it is actively checked in the water systems of hospitals and nursing homes. *Legionella pneumophilia* serotype 1 is the most frequent cause of Legionnaire’s disease and is the serotype most frequently isolated from environmental sources (McCarthy and

Hill, 2001). Pontiac fever is caused by the same bacterium, but produces a milder respiratory illness without pneumonia, which resembles acute influenza (Winn, 1996). Legionellosis is a disease of significant medical and public interest. In the United States, the disease affects 8000 to 18000 individuals a year (Fields et al., 2002).

Campylobacter is an important cause of acute bacterial gastroenteritis in human worldwide (Coker et al., 2002). *C. jejuni* infections can also cause Reiter syndrome, a reactive arthropathy, and Guillian–Barre syndrome, an acute neuromuscular paralysis (Tauxe, 1992). From 1996 to 2000, there were an estimated 2.4 million Campylobacter infections each year in the US, with 21.9 cases reported per 100,000 people (Nachamkin and Blaser, 2000). Although the trend of Campylobacter infections appears to be downward, there were still 5215 Campylobacter infections reported for 2003, which represented an incidence of 13 cases per 100000 people (CDC, 2004).

Salmonella enterica serovar Typhimurium (S. Typhimurium) has a diverse host range, which includes humans, cattle, pigs, sheep, horses, rodents and birds (Kingsley and Baumler, 2000). Salmonella spp. constitute a major public health concern because they are the second-most commonly isolated pathogens during the diagnosis of human diarrheal disease and S. Typhimurium accounts for most of these isolates (Hohmann, 2001; CDC, 2002). Food-producing animals carry S. Typhimurium and fecal contamination of poultry and meat, at slaughter, provide common sources of human infection (Sanchez et al., 2002). Environmental factors that enhance either the survivability or dispersion of the organism could result in a spatial pattern of disease risk. Identification of such a pattern would assist in elucidating the risk factors for salmonellosis in dairy herds and could lead to the development of targeted surveillance systems as well as recommendations for minimizing the impact of these risks and reducing the incidence of salmonellosis (Graham et al., 2005).

Due to the extensive presence of pathogens in especially water reservoirs, dairy and meat products and the diverse host range of some pathogen strains, pathogen detection is of the utmost importance primarily for health and safety reasons. The first control point in the prevention of water-borne outbreaks is monitoring the microbiological water quality parameters.

Level of contamination is commonly analyzed via culture dependent determination of indicator parameters such as total coliform and fecal coliform (Ainsworth, 2004). The coliform group consists of several genera of bacteria belonging to the family Enterobacteriaceae. Traditionally these genera included Escherichia, Citrobacter, Enterobacter and Klebsiella (Gleeson and Gray, 1997). Selection of these two parameters is a bias since they do not include viruses, protozoa and many other pathogenic bacteria, which are not included in the coliform group.

Table 2.2. Pathogen microorganisms represented by coliform index and their specifications (WHO, 2006).

Microorganism	**Specifications**
Escherichia coli	Causes three infectious diseases: urinary tract infections, neonatal meningitis, and gastroenteritis
Citrobacter spp.	Causes a wide spectrum of diseases including urinary tract, respiratory tract, wounds, bone, peritoneum, endocardium, meninges, intestines and bloodstream
Enterobacter spp.	Nosocomial pathogen, that causes of hospital acquired septicemias, nosocomial pneumonias, nosocomial urinary tract infections, postsurgical peritonitis cases
Salmonella spp.	Most common bacterial cause of diarrhea in the United States, and the most common cause of food-borne deaths. Responsible for 1.4 million cases of food-borne illness a year.
Shigella spp.	Causes an estimated 448,000 cases of diarrhea illnesses per year. Poor hygiene causes Shigella to be easily passed among people, infected individuals and food.
Klebsiella spp.	Causes severe infections
Yersinia spp.	Causes plague, ulcer, acute stomach and intestine infections

The most common bacteria represented and not represented by the coliform index and their health hazards are given in Tables 2.2 and 2.3 respectively. Ability of pathogens to live in different environments, short survival, and inability to identify the source of fecal contamination and low correlation with the presence of pathogens are other limitations related to the application of indicator bacteria. In addition, treatment processes are more effective on indicator bacteria compared to pathogenic bacteria, viruses or protozoa (Girones et al., 2010). A critical restriction of cultivation-based methods is that bacteria

can enter viable but non-culturable state (VBNC) under stress conditions such as lack of nutrients, oxygen, light or unstable temperature (Oliver, 2010).

Table 2.3. Pathogen microorganisms not represented by coliform index and their specifications (WHO, 2006).

Microorganism	**Specifications**
Listeria monocytogenes	Causes listeriosis, a serious disease for pregnant women, newborns, and adults with a weakened immune system.
Campylobacter	Second most common bacterial cause of diarrhea in the United States. Also causes fever, menengitis, Reiter syndrome, a reactive arthropathy, and Guillian–Barre syndrome, an acute neuromuscular paralysis
Legionella	Causes legioners disease and pontiac fever
Clostridium botulinum	Produces a toxin which causes botulism, a life-threatening illness that can prevent the breathing muscles from moving air in and out of the lungs
Staphylococcus aureus	Produces a toxin that causes vomiting shortly after being ingested. Causes infections in the wounds, toxic shock syndrome, blood poisoning
Vibrio vulnificus	Causes gastroenteritis, wound infection, and severe bloodstream infections.
Acinetobacter spp.	Causes infections in the urinary system, pneumonia, menegitis
Aeromonas spp.	Causes poisoning in blood, respiratory tract infections
Bacillus cereus	Causes food poisoning
Bacillus anthracis	Causes antrax
Burkholderia pseudomallei	Causes pneumonia, nervous system diseases, ulcer
Helicobacter pylori	Causes ulcer, gastric cancer
Mycobacterium spp.	Diseases in the respiratory tract, gastrointestinal and urinary tract, epiderma, skeleton and lymphs
Pseudomonas aeruginosa	Causes respiratory tract infections, poisoning in blood, meningitis
Tsukamurella spp.	Causes chronical lung diseases, weakening in the immune system, wound infections, menegitis, skin and bone infections
Vibrio spp.	Causes cholera, ulcer, acute stomach and intestine infections
Toxic *Cyanobacteria*	Effects of toxins on liver, neurotoxicity, and tumor formations

Drinking water regulations are limited to detection of collective parameters such as total coliform and fecal coliform and individual species/genus such as *Escherichia coli*, Enterococci, *Pseudomonas aeruginosa, Clostridium perfringens, Bacteroides fragilis, Legionella, Cryptosporidium, Giardia lamblia* (WPCR, 2004; EC, 1998; WHO, 2011; USEPA, 2009). Monitoring these individual species and total or fecal coliforms as indicator organisms for water quality analysis underestimates a vast amount of bacterial, viral and protozoan pathogens. Comprehensive analysis of microbiological quality of drinking water reservoirs and treated water and wastewater prior to discharge is essential for understanding the availability of contamination and suitability of water quality for domestic/beneficial uses.

2.3. Methods for Detection and Identification of Environmental Biodiversity

2.3.1. Conventional Methods

Conventional microbiological methods have been a standard practice for the detection and identification of pathogens in water and wastewater for nearly one century and continue to be a reliable standard for ensuring safety. The procedure of such a method usually includes microbiological culturing and isolation of the pathogen, followed by confirmation by biochemical and/or serological tests, taking up to 5–7 days to get a confirmed result for a particular pathogenic organism (Swaminathan and Feng, 1994; Vasavada, 1997). While reliable and unambiguous, these conventional methods are time consuming and labor intensive, and are therefore not suitable for modern water quality assurance to make a timely response to possible risks. Brief information about conventional methods are given as follows:

2.3.1.1. Multiple Tube Fermentation Method. The multiple tube fermentation (MTF) method relies on the principle of dilution to extinction. 10-fold dilutions are prepared and inoculated to a series of tubes containing liquid media. Gas production, acid formation or abundant growth are monitored after 24 and 48 hours of incubation at 37°C. The resulting positive and negative reactions in the dilution series are used to estimate the most probable number (MPN) of cells per 100 ml of sample. This is the presumptive stage of MTF, which gives an estimate of the number of coliforms. For confirmation, positive reactions are sub-

cultured to confirmatory media, either Brilliant Green Lactose Bile Broth or Lauryl Tryptose Lactose Broth and incubated at 37°C for another 48 hours. Any gas production at the end of the incubation period confirms the presence of coliforms in the original sample. This is known as the confirmatory stage. For routine water analysis it is generally sufficient to only proceed as far as the confirmatory phase for total coliforms (Köster et al., 2003, Rompre et al., 2010).

2.3.1.2. Membrane Filtration Method. Membrane filtration was developed as a practicable alternative to the MPN approach. In principle, a measured volume of water is filtered through a membrane with appropriate pore size such that the microorganisms are retained on or near the surface of the membrane. The membrane is then aseptically transferred to a solid agar base selective for the organism or to an absorbent pad saturated with a suitable liquid medium and incubated at a specific temperature for a specific time. The organisms retained by the membrane will form colonies of characteristic morphology and color depending on the medium. These colonies are counted and the number of organisms per 100 mL is calculated. The membranes are incubated for 18 hours at 37°C for total coliforms and 44°C for fecal coliforms (Köster et al., 2003, Rompre et al., 2010).

2.3.1.3. Pour and Spread Plate Method. These methods are used to culture, identify and enumerate the bacteria. In the pour plate method, sample is diluted. Small amounts of each dilution is mixed with warm liquid agar medium, poured into a culture dish, solidified and incubated. Separate bacterial colonies are counted after incubation and the results are reported as colony forming units (cfu) per unit volume of sample (cfu/ml). Appropriate dilutions allow the determination of total number of bacteria (Köster et al., 2003).

2.3.2. Molecular Methods

Molecular methods have been a reliable alternative to culture-dependent methods on the basis of reduced analysis time within hours, ability to distinguish serotypes in subspecies level and eligibility to detect species independent of coliform index. These major characteristics of culture-independent molecular methods allow better identification, determination and quantification of a broad range of bacterial, viral and parasitic pathogens. In addition, limitations of classical methods in identification of complex

microbial structure in environmental samples representing high genetic diversity are overcome by the use of molecular techniques.

Culture independent methods target different molecules such as DNA, RNA, proteins, and membrane lipids for investigation of the microbial communities. DNA codes for 16S rRNA for phylogenetic information and it carries the sequence information about metabolically important enzymes. However, DNA cannot differentiate between live and dead cells. RNA is the molecule that codes for active species and carries the information of metabolically active genes. RNA can distinguish between live and dead cells in contrast to DNA. Protein analysis is conducted for determination of metabolically important enzymes. Membrane phospholipids are alternative molecules used for phylogenetic analysis. Using these target molecules, molecular methods are eligible to answer questions about complex ecosystems such as (i) Which species are available in the ecosystem? (ii) What is the quantity of available species? (iii) What metabolic functions are they capable of? (iv) Are they active?

2.3.2.1. Polymerase Chain Reactions (PCR). The first step in most of the molecular methods is the extraction of genetic material followed by amplification of the desired region on the genome. Genetic material can be either deoxyribonucleic acid (DNA) or ribonucleic acid (RNA). PCR is based on exponential amplification of the target genes on double stranded DNA. In case RNA is extracted as genetic material, the complementary strain is synthesized by reverse transcriptase PCR (RT-PCR) to obtain a double stranded DNA named as complementary DNA (cDNA).

PCR is an enzymatic reaction that requires (i) template DNA/cDNA, which carries the information of the sequence to be amplified, (ii) oligonucleotide primer pair, which stick to specific complementary sequences on each strand of DNA and determine the target DNA region to be amplified, (iii) heat-stable DNA polymerase enzyme which is involved in the synthesis of new DNA, (iv) deoxyribonucleoside triphosphate (dNTP) mixture to form new DNA strands, (v) buffer solutions appropriate for DNA polymerase activity and (vi) Mg^{2+} ions to improve specificity and PCR efficiency.

PCR is based on three major stages known as denaturation, annealing and extension at different temperatures. First step is the initial denaturation of the entire template DNA at 95°C. Amplification consists of 20-40 cycles of denaturation of target DNA region at 95°C, annealing of primers to the target DNA at 48-72°C (depending on the primer), extension of newly synthesized DNA strand at 72°C (optimum activity temperature of DNA polymerase). The reaction terminates after a final extension of 5-15 minutes at 72°C to provide all the elongation of any remaining single stranded DNA. PCR provides exponential amplification, which produces 2^n copies (n = cycle number) of amplicons at the end of the reaction.

PCR is the most commonly applied method for quick determination of the organism of interest. It has also been modified to amplify more than one region of interest on the same genome using multiple primer pairs in the same reaction. This modified reaction is named as multiplex PCR. Both PCR and multiplex PCR have been successfully applied in fields of pathogen detection in clinical specimens (Aranda et al., 2007), food (Aslam et al., 2003; Marathe et al., 2012) and environmental samples (Bonetta et al., 2011; Calvo et al., 2008; Kong et al., 2002).

The PCR products can be analyzed by fingerprinting techniques which have the potential to separate the PCR products originating from different DNA sequences representing populations in the original samples. The PCR products can also be cloned and subsequently sequenced to allow identification of populations. Since the amount of DNA produced by PCR ideally increases exponentially during the amplification, errors occurring early in the process will result in biased results.

2.3.2.2. Quantitative Real-Time PCR (qPCR). qPCR is a modification of PCR which allows quantification of the initial and final amount of the template most specifically, sensitively and reproducibly. Real-time PCR monitors the fluorescence emitted during the reaction as an indicator of amplicon production during each PCR cycle as opposed to the endpoint detection (Higuchi et al., 1992). The real-time progress of the reaction can be viewed in some systems. Real-time PCR does not detect the size of the amplicon and thus does not allow the differentiation between DNA and cDNA amplification, however, it is not influenced by non-specific amplification unless SYBR Green is used. Real-time PCR

quantification eliminates post-PCR processing of PCR products. This helps to increase throughput and reduce the chances of carryover contamination. In comparison to conventional PCR, real-time PCR also offers a much wider dynamic range of up to 10^7-fold (compared to 1000-fold in conventional PCR). It means that the broader the dynamic range, the more accurate the quantification.

The real-time PCR system is based on the detection and quantification of a fluorescent reporter (Lee et al., 1993*;* Livak et al., 1995). This signal increases in direct proportion to the amount of PCR product in a reaction. By recording the amount of fluorescence emission at each cycle, it is possible to monitor the PCR reaction during exponential phase where the first significant increase in the amount of PCR product correlates to the initial amount of target template. The higher the starting copy number of the nucleic acid target, the sooner a significant increase in fluorescence is observed.

A fixed fluorescence threshold is set significantly above the baseline that can be altered by the operator. The parameter C_T (threshold cycle) is defined as the cycle number at which the fluorescence emission exceeds the fixed threshold. There are three main fluorescence-monitoring systems for DNA amplification (Wittwer et al., 1997a): (1) hydrolysis probes; (2) hybridizing probes; and (3) DNA-binding agents (Wittwer et al., 1997b; van der Velden et al., 2003). Hydrolysis probes include TaqMan probes (Heid, et al. 1996), molecular beacons (Mhlanga and Malmberg, 2001; Vet et al., 2002; Abravaya et al., 2003; Tan et al., 2004; Vet and Marras, 2005) and scorpions (Saha et al., 2001; Solinas et al., 2001; Terry et al., 2002). They use the fluorogenic 5' exonuclease activity of Taq polymerase to measure the amount of target sequences in cDNA samples.

TaqMan probes are oligonucleotides longer than the primers (20-30 bases long with a Tm value of 10^oC higher) that contain a fluorescent dye usually on the 5' base, and a quenching dye typically on the 3' base. When irradiated, the excited fluorescent dye transfers energy to the nearby quenching dye molecule rather than fluorescing (this is called FRET = Förster or fluorescence resonance energy transfer) (Hiyoshi, 1994; Chen, 1997). Thus, the close proximity of the reporter and quencher prevents emission of any fluorescence while the probe is intact. TaqMan probes are designed to anneal to an internal region of a PCR product. When the polymerase replicates a template on which a TaqMan

probe is bound, its 5' exonuclease activity cleaves the 5’ end of probe which contains the reporter dye (Holland, 1991). This ends the activity of quencher (no FRET) and the reporter dye starts to emit fluorescence which increases in each cycle proportional to the rate of probe cleavage. Accumulation of PCR products is detected by monitoring the increase in fluorescence of the reporter dye. TaqMan assay uses universal thermal cycling parameters and PCR reaction conditions. Because the cleavage occurs only if the probe hybridizes to the target, the origin of the detected fluorescence is specific amplification. The process of hybridization and cleavage does not interfere with the exponential accumulation of the product. One specific requirement for fluorogenic probes is that there be no G at the 5' end. A 'G' adjacent to the reporter dye quenches reporter fluorescence even after cleavage.

The cheaper alternative is the double-stranded DNA binding dye chemistry, which quantitates the amplicon production (including non-specific amplification and primer-dimer complex) by the use of a non-sequence specific fluorescent intercalating agent (SYBR-green I or ethidium bromide). It does not bind to ssDNA. SYBR green is a fluorogenic minor groove binding dye that exhibits little fluorescence when in solution but emits a strong fluorescent signal upon binding to double-stranded DNA (Morrison, 1998). Disadvantages of SYBR green-based real-time PCR include the requirement for extensive optimization. Furthermore, non-specific amplifications require follow-up assays (melting point or dissociation curve analysis) for amplicon identification (Ririe, 1997). Another controllable problem is that longer amplicons create a stronger signal. In Figure 2.1, fluorescence monitoring system for qPCR is depicted.

The efficiency of the PCR should be 90 - 100% ($-3.6 > \text{slope} > -3.1$). A number of variables such as length of the amplicon, secondary structure and primer quality can affect the efficiency of the PCR. Although valid data can be obtained that fall outside of the efficiency range, the qPCR should be further optimized or alternative amplicons designed. For the slope to be an indicator of real amplification (rather than signal drift), there has to be an inflection point. This is the point on the growth curve when the log-linear phase begins. It also represents the greatest rate of change along the growth curve. (Signal drift is characterized by gradual increase or decrease in fluorescence without amplification of the product.) The important parameter for quantification is the C_T. The higher the initial

amount of genomic DNA, the sooner accumulated product is detected in the PCR process, and the lower the C_T value. The threshold should be placed above any baseline activity and within the exponential increase phase (which looks linear in the log transformation). A C_T value of 40 or higher means no amplification and this value cannot be included in the calculations.

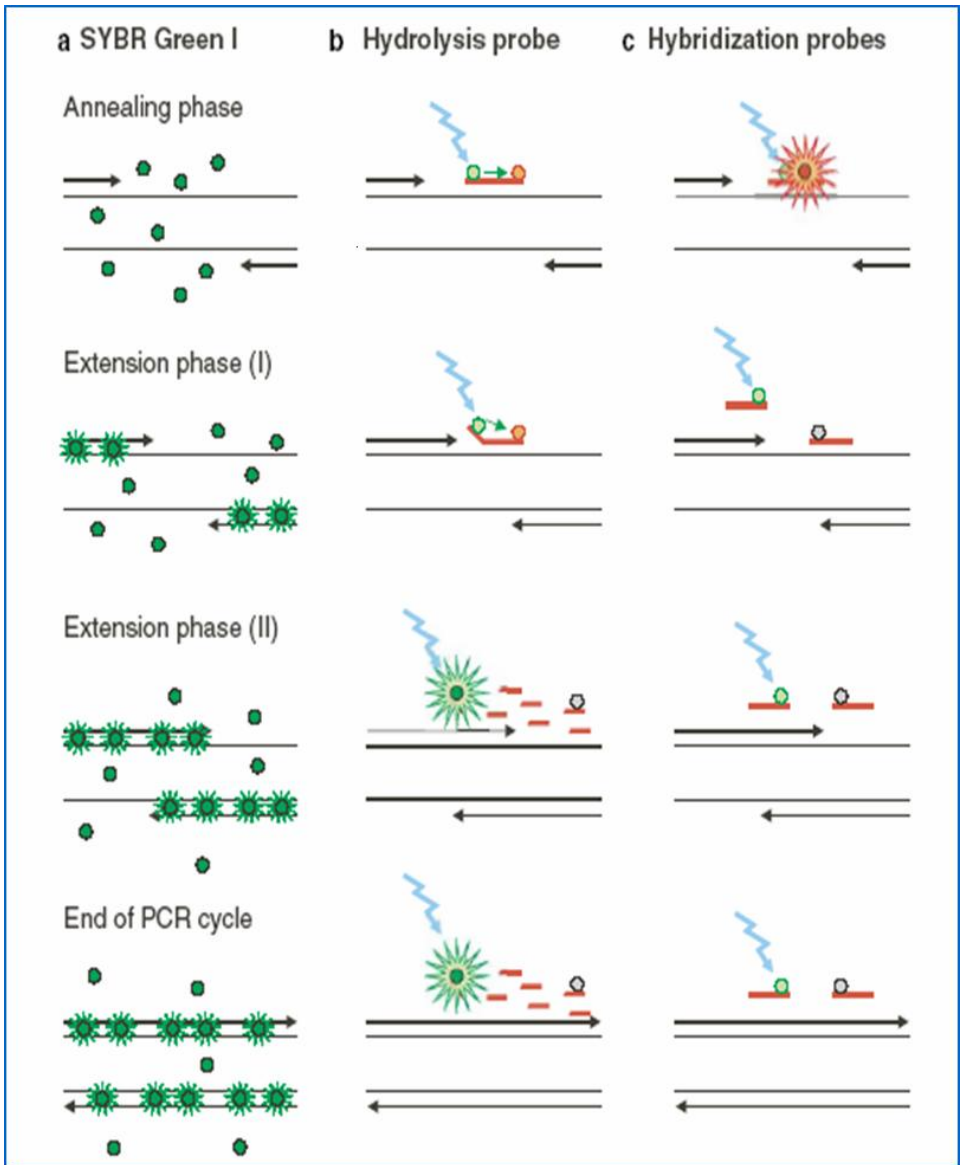


Figure 2.1. Fluorescence monitoring systems for qPCR.

2.3.2.3. Cloning and Sequencing. Cloning can produce large amounts of DNA segments originally isolated from environmental samples. The DNA fragments can be produced after digestion with restriction enzymes of the DNA extracted from a sample (i.e., shotgun

cloning), or after PCR or RT-PCR. Compared to cloning after PCR, shotgun cloning introduces less bias and produces clones of multiple genes at the same time. Cloning after PCR is rapid and convenient, but can be biased. The bias can be introduced during the PCR step or during cloning. For instance, the use of rare-cutting restriction enzymes during cloning might also cut amplified rDNA. In addition, it is possible that different rRNA gene fragments are cloned with different efficiencies.

Cloned DNA fragments can be sequenced to study the phylogenetic diversity of the microbial community from which the sample was originally obtained. The resulting sequences can be compared to sequences in databases to identify the closest phylogenetic relatives. There are a number of problems that need to be taken into consideration when using this technique. First, when the retrieved sequences exhibit high similarity to sequences available in databases (98% to 99% identity), it is difficult to rule out PCR errors. Secondly, it is difficult to convert the rRNA similarity to the nomenclature level of species or genus. In general, more than 97% 16S rRNA identity indicates that two sequences belong to the same species, which typically corresponds to DNA:DNA hybridization values above 70%. It has been observed that some species express different 16S rRNA genes at different growth stages, or multiple 16S rRNA genes are expressed at the same time.

Basic sequencing methods are known as Maxam - Gilbert sequencing and Sanger sequencing. Maxam - Gilbert sequencing is also known as chemical sequencing that is based on radioactive labeling and chemical modification of DNA (Maxam and Gilbert, 1977). Sanger sequencing involves modified nucleotides, known as dideoxynucleosidetriphosphates (ddNTPs) that terminate DNA strand elongation (Sanger et.al., 1977). Therefore, it is also known as chain-terminating sequencing. The ddNTPs may be radioactively or fluorescently labeled for detection in automated sequencing machines. This method is considered as the ‘first generation’ technology for sequencing. It has been a useful tool for revealing the microbial community in complex environmental samples such as drinking water reservoirs (Röske et al., 2012), marine (Kolukirik et al., 2011) and lake sediments (Song et al., 2011) and biofilms on drinking water system networks (Schmeisser, 2003).

2.3.2.4. Next Generation Sequencing. Despite the fact that automated Sanger sequencing has been widely used for more than 25 years, demand for low-cost and rapid analysis led to the development of next-generation or second-generation methods for genome sequencing. Recent high-throughput technologies are especially significant for community analysis of highly diverse environmental samples since the traditional Sanger sequencing is based on sequencing individual specimens and therefore is not adequate for deciphering the biodiversity of environmental samples that contain mixtures of DNA from hundreds to thousands of individuals (Shokralla, 2012). Next generation sequencing (NGS) technologies can effectively read multiple DNA sequences present in environmental samples concurrently as reliable as traditional sequencing with considerably low-cost.

NGS technologies can be classified into two main categories as PCR-based technologies and 'single-molecule' sequencing (SMS) technologies. PCR-based technologies currently include four commercially available platforms: Roche 454 Genome Sequencer (Roche Diagnostics Corp., Branford, CT, USA), HiSeq 2000 (Illumina Inc., San Diego, CA, USA), AB SOLiD™ System (Life Technologies Corp., Carlsbad, CA, USA) and Ion Personal Genome Machine (Life Technologies, South San Francisco, CA, USA). 'Single-molecule' sequencing (SMS) technologies, are non-PCR based and do not include an amplification step prior to sequencing. Two single-molecule sequencing systems have been announced recently: HeliScope (Helicos BioSciences Corp., Cambridge, MA, USA) and PacBio RS SMRT system (Pacific Biosciences, Menlo Park, CA, USA) (Shokralla et.al., 2012). Common steps applied in both PCR based and non-PCR based technologies can be grouped as (i) template preparation, (ii) sequencing and imaging, and (iii) data analysis (Metzker, 2010). However, different strategies and chemistries are involved in each method.

Sanger sequencing has been replaced by NGS methods in the field of metagenomics due to the ease of analyzing complex diversity. Applications with freshwater samples on detection of eukaryotes were reviewed by Shokralla (2012). Vaz-Moreira et al. (2011) successfully applied 454 pyrosequencing to reveal the bacterial community in river samples. Klindworth (2012) also used 454 pyrosequencing for bacterial community analyses in sea water.

2.3.2.5. Denaturing/Temperature Gradient Gel Elecrtophoresis. DGGE and TGGE were introduced by Muyzer and co-workers (1993) in the field of complex environmental samples. These fingerprinting methods have been routinely used for profiling the microbial diversity in natural and engineered ecosystems. It is possible to obtain qualitative and semi-quantitative estimations of biodiversity by DGGE/TGGE. They typically involve amplifying the genes encoding the 16S rRNA by PCR and then, separating these fragments in a polyacrylamide gel. DGGE/TGGE exploits the fact that DNA molecules that have the same length, but differ at least by one nucleotide, can be separated by electrophoresis through a linear gradient of increasing chemical denaturants of urea and formamide (DGGE), or through a linear temperature gradient (TGGE). The two strands of a DNA molecule separate or melt at a specific temperature, which depends on the hydrogen bonds formed between complementary base pairs (GC-rich domains melt at higher temperatures), and on the attraction between neighboring bases on the same strand. When run on a polyacrylamide gel, the mobility of the molecule is retarded when the first melting domain is reached, resulting in partial dissociation of the fragment. Complete strand separation is prevented by the presence of a high melting domain, known as a GC clamp, which is added to one primer. The DGGE/TGGE pattern obtained provides a rapid identification of the predominant species. A big advantage of these techniques is that they make it possible to obtain taxonomic information by excising, re-amplifying and sequencing specific DNA fragments or by hybridization analysis with taxon specific oligonucleotides probes (Heuer et al., 1999). An individual discrete band refers to a unique "sequence type" or phylotype or operational taxonomic unit (OTU). Most DGGE/TGGE studies focus on the number of different bands in order to get an estimate of the community richness, and there have been very few studies that also take into account the intensity of each band as providing an estimate of the abundance of each band-population (Nübel et al., 1999).

The choice of the primer set and the optimization of the gel running conditions before the technique can be used to screen for sequence polymorphism of a particular gene are the main limitations (Muyzer et al., 1993; Duarte et al., 2012), and the difficulty of comparing patterns across gels, when these patterns include numerous bands. This implies that multiple gels and different combinations of samples are required if numerous samples are being investigated. Other limitations associated with this technique are the fact that DNA fragments with differing sequences may migrate together, and its limited sensitivity of

detection of rare community members (Vallaeys et al., 1997). There are several different ways to carry out the statistical analysis of data obtained by DGGE/TGGE (Fromin et al., 2002).

DGGE/TGGE approaches have been used in a number of studies of eubacterial, archaeal and eukaryotic communities in aquatic environments. DGGE bands were excised and sequenced for a better understanding of the available community (Röling et al., 2001; Cetecioglu et al., 2009; Kolukirik et al., 2011).

2.3.2.6. Other Community Fingerprinting Methods. In addition to denaturing gradient gel electrophoresis (DGGE), temperature gradient gel electrophoresis (TGGE), several fingerprinting techniques, such as T-RFLP (terminal restriction fragment length polymorphism), single stranded conformation polymorphism (SSCP) or ribosomal intergenic spacer analysis (RISA) and automated ribosomal intergenic spacer analysis (ARISA), have been developed to screen clone libraries, to estimate the level of diversity in environmental samples, to follow changes in community structure (e.g., trace one or more populations over time) and to compare diversity and community characteristics in various samples. These techniques usually involve gel electrophoresis that can separate different DNA segments of a community rDNA library.

