Molecular Biology Techniques Applied to Food Industry Water Surveillance

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Zusammenfassung

Mit Hilfe molekularbiologischer und damit kultivierungsunabhängiger Methoden wurden pathogene Bakterien in Trinkwasser an hygienekritischen Kontrollpunkten entlang der Fertigungsstrecke eines deutschen Molkereiunternehmens und eines spanischen Betriebes für Rohschinken nachgewiesen. Mit der denaturierenden Gradienten - Gelelektrophorese (DGGE) konnten Veränderungen in der bakteriellen Population beschrieben werden, welche die biologische Instabilität in Trinkwasser und in Biofilmpopulationen aufzeigen. Autochthone Bakterien konnten durch Sequenzierung von DNA - Banden aus DGGE - Gelen identifiziert werden. Für genauere Untersuchungen wurden PCR und qPCR eingesetzt, um eine Anzahl pathogener Bakterien (d.h. *Listeria monocytogenes*, *Mycobacterium avium* subsp. *paratuberculosis*, *Campylobacter jejuni*, *Enterococcus* spp., *Salmonella* spp., *Escherichia coli*, und *Pseudomonas aeruginosa*) nachweisen zu können.

Eine spezifische Strategie wurde entwickelt, um hygienekritische Kontrollpunkte in den Lebensmittelbetrieben zu ermitteln zu können, an denen die technischen Voraussetzungen für Nachweise und die Erfassung und Vermeidung unerwünschter Polymerase - Inhibitoren betrachtet wurden.

Die Populationen autochthoner Bakterien an den meisten Trinkwasser-Kontrollpunkten stellten sich als äußerst stabil heraus. Nur ein Kontrollpunkt des deutschen Molkereiunternehmens zeigte Veränderungen in der Population. Enterokokken und *Pseudomonas aeruginosa* konnten in einigen Wasserproben dieser Unternehmen mit molekularbiologischen Methoden nachgewiesen werden, nicht jedoch mit den herkömmlichen Kultivierungsmethoden. Einige opportunistische Bakterien, wie *Enterobacter* sp., *Acinetobacter*, *Sphingomonas* sp. und apathogene Bacillus - Arten, wurden durch Sequenzierung von DNA - Banden aus DGGE - Gelen identifiziert. In dem spanischen Rohschinken - Unternehmen wurden keine Populationsverschiebungen gefunden, jedoch wurde *P. aeruginosa* - DNA im Trinkwasser - und Biofilm - Proben detektiert.

DNA - basierte Methoden, die für den Nachweis und die Charakterisierung von Bakterien in Trinkwasser und in Trinkwasserbiofilmen angewandt wurden, können nicht zwischen DNA von lebenden und toten Zellen unterscheiden. Eine Reihe kultivierungsun-

abhängiger Methoden wurden erprobt, um dieses Problem zu lösen.

Es wurden Behandlungen der Proben mit Desoxyribonuclease I (DNase I) oder Propidiummonoazid (PMA) vor der Untersuchung mit DNA - basierten Methoden getestet, optimiert und verglichen, um lebende von toten Bakterien in Trinkwasser und in Biofilmen unterscheiden zu können.

Die Vorbehandlung mit Desoxyribonuclease I / Proteinase K (DNase/PK) wurde für den Verdau von freier DNA und DNA von toten Zellen mit geschädigten Zellmembranen optimiert. Da diese Methode für den Nachweis von Bakterien im Trinkwasser verwendet werden soll, wurden verschiedene Membranfilter zur Aufkonzentrierung der Biomasse aus den Wasserproben getestet. Untersucht wurde, ob die Membranfilter die DNase/PK - Behandlung in irgendeiner Weise beeinflussen.

Nachdem die DNase/PK - Methode etabliert war, wurde sie mit lebenden und toten Zellen und mit freier DNA getestet. Dafür wurde eine Mischung aus lebenden Zellen von *S. aureus*, toten Zellen von *P. aeruginosa* und genomischer DNA von *S. enterica* hergestellt. Aliquots dieser Mischung wurden vorbehandelt und anschließend untersucht, um die verschiedenen Vorgehen zu vergleichen. Die Populationsanalysen der Bakterien wurde mit Hilfe der PCR - DGGE durchgeführt um die Proben ohne Vorbehandlung (Gesamt - DNA) und mit Vorbehandlung durch DNase/PK oder PMA (DNA lebender Zellen) zu vergleichen. Kultivierungsmethoden, quantitative PCR mit Sybr Green und 5 - Cyano - 2,3 - Ditoryltetrazoliumchlorid (CTC)/4' - 6 - Diamidin - 2 - Phenylindol (DAPI) - Färbung wurden angewandt, um die Fähigkeit dieser Behandlungen zu verifizieren, dass ausschließlich DNA von lebenden Zellen nachgewiesen werden kann.

Im nächsten Schritt wurden die vershiedenen physiologischen Stadien von Bakterien aus natürlichen Trinkwasserbiofilmen einer Pilotanlage in einem Wasserwerk bestimmt. Veränderungen im DNA - Muster, welche nach einer DGGE - Analyse sichtbar wurden, zeigten: (i) die Anwendbarkeit der Behandlung von PMA und DNase/PK bei der Untersuchung natürlicher Biofilme; (ii) dass der Nachweis von DNA toter Bakterien und extrazellulärer DNA durch die Vorbehandlung mit PMA oder DNase/PK erfolgreich unterbunden wird; und (iii) dass eine Behandlung mit DNase/PK eine deutlichere Auswirkung auf die Unterscheidung von lebend und tot hat, aufgrund der gleichmäßigen Wirkung des Enzyms und durch das Wegfallen von Waschschritten während des Vorgehens.

Diese Arbeit fasst in einer Diskussion die verschiedenen Methoden zusammen, die für den Nachweis möglicher hygienekritischer Kontrollpunkte verwendet wurden, einschließlich spezifischer Nachweise für Pathogene in Wasser - und Biofilm - Proben und

Veränderungen bakterieller Populationen der ausgewählten Kontrollpunkte innerhalb eines Lebensmittelbetriebes. Einige mögliche zukünftige Anwendungen wurden im Ausblick beschrieben.

Abstract

Culture - independent techniques were applied and optimized for the detection of pathogenic bacteria in drinking water at potentially critical control points along the production lines at a German dairy company and at a Spanish dry cured ham company. Denaturing gradient gel electrophoresis (DGGE) was used to describe bacterial population shifts indicating biological instability in drinking water and biofilm samples. Autochthonous bacteria were identified by sequencing the DNA bands excised from the DGGE gels. More specifically, polymerase chain reaction (PCR) and quantitative PCR (qPCR) were applied to detect a number of pathogenic bacteria, i.e. *Listeria monocytogenes*, *Mycobacterium avium* subsp. *paratuberculosis*, *Campylobacter jejuni*, *Enterococcus* spp., *Salmonella* spp., *Escherichia coli*, and *Pseudomonas aeruginosa*.

A specific strategy was established for the detection of possible water - derived critical control points at the food companies, where the technical detection requirements and the occurrence of unwanted polymerase inhibitions were contemplated.

Autochthonous bacterial populations were found to be highly stable at most of the drinking water sampling points. Only one sampling point exhibited population shifts at the German dairy company at the first sampling period. Enterococci and *Pseudomonas aeruginosa* were detected in some water samples from these companies by molecular biology detection methods, but not by conventional culturing methods. Some opportunistic bacteria as *Enterobacter* sp., *Acinetobacter*, *Sphingomonas* sp. and non - pathogenic *Bacillus*, were also detected after DNA sequencing of DGGE bands. No population shifts were found at the Spanish dry cured ham company, but DNA of *P. aeruginosa* was present in the drinking water and drinking water biofilm samples.

DNA - based methods were used for the detection and characterization of bacteria in drinking water and in drinking water biofilms. They cannot distinguish between DNA from live and dead cells. Further culture - independent methods were tested to face this problematic.

Treatments of the samples with deoxyribonuclease I (DNase I) or propidium monoazide

(PMA) before their analysis with DNA - based methods were tested, optimized and compared in this work. The inactivation of DNase I gained a great importance in the treatment. After testing different inactivation procedures, DNase I was finally inactivated with proteinase K. These treatments were used in order to detect and analyze only live bacteria in drinking water and biofilm samples.

The Deoxyribonuclease I/Proteinase K (DNase/PK) treatment was optimized for the digestion of free DNA and DNA from dead cells with injured cell membranes. Due to the fact that this technique should be used for the detection of live bacteria present in drinking water, this protocol was tested in the presence of different filter membranes to investigate if the filter membranes used for the concentration of biomass present in the water samples altered anyhow the DNase/PK treatment.