The PCR products can be analyzed by fingerprinting techniques such as denaturing gradient gel electrophoresis (DGGE), temperature gradient gel electrophoresis (TGGE), terminal restriction fragment length polymorphism (T-RFLP), single stranded conformation polymorphism (SSCP) or ribosomal intergenic spacer analysis (RISA) and automated ribosomal intergenic spacer analysis (ARISA) which have the potential to separate the PCR products originating from different DNA sequences representing populations in the original samples. The PCR products can also be cloned and subsequently sequenced to allow identification of populations. Since the amount of DNA produced by PCR ideally increases exponentially during the amplification, errors occurring early in the process will result in biased results.

SSCP detects sequence variations among DNA fragments, which are usually PCR-amplified, 16S rRNA gene sequences. SSCP was first used to assess the diversity of

natural microbial communities. At low temperatures, single-stranded DNA will adopt a three-dimensional conformation determined by the intramolecular interactions that influence their electrophoretic mobility in a non-denaturing polyacrylamide gel. PCR fragments of the same size, but with differing nucleotide sequences will be separated due to their differing electrophoretic mobility. Differences in mobility are detected on autoradiograms (radioactive detection), by silver staining the bands or using fluorescently labeled primers that are subsequently detected by an automated DNA sequencer (non-radioactive detection) (Dong and Zhu, 2005). Applications of SSCP for fingerprinting drinking water supply system (Eichler et al., 2006), surface water and tap water (Kahlisch et al., 2012) in combination with sequencing excised bands are available in the literature.

T-RFLP analysis is a community fingerprinting technique that is based on the restriction digest of double-stranded fluorescently end-labeled PCR fragments. One primer is labeled at the 50 terminus with a fluorescent dye. As a general rule, a single species will contribute a single terminal fragment of a given size, although several species may have terminal fragments of identical size. Fragments of the 16S rRNA gene are usually targeted. The fragments are separated by gel electrophoresis in non-denaturing polyacrylamide gels or by capillary electrophoresis, and distinguished by laser induced fluorescent detection. These fluorescence data are converted into electrophoregrams in which the peaks represent fragments differing in size, and the areas under the peaks indicate the relative proportions of the fragments. The advantage of this technique is its ability to detect even rarer members of a microbial community. In addition, phylogenetic assignments can be inferred from the sizes of the terminal restriction fragment (TRF) using web-based resources that predict TRF sizes for known bacteria(Liu et al., 1997). Like SSCP, T-RFLP analysis has been used to compare the dynamics both between and within microbial populations in lake sediments (Song et al., 2011) and salt marshes (Cordova-Kreylos et al., 2006).

RISA was developed by Borneman and Triplett in 1997 and was firstly applied to study the microbial diversity in soils. The method involves PCR amplification of the spacer region located between the small (16S) and large (23S) subunit rRNA genes in the rRNA operon. This region is extremely variable in size (ranging from 50 bp to more than 1.5 kb) and nucleotide sequence. Primers are thus defined to target to conserved regions in the 16S and 23S genes. In RISA, the polymorphism revealed is linked to the length

heterogeneity. Amplification products differing in length are separated on polyacrylamide gels on the basis of their size and visualized by silver staining. This tool has been used successfully to assess community fingerprints, each band corresponding to at least one organism.

Fisher and Triplett developed the automated version of RISA in 1999, which they named ARISA, in order to be able to assess community diversity more rapidly and more efficiently. PCR amplification of the 16S–23S region is performed using a fluorescently labeled, forward primer, which makes it possible to detect the amplicons by automated capillary electrophoresis. The total number of distinct fluorescent peaks in the ARISA data within a given sample is taken as an estimate of species diversity, and the sizes of the fragments can be compared to those in the GeneBank database.

2.3.2.7. Fluorescent in situ Hybridization (FISH). Whole cell or in situ hybridization is a method to enumerate specific microbial populations and to determine their spatial distribution directly in their natural environment without cultivation. Nowadays, FISH technique is widely used in environmental microbiology studies (De Long, 1992; Raskin et al., 1994; Wagner et al., 2003). Fluorescently-labelled rRNA-targeted oligonucleotide probes were shown to allow the detection of individual cells *in situ* (DeLong et al., 1999). This made whole-cell hybridization with rRNA–targeted probes suitable tool for not only determination of microorganisms but also quantitative analysis in environmental microbiology (Amann et al., 1990). Probes can be designed to be complementary to species-, group-, or kingdom-specific target sites. Cells are fixed to make them permeable to the probe, which then hybridizes its specific target site. Generally, a probe targeting the bacteria domain is used in combination with more specific probes. After hybridization, the microbial communities are typically examined by epifluorescent microscopy.

FISH has been used to explore bacterial communities in activated sludge, marine and freshwater environments, in both pristine and contaminated aquifer. Despite the numerous advantages of FISH, the application of the technique to investigate natural samples still produces some false positive and false negative results. One of the reasons is that all bacterial and archaeal cells may not be permeabilisied using standard fixation protocols (Amann et al., 1995). In addition, the choice of the probe and of the fluorochrome, the

stringency conditions, the hybridization temperature, autofluorescent sample or background and the physiological state of the target cells can all significantly influence the efficiency of this tool (Head et al., 1998). In addition, FISH is a strictly taxonomic method which cannot give specific information to strain level. In aquatic samples, the proportion of cells visualized with monolabeled oligonucleotide probes can be highly variable. In addition, many of the cells have low ribosome content which is generally below current detection limits using monolabeled oligonucleotides and standard epifluorescence microscopy (DeLong et al., 1999).

2.3.2.8. Fatty Acid Methyl Ester (FAME) Analysis. FAME can be created by an alkali-catalyzed reaction between fats or fatty acids and methanol. The molecules in biodiesel are primarily FAMEs, usually obtained from vegetable oils by trans-esterification.

More than 300 fatty acids and related compounds are found in bacteria and every microorganism has its specific FAME profile (microbial fingerprinting) Thus, it can be used as a tool for microbial source tracking. The wealth of information contained in these compounds is both in the qualitative differences (usually at genus level) and quantitative differences (commonly at species level). As the biochemical pathways for creating fatty acids are known, various relationships can be established. Gas chromatography is used to obtain fatty acid profiles. Fatty acids were quantified by comparison with known standard fatty acids by using peak width and area (Ritchie et al., 2000).

2.3.3. Immobilization-Based Methods

Immobilization of cells is a common approach for different purposes in various fields of industry and science. Enhancement of production in bioreactors (Kourkoutas et al., 2003), removal of contaminants in wastewater (Takeno et al., 2005), atomic force microscopy experiments (Louise Meyer et al., 2010, Vadillo-Rodríguez et al., 2004) and detection of bacteria by biosensors (Xu and Ying, 2011) are basic industrial, environmental and scientific applications of immobilization.

Immobilization of bacteria can be provided by different mechanical, physical and chemical methods. These methods can be categorized in four major classes (Kourkoutas,

2003; Suo, 2012): (i) physical adsorption to solid surfaces, (ii) physical entrapment within a porous matrix or encapsulation in hydrogels, (iii) self-aggregation by flocculation (natural) or with cross-linking agents (artificially induced), and (iv) covalent binding. Extensive schematic description of cell immobilization strategies is given in Figure 2.2.

Physical adsorption involves the attraction of bacteria by electrostatic interactions. Bacterial surfaces are typically negatively charged under physiological conditions and they can be adhered to a positively charged surface. Various molecules such as polylysine, polyethyleneimmine, gelatin and alginate have been used for providing a positively charged surface for physical adsorption of bacteria (Suo et al., 2012; Vadillo-Rodríguez et al., 2004; Louise Meyer et al., 2010).

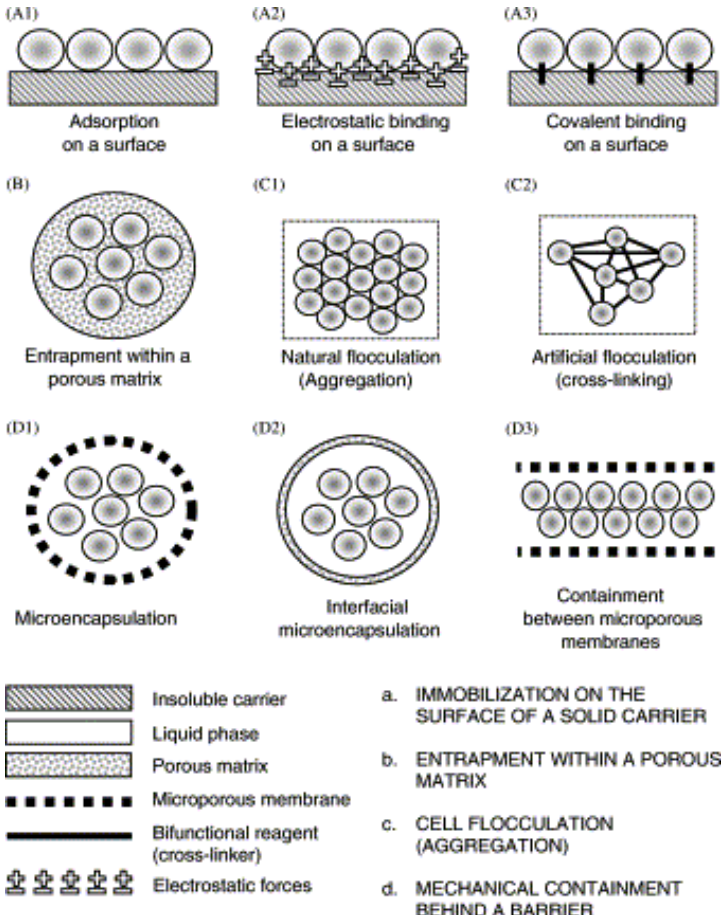


Figure 2.2. Basic cell immobilization strategies.

Physical entrapment is based on limitation of the mobility of bacteria. Bacterial motion is significantly slowed in viscous media of micro-cavities. Therefore, bacteria can be randomly trapped within a porous media or hydrogels while mass transfer of nutrients and metabolites is still allowed but cells are prevented from diffusing into the surrounding medium.

Flocculation is an immobilization technique generally observed in bioreactors either for industrial production or water and wastewater treatment.

Covalent binding takes advantage of the interaction between an analyte and an appropriate ligand immobilized on a flat surface. Since receptors often recognize specific types of ligands on a bacterial surface, such immobilization could achieve a high degree of specificity and efficiency. Due to the specificity, selectivity and efficiency of analyte-receptor interactions, covalent binding has been widely incorporated in construction of platforms for bacterial detection systems such as microarrays and biosensors. A wide range of recognition elements such as enzymes, DNA and antibodies, has been used for analyzing environmental samples.

Enzymes rely on catalytic conversion of the substrate for detection. Substrate production rate is related to the dose-dependent inhibition of the enzymatic reaction. Enzymes were historically the first molecular recognition elements included in biosensors and continue to be the most extensively studied area in general as well as environmental applications (Rogers, 2006; Choi, 2004).

DNA-based detection can be more specific than antibody based detection systems, and the sensitivity can be improved by combination with PCR. Gene probes are already finding application in the detection of disease-causing microorganisms in water supplies, food, or in plants, animal or human tissues (Rogers, 2006).

Antibody-based immobilization is faster and more robust than DNA detection. Moreover, it has the ability to detect not only contaminating organisms but also their biotoxins. Antibody-mediated immobilization is referred to as immunoimmobilization.

Sample Processing

Most immobilization based methods such as immunosensors and microarrays have a minimum detection limit of 10^3 cfu/ml for pure cultures. The limit of detection in environmental samples ranges from 10^3 to 10^7 cfu/ml. This is a major drawback for field applications and low-volume samples. A pre-concentration step is generally required for reliable results with biosensors and microarrays. Pre-concentration is generally provided by membrane filtration, centrifugation and PCR amplification for DNA microarrays. Pre-enrichment of bacteria in selective nutrient broths is another method for concentration of target. But this method is out of the concept of field applications. Centrifugation and filtration are methods that require extra instrumentation set-up which make the detection method far from being practical and portable. Therefore, a more compact pre-concentration system is necessary for combining with current detection systems.

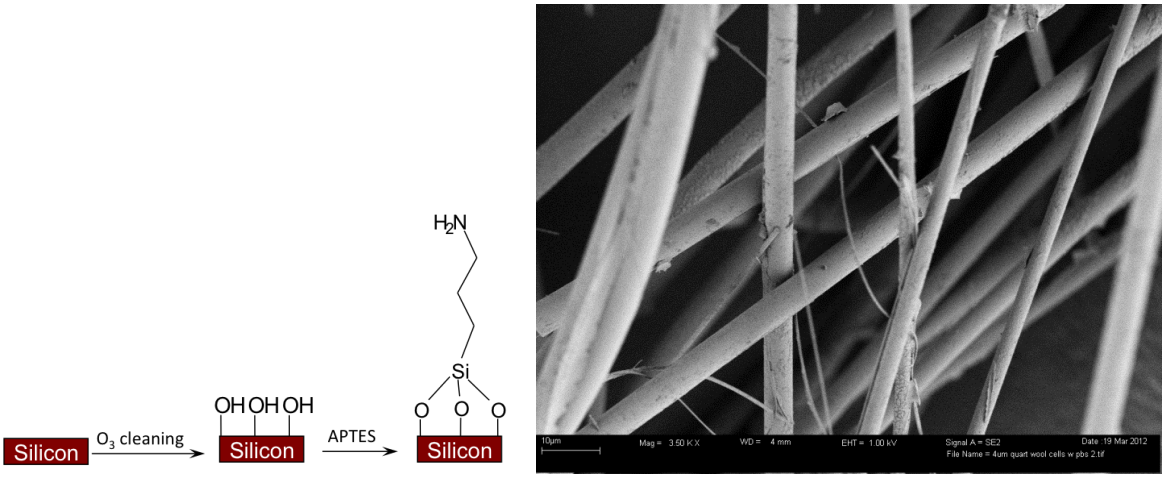


Figure 2.3. Structure of BiyoTrap (a) an example of surface chemistry (b) FESEM image.

BiyoTrap®, a recently patented system developed in ICAL, MSU, is a promising technology for concentration of bacteria available in food and environmental samples of different characteristics such as water, wastewater, marine and fuel. Biyotrapped samples can be used for a variety of applications such as cell viability assays (adenosine triphosphate (ATP) measurement), nucleic acid based assays (metagenome analysis) and immunoimmobilization assays. BiyoTrap uses chemically modified glass fibers to capture bacteria. The fibers act as a trap and not as a filter for bacteria. The chemical modification

covalently attaches charged amine groups to the fiber surface. The negatively charged bacteria are attracted to the positively charged fibers, trapped within the voids of the charged 3-D glass fiber network and remain in and around the activated fiber network. Surface chemistry and field emission scanning electron microscope (FESEM) image of BiyoTrap is given in Figure 2.3.

2.3.3.1. Immunoimmobilization. Antibodies have been widely used as recognition elements for their specificity, versatility, and strong and stable binding to a specific antigen. The large variety of bacterial surface antigens and corresponding antibodies offers a number of choices for immunoimmobilization, which could be highly specific for a given species.

Extensive research has been reported on the development of new detection methods that involve converting an already captured pathogen into an output signal by optical, electrochemical, mechanical or other means (Byrne et al., 2009). However, there has been little study of how to enhance the capture efficiency. Antigen-antibody interactions and surface chemistry are two key factors maximizing the efficiency of immunoimmobilization.

Antigen-Antibody Selection

A general guideline for antibody selection is to use antibodies targeting antigens on the surface of the bacterium (the outer membrane of Gram-negative and the peptidoglycan layer of Gram-positive bacteria). Antigens inside bacterial cells or embedded in cell wall components usually should be avoided since it is impossible for antibody molecules to reach them in living bacteria. Moreover, previous work using antibodies against whole bacterial cells often resulted in low immobilization efficiency (Premkumar et al. 2001; Rozhok et al. 2005). Therefore, a systematic evaluation and comparison of the immobilization efficiencies of selected antibody-antigen pairs associated with common bacterial surface antigens is still needed.

Suo et al. (2009) evaluated the immobilization efficiencies of immunoglobulin G (IgG) antibodies against four different types of surface antigens of S. Typhimurium and *E.*

coli: lipopolysaccharides (LPS), flagella, fimbriae, and a capsular protein. The results show that, with the exception of the capsular protein, all the surface antigens tested can in principle be targeted to achieve some degree of immobilization and that the immobilization efficiency is correlated to multiple factors, especially to the choice of antibody-antigen pairs. Other factors, such as the clonal type, the purity of the antibody, the antigen expression level, and the incubation time, also contribute to immobilization efficiency.

Surface Chemistry

Solid surfaces like gold, silver, silicon, aluminum, titanium and steel have been commonly used for constructing self-assembled monolayer (SAMs) to attach antibodies for immunoimmobilization purposes. Each surface has its unique chemistry for covalent linking of antibodies and antigens. In order to achieve a high immobilization efficiency, a substrate for bacterial immobilization should have a larger number of antibody molecules on the surface, with the paratope of each antibody molecule pointing away from the substrate. It would also be desirable for the antibody molecules to have sufficient freedom of movement to orient themselves in a proper binding direction towards the bacterial antigens.

As shown in Figure 2.4 (a), thiolated tethers including 16-mercaptohexadecanoic acid (MHA) and 11-mercapto-undecanoic acid (MUA) have been commonly used to activate gold and silver surfaces. The carboxyl terminal of MHA and MUA can link to amino groups after activation. Silanes with an active terminal are widely used for silicon oxide, silicon nitride, glass, aluminum, titanium and steel surfaces. Two popular silanes aminopropyltriethoxylsilane (APTES) and (3-glycidoxypropyl)- trimethoxysilane (GOPTMS) are shown in Figure 2.4 (b) and (c). APTES is further modified with N-(3-maleimidopropionyloxy) succinimide (BMPS), a short cross-linker, to provide an active maleimido terminal to link to the cysteine residue of antibodies. The glycidyl terminal of GOPTMS can react with amino or hydroxyl groups of an antibody. Another popularly used linkage is the biotin- avidin (streptavidin/neutravidin) system (Figure 2.4 (e)), which involves the covalent linking of biotin to antibodies followed by the binding of biotin-labeled antibodies to an avidin layer on the substrate (Taitt et al., 2004). Antibodies can

also be linked to substrate surfaces through protein A/G/L (Figure 2.4 (d)), which specifically binds to the Fc region of IgG (Choi et al., 2008; Gao et al., 2006).

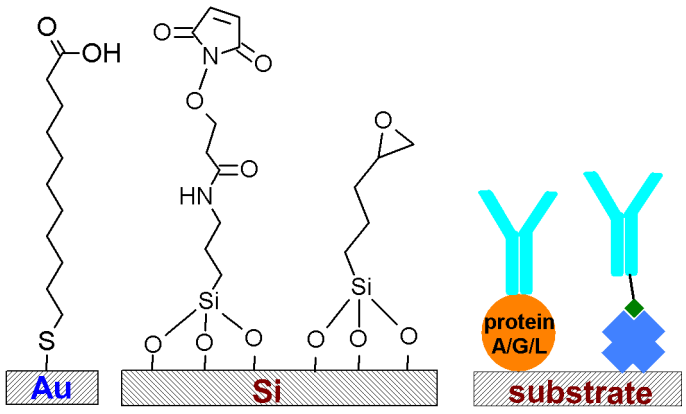


Figure 2.4. Chemistry of antibody linkage.

2.3.3.2. Surface Characterization Tools. In order to understand the composition of SAMs, sensitive surface characterization methods are required. X-ray photoelectron spectroscopy (XPS), Auger electron spectroscopy (AES), time-of-flight secondary ion mass spectrometry (ToF SIMS) and near-edge X-ray absorption fine structure spectroscopy (NEXAFS) are well known methods used for revealing the surface chemistry (Cheng, 2008, Suo, 2008 and 2009). These methods are capable of providing sub-monolayer depth resolution information about the atomic concentration of adsorbed molecules on the uppermost layers of the sample.

XPS is probably the most sensitive surface-sensitive analytical technique with a detection limit of about 0.1% of a monolayer. It is capable of identifying and quantifying all elements, except for hydrogen and helium, present in the uppermost atomic layers of a sample (Moulder et al., 1992). ToF-SIMS is another leading surface-sensitive analytical technique with trace element sensitivity, capable of sub-monolayer depth resolution (Vickerman, 2001). AES allows obtaining quantitative surface elemental distribution maps of samples at high spatial resolution of ~0.3 to 3 μm and a depth resolution anywhere from sub-nm to 10 nm (Hochella et al., 1986). With field emission primary electron sources the spatial resolution reaches low nm domains. The method is based on the analysis of energetic electrons emitted from an excited atom after a series of internal relaxation events.

NEXAFS is an element-specific and local bonding-sensitive spectroscopic analysis that reveals electronic structure and information on the orientation of adsorbed molecules (Hähner, 2006). In addition to these high sensitive characterization techniques, ellipsometry has been used for measuring the thickness of SAMs (Shäferling et al., 2003) and atomic force microscopy was used for determination of surface topography (Rozhok et al., 2005).

Principles of XPS and AFM are summarized in the following. XPS was extensively used for surface characterization in this thesis. AFM was used for 3-D imaging of surface antigens of bacteria. Detailed information about principles and applications of other methods are available in the literature (Hähner, 2006; Moulder et al., 1992; Vickerman, 2001; Hochella et al., 1986).

X-ray Photoelectron Spectroscopy (XPS)

XPS, more commonly known as electron spectroscopy for chemical analysis (ESCA), is a highly surface sensitive technique with a probing depth of ~1-10 nm (Tougaard, 2005) and ability to analyze 0.1% of o monolayer. (Chusuei and Goodman, 2003).

The principle of XPS is based on determination of the binding energy of electrons on the surface of the sample. This is accomplished by irradiating the surface with soft X-rays and analyzing emitted electrons. Usually AlKα (1486.6 eV, typically monochromatized) or MgKα (1253.6 eV) X-ray sources are used. Bombarding the surface with X-rays leads to the photoionization of atoms, molecules or ions on the specimen, which occurs as excitation and ejection of low-energy photoelectrons. Kinetic energies (E_K) of the emitted electrons are given by Formula 2.1, where hv is the energy of incoming photon (in this case 1486.6 eV and was monochromatized), E_B is the binding energy relative to the Fermi level and Φ is the work function of the spectrometer (Tougaard, 2005), which in this case was ~4 eV.

$$E_K = hv - E_B - \Phi \qquad (2.1)$$

E_K of the photoelectrons are determined by a hemispherical electron energy analyzer and Φ is a constant determined by calibration. This equation of conservation of energy allows calculating the E_B of core electrons. XPS excitation, electron energy levels, photoionization and Auger emission relaxation processes are illustrated in Figure 2.5.

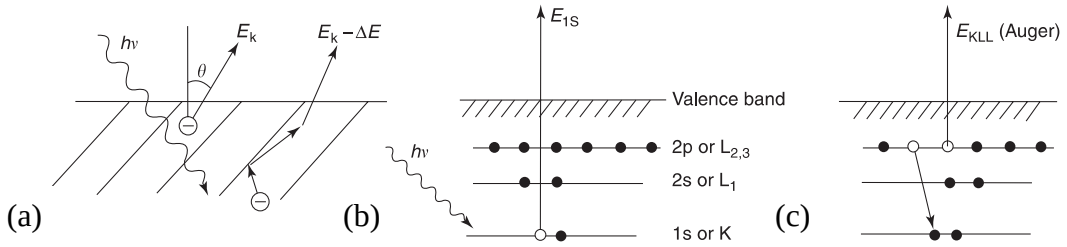


Figure 2.5. XPS excitation process and energy levels (a) Schematic representation of the XPS excitation process. (b) Schematic representation of the electron energy levels of an F atom and the photoionization of a F 1s electron (c) Auger emission relaxation process for the F 1s empty-state produced in (b) (Tougaard, 2005).

In addition to the photoelectrons emitted, Auger electrons may be emitted due to the relaxation of the energetic ions. In the Auger process, the outer shell electron falls into the inner empty core and a second electron (the same or outer shell) is emitted, carrying off the excess energy. The kinetic energy of this Auger electron is characterized by the energy of the initial ion and the doubly-charged final ion and is independent of the mode of the initial ionization process (Wagner et al., 1979).

XPS spectrum is displayed as a plot of electron binding energy versus the number of electrons in a fixed, small energy interval. Each element has a well-defined, unique core electron binding energy. Quantification of XPS data is performed according to these well-defined core electron binding energies of elements (Wagner et al., 1979). Intensity of XPS peak will be directly related to the abundance of the element on the sample surface. The peak intensity is generally reported as peak area instead of peak height. The intensity of the photoelectron transition of interest (I) is related to the atomic concentration (C) of the element within XPS and normalized by the sensitivity factor (S) of the spectrometer (Tougaard, 2005). Intensity of a single element X is calculated as Equation 2.2:

$$I_x = C_x \times S_x \qquad (2.2)$$

The relative atomic concentration of individual elements in a given sample is calculated as

$$C_x = (I_x/S_x) \Big/ \sum_n (I_n/S_n) \qquad (2.3)$$

In Figure 2.6, the X-ray photoelectron spectrum of a freshly gold-coated silicon oxide surface is demonstrated. The survey scan was performed in the range of 0 - 600 eV since all the elements of interest are expected to have a binding energy in this range. The intense peaks at binding energies of 284.6, 83.8 and 25.0 eV represent C 1s, Au 4f and O 2s, respectively.

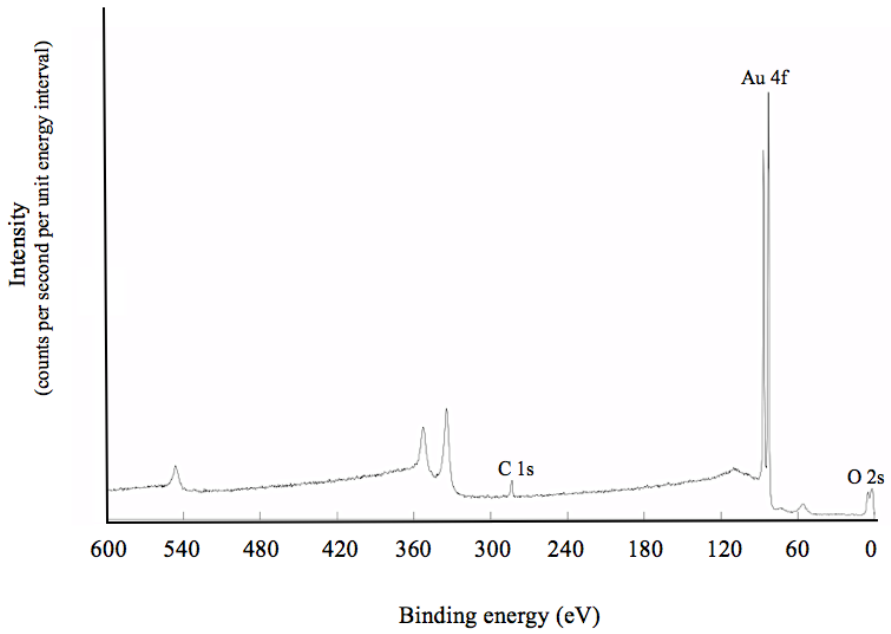


Figure 2.6. XPS spectrum of gold-coated surface.

Atomic Force Microscopy (AFM)

AFM enables topographical mapping of the sample with an extremely high-resolution. It has been commonly used in biological studies due to (i) high resolution (0.1 to 1.0 nm resolution in the x–y plane; 0.001 nm resolution (atomic resolution) in the z direction), (ii)

eligibility to use in ambient air and liquid media and (iii) simple and rapid sample preparation procedure.

AFM uses an ultra-small and sharp tip attached to a cantilever (~100 – 200 μm long and 20–60 nm radius of curvature) at the end of a cantilever to measure the forces between the tip and sample surface. As the cantilever tip scans the surface of the sample, the force between the tip and the sample varies with the difference in the surface height. The cantilever tip senses the change in force and bends. This bending of the cantilever is detected by a constant laser beam and gets reflected from the top of the cantilever towards a position-sensitive photodetector. Thus, three-dimensional image of the surface topography is recorded under a constant applied force (as low as nano Newton range), which provides a maximum resolution image without causing any damage to the sample surface.

AFM can be operated in either contact mode or tapping mode for surface analysis. In *contact mode*, the tip is in mechanical contact with the sample under a constant force in nano-Newton (nN) range. This contact may lead to the disruption and loss of integrity of soft surfaces and therefore not preferred for analysis of biological specimens. On the other hand, in *tapping mode*, the cantilever tip is stimulated to vibrations near the resonance frequency (~300kHz in air). On approach to the surface, the vibration amplitude of the cantilever will decrease. In this case, the surface is scanned at constant reduction of the oscillation amplitude and the tip is in intermitted contact with the surface during the scan. Forces applied to the sample are in pico-Newton (pN) range, which is less destructive to biological samples (Chatterjee et al., 2010).

2.3.3.3. Microarrays. Antibody-based assays focus on the detection of bacterial antigens, and it is possible to detect multiple pathogens in a single assay using microarray techniques (Cai et al. 2005; Choi et al. 2008).

During the past decades there have been great advances in micro-electromechanical systems (MEMS) and nano-electromechanical systems (NEMS) technologies. Most of the techniques, such as microcontact printing, focused ion beam (FIB) etching, micro-plotting,

e-beam lithography and dip-pen nanolithography (DPN), can be used to prepare micro- and nano- scale patterns on a flat substrate (Salaita et al. 2007).

The successful preparation of bacterial patterns relies mainly on the preparation of high-quality antibody microarrays, which can be made using two general approaches: (1) modifying the substrate surface chemically to form a chemical micropattern in which the antibody will bind only inside or outside the modified areas and (2) depositing the antibody directly onto designated locations on an activated substrate surface. Most patterning techniques, such as FIB etching, DPN, e-beam lithography and microcontact printing, fall into one of these categories. The advantage of the first approach is the convenience of preparing patterns at nanoscale resolution (except for microcontact printing, which has a practical resolution of around 1 μm (Huck 2007), which makes it possible to prepare single-cell arrays. However, it is difficult to represent multiple antibodies on the substrate using this approach since there are fairly limited chemical linkages available for antibody differentiation. The second approach (direct deposit approach) often refers to the microplotting method, which uses antibody solutions as ink for preparing antibody microspots on various substrates.

The microplotting approach has a great advantage in that there is, in principle, no upper limit on the number of antibodies that can be represented in the microarray. For both approaches, it is necessary to passivate the substrate areas to prevent the nonspecific absorption of bacterial cells. In the first approach, the passivation is done by modifying the substrates using PEGlyated tethers (Lahiri et al. 1999) before patterning the surface with antibodies. In the microplotting methods, the passivation is done by post-exposing the protein micropatterns to BSA or milk proteins before exposing them to bacterial cultures.

One problem with an antibody microarray is that generally the signal intensity of each antibody spot varies as a function of both the antibody concentration and the strain. This is actually an expected result, considering the immobilization efficiency is affected by multiple factors, including the substrate surface chemistry; the purity, clonal type and affinity of the antibody; the type and expression level of the bacterial antigen; the incubation duration; and the medium. Since microarrays employ multiple antibodies it is important to evaluate their cross-reactivities to avoid false positive results (Suo, 2012).

2.3.3.4. Biosensors. Nanotechnology has induced the collaboration of physics, chemistry, biology, engineering, and microelectronics that are capable of manipulating matter at nanoscale. This collaboration gave rise to the improvement of devices such as sensors and biosensors. Biosensor is a combination of two parts: a bioelement and a sensor element. The bioelement may be an enzyme, antibody, living cells, or tissue. The sensing element may be electric current, electric potential, and so on. The basic concept of a biosensor's operation is that a specific bioelement, recognizes a specific analyte and the sensor element transduces the change in the biomolecule into a quantitative/ semi-quantitative signal. The bioelement is very specific to the analyte to which it is sensitive. Depending on the transducing mechanism used, the biosensors can be of many types such as: resonant biosensors, optical detection biosensors, thermal-detection biosensors, ion-sensitive field-effect transistor (ISFET) biosensors, electrochemical biosensors and etc. Different combinations of bioelements and sensor-elements constitute several types of biosensors to suit a vast pool of applications (Marazuela and Moreno-Bondi, 2002; Rodriguez-Mozaz et al., 2004; González-Martínez et al., 2007).

Immunosensors are an important class of biosensors based on immunoimmobilization. The unique capacity of antibodies to bind specifically the analyte of interest is the key factor in their usefulness in immunosensor design. The higher the affinity of the antibody for the analyte, the better the sensitivity achieved; the selectivity is, on the other hand, a specific property of the antibody applied, rather than depending on the assay format (Marazuela and Moreno-Bondi, 2002). Fiber-optic and surface plasmon resonance immunosensors are among the most commonly commercialized immunosensors for detection of pathogens. In addition, single-use rapid test kits are also available for detection of antigens and antibodies in human blood and serum (SERO-Med Laborspezialitäten GmbH, 2008). Various methods for the detection of *E. coli* are compared in Table 2.4.

Attention is paid to optical immunosensors, because transducers based on optical properties are very popular in environmental immunochemistry due to the choice available and the versatility of optical transduction enhanced by fiber optic technology (González-Martínez et al., 2007). 2007 data of ISI Web of Science reports that 170 of 2500 articles on pathogen detection are about biosensor applications. Optical detection is involved in 35%

of those biosensors. They are probably the most popular bioanalysis technique, due to their selectivity and sensitivity (Lazcka et al., 2007). In any detection method, efficient capturing is always desired for bacterial detection, since it will facilitate converting captured pathogens into a detectable signal. Most importantly, higher capture efficiency will result in a higher sensitivity (lower detection limit).

As a result, over the past 30 years, numerous rapid methods have been developed for rapid detection and enumeration of microorganisms. Selection of the method depends on the range of testing, target microorganism, volume of sample and availability of trained personnel. Approaches that have been studied or are currently being studied include miniaturized biochemical tests, physicochemical methods that measure bacterial metabolites, highly specific nucleic acid-based tests, antibody-based methods, and some fully automated instrumental diagnostic systems (Swaminathan and Feng, 1994; Silley, 1994; Mandal 2011).

Table 2.4. Methods for *E. coli* detection (Lazcka et al., 2007).

Detection technique	**Sample type**	**Time of analysis**	**Detection limit (cfu/ml)**
Real-time PCR	Culture medium	5.5 hrs	5 cells
RT-PCR coupled to fluorescence	Drinking water	0.5 hrs	10^2
Fiber optic immunosensor	Culture	10 hrs	2.9×10^3
QCM Immunosensor	Culture/water	3 hrs	10^3
Impedimetric immunosensors	Culture/water	0.2 hrs	10^4 in culture 10^7 in water.

In order to construct an ideal automated system that can detect low cell numbers at shorter times, the new method should be *rapid* (so as the product processing could be in time regulated), *sensitive* (so as even trace contamination could be detected before the pathogen multiplies during the transport and storage), *specific* (so as a presence of other microbial contaminants does not interfere with the pathogen detection) (Koubova et al., 2001) and *deployable* (so as the system is miniaturized as a user friendly, hand-held device and eligible for field applications) The current strategy for developing a rapid method is to choose and combine some functions that can meet specific applications (Yang and Bashir, 2008).

3. MATERIALS AND METHODS

3.1. Sampling Points

Water samples were taken from 5 different drinking water treatment plants (DWTP) distributed in Asia and Europe continents of Istanbul as given in Table 3.1 and Figure 3.1. Triplicate water samples of reservoirs located in European and Asian sides of İstanbul were taken from water intake chambers in 5 DWTPs in July 2010, July 2011 and April 2013. These reservoirs, namely, Büyükçekmece, İkitelli and Kâğıthane DWTPs are located in Europe whereas Ömerli and Elmalı are located in Asian side. Büyükçekmece DWTP is fed by only Büyükçekmece Lake. Terkos Lake is the supply for both İkitelli and Kâğıthane DWTPs. In addition, Sazlıdere and Alibeyköy Dam Lakes supply raw water for İkitelli and Kâğıthane DWTPs, respectively. In Asian continent, Elmalı DWTP receives water from only Elmalı Dam. Ömerli DWTP has 5 sources namely Ömerli, Darlık, Emirli, İsaköy Dams and Melen River. Ömerli DWTP has three sampling points namely Ömerli 1, Ömerli 2 and Emirli. Ömerli 1, Ömerli 2 stand for sampling points at two distant locations within Ömerli Dam, expected to have different characteristics. Emirli pipeline receives water from Darlık, Melen and İsaköy reservoirs. In July 2010, Emirli received water from only Darlık and therefore, the sample was named as Darlık 2010. In July 2011 and April 2013, Emirli pipeline was supplied from Melen and İsaköy. The samples were labeled as Emirli 2011 and Emirli 2013.

Samples were taken into sterile polypropylene bottles, transferred to laboratory in cool boxes, kept at 4ºC until analysis and analyzed within one week. Samples were stored under appropriate conditions for chemical and microbiological analyses.

Table 3.1. Drinking water treatment plants, water reservoirs and locations.

Drinking Water Treatment Plant	Water Reservoirs	Continent
Büyükçekmece DWTP	Büyükçekmece	Europe
İkitelli DWTP	Terkos Sazlıdere	Europe
Kâğıthane DWTP	Terkos Alibeyköy	Europe
Ömerli DWTP	Darlık Emirli Ömerli İsaköy Melen	Asia
Elmalı DWTP	Elmalı	Asia

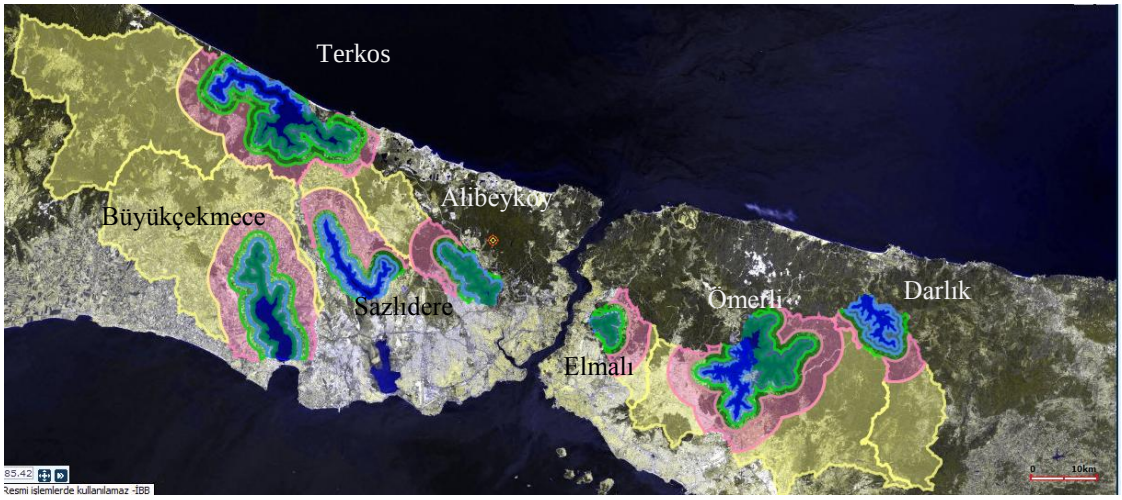


Figure 3.1. Drinking water reservoirs in İstanbul.

Figure 3.2. Drinking water treatment plants in İstanbul.

3.2. Physicochemical Analyses

pH was measured using WTW pH 320, total dissolved solids, conductivity and salinity were measured by WTW LF 320 according to manufacturer's instructions (Weilheim, Germany). Anion analyses were performed using a Dionex ICS 1500 Ion Chromatograph (Bannockburn, IL, USA). 10 ml of water samples were filtered through a 0.2 µm membrane filter into clean tubes and stored at +4°C. Heavy metal analyses were Perkin Elmer AAnalyst 300 atomic absorption spectrophotometer (AAS) and Perkin Elmer Optima 4300 DV inductively coupled plasma - optical emission spectrometer (ICP-OES), (Waltham, MA, USA). Dissolved organic carbon was measured using Total Organic Carbon Analyzer (Shimadzu TOC-VWP, Japan) Samples were prepared according to Standard Methods (Clesceri et al., 1998).

3.3. DNA-Based Analyses

3.3.1. Genomic DNA Extraction

Genomic DNA (gDNA) was extracted from filters using Meta-G-Nome DNA Isolation Kit (Epicentre Biotechnologies, Madison, WI, USA) following the manufacturer's instructions. The procedure of DNA extraction is summarized as follows: Triplicate water samples of 100 ml were filtered through 0.2 µm pore size, sterile polycarbonate filters (Merck Millipore GTTP04700) to trap the cells. Filter was transferred into a sterile falcon tube and the suspended cells were washed off from the filter with 1 ml filter wash buffer containing 0.2% Tween20 mix by vortexing. The cells were decanted by centrifugation, resuspended in a mixture of Tris-EDTA (TE) buffer, lysozyme and RNaseA and incubated ate 37ºC for 30 minutes. This step was followed by Meta-Lysis Solution (2x) and Proteinase K treatment at 65ºC for 15 minutes. The mixture was cooled to room temperature and placed on ice for 3-5 minutes. MPC Protein Precipitation Reagent was added to the tube and vortexed for 10 seconds. The debris was pelleted by centrifugation for 10 minutes at 14000 g at 4ºC. The supernatant was transferred into a clean tube, treated with isopropanol and centrifuged for 10 minutes at 14000 g at 4ºC. Isopropanol was removed and the remaining pellet was treated with 70% absolute ethanol, precipitated by

centrifugation for 10 minutes at 14000 g at 4ºC. Finally, the pellet was air dried and resuspended in 50 µl TE buffer.

Prior to DNA extraction from all samples, cellulose acetate and polycarbonate filters were tested to determine genomic DNA extraction efficiency. Same amount of water was filtered from cellulose acetate and polycarbonate filters and DNA was extracted according to manufacturer's instructions for Meta-G-Nome DNA Isolation Kit. It was observed that cellulose acetate filter was not able to trap enough DNA compared to polycarbonate filters.

3.3.2. Polymerase Chain Reaction (PCR) Amplification

16S ribosomal DNA (rDNA) was amplified with primers specific for bacteria. Bac8f-Bac1541r, Bac341f_GC-Bac534r primer pairs were used for amplifying ~1500 bp and 200 bp regions of bacterial 16S rDNA. 1500 bp PCR products were used for nested PCR with Bac341f_GC-Bac534r primer pairs. 200 bp products were used for DGGE analysis for determination of bacterial biodiversity.

Primer pairs used for PCR amplification of water sample DNAs are given in Table 3.2. Amplification was done in a 50 µl reaction volume containing 200 ng of DNA, 10 pmol of each primer, 10 mM of each deoxynucleoside triphosphate (dNTP), 1.5 mM $MgCl_2$, 5 µl of 10×Taq buffer and 4 U of Taq DNA polymerase (Fermentas, Latvia). PCR amplification was performed in a Techne TC-512 thermal cycler (Barloworld Scientific Ltd., U.K.) with an initial denaturation at 94°C for 5 min followed by 35 cycles of denaturation at 94°C for 30 sec, annealing for 20 sec, extension at 72°C for 30 sec and a final extension at 72°C for 5 min. PCR products were visualized on 1% (w/v) agarose gel pre-stained with ethidium bromide in 1x Tris-Acetate- EDTA (TAE) buffer (40 mM Tris, 20 mM Acetic Acid, 1 mM EDTA; pH 8) (Thermo-Scientific Ltd., U.K.) and the images were recorded using a Chemi-Smart 3000 gel documentation system (Vilber Lourmat, France).

Table 3.2. PCR Primers used for bacterial DNA amplification in water samples.

Primer	Experimental stage	Annealing temperature (°C)	Position[a]	Reference
Bact8f	First round of nested PCR	55	8-27	Lane, 1991
Bact1541r			1541-1522	
Bact341f_GC[b]	DGGE		341-357	Muyzer et al., 1993
Bact 534r			534-518	
Bact342f	Sequencing		342-361	Edwards et al., 1988

[a]*Escherichia coli* numbering.
[b]5′-GC clamp on Bact341f
(GCCCGCCGCGCGCGGCGGGCGGGGCGGGGGCACGGGGGGACGGGG).

3.3.3. Denaturing Gradient Gel Electrophoresis (DGGE)

Bacterial community fingerprint of water samples was revealed by DGGE analysis of 200 bp PCR products amplified using bacteria-specific primers as described by Muyzer et al. (1999). Electrophoresis was performed using the phorU (Ingeny, Leiden, NL) system at 100 V constant current at 60°C, for 17 h. PCR products (10 µl) mixed with equal volumes of loading buffer (0.25% bromophenol blue, 0.25% xylene cyanol FF, 15% Ficoll in water) were run on a 10% polyacrylamide gel (acrylamide : N,N'-methylenebisacrylamide ratio 37.5:1) in 1xTAE buffer (40 mM Tris, 20 mM acetic acid, 1 mM EDTA; pH 8) over a chemical denaturing gradient of urea and formamide equivalent to 40–70% denaturant. 10% (w/v) acrylamide:bisacrylamide 40% denaturant solution was prepared by mixing 25 mL of 40% acrylamide:bisacrylamide with 2 ml 50x TAE (2 M Tris, 1 M Acetic acid and 50 mM EDTA), 16 ml formamide and 16.8 g urea. 70% of denaturant concentration was reached by adding 28 ml formamide and 29.4 g urea to 33.3 ml of 30% acrylamide: bisacrylamide and 2 ml 50x TAE (2 M Tris, 50 mM EDTA, and 1 M Acetic acid). 100% denaturant solution is defined as 40% (v/v) formamide and 7 M urea. Both solutions were added distilled water up to final volume of 100 ml. A marker consisting of mixed bacterial 16S rDNA was used for normalization. Gels were stained with SybrGold (1:10000 diluted; Molecular Probes Inc., UK) and gel images were recorded using an Infinity 1500/3M gel documentation system (Vilber Lourmat, France).

3.3.4. Statistical Analysis and Diversity Indices

DGGE gel images were converted, normalized and analyzed by using the Bionumerics 6.1 software (Applied Maths, Kortrijk, Belgium). Presence-absence data for DGGE bands were obtained according to relative densities. Band position tolerance of 0.7% was applied. This was the minimum tolerance at which all marker lanes clustered at 100%. Cluster analysis was conducted using band-independent, whole-densitometric-curve-based Pearson product-moment correlation coefficients I and UPGMA clustering. Minitab 14 was used for principal component and correlation analysis.

Shannon-Weaver diversity indices were calculated according to the formula $H = -\sum_{i=1}^{s} p_i \log p_i$ where s indicates total number of operational taxonomic units (OTUs), n_i is the number of individuals represented by relative densities of the DGGE band of the i^{th} I, N is the total number of individuals and $p_i=n_i/N$ (Pielou, 1966; Shannon and Weaver 1949). The I richness was calculated using Margalef's Species Richness (SR) index (Margalef, 1958) using the formula $SR = (S-1)/\ln N$ where S is the number of taxa and N is the number of individuals. Species evenness index was calculated as $J' = H'/\log S$ (Pielou, 1966), where H' is Shannon-Weaver index and S is the number of OTUs.

3.3.5. Sequencing and Bioinformatics

Selected bands from the DGGE gels were excised, eluted from the gel fragments in 50 µl of 1 x TAE at -20°C overnight and re-amplified by sequence PCR using excised DGGE bands as template. Reaction was held by mixing 4.7 µl molecular grade water, 1 µl template, 0.3 µl primer, 2 µl Big Dye Reaction mix and 2 µl Big Dye Reaction buffer. PCR program was as follows: initial denaturation at 95°C for 5 minutes, 30 cycles of denaturation at 95°C for 30 seconds, annealing at 55°C for 30 seconds and elongation at 60°C for 4 minutes. PCR products to be sequenced were purified by ethanol precipitation. Briefly, PCR products were mixed with 2 µl 3 M Sodium Acetate (NaOAc) and 50 µl of 95% ice-cold ethanol. The mixture was incubated for 30 min on ice and centrifuged for 30 min at 14000 rpm. Supernatant was discarded and pellet was resuspended in 250 µl 70% ice-cold ethanol and centrifuged for 30 min at 14000 rpm. Supernatant discarded and pellet

was dried at 95°C for 5 minutes. Pellet was resuspended in 20 µl formamide and denatured at 95°C for 3 minutes. Then the mixture were sequenced using the ABI prism Big Dye Terminator Cycle Sequencing Ready Reaction Kit on an ABI Prism 377 DNA sequencer (Applied Biosystems, USA) using primer Bact342f generating 190 bp of bacterial sequence data. Partial 16S rRNA gene sequences were analyzed and manually edited in Chromas software package version 1.45 (http://www.technelysium.com/au/ chromas.html). Homology searches of the EMBL Nucleotide Sequence Database for the 16S rRNA gene sequences were performed with FASTA provided by the European Bioinformatics Institute (http://www.ebi.ac.uk/Tools/sss/fasta/nucleotide.html) to identify putative close phylogenetic relatives.

3.3.6. Quantitative Real-Time PCR (qPCR)

Specific pathogenic genes were quantified using the primer pairs given in Table 3.3. Light Cycler 480, LightCycler® FastStart DNA Master SYBR Green I (Roche Diagnostics GmbH, Mannheim, Germany) and SsoFast™ EvaGreen® Supermix (Hercules, CA; USA) were used for amplification. Reaction mix contained in a 20 µl reaction volume containing 25 ng of DNA, 0.5 µM of each primer and 14 µl of SsoFast™ EvaGreen® Supermix. qPCR amplification. 10^5 to 10^9 copies of the standard sequences were used to obtain the calibration curves. The following programme was applied for qPCR amplification: 95°C, 10 min; 45 cycles of 10 s at 95°C, 5-10 s at primer-dependent annealing temperature, 15 s at 72°C. A melt curve analysis was performed from 55°C to 95°C to determine if only one amplified product was generated during qPCR. The efficiencies were between 1.8 and 2.0, and the correlation factors (r^2) were not lower than 0.97 in all reactions.

Table 3.3. qPCR primers used for pathogen quantification.

Species	Target gene	Primer pair	Annealing temperature (°C)	Reference
ETEC	LT	LT-F LT-R	60	Vidal et al., 2005
Legionella pneumophila	*mip*	mip-F mip-R	57	Wellinghausen et al., 2001
Salmonella	*hilA*	hilA-F hilA-R	66	Marathe et al., 2012
EHEC	*stx2*	stx2-F stx2-R	60	Sharma et al., 1999

3.4. Immunoimmobilization Experiments

3.4.1. Specifications of *E. coli* Strains

Wild-type strains of *E. coli* having different target antigens and specification were used in this study. The strains obtained from Dr. D. Francis at South Dakota State University (SDSU) and the *E. coli* Reference Center at Pennsylvania State University (PSU). *E. coli* O157:H7 was obtained from Dr. T. Khan and Dr. B. Klayman at the Center for Biofilm Engineering, Montana State University (MSU). The antigen type and properties of these strains are given in Table 3.4. Presence of heat labile enterotoxin (LT), heat stable enterotoxin-a (Sta), and enterotoxin-b (STb) are indicated for *E. coli*.

Table 3.4. Properties of *E. coli* strains and target antigens.

Strain name	Target antigens	Properties	Source	Reference
1194	Fimbriae: F4 (987P)	Wild-type, O141:987P, Sta^+, STb^+	SDSU	Koh et al., 2008
1836-2	Fimbriae: F4 (K88ab)	Wild-type $K88ac^+$; LT^-, ST^-, $astA^+$	SDSU	Koh et al., 2008
263	Fimbriae: F4 (K88ab)	Wild-type O8:K87:K88ab, LT^+, STb^+	SDSU	Koh et al., 2008
3030-2	Fimbriae: F4 (K88ac)	Wild-type O157:K87:K88ac, LT^+, STb^+	SDSU	Koh et al., 2008
2.0961	Fimbriae: F7 (F41)	Wild-type O101:$F41^+$	PSU	-
3.1012	Fimbriae: F6 (987P)	Wild-type $987P^+$	PSU	-
9.1360	Fimbriae: F5 (K99)	Wild-type O64:$K99^+$	PSU	-
B41	Fimbriae: F4; F7 (K99; F41)	Wild-type O101:K99/F41, $StaP^+$	MSU	Ascon et al., 1998
H10407	Fimbriae: CFA/I LPS: LPS Flagella: Flagellin	Wild-type O78:H11:CFA/I, LT^+, ST^+	MSU	Byrd et al., 2003
H681-K99	Fimbriae: F5 (K99)	Parent strain *X*6212 asd^-, plasmid pMAK99-asd^+ expressing K99	MSU	Ascon et al., 2005
O157:H7	LPS: LPS- Flagella: Flagellin	Wild-type O157:H7; LT^-, ST^-	MSU	Bansal et al, 2007

All bacterial strains were inoculated from bacteria stocks frozen at -80°C and grown on Lysogeny broth (LB) plate overnight at 37°C. For *E. coli* O157:H7, trypticase soy agar (TSA) and broth (TSB) with kanamycin was used as growth medium. One colony was scraped from bacteria plate and grown in 3 ml LB or TSB for 3 hrs at 37°C at 150 rpm in an orbital shaker incubator. Viable cell number was determined by plating 10-fold serial dilutions. The dilutions were prepared in LB or TSB. 100 μl of each dilution was plated and incubated overnight in 37ºC. Average viable cell number was calculated according to

the dilutions showing 30 colonies per plate and reported as colony forming unit per ml (cfu/ml).

3.4.2. Antibody-Antigen Pairs for *E. coli*

The commercially available antibodies were purchased. For CFA/I antigen with no commercially available antibody, CFA/I antigens were purified from pure cultures of ETEC H10407 and polyclonal antibodies were prepared by immunizing rabbit. Table 3.5 summarizes the antigen-antibody pairs targeting fimbriae, lipopolysaccharides (LPS) and flagella for immunoimmobilization of *E. coli*. Different from the previous experiments, antibody specific to LPS O157 was used for EHEC O157:H7 strain.

Table 3.5. Antibody-antigen pairs tested for the immobilization of *E. coli.*

Antigen		**Antibody**				**Bacterial strain**
		Host	Type	Source	Cat. Number	
Fimbriae (Pili)	CFA/I	Rabbit	pAb*	MSU	N/A	ETEC** H10407
	K99	Mouse	mAb*	Santa Cruz	sc-66038	*E. coli* B41; *E. coli* H681; *E. coli* 9.1360
	K88ab	Sheep	pAb	Novus	NB100-62532	*E. coli* 263; *E. coli* 1836-2
	K88ac	Sheep	pAb	Novus	NB100-62533	*E. coli* 3030-2
	F41	Sheep	pAb	Abcam Inc.	ab35291	*E. coli* B41; *E. coli* 2.0961
	987P	Sheep	pAb	Novus	NB100-62530	*E. coli* 1194; *E. coli* 3.1012
LPS	LPS (Lipid A)	Goat	pAb	Biodesign	B656926	ETEC H10407, EHEC** O157:H7
	LPS O157	Mouse	pAb	Santa Cruz	sc-710004	EHEC O157:H7
Flagella	Flagellin	Mouse	mAb	Inotek	N/A	ETEC H10407, EHEC O157:H7

*pAb: Polyclonal antibody, mAb: monoclonal antibody;

** ETEC: Enterotoxigenic *E. coli*, EHEC: Enterohemorrhagic *E. coli*

3.4.3. Substrate Modification

3.4.3.1. Surface Activation. Gold substrates were modified via thiol chemistry using 11-mercaptoundecanoic acid **(**MUA), and activated with 1-Ethyl-3-[3-dimethyl aminopropyl] carbodiimide hydrochloride (EDC), a zero-length crosslinker, and *N*-Hydroxysuccinimide (NHS) prior to antibody modification. The thiol chemistry is illustrated in Figure 3.3.

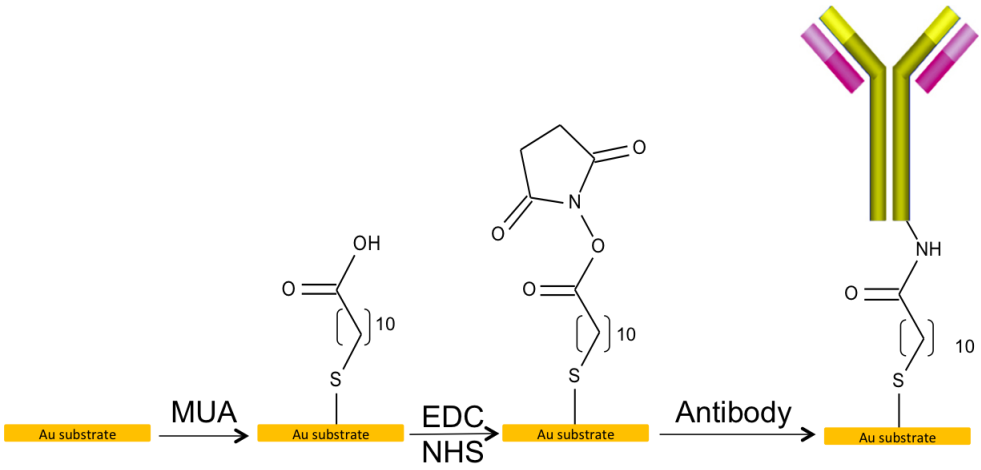


Figure 3.3. Thiol chemistry.

Surface activation steps are as follows: 100 mm diameter silicon wafers (Thickness: 500-550 μm; Resistivity: 0.002-0.005 ohm-cm; Type, Dopant: p, Boron; Orientation: 100; Back surface: Alkaline etched. Silicon Inc, Boise, ID, USA) were diced as 1 x 1 cm chips. The chips were precleaned in distilled water, ethanol and chloroform by ultrasonication. After precleaning, the chips were first coated with a thin film of chromium (~4-5 nm) followed by a thin film of gold (~35-40 nm) using Emitech K575X Sputter Coater system (Quorum Technologies, UK). Freshly prepared gold chips were soaked into 4 mM 11-mercaptoundecanoic acid (MUA) prepared in ethanol for thiol linkage and incubated overnight. The surface was activated with 100 mM 1-Ethyl-3-[3-dimethyl aminopropyl] carbodiimide hydrochloride (EDC), a zero-length crosslinker, and 40 mM *N*-Hydroxysuccinimide (NHS) in 2-(*N*-morpholino) ethanesulfonic acid (MES) buffer (pH 6.5) for 1h. Chips were stored at room temperature during incubation steps.

3.4.3.2. Antibody Linking. 4 drops of 1 μl antibody with a concentration of 10-20 μg/ml were deposited on activated gold-coated substrates. Substrates were incubated with antibody solution for 45 min at room temperature. Excess antibody was rinsed off using 0.01 M phosphate buffered salt solution (PBS, pH 7). 1 mg/ml bovine serum albumin (BSA) was used as a blocking agent after antibody modification. Excess BSA was rinsed off using PBS buffer.

3.4.3.3. Immobilization of Bacteria. Substrates with antibody were incubated with bacteria 10x diluted (corresponding to a concentration of ~$4x10^7$ cfu/mL) in PBS (pH 7) for 45 min at room temperature. Loosely attached cells were gently rinsed with PBS.

3.4.4. Antibody Titration Experiments

Antibody titration experiments were performed in order to determine the minimum concentration of antibody required for effective immunoimmobilization. The experiment was conducted using anti-LPS O157 antibody. In the last step of surface activation, gold-coated chips were decorated with different antibody concentrations in the range of 0.5 to 20 μg/ml and incubated with ~$4x10^7$ cfu/ml of *E. coli* O157:H7. The substrate surface was scanned using XPS after every step of surface modification.