Once the DNase/PK protocol was established a test was done with live and dead bacteria and free DNA. For this, defined mixtures of live *S. aureus*, dead *P. aeruginosa* and genomic DNA of *S. enterica* were mixed in a sample. Aliquots of this sample were treated and then analyzed to compare the different procedures. Bacterial population analysis was done by PCR - DGGE, comparing samples without treatment (total DNA) and samples treated with DNase/PK or propidium monoazide (DNA from live cells). Cultivation methods, Sybr Green quantitative qPCR, and 5 - cyano - 2,3 - ditoryl tetrazolium chloride (CTC)/4' - 6 - diamidino - 2 - phenylindole (DAPI) staining were used to verify the ability of the treatments to detect only DNA from live cells. This experiment demonstrated the usefulness of the DNase/PK method.

The different physiological stages of the bacteria present in natural drinking water biofilm samples from a pilot scale built up at a waterworks were analyzed. Shifts in the DNA patterns observed after DGGE analysis, demonstrated: (i) the applicability of PMA and DNase/PK treatment in natural biofilm investigation; (ii) the detection of DNA from dead bacteria and extracellular DNA (eDNA) could be successfully blocked by treatment with PMA or DNase/PK; and (iii) DNase/PK treatment demonstrated a clearer effect on live/dead differentiation due to a more homogeneous effect of the enzyme and to the absence of washing steps in the procedure.

This work concludes with a discussion about the different methods that were used for the detection of possible water - derived critical control points, including specific pathogen detection in water and biofilm samples, and bacterial population shifts of the chosen sampling points within a food company. Some possible future applications were described in the outlook.

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Abbreviations

A adenin

ANV amphotericin B, nalidixic acid and vancomycin

ATCC American type culture collection

BHI Brain heart infusion

bp base pair

BSA bovine serum albumin

C cytosine

CCPs critical control points

CFU colony forming units

C_T cycle threshold

CTC 5 - cyano - 2,3 - ditoryl tetrazolium chloride

DAPI 4' - 6 - diamidino - 2 - phenylindole

ddNTPs dideoxyribonucleotide triphosphate

DGGE denaturing gradient gel electrophoresis

DNA deoxyribonucleic acid

DNase I deoxyribonuclease I

DNase/PK Deoxyribonuclease I/Proteinase K

dNTPs deoxyribonucleotide triphosphate

dsDNA double - stranded DNA

DSMZ German collection of microorganisms and cell cultures

eDNA extracellular DNA

EDTA Ethylenediaminetetraacetic acid

EPS extracellular polymeric substance

EMA ethidium monoazide

FAM carboxyfluorescein

G guanine

HACCP Hazard analysis and critical control points

HPC Heterotrophic plate count

MAP *Mycobacterium avium* subsp. *paratuberculosis*

ME mixed cellulose ester

MF - HPC Membrane filtration - heterotrophic plate count

mRNA messenger ribonucleic acid

NTC no template control

PC polycarbonate

PCR polymerase chain reaction

PI propidium iodide

PMA propidium monoazide

PVC polyvinyl chloride

PVPP polyvinylpolypyrrolidone

qPCR quantitative PCR

R2A Reasoner's 2A agar

rDNA ribosomal DNA

RNA ribonucleic acid

ssDNA single stranded DNA

T thymine

TAE buffer solution containing tris base, acetic acid and EDTA

TAMRA Carboxytetramethylrhodamine

Tris base tris (hydroxymethyl) aminomethane

Tris - HCl solution of Tris base and concentrated hydrochloric acid

U Unit

US - NASA United States of America - National Aeronautics and Space Administration

UV Ultraviolet

VBNC Viable but non - culturable

1. Introduction

1.1. Motivation

Food safety is of fundamental importance worldwide. Despite significant investment the incidence of food derived disease still increases. The European Union food hygiene guidelines, according to the principles of the Hazard analysis and critical control points (HACCP), stipulate the introduction of self - control systems for food companies. The HACCP concept confers an important contribution to consumer's health protection by controlling the production, treatment, processing, transport, storage and sale of food. According to the international definition this concept aims to identify important possible health threaten dangers, these dangers are then analyzed, their occurrence are determined and the importance for health is measured. Finally, critical control points during the food production process are specified, steps in the process that could carry a danger are avoided or reduced to an acceptable level.

Drinking water coming from public suppliers is not sterile, but contains a number of autochthonous and mostly harmless bacteria (Szewzyk et al., 2000; WHO, 2004a). Process water is used for many purposes in the food industry, i.e., as an ingredient, as part of the manufacturing process and in direct contact with the foodstuff, or in any indirect contact with the food product (Casani and Knøchel, 2002). Pathogenic or opportunistic bacteria may enter drinking water facilities under irregular operating conditions. In this case, some of these bacteria are able to persist and distribute across the production lines at food companies (Allen et al., 2004; USEPA, 1992). Various scenarios may influence microbial drinking water quality, e.g. rupture of pipelines, water stagnation, pipeline material, etc. (Bartram et al., 2004b).