3.4.5. Limit of Detection

Dilution experiments were conducted for determination of minimum detection limit of immobilization method. Pure cultures of bacteria were serially diluted in PBS in the range of (10^1) to (10^7) times. Identical antibody-modified gold-coated chips were prepared and each of them was incubated in a different dilution of bacterial culture.

3.4.6. Antibody Microarray

3.4.6.1. Cross-Reactivity Experiments. Cross-reactivity experiments were conducted in order to determine the affinity of *E. coli* cells and non-target antibodies. This is the basic experiment prior to designing the antibody microarray. Pure cultures of target *E. coli* strains were incubated on gold chips modified with relevant and irrelevant antibodies. The

chips were incubated in pure cultures of bacteria 10x diluted in PBS for 45 min at room temperature. Loosely attached cells were gently rinsed with PBS.

3.4.6.2. Microarray Preparation. Non-cross-reactive antigen antibody pairs were selected according to the cross-reactivity experiment results. Antibody microarray was prepared using a microplotter (BioRad VersArray Chipwriter Compact System). Antibodies were automatically plotted onto substrates as a matrix of 9 x 9, each row plotted with a different antibody, having an average spot size of ~60 μm. Relative humidity inside the microplotter's chamber was maintained at 90-92% during plotting and increased to 98% during incubation so that the antibodies would not dry on the surface until incubation with bacteria. Antibody-modified surface was blocked with 1 mg/ml of BSA.

3.4.6.3. Sorting Experiments. Sorting experiments involve the incubation of microarray with a mixed culture of bacteria. Fluorescent characteristics were used for differentiation of attached cells on the microarray surface. Two wild-type *E. coli* strains (H10407 and 3030-2) were genetically modified to express red and green fluorescent proteins by transforming pDsRed-Express plasmid (Clontech, Mountain View, CA) to ETEC H10407 and pQGgfp plasmid (laboratory constructed) to wild-type *E. coli* 3030-2. In addition, plain EHEC O157:H7 and *E. coli* 1836-2 cultures were stained with green-fluorescent SYTO® 9 dye (Invitrogen, Eugene, OR, USA) and blue-fluorescent 4′,6-diamidino-2-phenylindole, dihydrochloride (DAPI) (Invitrogen, Eugene, OR, USA) respectively, prior to incubation with antibody-modified chips.

3.4.7. Optical Microscopy

Immunoimmobilized cells on gold-coated chips were visualized *in situ* by optical microscopy. Optical microscopy is capable of examining samples in ambient conditions or water environments at a resolution of ~ 1 μm. Depending on the characteristics of the specimen, optical microscope can be operated at bright field reflection, bright field transmission, dark field, phase contrast, Nomarsky differential interference contrast (DIC) and fluorescence modes.

In this study, Olympus BX61 microscope, equipped with a motorized z-stage, a DP71 charge-coupled device (CCD) camera, was used for optical imaging of immunoimmobilized bacterial cells in bright field and fluorescence modes. Bright field images were taken at reflection mode since the specimens were non-transparent. The samples were imaged using 10x and 60x water immersion objective lenses in PBS buffer. Three filter sets, fluorescein isothiocyanate (FITC; excitation maximum 495 nm, emission maximum 521 nm), tetramethyl rhodamine isocyanate (TRITC; excitation maximum 540 nm, emission maximum 604 nm) and 4′,6-diamidino-2-phenylindole, dihydrochloride (DAPI; excitation maximum 358 nm, emission maximum 461 nm) were used for imaging the green, red and blue stains of the fluorescent stains, respectively.

3.4.8. Atomic Force Microscopy (AFM)

Bacterial surface antigens such as fimbria and flagella were revealed by a Veeco, Multimode V atomic force microscope in tapping mode in air (CA, USA) with a J-type scanner and using NSC36 AFM probes from MikroMasch (SC, USA). 100 µl of fresh bacterial culture were immobilized on freshly cleaved mica disks for 1 h at room temperature, rinsed with PBS buffer and water and dried gently with nitrogen. The images were recorded and analyzed with NanoScope software (CA, USA).

3.4.9. X-ray Photoelectron Spectroscopy (XPS)

In this study, XPS analysis was conducted to reveal the atomic characterization of the gold substrates after each step of chemical modification and finally confirm the deposition of antibody molecules on the surface by monitoring N1s content on the surface. N1s is directly related to the presence of NH_2 groups deposited on the surface. Physical Electronics PHI 5600ci system equipped with monochromatized Al Kα X-rays (1486.6 eV) was used for this purpose. Data acquisition and data analysis were performed with RBD AugerScan3 software (RDB Instruments, OR, USA).

3.4.10. Field Sample Applications: BiyoTrap® and Immunoimmobilization

In this study, field samples were taken from Bozeman Creek and Alibeyköy Dam and applications were conducted using two types of samples: (i) raw sample, as taken from contaminated water source and (ii) 100x pre-concentrated. Samples were immobilized onto gold-coated chips modified with anti-CFA/I and anti-LPS O157 antibodies targeting fimbriae and LPS of *E. coli*. Pre-concentration of samples was provided by BiyoTrap®.

The sample can be driven through the BiyoTrap column by either peristaltic pump or syringe pump. Syringe pump set up is depicted in Figure 3.4. Both set ups were used in this study. 500 ml water sample was passed through the BiyoTrap column with a pumping speed of 50 ml/min. After trapping cells, the glass wool was transferred into a sterile 15 ml tube aseptically and re-wetted with 0.5 ml of BiyoTrap effluent and mixed manually. By the shear force applied during mixing, bacteria captured on BiyoTrap wool were detached from fibers. The suspension of fibers and water sample were used for analysis.

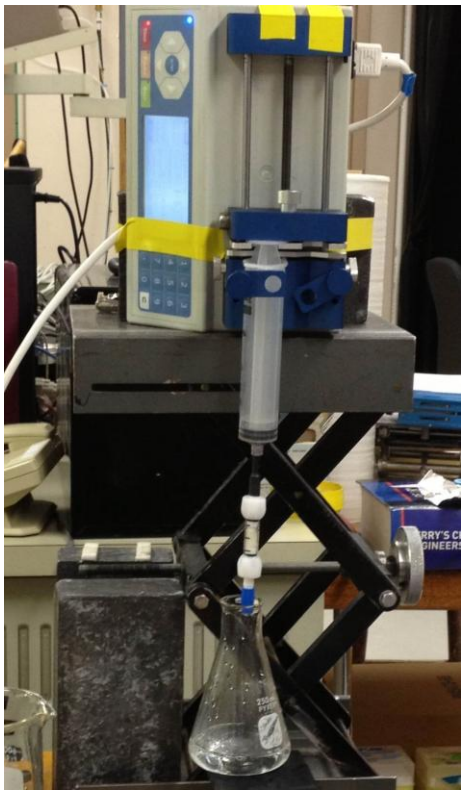


Figure 3.4. BiyoTrap® set-up with syringe pump (ICAL, MSU).

Cell viability on BiyoTrap fibers was confirmed by bioluminescence test. BacTiter-Glo™ Microbial Cell Viability Assay kit (Promega Corporation, Madison, WI, USA was used for measuring ATP bioluminescence enzymatically. The principle of the method is summarized in Figure 3.5. Luciferin is Mono-oxygenized by catalysis of luciferase in the presence of Mg^{2+}, ATP and molecular oxygen. A photon of light is released as luciferin is converted to oxyluciferin. The intensity of this light is measured in a plate reader.

The kit has two components: BacTiter-Glo™ Buffer and lyophilized substrate. First of all, the buffer and the substrate were thawed and equilibrated to room temperature. The substrate was dissolved in appropriate volume of the buffer. This mixture is forms the BacTiter-Glo™ reagent. Equal volumes of the sample and the reagent (100 µl each) were mixed in an opaque-walled 96 well plate and incubated for 5 minutes at room temperature. The luminescence was recorded using Synergy H1 Hybrid Multi-Mode Microplate Reader (BioTek Winooski, VT, USA).

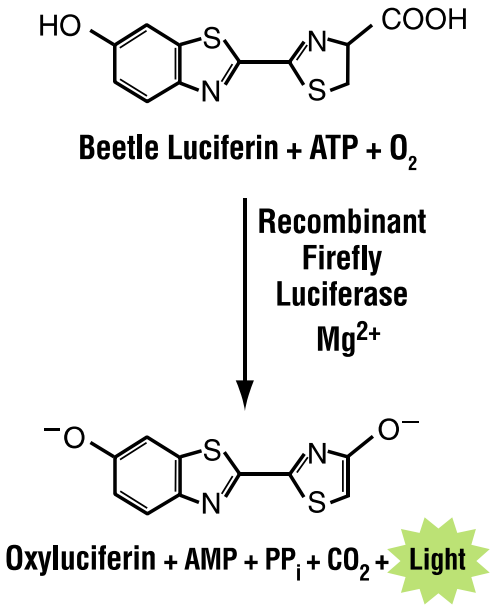


Figure 3.5. The luciferase reaction.

4. RESULTS AND DISCUSSIONS

This study covers physicochemical analysis, DNA-based bacterial characterization, diversity analysis, and quantification of selected pathogens in drinking water reservoirs in İstanbul. In addition, an effective immunoimmobilization method was used for selective detection of *E. coli* in water samples.

In Section 4.1, physicochemical characterization of water samples taken from 5 DWTPs in İstanbul was outlined. pH, conductivity, TDS, salinity, DOC, heavy metals, anions and cations were monitored. Water quality parameters were generally within the desired limits of regulations with some exceptions in heavy metal and anion analyses.

Section 4.2 covers information about DNA-based characterization of the bacterial community via DGGE and sequencing, and quantitative information about selected pathogens using qPCR in water reservoirs. Principal component and correlation of the diversity and chemical characteristics of water samples were reported.

In Section 4.3, detailed information about improvement of the surface chemistry for efficient immunoimmobilization of bacteria highlighted as: (i) revealing 3-D morphology and surface antigens i.e., flagella and fimbriae, of bacteria using AFM. (ii) determination of efficiently binding antigen-antibody pairs. (ii) optimization of surface chemistry (iii) antibody titration experiments for determination of the minimum antibody requirement (iv) dilution experiments for determination of detection limit (v) cross-reactivity experiments, and construction of antibody microarray for sorting different *E. coli* strains. (vi) XPS characterization of elemental composition on gold-coated surfaces and (vii) field applications of immunoimmobilization method combined with BiyoTrap® technology.

4.1. Physicochemical Analyses

Water samples taken from Terkos, Sazlıdere, Büyükçekmece, Alibeyköy, Darlık, Elmalı and Ömerli Reservoirs in July 2010, July 2011 and April 2013 were monitored for physicochemical parameters such as pH, conductivity, total dissolved solids (TDS), salinity, anions, cations, heavy metals and dissolved organic carbon (DOC). Results of analysis were compared to Turkish Regulation on Quality of Surface Waters used as Drinking Water Supply (TR QSWDS). In this regulation, water quality is classified in three levels (A1-3) according to the requirements of treatment. A1 level quality waters require simple physical treatment and disinfection. A2 level quality water requires physical and chemical treatment and disinfection. A3 level quality waters require physical, chemical and advanced treatment and disinfection in order to reach drinking water quality. In application, drinking water treatment plants in Istanbul apply physical and chemical treatment and disinfection before the distribution of water. This means that water samples need to meet A2 level quality parameters.

4.1.1. pH, Conductivity, TDS and Salinity Measurements

pH of water samples were measured in the range of 6.2-7.8 which can be accepted as neutral. Water samples had a conductivity of 0.4 to 0.8 mS/cm. pH and conductivity values were within the desired limits of TR QSWDS from A1 to A3 level quality. TDS values were measured in the range of 200-450 ppm. Salinity is an important parameter which indicates the interaction of reservoir with sea water. It was possible to observe salinity in this case since Büyükçekmece Lake, which used to be a part of Marmara Sea, Elmalı Dam and Terkos Lake are very close to Marmara Sea and Black Sea. Salinity values of water samples were measured as zero which shows that there is no or trace mixing with sea water.

Table 4.1. Chemical characterization of water samples taken in 2010, 2011 and 2013.

Sampling sites	Alibeyköy	Büyükçekmece	Darlık	Elmalı	Emirli	Ömerli 1	Ömerli 2	Sazlıdere	Terkos-İkitelli	Terkos-Kâğıthane
	Concentration range (ppm)									
Heavy metals										
Cr	<0.005-0.016	<0.005-0.011	0.01	<0.005-0.005	<0.005-0.005	<0.005-0.020	<0.005-0.005	0-0.047	0-0.004	0.001-0.004
Mn	<0.005-0.062	<0.005-0.008	<0.05	0-0.062	0.010-0.023	0-0.014	0.012	0-0.041	0.008-0.013	0.008-0.023
Fe	<0.005-0.025	<0.005-0.060	0.058	<0.005-0.051	0.051-0.226	0.014-0.100	0.010-0.021	0.017-0.038	0.016-0.055	0.016-0.036
Ni	<0.004-0.006	<0.004-0.279	0.223	0.009-0.054	<0.004-0.002	<0.004-0.247	<0.004-0.001	<0.004-0.002	0.001-0.002	<0.004
Cu	0.002-0.019	0.004-0.025	0.007	<0.004-0.004	0.001	<0.004-0.005	<0.004-0.002	<0.004-0.003	<0.004-0.007	<0.004-0.005
Zn	0.006-0.047	<0.004-0.012	0.005	0.002-0.037	0.011-0.014	0.011-0.071	0.010-0.023	0.002-0.010	0.014-0.029	0.007-0.008
Al	<0.004-0.054	0.014-0.028	n.m.	<0.004-0.032	<0.004-0.198	<0.004-0.020	<0.004-0.077	0.019-0.038	<0.004-0.014	<0.004-0.038
Cd	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	0-0.001	<0.001	<0.001
Pb	<0.001-0.003	<0.001	<0.001	<0.001-0.003	<0.001-0.002	<0.001-0.001	<0.001-0.003	<0.001-0.003	<0.001-0.001	0.003-0.004
Co	<0.002-0.006	<0.002	0.010	<0.002-0.009	<0.002-0.002	<0.002-0.002	<0.002-0.002	<0.002-0.002	<0.002-0.002	<0.002-0.002
Cations										
Na^{+}	8.65-46.80	7.00-40.60	67.150	19.20-30.90	12.60-13.11	7.75-18.25	11.75-14.15	8.90-20.50	11.78-18.75	11.87-19.60
K^{+}	0.86-4.41	0.87-3.93	0.960	1.90-3.81	1.18-36.34	1.73-4.44	1.82-2.93	1.01-3.80	1.54-2.18	2.68-40.74
Mg^{2+}	2.77-16.62	6.97-12.44	7.330	3.92-12.33	1.11-5.25	4.10-4.35	4.08-4.35	4.01-5.16	3.14-3.78	3.17-3.73
Ca^{2+}	26.93-89.46	41.03-82.31	93.700	42.89-58.56	53.00-91.36	20.03-41.77	30.57-42.67	40.70-83.46	48.68-49.94	48.20-48.94
Anions										
F^{-}	0.03-0.16	0.14-0.25	0.194	0.07-0.20	0.09-0.12	0.06-0.11	0.04-0.11	0.05-0.19	0.04-0.11	0.05-0.10
Cl^{-}	10.38-86.95	33.54-48.49	30.907	35.21-67.84	9.21-22.52	16.54-54.03	17.24-20.59	7.72-24.76	21.56-23.56	21.44-22.65
NO_2^{-}	<0.005	<0.005 – 0.31	<0.005	<0.005-0.52	<0.005	<0.005-0.15	<0.005	<0.005-0.35	<0.005	<0.005
Br^{-}	<0.012-0.5	<0.012	<0.012	<0.012	<0.012	<0.012-0.30	<0.012	<0.012-0.33	<0.012	<0.012
NO_3^{-}	<0.023-5.32	<0.023-6.27	<0.023	<0.023-5.83	1.66-2.48	<0.023-3.97	1.59-3.69	<0.023-4.51	0.70-0.92	0.66-1.12
SO_4^{2-}	8.17-131.95	49.55-63.54	40.598	39.19-135	12.89-28.55	16.67-103.67	17.74-19.18	11.26-33.58	13.73-16.48	13.78-16.25
PO_4^{3-}	<0.011	<0.011	0.623	<0.011-0.96	<0.011	<0.011	<0.011	<0.011	<0.011	<0.011
Dissolved Organic Carbon										
DOC	4.24	4.70	-	5.54	2.48	3.34	3.45	4.34	5.22	5.38

4.1.2. Anion and Cation Analysis

Anion, cation, heavy metal and dissolved organic carbon measurements were given in Table 4.1. All anion concentrations were within the A2 level quality of water except for PO_4^{3-}. Cl^- and SO_4^{2-} were measured as the most abundant anions in sampled reservoirs. In the literature, Cl^- and SO_4^{2-} concentrations for rivers and reservoirs were in the range of 0.60-70.00 and 1.20-160.00 mg/l, respectively (Pehlivan and Yılmaz, 2005; Li and Zhang 2008, 2010). The measurements in this study were in accordance with literature values. High range of sulfate might be an indicator of sulfate reduction mechanism. NO_2^- was measured in Büyükçekmece, Elmalı, Ömerli and Sazlıdere in the range of 0.07-0.52 mg/l. NO_3^- levels were between 0 to 6.27 mg/l. NO_2^- and NO_3^- measurements were in good agreement with literature (Chueng et al., 2003). PO_4^{3-} was measured in Darlık and Elmalı and the levels of PO_4^{3-}were close to and higher than the limit of 0.7 mg/l desired for A2 level water quality. F^- levels were in the range of 0.03-0.25 mg/l. Br^- was measured in Alibeyköy, Ömerli-1 and Sazlıdere in the range of 0 - 0.5 mg/l.

Ca^{2+} and Na^+ were measured as the most abundant cations followed by Mg^{2+}. These cations were not listed in TR QSWDS. In previous studies (Pehlivan and Yılmaz, 2005; Li and Zhang 2008, 2010), Na^+, K^+, Ca^{2+} and Mg^{2+} concentrations were measured in the ranges of 0.3-28 mg/l. 0.2-7 mg/l. 13-84 mg/l and 1.9-26 mg/l respectively. Most of the measurements in this study are parallel to those found in literature except for Na^+ and Ca^{2+}. Concentrations up to 67.15 mg/l Na^+ and 93.70 mg/l Ca^{2+} were measured. Pehlivan and Yılmaz (2005) published that richness of Na^+ and Cl^- in Elmalı reservoir was due to interaction with volcanic rocks.

4.1.3. Heavy Metal Analysis

Heavy metal analyses showed that Cr, Mn, Fe, Ni, Cu, Zn, Al, Cd, Pb and Co were available in water samples. Most of the measurements were in agreement with TR QSWDS limits. However, there were peak measurements of Ni, Co and Cd above the regulatory limitations. Ni was measured 0.279, 0.223 and 0.247 mg/l for Büyükçekmece 2010, Darlık 2010 and Ömerli 1 2010 samples respectively, where the regulation limit value was 0.05 mg/l. Regulation limit for Co is 0.002 mg/l, however, Co concentrations in

Alibeyköy, Elmalı and Darlık 2010 samples were measured as 0.006, 0.009 and 0.10 mg/l respectively. In addition Cr concentration in Sazlıdere 2010 sample was measured as 0.047 mg/l, which is very close to the limit of 0.05 mg/l. Cd concentration of Sazlıdere 2013 sample was measurement as 0.001 mg/l which was the limit concentration.

Ni level of 0.200 mg/l was found in a river sample previously (Cheung et al., 2003). Other studies reported Cu concentrations of 0-0.030 mg/l and Cr concentrations of 0-0.380 mg/l (Cheung et al., 2003; Altındağ and Yiğit, 2005; Tonguç-Yayıntaş et al., 2007) which were in accordance with our results.

Overall chemical analyses show that heavy metal and anion concentrations were within the desired range of regulation of TR QSWDW except for Co, Ni and Cd. Cr concentration is close to the upper allowed limit of 0.050 mg/l.

4.1.4. Dissolved Organic Carbon Analysis

Organic carbon is an important food and energy source in aquatic environments. Organic carbon can be dissolved in water through decomposition of biota in the water body. External sources such as atmospheric deposition, soil and plant roots may also contribute to the presence of organic carbon. DOC might affect the pH of surface waters and light penetration. It may also form water-soluble complexes with metal ions and helps their mobility and transportation.

DOC levels desired for A1 and A2 level quality were declared in the regulation as 5 and 8 mg/l respectively. DOC measurements in this study were in the range of 2.38-5.54 mg/l desired for A1 level quality of drinking water supply. This range was in agreement with literature data of 0.2-12 mg/l, reported by Cheung et al. (2003).

4.2. DNA-Based Experiments

4.2.1. Genomic DNA Extraction and PCR Amplification

Genomic DNA was extracted from triplicate samples and ~ 200 bp region of DNA was amplified using GC-clamped primer pair for DGGE analysis.

4.2.2. DGGE Analysis

4.2.2.1. Cluster Analysis. Bacterial community fingerprints of water samples were revealed by DGGE. Cluster analysis of DGGE profiles were completed using band position based Dice coefficient and and band-independent, whole-densitometric-curve-based Pearson correlation coefficients. A total of 66 different bacterial species were detected. Shannon-Weaver index, Margalef's species richness and species evenness values are given in Table 4.2. The results show that there was a dynamic community in all of the sampling points. Considering that the samples were taken in July 2010 (summer), July 2011 (summer) and April 2013 (spring), the abundance of bacteria in the sampling points on different sampling times do not have a consistent trend. Nevertheless, average number of taxa was calculated as 20, 20 and 23 for July 2010, July 2011 and April 2013 samples, respectively, which indicates that the overall diversity might be correlated to season. Alibeyköy sample had the highest diversity of 33 taxa followed by Sazlidere (29 taxa) and Ömerli (28 taxa) in April 2013. Darlık (2010), and Emirli (2011) were the least diverse sites with 11 taxa. These results were parallel to Shannon-Weaver indices that vary between 2.398 and 3.497. Margalef's Richness is also in accordance with number of taxa. Species evenness indices were calculated as 1, which shows that the species are equally distributed. Bacterial diversity detected in Istanbul's drinking water reservoirs is in the range of 11 to 37 taxa which was previously determined for other worldwide drinking water reservoirs such as Australia, Germany and Portugese (Hoefel et al., 2005, Eichler et al., 2006, Vaz-Moreira et al., 2011).

38 distinct bands were excised and sequenced from July 2010 samples. 2010, 2011 and 2013 samples were analyzed with Bionumerics and the presences of sequenced bands were determined in all samples. Dendograms were obtained by analysis of DGGE band

patterns based on whole-densitometric-curve-based Pearson correlation coefficient and UPGMA. Analyses were performed for all samples and 2010, 2011 and 2013 samples separately. Samples were clustered according to the sampling periods.

Table 4.2. Shannon-Weaver index, Margalef's species richness and species evenness measurements.

Sampling site	Sampling time	Taxa	Shannon-Weaver index	Margalef's richness	Evenness
Alibeyköy	**July 2010**	16	2.773	5.41	1
	July 2011	23	3.135	7.016	1
	April 2013	33	3.497	9.152	1
Büyükçekmece	**July 2010**	22	3.091	6.794	1
	July 2011	19	2.944	6.113	1
	April 2013	17	2.833	5.647	1
Darlık	**July 2010**	11	2.398	4.17	1
Elmalı	**July 2010**	25	3.219	7.456	1
	April 2013	12	2.485	4.427	1
Emirli	**July 2011**	11	2.398	4.17	1
	April 2013	21	3.045	6.569	1
Ömerli 1	**July 2010**	20	2.996	6.342	1
	July 2011	21	3.045	6.569	1
	April 2013	28	3.332	8.103	1
Ömerli 2	**July 2011**	23	3.135	7.016	1
	April 2013	25	3.219	7.456	1
Ömerli Lake	**April 2013**	27	3.296	7.889	1
Sazlıdere	**July 2010**	26	3.258	7.673	1
	July 2011	21	3.045	6.569	1
	April 2013	29	3.367	8.315	1
Terkos İkitelli	**July 2011**	26	3.258	7.673	1
	April 2013	16	2.773	5.41	1
Terkos Kâğıthane	**July 2011**	14	2.639	4.926	1
	April 2013	24	3.178	7.237	1

4.2.2.2. Sequencing and Phylogenetic Analyses. The excised DGGE bands were numbered on DGGE gel given in Figure 4.1. Similarity research of 38 sequenced DGGE bands was conducted. Although 200 bp DNA data was sequenced, 68% of the sequences showed a similarity value higher than 90%. Phylogenetic affiliation of bacteria is given in Table 4.3.

Band 5 represents an uncultured bacterial isolate form a DGGE gel band which is responsible for algal outbreaks and available in Alibeyköy, Darlık and Ömerli samples. It was isolated from a reservoir water sample (Chen and Yang, 2009). Band 19 is a methane oxidizing bacterium. *Clonothrix fusca* strain AW-b which is observed in Alibeyköy and Sazlıdere samples previously isolated from an artesian well (Vigliotta et al., 2007). Band 20 was an Uncultured bacterium DW_NsrE7-9 which was isolated from a pilot-scale chloraminated drinking water distribution system (Regan et al., 2002). The bacterium is responsible for nitrite oxidation and was observed in Darlık and Büyükçekmece reservoirs. Similarly, a nitrate respirating uncultured bacterium clone 95 (band 32) was observed in Elmalı and Büyükçekmece samples. The bacterium was isolated from a lake sediment where arsenic sequestration is performed by nitrate respiring bacteria (Gibney and Nüsslein, 2007). This could be an indirect indicator of arsenic availability in Elmalı and Büyükçekmece reservoirs. *Methylosinus sp.* LW8 represented by band 22 was a methanotroph isolated from freshwater lake sediment and available in Sazlıdere. Ömerli and Darlık samples (Auman et al., 2000). An uncultured bacterium clone, previously isolated from river sediment was observed in Sazlıdere sample (band 33), reported to be a dechlorinating microorganism in charge of hexachlorobenzene and chloroethene transformation (Taş et al., 2010).

Sequenced species that belong to the *Bacteria* given in Table 4.3 were reported to be in charge of nitrate respiration (Gibney and Nüsslein, 2007), nitrite oxidation (Regan et al., 2002) and dechlorination (Taş et al,. 2010). Overall number of species belonging to *Bacteria* has an abundance of 53% most of which were isolated from environmental samples such as groundwater, river, lake, bay, surface water, artesian basin and tap water.

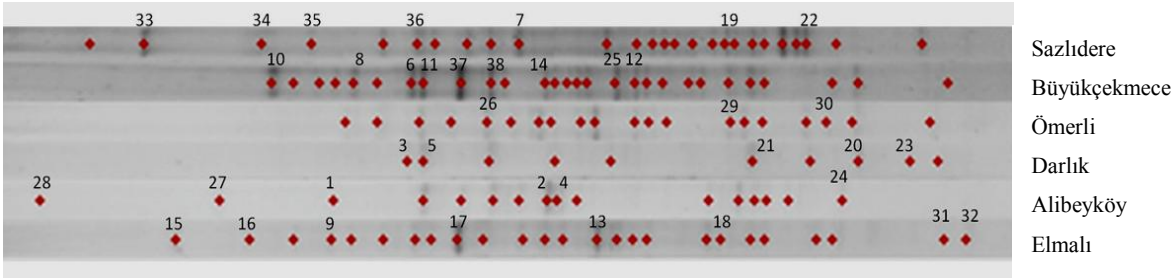


Figure 4.1. Excised DGGE bands.

Cyanobacteria and *Proteobacteria* were observed as the dominant phyla sharing 24% and 16% of the bacterial sequences, respectively. Isolation sources of *Cyanobacteria* were reported as surface water, lake, bay, estuarine, seagrasses and water column. They were in charge of non-toxic algal blooms in some cases (Lyra et al., 2001). *Proteobacteria* phylum covers species that belong to subclasses of *Alpha-*. *Beta-* and *Gammaproteobacteria,* isolated from river, freshwater lake sediment and organic aggregates. They include methanotrophs such as Methylosinus sp. LW8 (Vigliotta et al., 2007) and *Clonothrix fusca* strain AW-b (Auman et al., 2000). Strains affiliated to *Actinobacteria* and *Bacteroidetes* shared 5% and 3% of the community respectively. They were isolated from drinking water basin, tidal freshwater sediment and soil (Roes-Hill et al, 2009; Song et al., 2009; Edmonds et al., 2009; Davis et al., 2011). Beta-proteobacteria, Actinobacteria and Planctomycetes were reported as classical freshwater algae (Eichler et al, 2006). In addition, Actinobacteria, Firmicutes and Proteobacteria were found to have a total abundance of 97% (Hoefel et al., 2005). The results of this study are partly in accordance with previous reports since Proteobacteria has an abundance of 18%. However, it is not possible to determine the phyla of 54% of the uncultured bacteria due to lack of phylogenetic information. In addition, dominance of Cyanobacteria was observed in this study.

Cluster analysis for July 2010, July 2011 and April 2013 samples were given in Figure 4.2. It was observed that the samples were clustered according to sampling periods. Cluster analyses within each sampling period were given separately in Figures 4.3 to 4.5. The diversity and similarity of sampling points were unique to each sampling point and did not have consistency.

Table 4.3. Pylogenetic affiliation of bacteria identified by DGGE.

Phylogenetic affiliation	DGGE Band no	Closest relative (FASTA search)	Accession number	Similarity (%)
Actinobacteria	25	Streptomyces hypolithicus strain HSM#10	EU196762.1	78
	29	Uncultured actinobacterium clone DJ-G	FJ601383.1	92
Bacteria	6	Bacterium A8	EF016524.1	90
	1	Uncultured bacterium clone 2K76	GU074261.1	90
	3	Uncultured bacterium isolate DGGE gel band 0.22-37	GQ342221.1	98
	5	Uncultured bacterium isolate DGGE gel band 1	GU305691.1	92
	9	Uncultured bacterium clone RW0270	GU635614.1	92
	12	Uncultured bacterium clone DXH4-32	FJ558929.1	90
	13	Uncultured bacterium isolate DGGE gel band 3-40	GQ342197.1	96
	14	Uncultured bacterium. specimen voucher bandDNA9	AM040900.1	95
	23	Uncultured bacterium clone 3C002651	EU801380.1	95
	26	Uncultured bacterium clone 3C002524	EU801272.1	89
	27	Uncultured bacterium. clone Sta0-25	AJ416164.1	84
	30	Uncultured bacterium clone B79	AF407727.1	91
	32	Uncultured bacterium clone 95	DQ165159.1	98
	33	Uncultured bacterium clone P2_F01003_HCB	FJ810760.1	81
	35	Uncultured bacterium clone 2B226	GQ164883.1	78
	36	Uncultured bacterium isolate DGGE gel band QCS357F 8	JF431093.1	100
	38	Uncultured bacterium isolate DGGE gel band QCS357F 11	JF431096.1	97
	20	Uncultured bacterium DW_NsrE7-9	AY048959.1	76
	31	Uncultured bacterium DSSD9	AY328708.1	93
	21	Bacterium Ellin6529	HM748677.1	72
Bacteroidetes	16	Uncultured Bacteroidetes bacterium clone C08_E_RAMPD35	GQ242443.1	80
Cyanobacteria	4	Microcystis sp. strain GL280641	AJ133173.1	88
	2	Synechococcus sp. ECS-18	DQ023295.1	99
	17	Uncultured Synechococcus sp. clone LS190_CD1_May_5m_6-F12	DQ519774.1	95
	7	Uncultured cyanobacterium clone SLc2.B05_L2	EF555720.1	89
	8	Uncultured cyanobacterium isolate DGGE gel band B2C	AY525671.1	90
	11	Uncultured cyanobacterium clone LPSB70	FJ901831.1	91
	15	Uncultured cyanobacterium clone FALLScyano02F07	DQ398215.1	98
	28	Uncultured cyanobacterium isolate DGGE gel band MB-06-MIC-B18	EU882211.1	95
	10	Uncultured Xenococcus sp. clone CrV-P5	DQ072929.1	80
Proteobacteria	18	Uncultured alpha proteobacterium clone SepB-9	EF032661.1	96
	37	Uncultured alpha proteobacterium clone TH_b268	EU373109.1	92
	22	Methylosinus sp. LW8	AY007294.1	97
	24	Magnetospirillum gryphiswaldense magnetosome island	AM085146.1	92
	34	Uncultured beta proteobacterium clone PRD01b009B	AF289169.1	97
	19	Clonothrix fusca strain AW-b	DQ984190.1	89

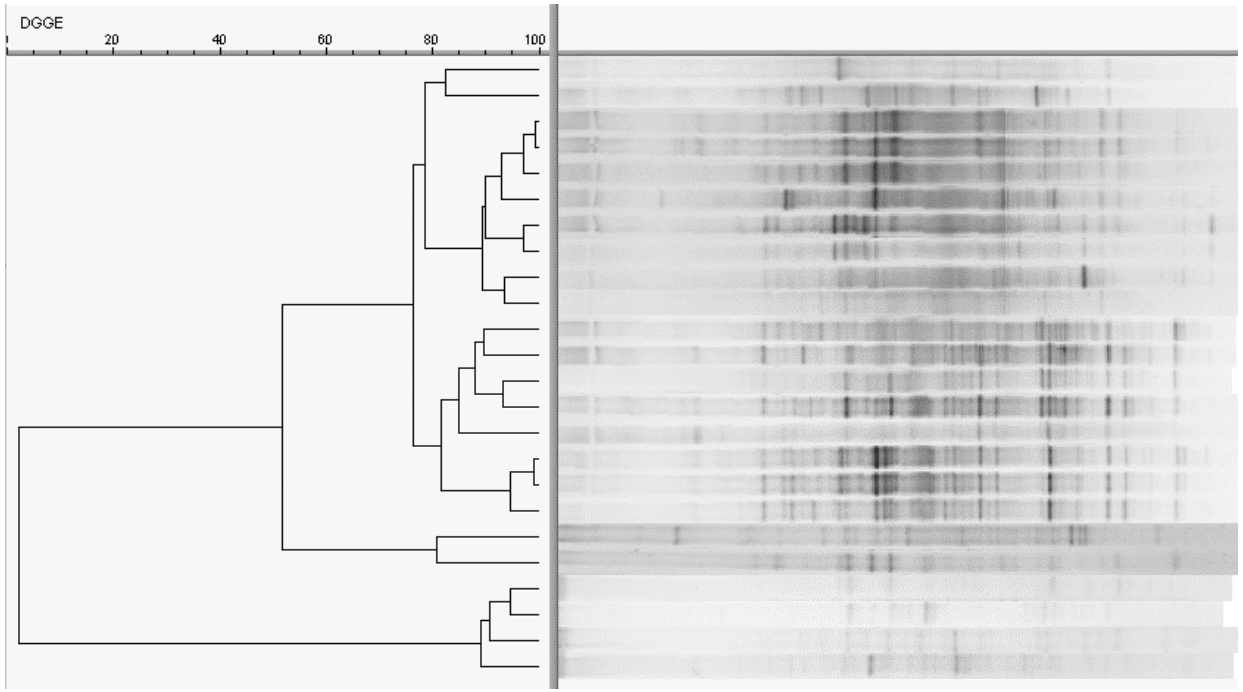


Figure 4.2. Cluster analysis of DGGE band patterns for 2010, 2011 and 2013 samples.

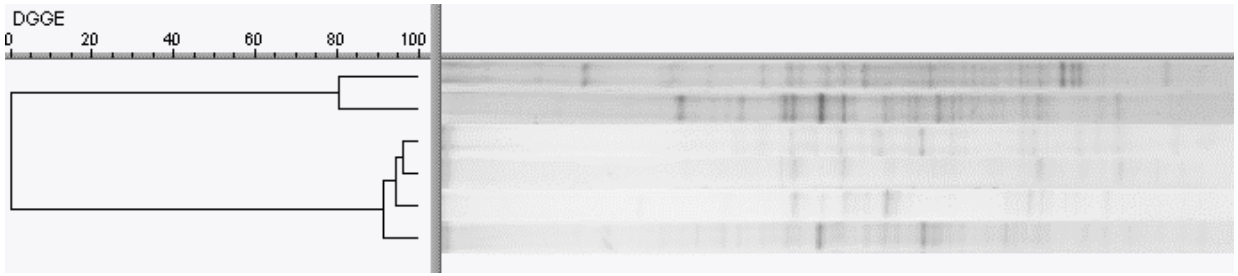


Figure 4.3. Cluster analysis of DGGE band patterns for July 2010 samples.

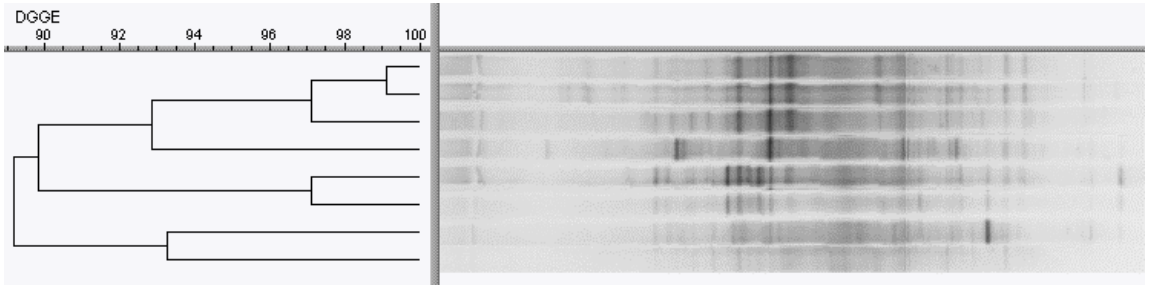


Figure 4.4. Cluster analysis of DGGE band patterns for July 2011 samples.

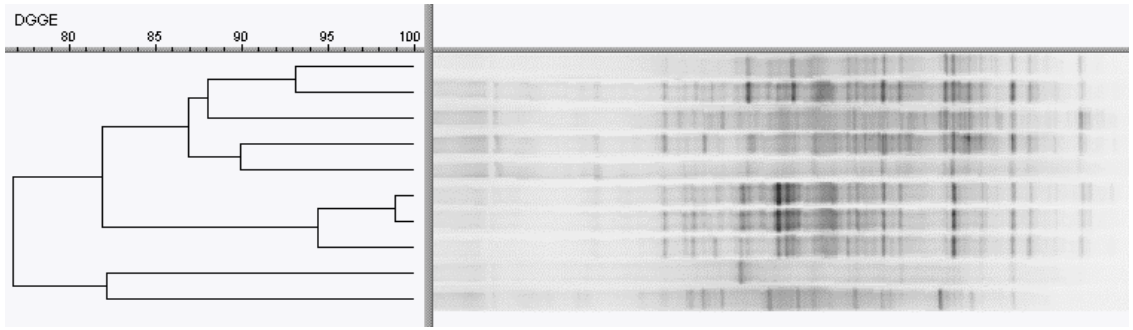


Figure 4.5. Cluster analysis of DGGE band patterns for April 2013 samples.

Table 4.4. Matching sequenced species on samples.

	Closest relative		Uncultured cyanobacterium isolate DGGE gel band MB-06-MIC-B18			Uncultured bacterium clone P2_F01003_HCB		Uncultured cyanobacterium clone FALLScyano02F07		Uncultured bacterium, clone Sta0-25		Uncultured Bacteroidetes bacterium clone C08_E_RAMPD35	
	Accession number (FASTA)		EU882211.1			FJ810760.1		DQ398215.1		AJ416164.1		GQ242443.1	
	Similarity (%)		95			81		98		84		80	
	Band no		28			33		15		27		16	
	Location on DGGE gel (%)		15.6	19.7	20.6	24.5	25.4	26.7	27.7	30.2	31.9	32.6	33.8
Sampling site - Year	**Alibeyköy**	**2010**	1	0	0	0	0	0	0	1	0	0	0
		2011	0	0	1	0	0	0	0	1	0	0	0
		2013	0	0	0	0	0	0	0	0	0	0	0
	Büyükçekmece	**2010**	0	0	0	0	0	0	0	0	0	0	0
		2011	0	0	0	0	0	0	0	0	0	0	0
		2013	0	0	0	0	0	0	0	0	0	0	0
	Darlık	**2010**	0	0	0	0	0	0	0	0	0	0	0
	Elmalı	**2010**	0	0	0	0	0	1	0	0	0	1	0
		2013	0	0	0	0	0	0	0	0	0	0	0
	Emirli	**2011**	0	0	0	0	0	0	0	0	0	0	0
		2013	0	1	0	1	1	0	0	0	0	1	0
	Ömerli 1	**2010**	0	0	0	0	0	0	0	0	0	0	0
		2011	0	0	0	0	1	0	1	0	1	0	0
		2013	0	0	0	0	0	1	0	0	0	0	0
	Ömerli 2	**2011**	0	0	0	1	0	0	1	0	1	0	0
		2013	0	0	0	0	0	1	0	0	0	0	0
	Ömerli Lake	**2013**	0	0	0	0	0	1	0	0	0	0	0
	Sazlıdere	**2010**	0	0	0	1	0	0	0	0	0	0	1
		2011	0	0	0	0	0	0	0	0	0	0	0
		2013	0	0	0	1	0	1	0	0	0	0	0
	Terkos-İkitelli	**2011**	0	0	0	0	0	0	0	0	0	1	0
		2013	0	0	0	0	0	1	0	1	0	0	0
	Terkos-Kâğıthane	**2011**	0	0	0	0	0	0	0	0	0	0	0
		2013	0	0	0	0	0	1	0	0	0	0	0

Table 4.4. Matching sequenced species on samples (cont.).

	Closest relative		Uncultured beta proteobacterium clone PRD01b009B	Uncultured Xenococcus sp. Clone CrV-P5			Uncultured bacterium clone RW0270	Uncultured cyanobacterium isolate DGGE gel band B2C			Bacterium A8	Uncultured bacterium isolate DGGE gel band QCS357F 8
	Accession number (FASTA)		AF289169.1	DQ072929.1			GU635614.1	AY525671.1			EF016524.1	JF431093.1
	Similarity (%)		97	80			92	88			88	100
	Band no		34	10			9	8			6	38
	Location on DGGE gel (%)		34.9	35.9	37.6	38.5	39.7	40.8	42.3	43.4	45.5	46.7
Sampling site - Year	**Alibeyköy**	**2010**	0	0	0	0	1	0	0	0	0	1
		2011	0	0	0	1	0	0	1	0	0	1
		2013	0	1	1	0	1	1	1	1	1	1
	Büyükçekmece	**2010**	1	0	1	0	0	0	1	1	1	1
		2011	0	1	0	1	0	1	1	0	1	1
		2013	0	1	0	1	0	1	0	0	1	1
	Darlık	**2010**	0	0	0	0	0	0	0	0	1	1
	Elmalı	**2010**	0	1	0	0	1	1	0	1	0	1
		2013	0	0	0	0	0	0	0	0	1	0
	Emirli	**2011**	0	0	0	0	0	1	0	1	0	1
		2013	0	1	0	0	1	1	0	1	0	0
	Ömerli 1	**2010**	0	0	0	0	0	1	0	1	0	1
		2011	0	1	0	1	0	0	1	0	1	1
		2013	0	1	0	1	1	1	1	0	1	1
	Ömerli 2	**2011**	0	1	0	1	0	0	1	0	1	1
		2013	0	1	0	1	1	0	1	0	1	1
	Ömerli Lake	**2013**	0	1	0	1	1	1	1	0	1	1
	Sazlıdere	**2010**	0	0	1	0	0	0	0	1	0	1
		2011	0	1	1	1	0	1	0	1	1	1
		2013	0	1	1	1	1	1	1	1	0	1
	Terkos-İkitelli	**2011**	0	1	1	0	1	1	1	1	1	1
		2013	0	1	0	1	1	0	0	0	0	1
	Terkos-Kâğıthane	**2011**	0	1	1	0	0	0	0	0	1	1
		2013	0	1	0	1	0	1	0	1	1	1

Table 4.4. Matching sequenced species on samples (cont.).

	Closest relative		Uncultured cyanobacterium clone LPSB70		Uncultured Synechococcus sp. Clone LS190_CD1_May_5m_6-F12	Uncultured bacterium isolate DGGE gel band 0.22-37		Uncultured bacterium isolate DGGE gel band 1	Uncultured cyanobacterium clone SLc2.B05_L2			Synechococcus sp. ECS-18
	Accession number (FASTA)		FJ901831.1		DQ519774.1	GQ342221.1		GU305691.1	EF555720.1			DQ023295.1
	Similarity (%)		88		95	98		92	87			99
	Band no		11		17	3		5	7			2
	Location on DGGE gel (%)		48.1	49.1	49.6	50.5	51.8	52.8	54.6	55.3	56.3	57.2
			0									
Sampling site - Year	**Alibeyköy**	**2010**	0	0	0	1	0	1	1	0	0	1
		2011	1	0	0	1	1	0	1	0	0	0
		2013	0	0	1	1	1	1	0	1	0	0
	Büyükçekmece	**2010**	0	0	1	0	0	1	0	0	0	1
		2011	0	0	0	1	0	1	0	0	0	0
		2013	0	0	1	0	1	0	1	0	0	0
	Darlık	**2010**	1	0	0	0	0	1	0	0	0	0
	Elmalı	**2010**	0	0	1	0	1	0	0	1	0	1
		2013	0	0	0	1	0	0	0	1	0	0
	Emirli	**2011**	0	0	0	0	0	1	0	0	0	0
		2013	0	0	1	0	0	1	0	1	0	0
	Ömerli 1	**2010**	0	0	1	0	1	0	1	0	1	0
		2011	1	0	0	1	0	1	0	0	0	0
		2013	0	0	0	1	1	0	0	0	0	0
	Ömerli 2	**2011**	0	1	1	1	0	1	0	0	0	0
		2013	1	0	0	1	1	1	0	1	0	0
	Ömerli Lake	**2013**	1	0	0	1	1	0	0	0	0	0
	Sazlıdere	**2010**	0	0	0	1	0	1	1	0	0	0
		2011	0	0	0	1	1	0	0	0	0	0
		2013	0	0	1	1	0	1	0	0	0	0
	Terkos-İkitelli	**2011**	0	1	0	1	0	1	0	0	1	0
		2013	0	0	0	0	0	0	0	1	0	0
	Terkos-Kâğıthane	**2011**	0	1	0	1	0	1	0	0	0	0
		2013		0	0	0	0	0	0	1	0	0

Table 4.4. Matching sequenced species on samples (cont.).

	Closest relative		Uncultured bacterium, specimen voucher bandDNA9		Uncultured bacterium isolate DGGE gel band 3-40		Uncultured bacterium clone DXH4-32					Streptomyces hypolithicus strain HSM#10			
	Accession number (FASTA)		AM040900.1		GQ342197.1		FJ558929.1					EU196762.1			
	Similarity (%)		95		96		90					78			
	Band no		14		13		12					25			
	Location on DGGE gel (%)		57.9	59.8	60.9	62.1	63.1	63.6	64.4	65.4	66.7	67.8	68.2	68.9	69.6
Sampling site - Year	**Alibeyköy**	**2010**	1	1	0	0	0	0	0	0	0	0	0	0	0
		2011	1	0	0	0	0	0	0	1	0	1	1	1	1
		2013	0	1	1	1	0	0	1	0	1	1	0	0	1
	Büyükçekmece	**2010**	1	1	0	0	1	1	1	0	0	1	0	1	0
		2011	0	0	0	0	0	0	1	0	1	1	1	0	0
		2013	0	0	0	0	0	0	0	0	0	0	0	0	0
	Darlık	**2010**	1	0	0	1	0	0	0	0	0	0	0	0	0
	Elmalı	**2010**	1	0	1	0	1	0	1	1	0	0	0	0	0
		2013	0	1	1	0	0	0	0	1	1	0	0	0	0
	Emirli	**2011**	1	0	0	0	0	0	1	0	0	1	1	0	0
		2013	0	0	1	0	0	0	1	1	1	0	0	0	0
	Ömerli 1	**2010**	1	1	1	0	0	0	1	1	1	0	0	0	0
		2011	0	0	0	0	0	0	0	0	0	1	1	1	0
		2013	1	1	1	1	1	0	1	0	1	0	0	0	0
	Ömerli 2	**2011**	0	0	0	0	0	0	1	0	0	1	1	1	0
		2013	1	1	1	1	1	0	1	0	1	0	0	0	0
	Ömerli Lake	**2013**	1	1	1	1	1	0	1	0	1	0	0	0	0
	Sazlıdere	**2010**	0	0	0	1	0	0	1	1	1	1	0	1	0
		2011	0	0	1	0	0	0	0	0	1	1	1	0	1
		2013	0	1	1	1	1	0	1	0	1	0	0	0	0
	Terkos-İkitelli	**2011**	0	1	0	0	0	0	1	0	0	0	1	0	1
		2013	1	1	1	0	0	0	1	0	1	0	0	0	0
	Terkos-Kâğıthane	**2011**	0	0	0	0	0	0	1	0	0	1	1	0	1
		2013	1	1	1	1	0	0	1	0	1	1	0	0	0

Table 4.4. Matching sequenced species on samples (cont.).

	Closest relative		Uncultured alpha proteobacterium clone SepB-9	Uncultured actinobacterium clone DJ-G	Clonothrix fusca strain AW-b	Bacterium Ellin6529					Methylosinus sp. LW8	Uncultured bacterium clone B79	
	Accession number (FASTA)		EF032661.1	FJ601383.1	DQ984190.1	HM748677.1					AY007294.1	AF407727.1	
	Similarity (%)		96	92	87	72					97	88	
	Band no		18	29	19	21					22	30	
	Location on DGGE gel (%)		70.6	71.8	72.7	73.7	74.7	76	77.3	78.2	79.2	80.4	81.4
Sampling site - Year	**Alibeyköy**	**2010**	1	0	1	1	1	0	1	0	0	0	1
		2011	1	1	1	1	0	0	0	1	0	1	0
		2013	0	1	1	1	1	1	1	1	1	0	1
	Büyükçekmece	**2010**	0	1	0	1	1	0	0	0	0	1	0
		2011	0	1	1	0	1	1	1	0	0	1	0
		2013	0	0	1	1	0	1	1	0	1	0	1
	Darlık	**2010**	0	0	0	1	0	0	0	0	1	0	0
	Elmalı	**2010**	1	1	0	1	1	0	0	0	1	1	0
		2013	0	0	1	1	1	0	1	0	0	0	1
	Emirli	**2011**	0	0	1	0	0	1	0	0	0	1	0
		2013	0	1	0	1	1	1	1	0	0	0	1
	Ömerli 1	**2010**	0	1	0	1	1	0	0	1	0	1	0
		2011	0	0	1	0	1	0	1	1	0	1	0
		2013	0	0	1	1	0	0	1	1	0	0	1
	Ömerli 2	**2011**	0	0	1	0	1	0	1	1	0	1	0
		2013	0	0	1	1	0	0	1	0	0	0	1
	Ömerli Lake	**2013**	0	0	1	1	0	0	1	1	0	0	1
	Sazlıdere	**2010**	1	1	1	1	1	1	1	1	0	1	0
		2011	0	0	0	0	1	1	0	1	0	1	0
		2013	0	0	1	1	1	1	1	0	0	0	1
	Terkos-İkitelli	**2011**	0	0	1	1	1	0	0	1	0	1	0
		2013	0	0	0	1	0	0	1	0	0	0	1
	Terkos-Kâğıthane	**2011**	0	0	0	0	1	0	0	1	0	0	0
		2013	0	0	1	1	1	1	1	0	0	0	1

Table 4.4. Matching sequenced species on samples (cont.).

	Closest relative		Magnetospirillum gryphiswaldense magnetosome island	Uncultured bacterium DW_NsrE7-9					Uncultured bacterium clone 3C002651	Uncultured bacterium DSSD9	Uncultured bacterium clone 95	
	Accession number (FASTA)		AM085146.1	AY048959.1					EU801380.1	AY328708.1	DQ165159.1	
	Similarity (%)		89	76					95	93	98	
	Band no		24	20					23	31	32	
	Location on DGGE gel (%)		82.4	82.9	83.6	84.7	85.9	87.1	87.9	89	90.3	91.4	94.8
Sampling site - Year	**Alibeyköy**	**2010**	0	0	0	0	0	0	0	0	0	0	0
		2011	0	1	0	0	0	0	0	0	1	1	0
		2013	0	0	1	0	0	1	0	0	1	0	0
	Büyükçekmece	**2010**	1	0	0	0	0	0	0	0	1	0	0
		2011	0	1	0	0	0	0	0	0	0	0	0
		2013	0	0	1	1	1	0	0	0	0	0	0
	Darlık	**2010**	1	1	0	0	0	1	0	1	0	0	0
	Elmalı	**2010**	0	0	0	0	0	0	0	0	1	1	0
		2013	0	0	0	0	0	0	0	0	0	0	0
	Emirli	**2011**	0	0	0	0	0	0	0	0	0	0	0
		2013	0	0	0	0	0	0	0	0	0	0	0
	Ömerli 1	**2010**	1	0	0	0	0	0	0	1	0	0	0
		2011	0	1	0	0	0	0	0	0	1	0	1
		2013	0	0	1	0	0	1	0	0	1	1	1
	Ömerli 2	**2011**	0	1	0	0	0	0	0	0	1	0	0
		2013	0	0	1	0	0	1	0	0	1	0	0
	Ömerli Lake	**2013**	0	0	1	0	0	1	0	0	1	1	0
	Sazlıdere	**2010**	0	0	0	0	0	0	1	0	0	0	0
		2011	1	0	0	0	0	0	0	0	0	1	1
		2013	0	0	1	1	0	1	0	0	1	0	0
	Terkos-İkitelli	**2011**	0	1	0	0	0	0	0	0	1	1	1
		2013	0	0	0	0	0	0	0	0	1	0	0
	Terkos-Kâğıthane	**2011**	0	0	0	0	0	0	0	0	0	0	1
		2013	0	0	1	0	0	1	0	0	1	0	0

4.2.2.3. Statistical Analysis. PCA of bacterial DGGE profile gave 40% of variability explained by the first two components. Figure 4.6 shows the loadings on first two principal components, PC1 and PC2. Similar to cluster analysis, 2011 and 2013 samples were grouped separately whereas 2010 samples were dispersed between 2011 and 2013 samples. Correlation coefficients for chemical analyses and principal components are given in Table 4.5. According to correlation analysis, it was observed that changes in Cr, Zn, Co, F^-, and NO_3^- were related with changes in bacterial community. PC1 has positive loadings on Cr, Zn, Co, and NO_3^- concentrations whereas F^- concentration is positively correlated to PC2. Impact of SO_4^{2-} on community structure in water reservoir sediment was recently reported (Röske et al., 2012). Correlation of non-coliforms and oxidized nitrogen compounds in a drinking water distribution system was also revealed (Power and Nagy, 1999).

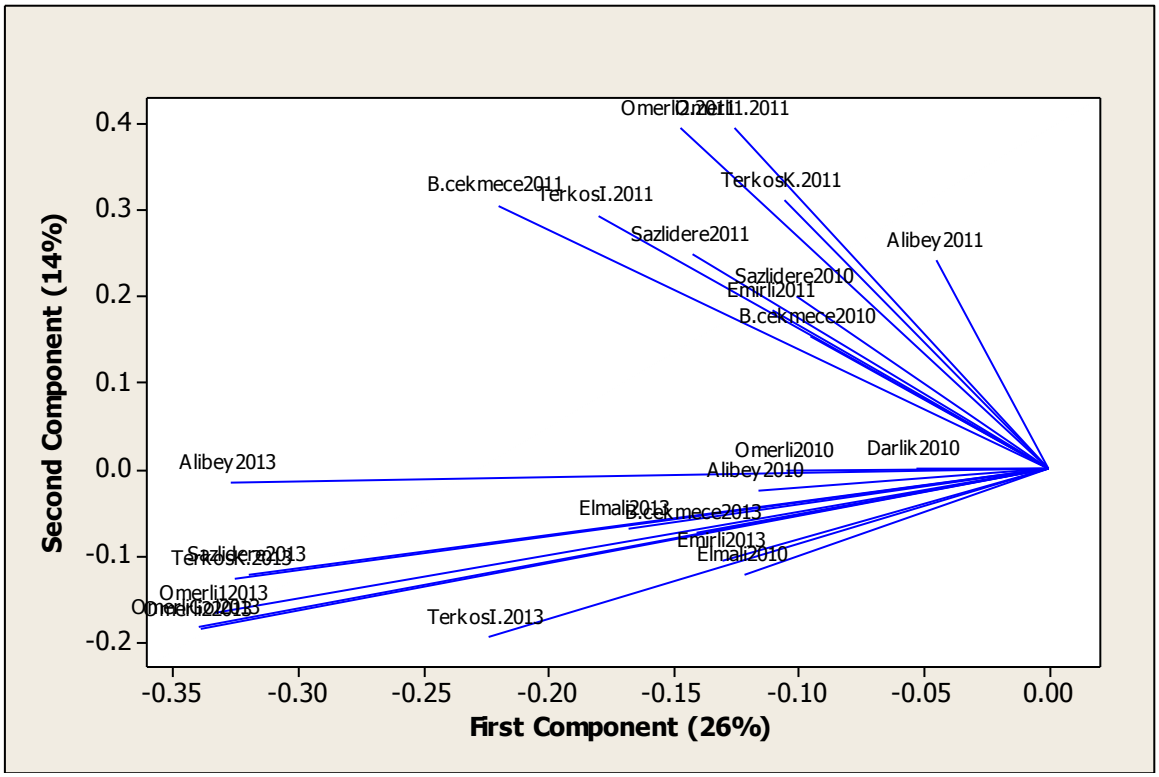


Figure 4.6. Loadings on principal components.

Table 4.5. Correlation coefficients for chemical analyses and principal components.

Variable (mg/l)	PC1 (26%)	PC2 (14%)
Cr	**0.030***	0.154
Mn	0.474	0.604
Fe	0.310	0.749
Ni	0.065	0.932
Cu	0.225	0.554
Zn	**0.039***	0.808
Cd	0.068	0.000
Pb	0.101	0.000
Co	**0.017***	0.685
Na	0.276	0.619
K	0.317	0.104
Mg	0.676	0.595
Ca	0.965	0.432
F^-	0.868	**0.049***
Cl^-	0.564	0.668
NO_2^-	0.829	0.371
Br	0.094	0.951
NO_3^-	**0.011***	0.299
SO_4^{2-}	0.896	0.964
PO_4^{3-}	0.247	0.258

*: Significance at P = 0.05, 0.01 and 0.001; significant coefficients are printed in bold.

4.2.3. qPCR Quantification

Quantification of selected pathogens in water samples was performed using qPCR. ETEC H10407 and EHEC O157:H7 strains are two severe human pathogens. These strains were also used for immunoimmobilization experiments. For a better correlation and comparison of detection limits, LT and *stx2* genes for ETEC and EHEC, respectively were quantified. Detection of EHEC O157:H7 via non-culture dependent methods is also important since this strain cannot be detected by coliform index due to its non-lactose fermenting characteristic. Legionella is one of the pathogenic threats that survive in hot water systems, spas, fountains very close environments such as air conditioners. This is also a non-coliform pathogen. Salmonella spp. are the second most common pathogen causing human diarrheal disease that have a diverse host range. *mip* and *hilA* genes were amplified for quantification of Legionella and Salmonella respectively.

Quantification results are reported in Table 4.6. Due to the fact that variable number of genes might be available in one cell, the results are reported in terms of gene copy number per 25 ng DNA which was used for qPCR amplification.

Table 4.6. Quantification of pathogens using qPCR.

		QPCR results (gene copy number/25 ng DNA)			
Sampling site	**Sampling time**	**EHEC**	**Legionella**	**Salmonella**	**ETEC**
		stx gene	***mip gene***	***hilA gene***	***LT gene***
Alibeyköy	**April 2013**	2.50E+002	6.12E+003	1.10E+003	2.22E+001
	July 2010	0	2.43E+003	0	5.16E+11
	July 2011	1.52E+004	4.90E+003	2.94E+003	2.54E+001
Büyükçekmece	**April 2013**	0	5.39E+003	4.89E+003	5.13E+000
	July 2010	0	9.74E+003	0	5.37E+10
	July 2011	2.92E+001	1.95E+003	0	0
Darlık	**July 2010**	0	4.18E+003	0	0
Elmalı	**April 2013**	0	1.28E+004	4.27E+003	0
	July 2010	0	6.63E+003	0	6.74E+11
Emirli	**April 2013**	2.28E+002	1.78E+004	0	9.71E+001
	July 2011	0	4.29E+003	0	0
Ömerli 1	**April 2013**	2.10E+002	7.74E+003	7.72E+003	0
	July 2011	1.72E+004	4.25E+003	2.61E+003	3.81E+001
	July 2010	0	3.41E+003	1.33E+06	0
Ömerli 2	**April 2013**	2.08E+002	4.67E+003	4.18E+002	2.91E+001
	July 2011	2.34E+003	7.86E+004	3.03E+003	0
Ömerli Lake	**April 2013**	4.39E+001	1.17E+004	3.12E+003	1.44E+000
Sazlıdere	**April 2013**	3.84E+002	5.39E+003	2.63E+003	6.58E+000
	July 2010	0	1.48E+006	0	0
	July 2011	4.23E+003	3.68E+004	1.17E+003	1.69E+001
Terkos İkitelli	**April 2013**	0	2.76E+003	6.04E+000	0
	July 2011	7.81E+002	5.65E+003	5.25E+003	2.07E+001
Terkos Kâğıthane	**April 2013**	0	7.14E+004	1.30E+003	0
	July 2011	1.63E+004	1.94E+004	1.11E+004	1.19E+001

Maximum gene copy numbers for related genes of ETEC, EHEC, Legionella and Salmonella were quantified as 5 to $6.74x10^{11}$, $2.92x10^{1}$ to $1.63x10^{4}$, $1.95x10^{3}$ to $1.48x10^{6}$ and 1 to $1.33x10^{6}$ per 25 ng DNA/µl respectively.

qPCR data showed that Legionella was a significant problem during all sampling times at every sampling point. Salmonella was observed as the second most common pathogen followed by ETEC and EHEC.

Parallel to the data in this study, Kao and co-workers (2013) detected Legionella spp. In the ranges of $9.75x10^4$ - $3.47x10^5$ cells/L in river water and $6.92x10^4$ - $4.29x10^5$ cells/L in raw drinking water samples. Lothigius et al. (2008) quantified 3-1332 cells/ml in water samples in an endemic area by LT gene. Ahmed et al (2009) detected $1.2x10^2$ gene copies of Salmonella and 1-10 gene copies of EHEC per 100 ml lake water.

4.3. Immunoimmobilization Experiments

4.3.1. AFM Imaging

AFM imaging of the *E. coli* strains were performed in order to reveal their fimbriae and flagella. AFM images of *E. coli* strains are given in Figures 4.7 and 4.8.

Fimbriae may show different morphology for different strains. It was observed that fimbriae expression level was low for 3.1012 and 9.1360 strains since no visible fimbriae could be imaged in most of the bacteria. This could be related to the fact that the expression level of fimbriae under in vitro conditions may not be as high as the expression level in living cells (Moon et al., 1979). Similarly, it was hard to observe flagella for O157:H7.

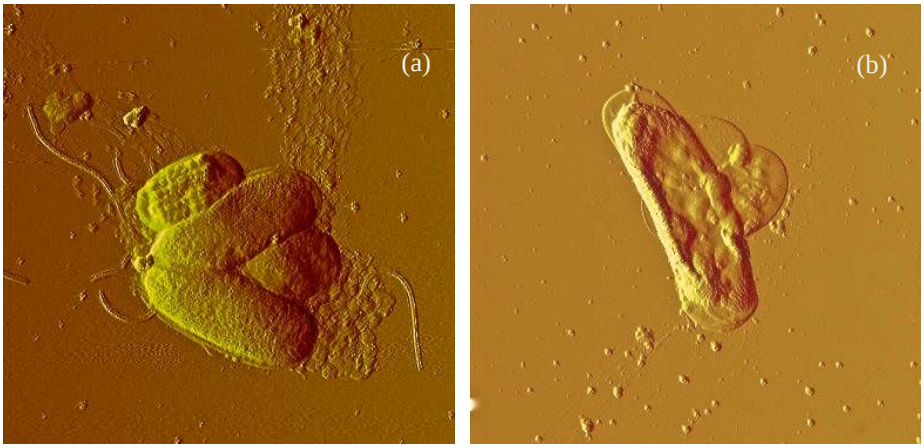


Figure 4.7. AFM images of *E. coli* strains with fimbriae and flagella (a) H10407 with CFA/I fimbriae and flagella and (b) B41 with K99 fimbriae (8 μm x 8 μm images).

4.3.2. Determination of Efficiently Binding Antigen-Antibody Pairs

This part of the study covers the development of immobilization method for selected pathogenic *E. coli* strains using thiol chemistry, optimization of the immobilization conditions and construction of an antibody microarray. Antibodies targeting fimbrial, flagellar and lipopolysaccharide (LPS) antigens were used.

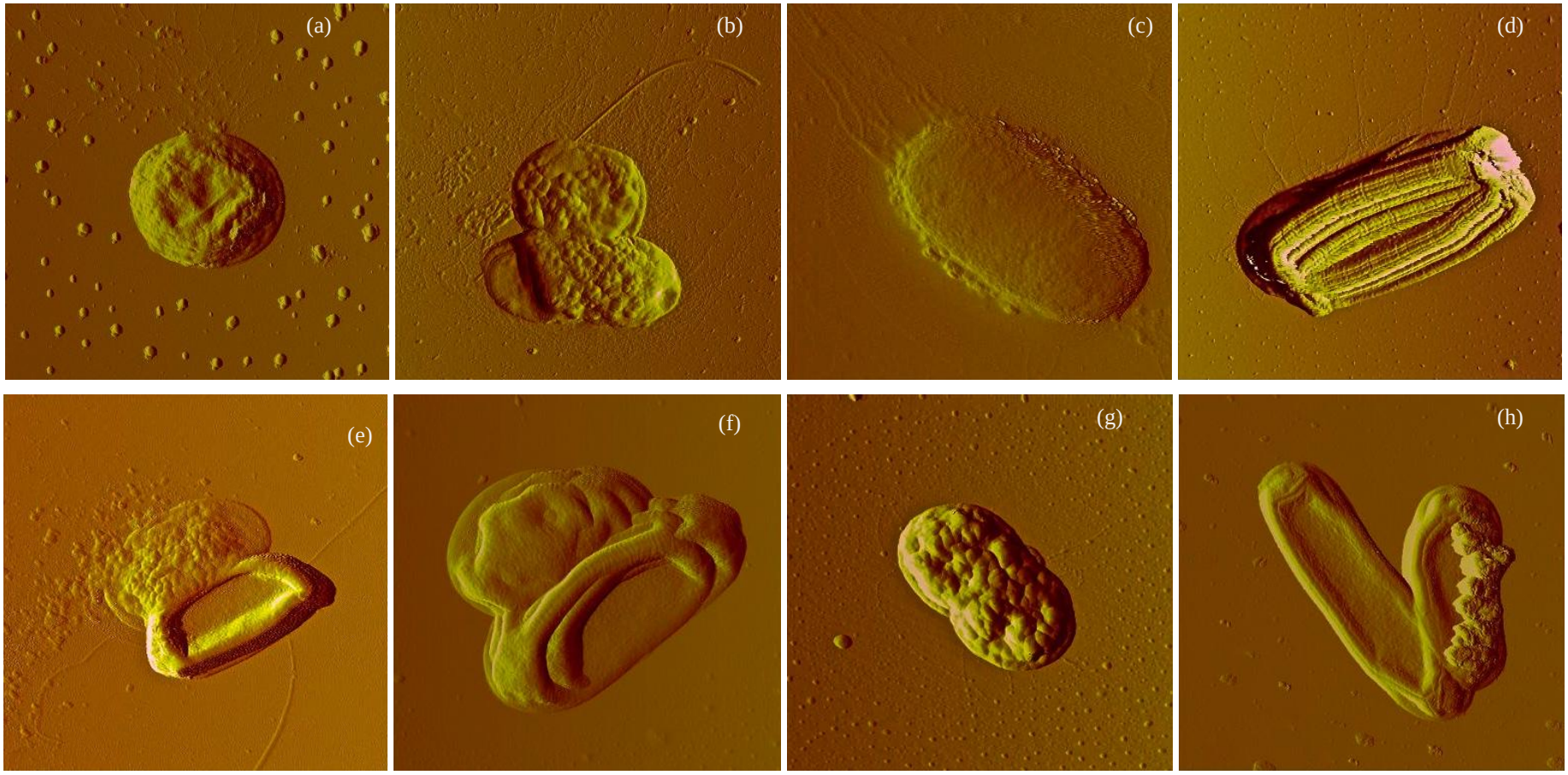


Figure 4.8. AFM images of *E. coli* strains with fimbriae (a) 1194 with 987P fimbriae, (b) 1836-2 with K88ab fimbriae, (c) 263 with K88ab fimbriae, (d) 3030-2 with K88ac fimbriae, (e) 2.0961with F41 fimbriae, (f) 3.1012 with 987P fimbriae, (g) 9.1360 with K99 fimbriae (h) H681 with K99 fimbriae (4.5 μm x 4.5 μm images).

Flagellar antigens were targeted by antibody against flagellin protein. Fimbrial antigens K88ab, K88ac, K99, F41 and 987P are animal colonization factors whereas colonization factor antigen I (CFA/I) is human mediated adhesin. Imaging was done by Olympus BX61 optical microscope.

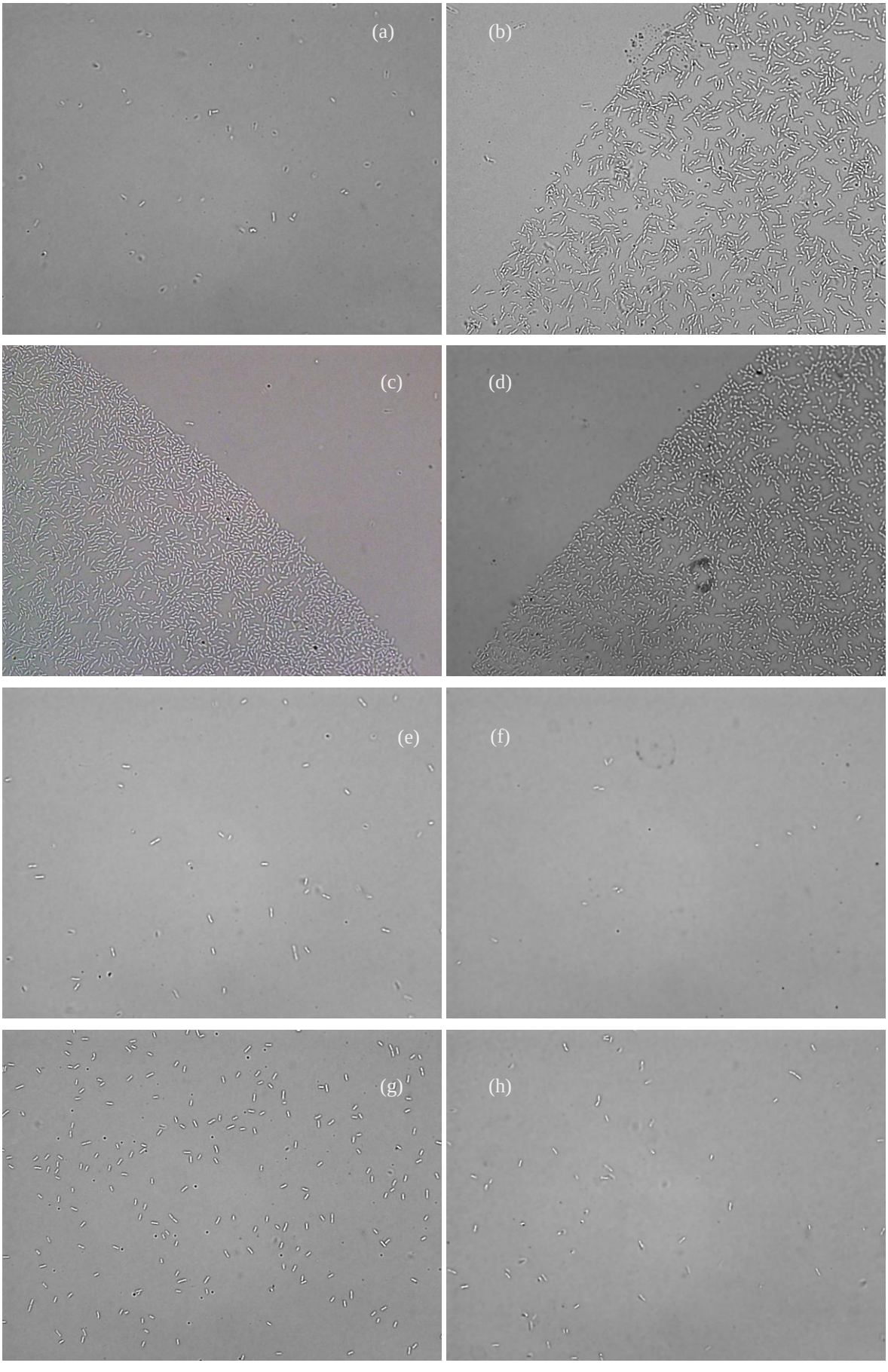
Gold substrates were modified via thiol chemistry using 11-mercaptoundecanoic acid (MUA), and activated with 1-Ethyl-3-[3-dimethyl aminopropyl] carbodiimide hydrochloride (EDC), a zero-length crosslinker, and *N*-Hydroxysuccinimide (NHS) prior to antibody modification.

Figure 4.9 depicts the *E. coli* strains immobilized from their fimbrial antigens. Silicon surface was coated with gold, modified with MUA, EDC, and NHS and decorated with relevant antibodies. *E. coli* 263, 3030-2 and 1836-2 strains show the best immunoimmobilization efficiency. The number of immobilized bacteria for the other strains was not satisfactory.

E. coli O157:H7 was targeted by its LPS and flagellar antigens. Figure 4.10 represents the immobilization status of this bacterium for both cases. Use of antibody specific to LPS of *E. coli* O157 was particularly effective on immobilization efficiency. This was the most efficient antibody-antigen pair as compared to the other combinations.

For ETEC H10407, three different antigens were targeted. Figure 4.11 gives a comparative result of immobilization efficiency for these antigen-antibody pairs. Immobilization with flagellar antibody, anti-CFA/I shows considerably better immobilization than LPS and flagellar antigens.

As a result of immobilization of the target strains with specific antigens, five pathogenic *E. coli* strains given in Table 4.7 were found to have the best immobilization efficiency. Approximately 3000-4000 cfu were immobilized onto (~170 μm)^2 area out of 10^7-10^8 cfu/ml.


(a)
(b)
(c)
(d)
(e)
(f)
(g)
(h)

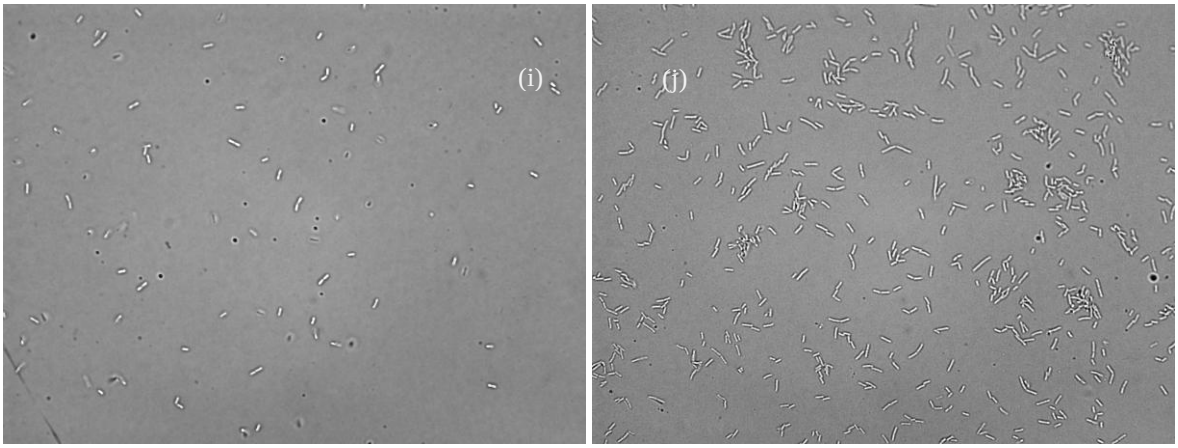


Figure 4.9. Immunoimmobilization of *E. coli* strains via fimbriae (a) 1194 via anti 987P (b) 1836-2 via anti K88ab (c) 263 via anti K88ab (d) 3030-2 via anti K88ac (e) 2.0961 via anti F41 (f) 3.1012 via anti 987P (g) 9.1360 via anti K99 (h) B41 via anti K99 (i) B41 via anti F41 (j) H681 via anti K99.

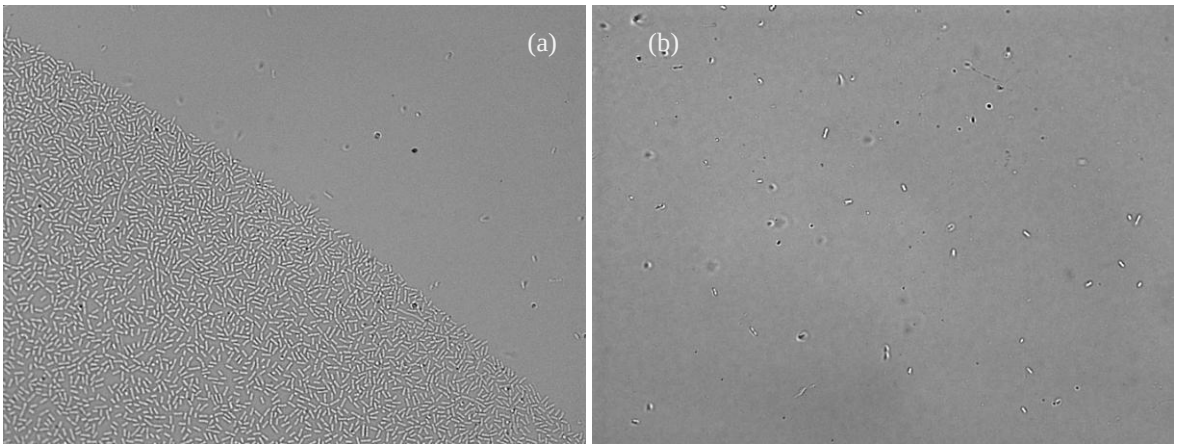


Figure 4.10. Immunoimmobiloization of *E. coli* O157:H7 via (a) anti LPS O157 (b) anti flagellin.

Table 4.7. Efficiently immobilized antibody-antigen pairs of *E. coli* strains.

Target antigen		**Bacterial strain**	**Host**	**Antibody**	
				Source	**Cat. number**
Fimbriae (Pili)	CFA/I	ETEC* H10407	Human	MSU	N/A
	K88ac	*E. coli* 3030-2	Animal	Novus	NB100-62533
	K88ab	*E. coli 1836-2*	Animal	Novus	NB100-62532
	K88ab	*E. coli 263*	Animal	Novus	NB100-62532
LPS	LPS-O157	EHEC* O157:H7	Human	Santa Cruz	sc-710004

ETEC: Enterotoxigenic *E. coli*; EHEC: Enterohemorrhagic *E. coli*

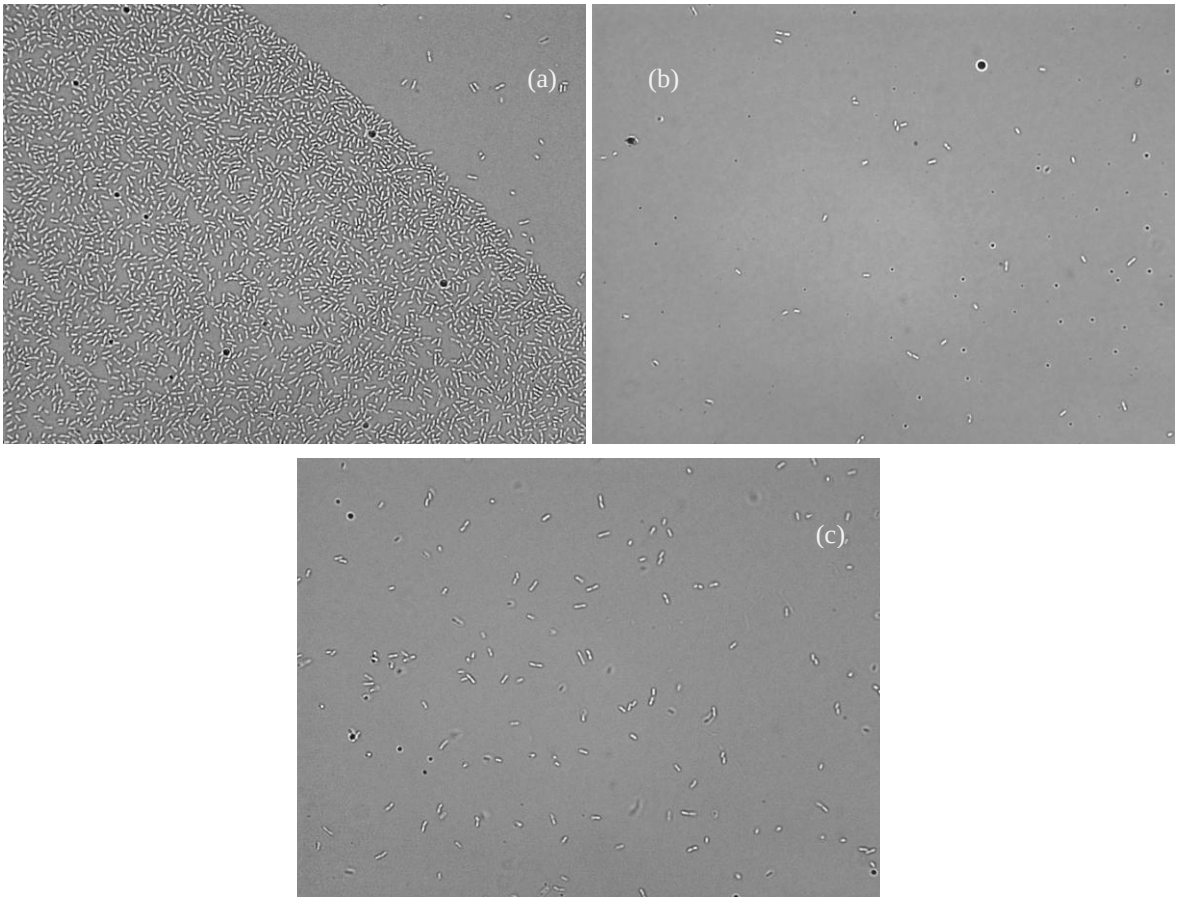


Figure 4.11. Immunoimmobiloization of *E. coli* H10407 via (a) anti CFA/I (b) anti flagellin (c) anti LPS.

4.3.3. Optimization of Surface Chemistry and XPS Analysis

Surface modification involves formation of self-assembled monolayers via thiol tethers, activation by succinimide esters and finally attachment of antibody molecules. Although modification with thiol tethers such as MUA has high reaction efficiency, NHS esterification is a critical stage that prepares the surface for coupling with antibody. Therefore, it is important to determine the optimum NHS – EDC concentration. In addition to NHS-EDC ratio, solvent type, pH and temperature are important factors for constructing the surface chemistry, providing efficient antibody linkage and bacteria immobilization.

In this part of the study, effects of (i) NHS-EDC concentration, (ii) solvent and (iii) temperature on antibody linking and self-assembled monolayer formation on gold were assessed. Gold-coated surface was analyzed with XPS after each step of MUA, EDC/NHS and antibody addition by means of nitrogen (N), sulfur (S), carbon (C), oxygen (O) and

gold (Au) composition. Changes in C1s, N1s, O1s, S2p and Au4f signals in XPS spectra were monitored. N is the indicator of free amine groups attached to the surface, which are involved in trapping antibodies. Increasing N1s signal indicates the attachment of antibody on the surface. S is observed in thiol link between Au and MUA. C and O content is dependent on the organic molecules attached to the surface. This part of the study was conducted using anti-CFA/I antibody, specifically raised against common factor antigen (CFA)/I of genetically modified Salmonella Typhimurium strain which expresses CFA/I fimbriae.

Different NHS-EDC concentrations were applied to achieve an efficient NHS esterification. NHS and EDC are both water-soluble crosslinkers. MES buffer or nanopure water (NP) was used as solvents (Moldovan et al., 2009; Y. Jung et al., 2008; Sam et al., 2010). MES buffer and nano pure water were used as solvents where NHS/EDC ratios of 2.6/1, 1/1 and 1/2.5 corresponding to concentrations of 260 mM/100 mM, 5 mM/5 mM and 40 mM/100 mM were used respectively. According to XPS results, it was determined that NHS/EDC ratio of 1 to 2.5 in nano pure water and MES buffer resulted in the most successful antibody linking. Furthermore, a higher antibody signal, based on the N1s XPS peak area, was observed for NHS/EDC (in 1/2.5 ratio) in MES buffer. This can be explained on the basis that nano pure water is prone to contaminants such as CO_2, in the environment, which might drastically alter its pH. Since EDC reaction is pH dependent, use of a buffer solution is expected to yield higher reaction efficiencies which leads to more efficient linking of antibody molecules. In the literature, optimum NHS-EDC concentration for complete activation of acid terminal groups was reported as 5 mM-5 mM (Sam et al., 2010). Although these concentrations and ratio were theoretically proven, various NHS-EDC concentrations and ratios were reported in application where either [NHS]>[EDC], [NHS]<[EDC] or [NHS]=[EDC]. For example, 100 mM NHS and 100 mM EDC were used for activation of carboxyl groups on gold wires (Baldrich et al., 2008), whereas 500 mM NHS and 200 mM EDC were used in QCM applications (Tlili et al., 2004). On the other hand, NHS-EDC ratio was variable within different SPR applications where combinations of 50 mM - 200 mM (Jung et al., 2008), 100 mM - 100 mM (Lee et al., 2005) were used. Effect of temperature on self-assembled monolayer (SAM) formation via alkane thiols was also reported (Baldrich et al., 2008; Love et al., 2005). In this study,

optimum NHS-EDC concentration was determined and applied as 40-100 mM in MES buffer.

It was published that temperatures above 25°C could enhance SAM formation kinetics (Love et al., 2003). Regarding this, two sets of experiments were run in SAM formation step, one of which was at room temperature (RT) and the other set at 30°C. The results showed that high temperature of 30°C had a negative impact on antibody attachment compared to that of RT. N1s relative concentration decreased from 8.2 to 4.6% at 30°C. Baldrich and coworkers (2008) observed that SAM formation at RT and 37°C temperature did not have a significant effect.

Table 4.8 summarizes XPS analysis for NHS-EDC reaction. Since the overall N content per molecule of this compound is only one N atom, it was below the detection limit of XPS during survey analysis. Similarly, MUA has one only S in its structure which does not give a significant S 2p peak. XPS survey analyses were given in Figure 4.12.

Table 4.8. Impacts of solvent, concentration and temperature on antibody linking.

				Relative atomic concentration %				
NHS/EDC ratios	**Solvent**	**Temperature**	**Antibody**	**C 1s**	**N 1s**	**O 1s**	**Au 4f**	**S 2p**
2.6/1	MES	Ambient (20-22°C)	Anti-CFA/I	62.2	1.8	9.3	26.7	0
1/1	MES			61.7	2.2	11.0	25.1	0
1/2.5	MES			61.5	8.2	15	15.3	0
1/2.5	Nano Pure water			62.6	6.6	15.5	15.1	0
1/2.5	MES	30°C		56.9	4.6	12.2	24.8	1.4

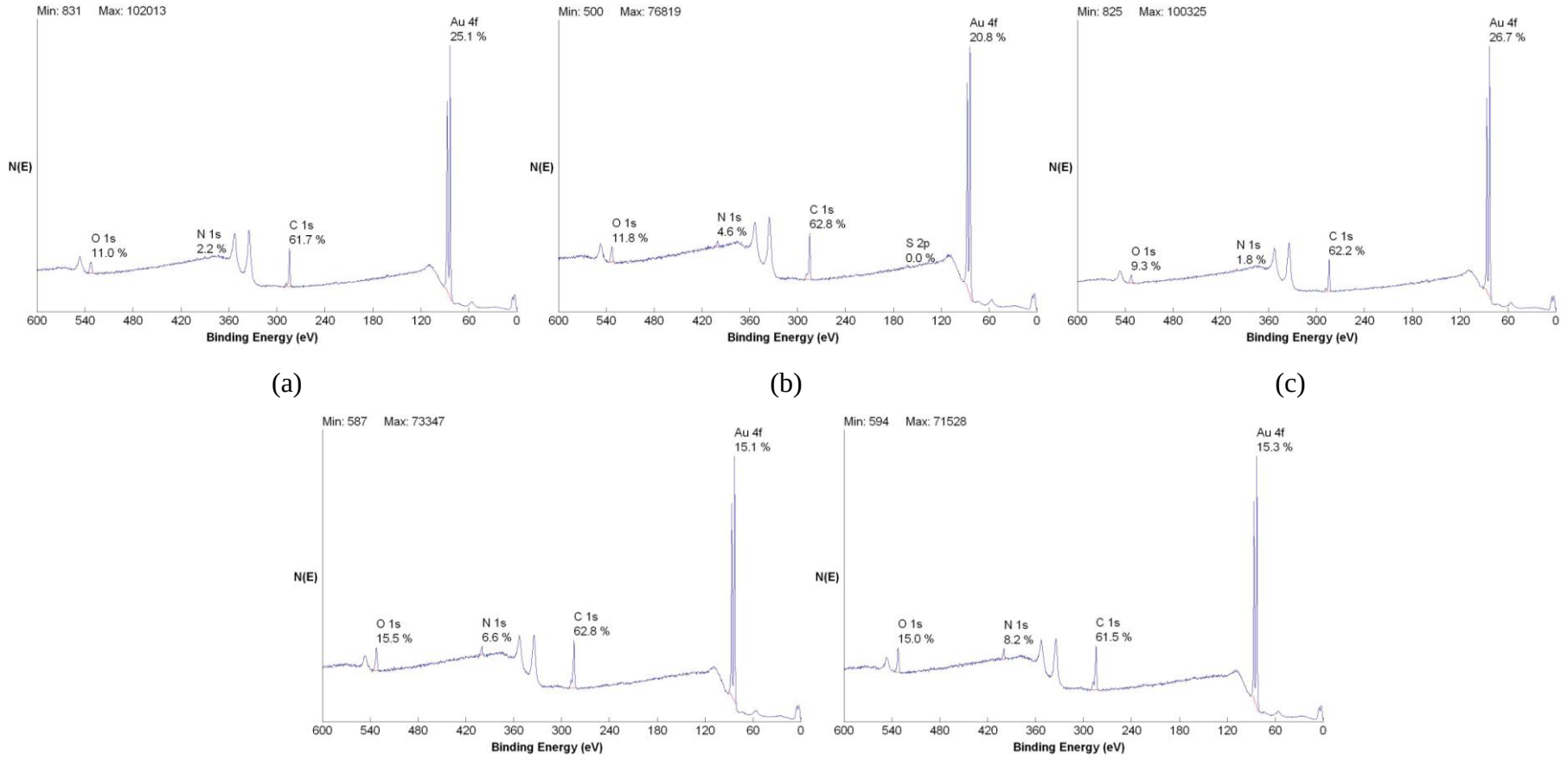


Figure 4.12. XPS survey analyses of different NHS-EDC concentrations in MES and NP: (a) 5 mM – 5 mM in MES (b) 5 mM – 5 mM in NP (c) 260 mM – 100 mM in MES (d) 40 mM – 100 mM in NP (e) 40 mM – 100 mM in MES.

XPS analysis was performed for gold-coated surfaces modified with other *E.coli* antibodies, namely anti flagellin, anti 987P, anti F41, anti K88ab, anti K88ac, anti K99 and anti LPS-O157. The results were given in Table 4.9. Surfaces modified with anti CFA/I, anti K88ab, anti K88ac and anti LPS-O157 have a high N1s signal within the range of 5.9 – 7.9%. This result is in good agreement with efficiently immobilized antigen-antibody pairs.

Table 4.9. XPS analysis of antibody-modified gold-coated surfaces.

Surface modification	**Atomic concentration (%)**				
	C 1s	**N 1s**	**O 1s**	**Au 4f**	**S 2p**
Cr-Au-MUA-NHS-EDC	55.5	1.4	12.2	29.2	1.7
Cr-Au-MUA-NHS-EDC-anti CFA/I	60.4	7.9	13.8	17.0	0.8
Cr-Au-MUA-NHS-EDC-anti Flagellin	56.9	2.1	9.4	30.1	1.6
Cr-Au-MUA-NHS-EDC-anti 987P	54.4	1.8	9.9	32.6	1.3
Cr-Au-MUA-NHS-EDC-anti F41	55.6	2.2	9.2	31.2	1.8
Cr-Au-MUA-NHS-EDC-anti K88ab	59.3	5.9	13.0	20.8	1.0
Cr-Au-MUA-NHS-EDC-anti K88ac	60.0	6.8	14.8	17.6	0.9
Cr-Au-MUA-NHS-EDC-anti K99	57.3	1.8	7.3	31.9	1.7
Cr-Au-MUA-NHS-EDC-anti LPS-O157	55	6.3	13.4	24.1	1.4

4.3.4. Antibody Titration Experiments

Titration experiments were conducted by immobilizing ~$4x10^7$ cfu/ml of *E. coli* O157:H7 on varying anti LPS O157 concentrations of 0.5 to 20 μg/ml.

In this experiment, N1s signal has reached a relative saturation concentration of 5.3% and did not show appreciable increase after modification with 10 μg/ml anti-LPS O157 antibody. This saturation value is in good agreement with study of Lai and coworkers (2009) who reported 5.4% N1s value after deposition of 5 mg/ml IgG. They observed that deposition of both 10 μg/ml and 5 mg/ml antibody concentrations resulted in the same N1s signal. This suggests that they have used 2-3 orders of magnitude more concentrations of antibody than needed. As shown in Figure 4.13, 10 μg/ml IgG is enough concentration to lay down a monolayer of antibody on the surface. Increasing the antibody concentration above this value is simply a waste of antibody.

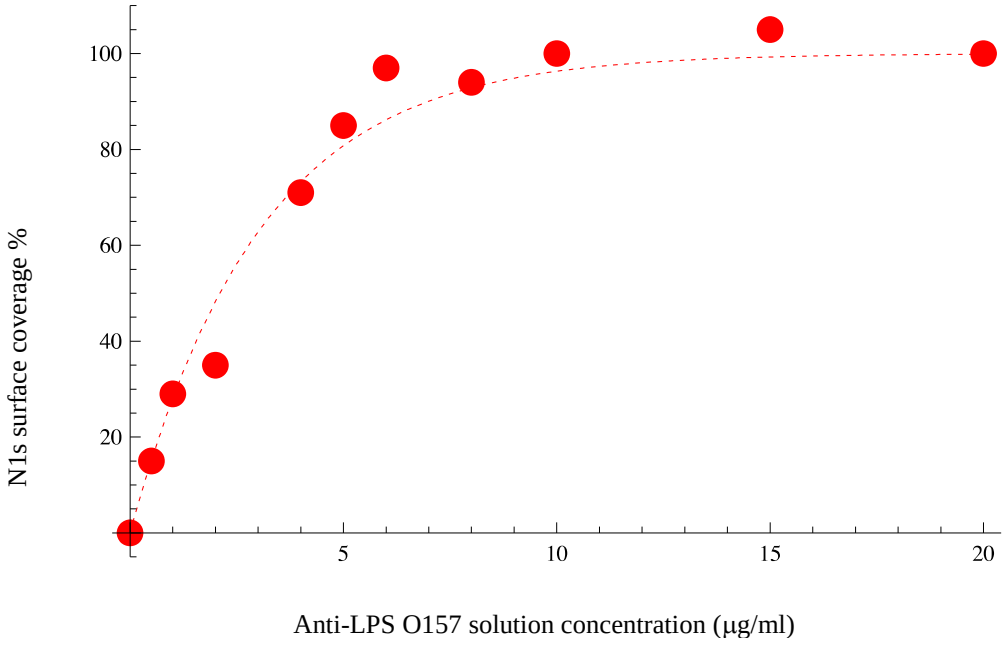


Figure 4.13. Saturation curve for N1s coverage %.

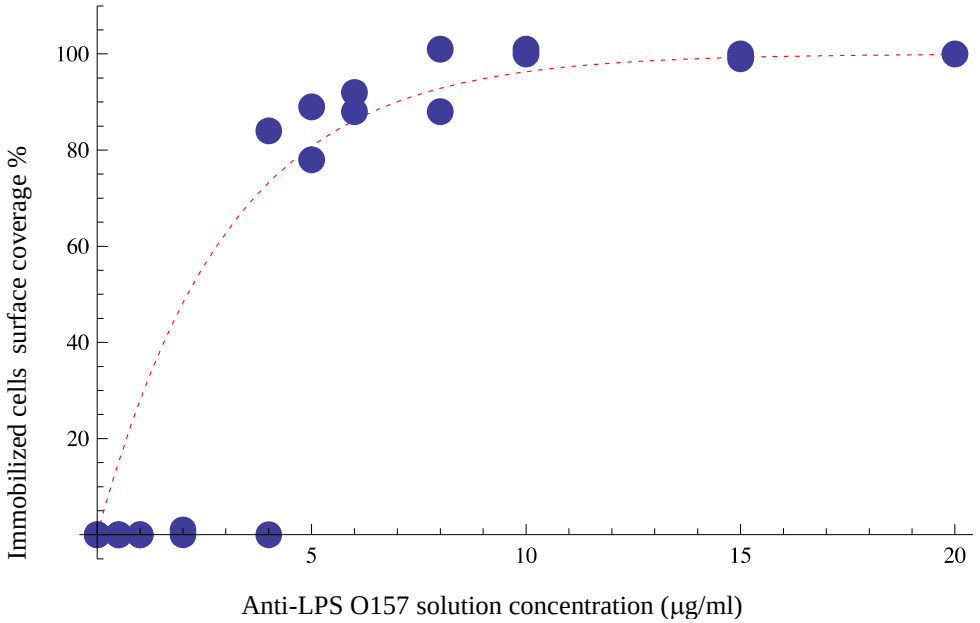


Figure 4.14. Saturation curve for immobilized cell coverage %.

Negligible amount of immobilization was observed for antibody concentrations of 0.5-4 µg/ml. Bacteria immobilization was observed at a minimum antibody concentration of 4 µg/ml and reached a plateau between 8-20 µg/ml. Stabilization of N1s% at antibody

concentrations higher than 8 μg/ml confirms that substrate surface was saturated and excess antibody was washed out.

Number of immobilized cells was determined in the range of 3400-3850 cfu per (~140 x 200 μm^2) area for 4-20 μg/ml antibody. Highest immobilization efficiency was observed with 10 μg/ml antibody which corresponds to a full coverage of antibody on the surface where relative N1s signal was 6.3%.

A linear relationship was observed between N1s signal and antibody concentrations of 0.5-6 μg/ml. N1s signal was stable after depositing antibody concentrations of 8 μg/ml and higher which suggests a saturation of antibody concentration on the surface between 8 - 20 μg/ml. Given in Figure 4.13, the experimental data points were fitted to a Langmuir isotherm.

The step function behavior of the bacteria immobilization (Figure 4.14) vs. antibody coverage is surprising; it is hypothesized that sudden onset of large number of bacteria immobilization is due to the requirement of minimum number of antibody-antigen pairs to hold a bacterium in place.

4.3.5. Limit of Detection

In order to determine the limit of detection, spots of antibody were deposited on modified gold-coated chips and incubated with serial dilutions of ~$1x10^8$ CFU/ml of pure cultures. Dilution experiments were conducted with most efficiently immobilized *E. coli* strains. ETEC H10407, EHEC O157:H7, *E. coli* 3030-2, *E. coli* 263 and *E. coli* 1836-2 strains were immobilized on corresponding anti CFA/I, anti LPS-O157, anti K88ac, anti K88ab and anti K88ab antibodies respectively. Serial dilutions were prepared from the stock culture (~$1x10^9$ cfu/ml) to a concentration of 10 cfu/ml, diluting 10 times at each step. Gold-coated chips were incubated with 1 ml of diluted cultures for 45 min at room temperature. Cell counts for stock *E. coli* cultures, dilution ratios and cell concentrations used for incubation of gold-coated substrates are given in Table 4.10.

ImageJ 1.440 software (National Institute of Health (NIH), USA) was used for the quantification of immobilized cells. Representative images for 10^1 and 10^2 dilutions of ETEC H10407, EHEC O157:H7, *E. coli* 3030-2, *E. coli* 263 and *E. coli* 1836-2 strains and dilution curves were given in Figures 4.15 to 4.20. The average number of immobilized cells per 140 x 200 μm^2 area is calculated in the range of 3000-4000, 300-400, 30-40 and 1-2 cells out of $10x10^7$ cfu/ml, $10x10^6$ cfu/ml, $10x10^5$ and $10x10^4$ cfu/ml respectively. Immobilization could not be observed for incubations with cultures below $10x10^3$ cfu/ml.

Table 4.10. Cell counts and incubated cell concentrations for *E. coli* strains.

	ETEC H10407	**EHEC O157:H7**	***E. coli* 3030-2**	***E. coli* 263**	***E. coli* 1836-2**
Stock cell culture concentration (cfu/ml)	8×10^8	10×10^8	7×10^8	8×10^8	8×10^8
Dilution ratio	**Incubated cell concentration (cfu/ml)**				
10^1	8×10^7	10×10^7	7×10^7	8×10^7	8×10^7
10^2	8×10^6	10×10^6	7×10^6	8×10^6	8×10^6
10^3	8×10^5	10×10^5	7×10^5	8×10^5	8×10^5
10^4	8×10^4	10×10^4	7×10^4	8×10^4	8×10^4
10^5	8×10^3	10×10^3	7×10^3	8×10^3	8×10^3
10^6	8×10^2	10×10^2	7×10^2	8×10^2	8×10^2
10^7	8×10^1	10×10^1	7×10^1	8×10^1	8×10^1

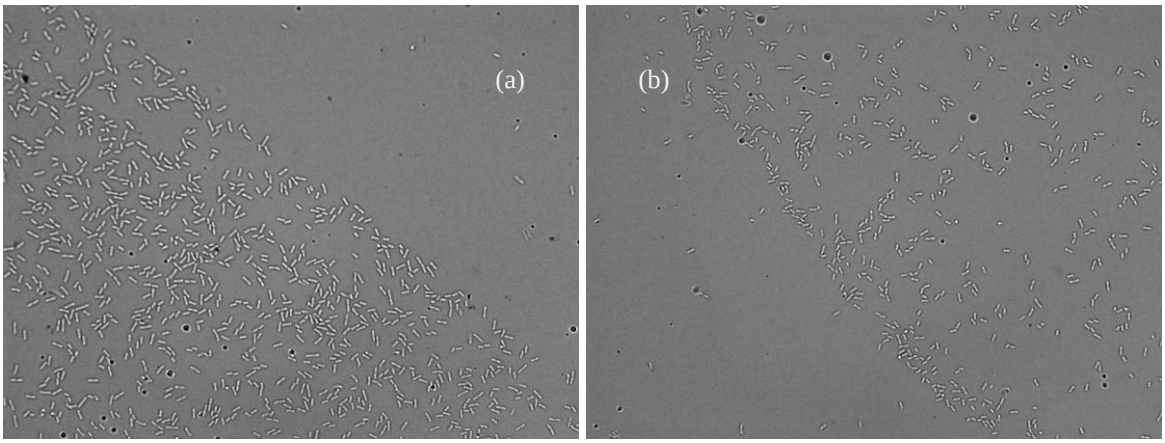


Figure 4.15. Images for 10^1 (a) and 10^2 (b) dilutions of ETEC H10407.

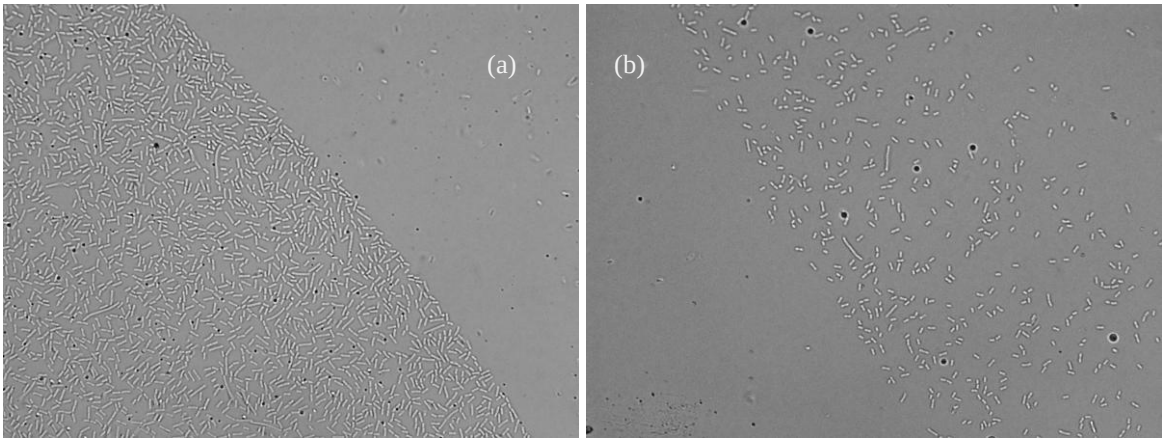


Figure 4.16. Images for 10^1 (a) and 10^2 (b) dilutions of EHEC O157:H7.

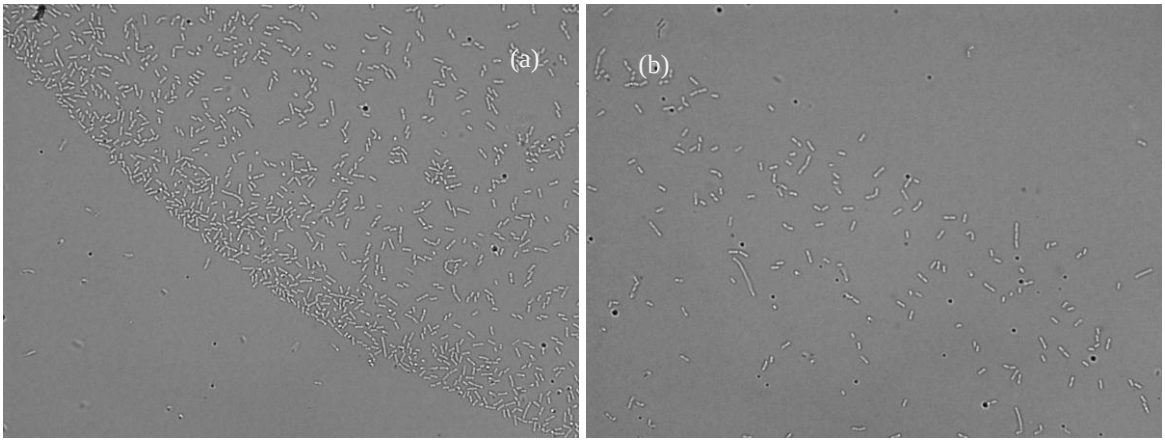


Figure 4.17. Images for 10^1 (a) and 10^2 (b) dilutions of *E. coli* 3030-2.

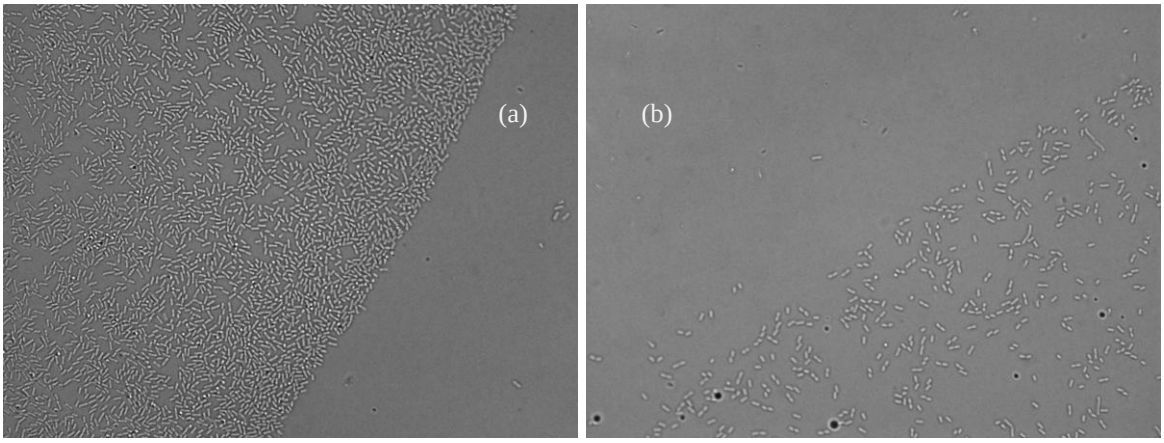


Figure 4.18. Images for 10^1 (a) and 10^2 (b) dilutions of *E. coli* 263.

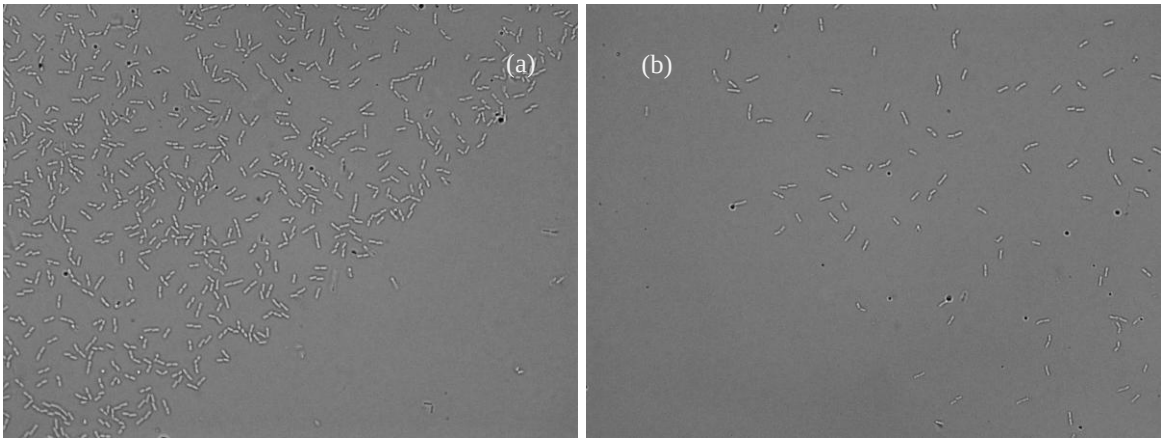


Figure 4.19. Images for 10^1 (a) and 10^2 (b) dilutions of *E. coli* 1836-2.

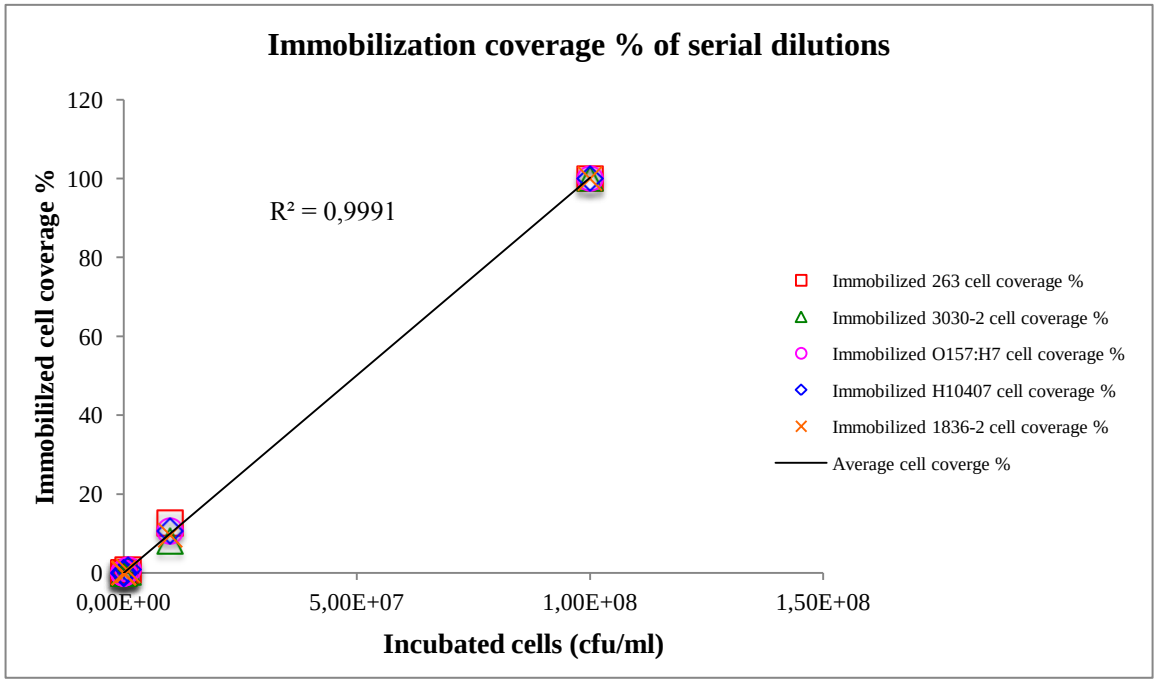


Figure 4.20. Dilution curve for *E. coli* strains.

4.3.6. Cross-Reactivity Experiments

Cross-reactivity experiments were conducted by incubating target bacteria with target and non-target antibodies on gold-coated substrates. The results showed that ETEC H10407 and EHEC O157:H7 are efficiently immobilized on anti CFA/I and anti LPS-O157 antibodies, respectively. However, anti K88ab and anti K88ac antibodies can immobilize *E. coli* 3030-2, *E. coli* 263 and *E. coli* 1836 strains with same efficiency. This can be explained on the basis that both antibodies target F4 fimbriae of *E. coli*. In Table

4.11, cross-reactivity is represented by “X” where target are denoted by “1” and non-target antigen-antibody pairs are denoted by “0”.

Table 4.11. Target, non-target and cross-reactive antibody-antigen pairs.

Strain	**Antibody**			
	anti CFA/I	**anti LPS O157**	**anti K88ab**	**anti K88ac**
EHEC O157:H7	0	1	0	0
ETEC H10407	1	0	0	0
***E. coli* 3030-2**	0	1	X	1
***E. coli* 263**	X	X	1	X
***E. coli* 1836-2**	0	0	1	X

4.3.7. Construction of Microarray and Sorting Experiments

Considering the cross-reactivity experiment results, three sets of microarray experiments were conducted. Antigen-antibody combinations on microarrays are given in Table 4.12. Microplotter was used for blotting antibodies in the desired order and amount. Cells were fluorescently labeled for differentiation on the microarray surface, as previously explained in Materials and Methods. ETEC H10407 RFP, expressing red fluorescent protein, EHEC O157:H7 stained with SYTO®9 and *E. coli* 1836-2 stained with DAPI were used for immobilization on the microarray.

Table 4.12. Microarray combinations.

Microarray 1	ETEC H10407 RFP – anti CFAI EHEC O157:H7 SYTO9 – anti LPS-O157 *E. coli* 1836-2 DAPI– anti K88ab
Microarray 2	ETEC H10407 RFP – anti CFA/I *E. coli* 3030-2 GFP – anti K88ac
Microarray 3	ETEC H10407 RFP – anti CFAI EHEC O157:H7 SYTO 9 – anti LPS-O157

In Figure 4.21, representative images for Microarray 1 with ETEC H10407 RFP-EHEC O157:H7 SYTO9 – *E. coli* 1836-2 DAPI on anti CFAI - anti LPS-O157 - anti K88ab respectively, are given. ETEC H10407 RFP and *E. coli* 1836-2 DAPI were successfully immobilized and sorted on the microarray. However, EHEC O157:H7 strain

had no incidence of immobilization or fluorescence although it was successfully immobilized in previous experiments.

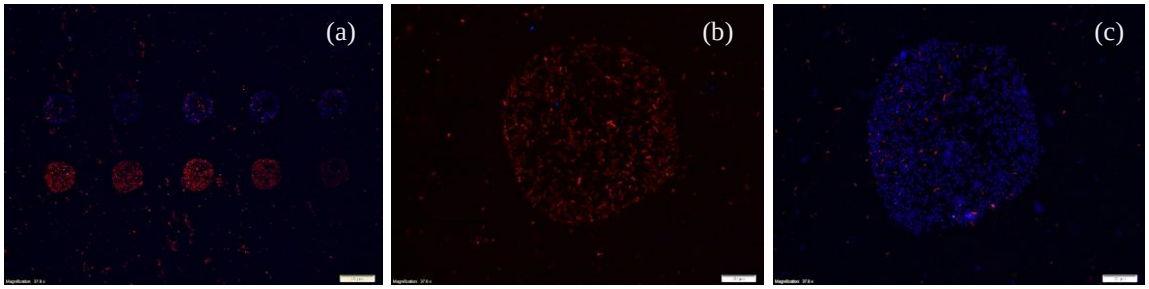


Figure 4.21. Fluorescence images of Microarray 1 (a), ETEC H10407 RFP on anti CFA/I spot (b), *E. coli* 1836-2 on anti K88ab spot (c).

On Microarray 2, ETEC H10407 RFP and *E. coli* 3030-2 GFP were efficiently immobilized and sorted by anti CFAI and anti K88ac antibodies. Figure 4.22 depicts the microarray and single cells sorted on the array.

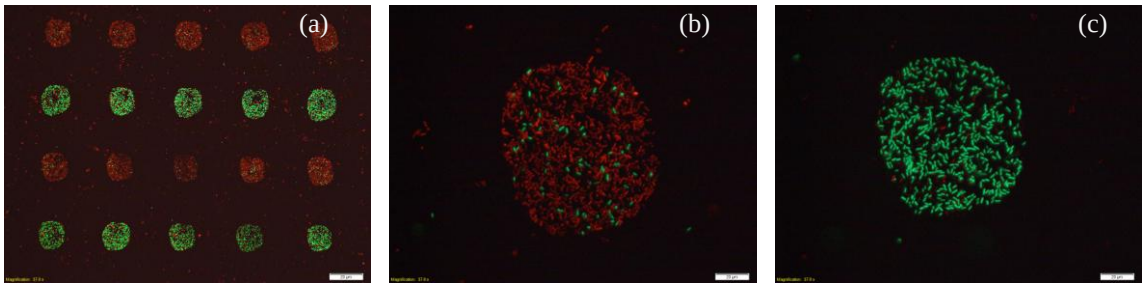


Figure 4.22. Fluorescence images of Microarray 2 (a), ETEC H10407 RFP on anti CFA/I spot (b), *E. coli* 3030-2 on anti K88ac spot (c).

Microarray 3 was the set of ETEC H10407 RFP and EHEC O157:H7 on anti CFA/I anti LPS-O157 respectively. Unlike Microarray 1, EHEC O157:H7 cells were successfully immobilized. However, fluorescence signal of EHEC O157:H7 was very low. The results are given in Figure 4.23.

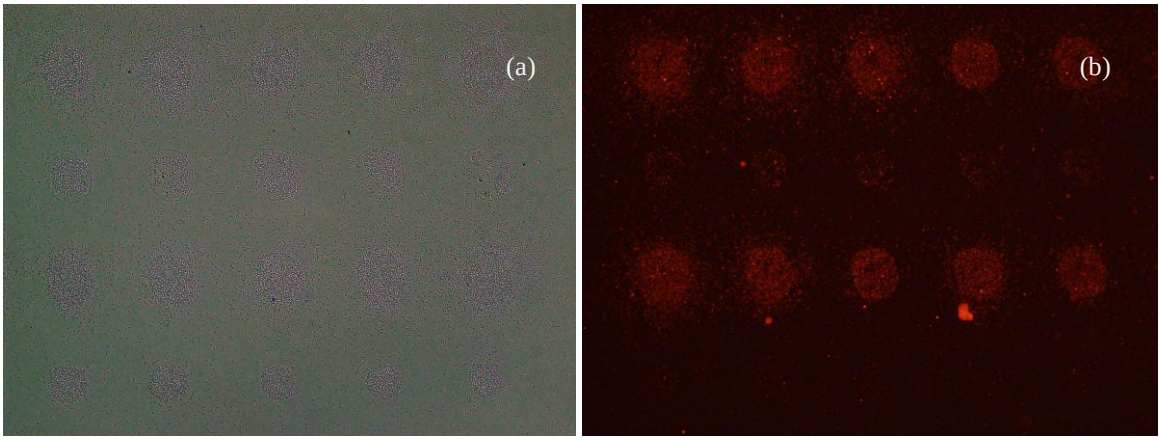


Figure 4.23. Bright field (a) and fluorescence (b) images for Microarray 3, ETEC H10407 RFP on anti CFA/I spots and EHEC O157:H7 on anti LPS-O157 spots.

4.3.8. Field Sample Applications: BiyoTrap® and Immunoimmobilization

This part of experiments is the proof-of-concept that immunoimmobilization can be used as a pathogen detection tool in real life applications. For this purpose, samples taken from Bozeman Creek and Alibeyköy Dam were pre-concentrated using BiyoTrap and directly immobilized on gold-coated chips activated with anti-CFA/I and anti-LPS O157 as described earlier.

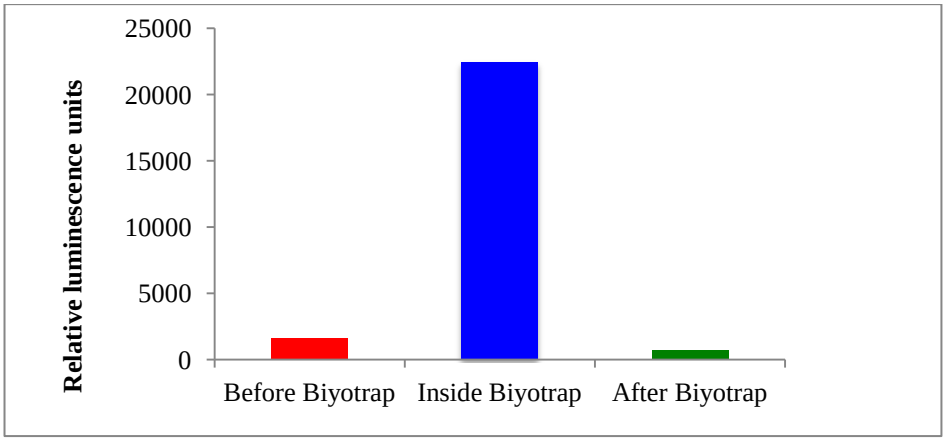


Figure 4.24. Bioluminescence measurement of before, inside and after BiyoTrap.

4.3.8.1. Cell Viability Measurement. Cell viability measurements before Biyotrap (raw sample), after Biyotrap (Biyotrap effluent) and inside Biyotrap measured by BacTiter-

Glo™ were given in Figure 4.24. Initial concentration of microbes was considerably low when compared to the luminescence values inside the Biyotrap. The results indicate that BiyoTrap is an efficient method for concentration of water samples.

4.3.8.2. Pre-concentration via Vacuum Filtration. For comparison of methods, a parallel set of experiment was performed by pre-concentration via vacuum filtration. 0.2 μm polycarbonate filter was used for filtration of 200 ml of Alibeyköy Dam sample. The filter was rinsed with 200 μl of filtrate and used for immunoimmobilization. However, immobilization efficiencies on both anti-LPS O157 and anti-CFA/I were similar to that of raw sample. Representative image was given in Figure 4.25. This experiment approves the requirement of an effective pre-concentration method for environmental samples.

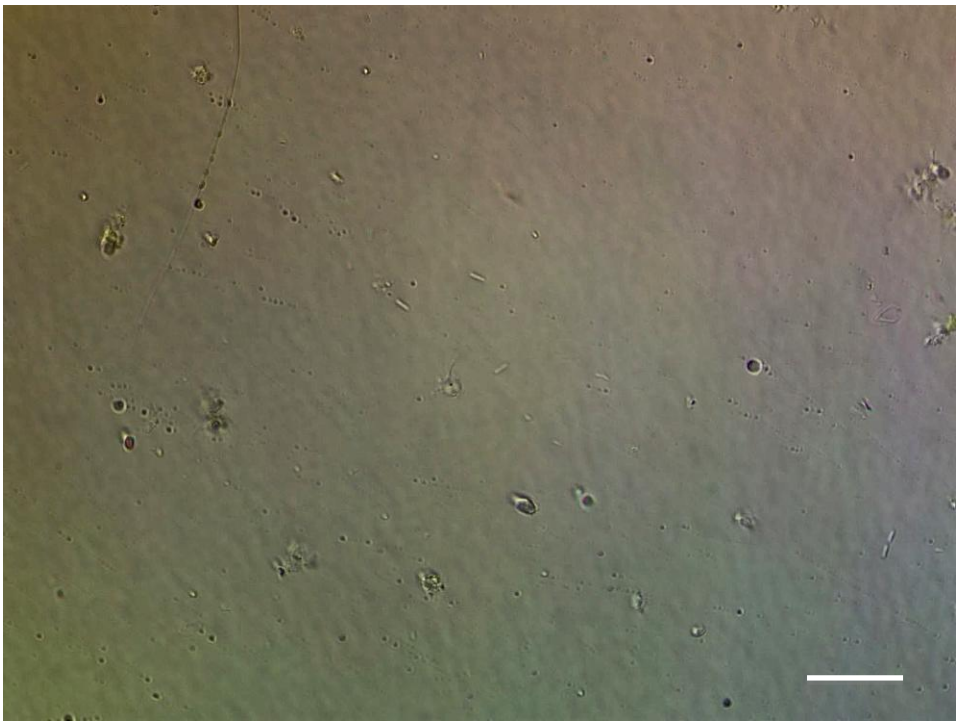


Figure 4.25. Alibeyköy Dam sample pre-concentrated via vacuum filtration and immobilized on anti-LPS O157 (the scale bar is 20 μm).

4.3.8.3. Pre-concentration via BiyoTrap®. Immunoimmobilization experiments were conducted using raw water and biyotrapped samples. 1 ml of raw Bozeman Creek and Alibeyköy Dam samples were incubated with anti-CFA/I and anti-LPS O157 modified

gold-coated chips. Since the sample was directly used as taken from the environment, all micro- and macro-organisms were visualized.

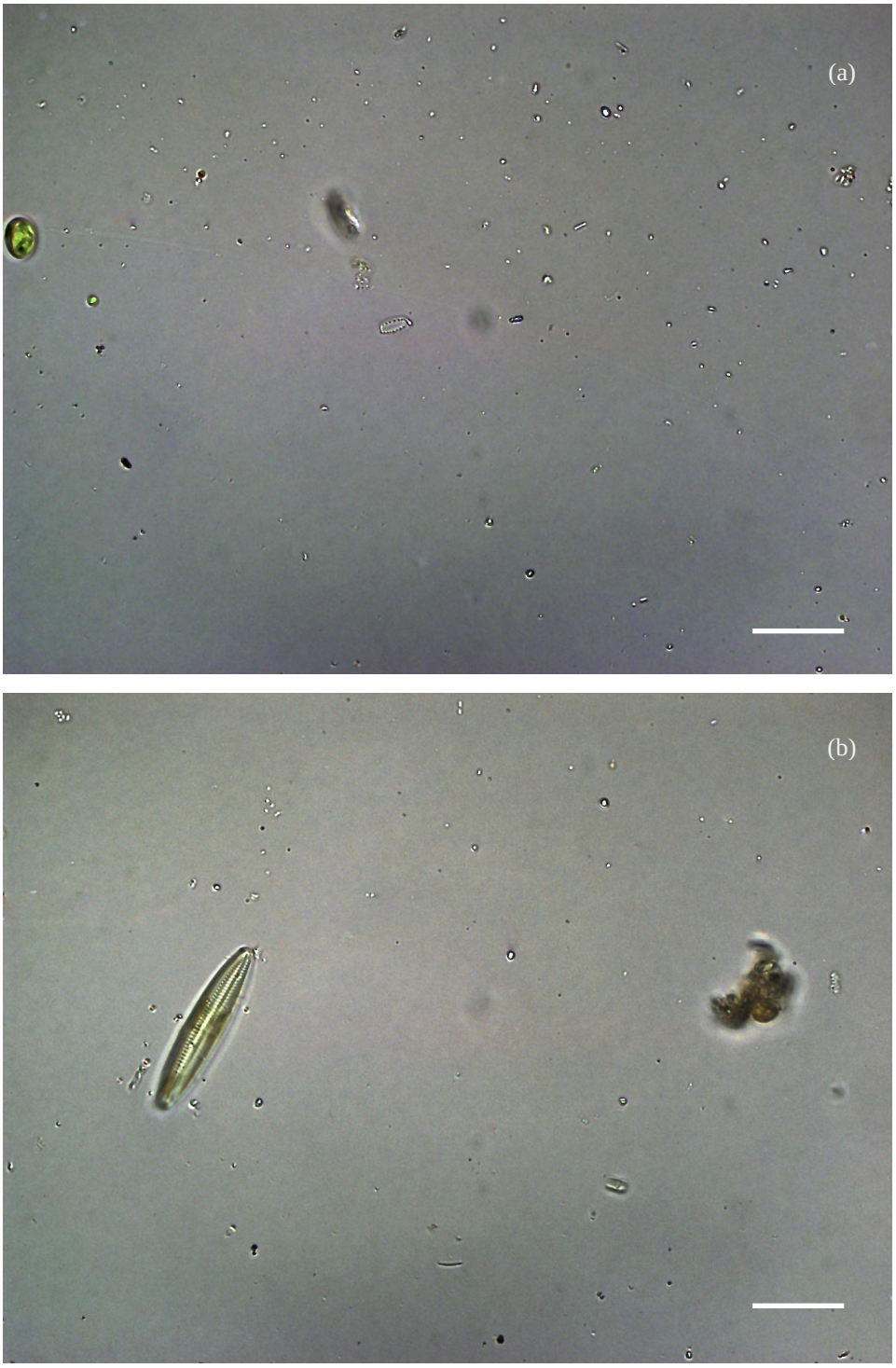


Figure 4.26. Bozeman Creek raw sample immobilized on (a) anti LPS O157 and (b) anti-CFA/I (the scale bar is 20 μm).

Representative images were given in Figure 4.26. Cell density was not as rich as immobilized pure cultures and sizes of most the cells were so small that they were barely visible under the microscope with 60x objective lens. This can be explained on the basis that the microorganisms were surviving under nutrient-poor and stressful conditions at low temperatures and they did not have enough food. Hence, they shrunk in size. Accurate cell counting could not be performed due to the lack of visibility.

Bozeman Creek and Alibeyköy Dam samples were pre-concentrated using biyotrap and immunoimmobilized on anti-LPS O157 and anti-CFA/I modified gold chips. Representative images were given in Figure 4.27. Samples were perfectly immobilized on anti-LPS O157 chips, fulfilling the antibody spot. Glass fibers from the BiyoTrap were also attached on the surface. This can be based on two theories: (i) the bacteria trapped on the surface of the glass fibers mediate their transfer onto the antibody spot, (ii) glass fibers are modified with positively charged amine groups which may naturally react with antibody.

In order to understand the attachment mechanism of glass fibers, 500 ml of nanopure water was filtered through the BiyoTrap, resuspended in 500 μl of BiyoTrap effluent and incubated on anti-LPSO157 and anti-CFA/I modified chips. It was observed that the glass fibers did not have a strong tendency to attach to the antibody spot.

In contrast to the immobilization efficiency on anti-LPS O157, cell density of biyotrapped samples immobilized on anti-CFA/I, were as low as cell density of immunoimmobilized raw samples. This case does not mean that Biyotrap did not have a positive effect on immobilization efficiency. BiyoTrap is a nonspecific concentration approach while antibody-antigen interactions are highly specific. Cells were expected to attach to anti-CFA/I through their fimbriae. However, bacteria may not synthesize fimbriae under stress conditions, which may lead to poor immobilization of biyotrapped field samples on anti-CFA/I chips. This is proved by an experiment where Bozeman Creek samples were spiked with ~ 4 x 10^4 cfu/ml ETEC H10407 RFP. Since ETEC H10407 RFP is capable of synthesizing red fluorescent protein, the handicap of staining other cells in the sample using DAPI or SYTO®9 was eliminated.

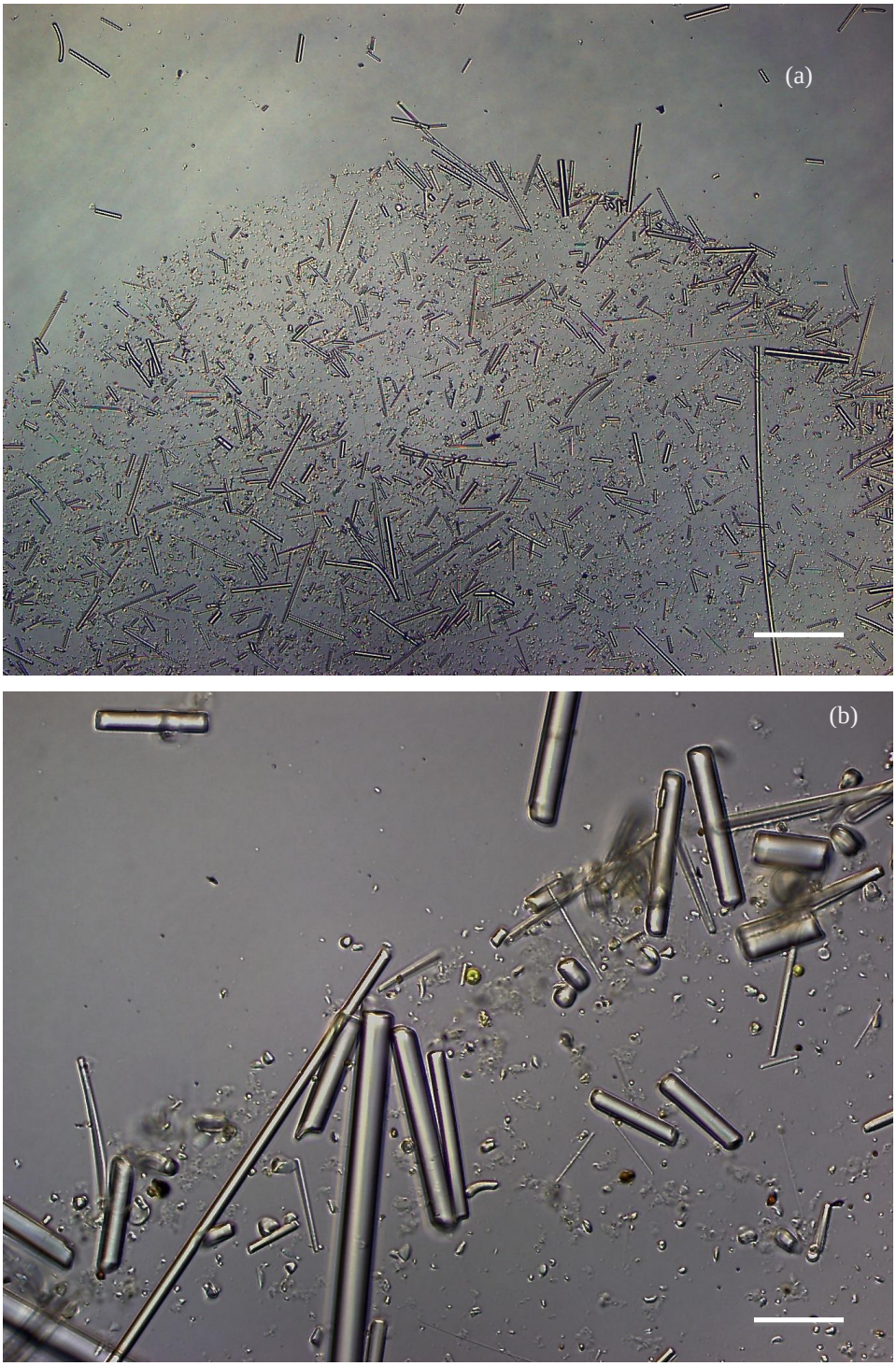


Figure 4.27. Bozeman Creek sample biyotrapped and immunoimmobilized on anti-LPS O157 (the scale bar is (a) 60 μm and (b) 20 μm).

In Figure 4.28, (a) bright field and (b) fluorescence images Bozeman Creek sample spiked with ~ $4x10^4$ cfu/ml ETEC H10407 RFP, biyotrapped and immunoimmobilized on anti-CFA/I is given.

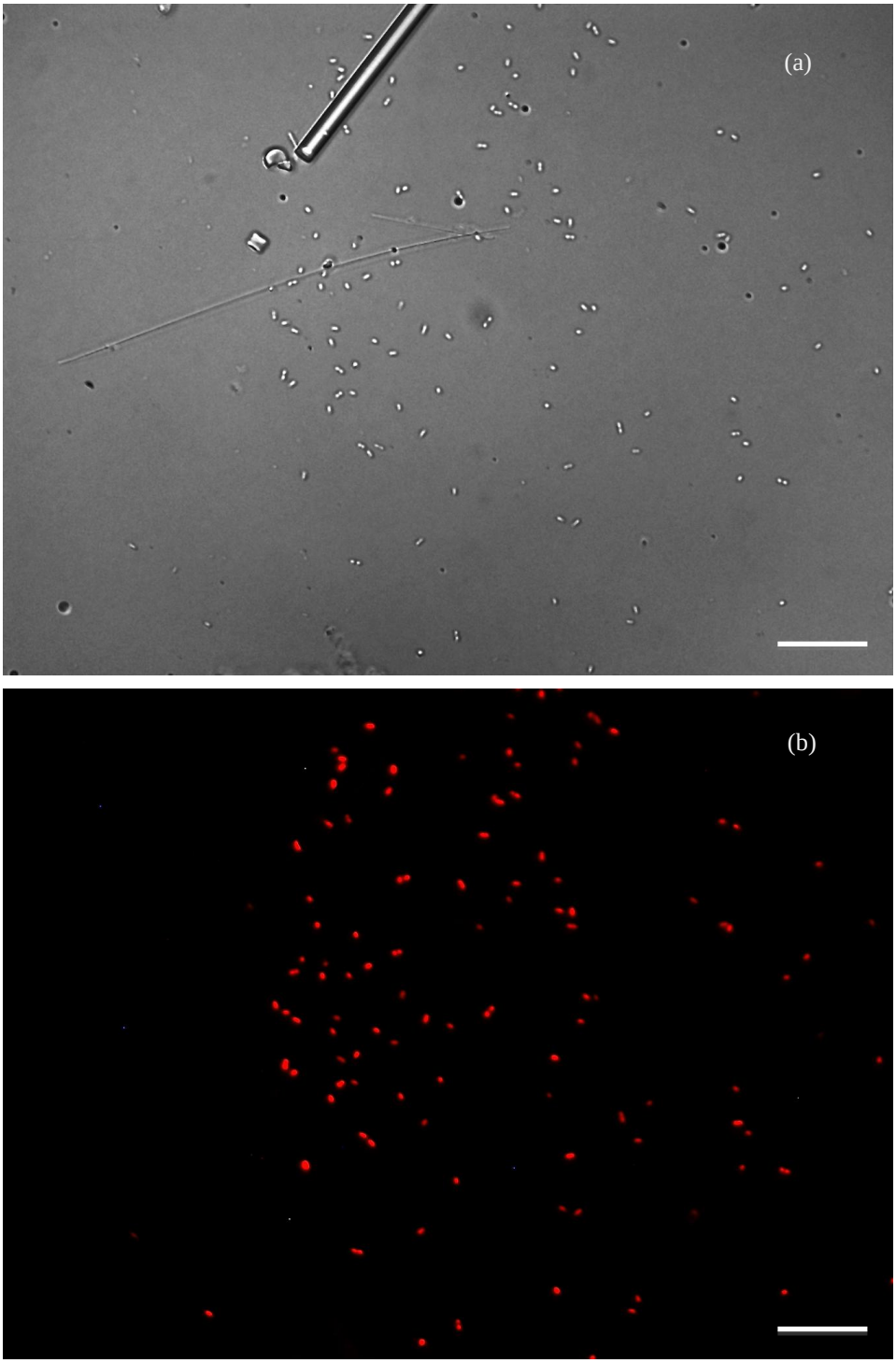


Figure 4.28. Biyotrapped and immunoimmobilized sample on anti CFA/I (a) Bright field and (b) fluorescence images of Bozeman Creek sample spiked with ~ $4x10^4$ cfu/ml ETEC H10407 RFP (the scale bar is 20 μm).

It was observed that almost all of the immobilized cells were fluorescing which indicates that they are ETEC H10407 RFP strain. In addition, there is a clear border of the antibody spot filled with ETEC H10407 RFP. A few, very tiny cells that do not fluoresce were available on the surface which explains the scarcity of cells having CFA/I fimbriae.

This experiment also proves the impact of BiyoTrap® on immobilization efficiency. Referring to the limit of detection experiments, it was found that the antibody modified surface was capable of immobilizing maximum 2 cells out of ~4 x 10^4 cfu/ml. In this experiment, capturing the cells in BiyoTrap boosted the limit of detection up to 115 cells out of ~4 x 10^4 cfu/ml. This is a significant capability of BiyoTrap, which makes it an essential tool for concentrating low number of microorganisms in field applications.

5. CONCLUSIONS

In the research for this dissertation (1) the bacterial diversity in İstanbul's drinking water reservoirs was investigated and correlated with chemical characterization of the water and (2) an efficient immunoimmobilization-based detection method was developed for the detection of pathogenic bacteria. The main findings of the study are as follows:

Physicochemical characterization of water samples taken from raw water intake structures of drinking water treatment plants in İstanbul showed that:

- The pH, conductivity, TDS, salinity and DOC were in the ranges required by regulations. The pH of water samples was in the range of 6.2-7.8. Conductivity was between 0.4 and 0.8 mS/cm. TDS values were measured in the range of 200-450 ppm. Salinity was not recorded.

- Cl^- and SO_4^{2-}, PO_4^{3-}, were the most abundant anions in sampled reservoirs. The levels of PO_4^{3-} were close to and higher than the limit of 0.7 mg/l for A2 level water quality.

- Na^+, K^+, Ca^{2+} and Mg^{2+} concentrations were measured in the ranges of 0.3 – 28 mg/l, 0.2 - 7 mg/l, 13 - 84 mg/l and 1.9 - 26 mg/l, respectively.

- Heavy metal concentrations were mostly within the desired range of regulation of TR QSWDW. Peak measurements of Ni exceeding the limit value of 0.005 mg/l were observed. Ni measurements in Büyükçekmece, Darlık and Ömerli 1 2010 samples were 0.0279, 0.0223 and 0.0247 mg/l respectively. Co in Alibeyköy, Elmalı and Darlık 2010 samples were also above the regulation limit of 0.002 mg/l. Measured Co concentrations for Alibeyköy, Elmalı and Darlık 2010 samples were 0.006, 0.009 and 0.010 mg/l respectively. Cd and Cr concentrations were measured as 0.001 and 0.047 mg/l in Sazlıdere 2013 and Sazlıdere 2010 samples respectively, which were close to the regulation limits.

Characterization using DGGE, sequencing and qPCR of microbial community structure in drinking water sources, including a correlation of diversity and the chemical composition of the water samples (revealed by principal component analysis), showed that

- The water reservoirs had a diverse population of bacteria. A total of 66 OTUs were present in the water samples taken from all DWTPs in 2010, 2011 and 2013.

- The species mostly belonged to uncultured bacteria that were previously observed in aquatic environments. (Forty-five bands were excised from DGGE gels, and 38 of them were sequenced.)

- Forty percent of the variability of DGGE bands was represented by the first two components. In correlation analysis, the changes in bacterial diversity were found to be parallel to the changes in Cr, Zn, Co, F^-, and NO_3^-.

- The presence of the LT gene representing ETEC in the qPCR results was observed in all of the samples in the range of 5.37 to 6.74 x 10^{11} gene copy number/25 ng DNA. STEC was detected via the *stx* gene in the range of 2.92 x 10^1 to 1.72 x 10^4 gene copy number/25 ng DNA. *Legionella* was detected in the range of 1.95 x10^3 – 1.48x10^6 gene copy number/25 ng DNA. *Salmonella* was detected in Büyükçekmece, Emirli, Ömerli and Terkos samples with a quantity of 6 to 1.3 x 10^6 gene copy number/25 ng DNA.

Surface-sensitive XPS was used to determine the bacteria immobilization efficiency vs. the solution concentration of antibodies preselected to be tethered to a gold-coated flat surface by monitoring the area under the N 1s peak, which is proportional to the density of the covalently tethered antibodies on the surface. These studies revealed that

- The minimum solution concentration of antibodies for the effective immobilization of bacteria is ~4 μg/ml. Saturation of the surface concentration of an antibody was observed at ~8 μg/ml, above which a larger antibody concentration in solution did not increase the N 1s signal. While the N 1s signal vs. the solution concentration of antibody behaved like an Langmuir isotherm

saturation, the corresponding immobilized bacterial density behaved like a step function in that the density of the bacteria immobilized on the surface jumped from a negligible value to nearly full saturation at an antibody concentration of ~4 μg/ml; above this value the density of immobilized bacteria also behaved like a Langmuir isotherm. This was interpreted as indicating that it takes a minimum number of antibody-antigen interactions to immobilize a bacterium on a surface: below this minimum very few bacteria are immobilized on the surface, and above this value the immobilization is proportional to the density of the antibodies tethered to the surface.

Studies conducted on the immunoimmobilization of various *E. coli* strains using anti-fimbrial and anti-LPS antibodies as well as on the cross-reactivity of various antigen-antibody pairs (revealed by microarray technology) showed that :

- The pure cultures of *E. coli* strains, i.e. *E. coli* 263, *E. coli* 1836-2, *E. coli* 3030-2, ETEC H10407 and EHEC O157:H7, were efficiently immobilized on anti-fimbrial and anti-LPS antibodies. An average of 4000 cells out of 4 x 10^7 cfu/ml pure culture were captured on a (170 μm)^2 area. The minimum detection limit of the immobilization method was determined to be ~10^3 to 10^4 cfu/ml.

- Combinations of ETEC H10407 (expressing RFP), EHEC O157:H7 (post-stained with SYTO9®), *E. coli* 1836-2 (post-stained with DAPI) and *E. coli* 3030-2 (expressing GFP) were successfully sorted on the same microarray using anti-CFA/I anti-LPS-O157, anti-K88ab and anti-K88ac antibodies, respectively. *E. coli* 1836-2 and *E. coli* 3030-2 were cross-reactive against anti-K88ac and anti-K88ab antibodies. Therefore, they were not immobilized on the same microarray. Cells were distinguished by their fluorescence characteristics via either the expression of red or green fluorescent proteins (RFP and GFP) or post-staining with DNA dyes such as DAPI or SYTO9®.

- As confirmed in microarray experiments, immunoimmobilization is a sensitive method that can discriminate among strains of the same species of bacteria provided that specific antigen-antibody pairs are selected.

The application of immunoimmobilization to capture environmental bacteria on antibody-modified chips indicated that it is necessary to pre-concentrate organisms before immunoimmobilization technology can be useful. Studies revealed that:

- The pre-concentration of environmental samples using BiyoTrap® technology considerably enhances the immobilization of target bacteria on antibody-modified chips. In addition, the limit of detection is improved, from 10^4 to 10^2 cfu/ml, by using BiyoTrap®. In principle, this limit can be improved even further.

- Fimbriae and LPS are equally efficient targets for the immobilization of pure cultures. However, LPS is a better target for the immobilization of environmental samples because pili/fimbriae production may not be favored under stress conditions.

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