According to the Drinking Water Directive 98/83/EC (EU Council decision, 1998) of the European Union, indicator microorganisms should be routinely monitored in drinking water in order to control microbial water quality of public distribution systems. The standard detection method described in these guidelines is the conventional plating on

defined media. In the last decades, the scientific community has accepted that culture-dependent methods do not reflect the real number of bacteria present in a sample. Viable but non-culturable (VBNC) or injured bacteria fail to grow on the routine bacteriological media, but are alive and metabolically active (Oliver, 2000). Only bacteria capable of growing on culture media will be detected, therefore false negative results might be obtained when traditional plating methods are used.

Additionally, molecular biology DNA-based methods detect total DNA present in the sample without discriminating DNA from live or dead cells. It is considered that only live bacteria represent a risk for the food industry, therefore the established methods for water surveillance of food companies should be able to detect only DNA from live cells.

1.2. Objectives

The purposes of this work were:

1. Application of culture-independent techniques for the quantification of different hygienic relevant bacteria in drinking water at food companies.
2. Establishment of a strategy based on culture-independent techniques to look for possible water-derived critical control points in production lines at food companies.
3. Development of culture-independent techniques able to discriminate live bacteria from dead bacteria in drinking water and in drinking water pipeline biofilms.

1.3. Overview of the Thesis

This work is organized as follows:

Chapter 1: The motivation and importance of studying the drinking water and drinking water facilities used in food companies under the HACCP concept are briefly described. Then, the main objectives to be accomplished at the end of the work, are mentioned.

Chapter 2: Relevant concepts about water surveillance as quality control parameter used at food companies are described. Then, an overview of the state of the art of the methodologies used for monitoring of pathogens in drinking water is presented.

Chapter 3: The material and methods used in the present work are described.

Chapter 4: The results of the sensitivity of the polymerase chain reaction (PCR), semi-nested PCR and quantitative PCR tests are shown. The protocol developed to detect and remove PCR inhibitors is described here. The analysis of drinking water systems of a German dairy and a Spanish dry cured ham company are shown. A toolbox used for the determination and analysis of the live bacterial fraction of samples is shown, giving special attention to the DNase I treatment procedure. An optimized DNase/PK treatment protocol is here exposed. DNase/PK and PMA treatments of drinking water samples after deoxyribonucleic acid (DNA) quantification are compared and evaluated. Finally, the results of the application of this toolbox for the analysis of drinking water pipeline biofilm samples of waterworks are presented.

Chapter 5: A summary of the results is here presented.

Chapter 6: A discussion about the methods used in this work to determine pathogens and bacterial population shifts in drinking water in order to perform a quality risk assessment of the water used in the production lines at food companies is done here.

Chapter 7: Final conclusions and a brief outlook for future works conclude this study.

2. Background and State of the Art

2.1. Hazard Analysis and Critical Control Points

Hazard analysis and critical control points (HACCP) is a preventive system used by the food industry to help ensure food safety. This concept started in 1959 when the United States of America - National Aeronautics and Space Administration (US - NASA) began a project in order to produce safe food for persons in space. The presence of hazardous elements in foods as contaminants, pathogenic microorganisms, objects, and chemicals could be controlled by this system.

The HACCP system consists of the following seven principles:

1. Hazard analysis: identification of hazards that must be prevented, eliminated or reduced to acceptable levels;
2. Determination of critical control points (CCPs): the identification of CCPs at the steps in a process where control is essential to prevent or eliminate a hazard or to reduce it to acceptable levels;
3. Establishment of critical limits: these limits separate acceptability from unacceptability for the prevention, elimination or reduction of identified hazards;
4. Establishment and implementation of effective monitoring procedures at CCPs;
5. Establishment of corrective actions when monitoring indicates that a CCP is not under control;
6. Establishment of procedures to verify that the HACCP system is working effectively;
7. Appropriate documentation of procedures and records to demonstrate the effective application of the already named measures.

HACCP is a tool used to assess hazards and to establish control systems that are

focused on prevention rather than testing the quality and safety of end-products (EU, 2005).

The success of HACCP in controlling hazards in food processing establishments led consumers and regulators to apply HACCP from "farm to table" and eventually raised the expectations of having pathogen-free foods. But such expectations are utopic, in view of the fact that the definitive process controls that can be applied by food processors cannot be applied at the "farm" and "table" ends of the food supply chain. Nevertheless, the food companies have to be able to assure safe food for the consumers.

Due to the high use and relevance of water in food companies, water was analyzed in this work to evaluate if it should be considered as a possible critical control point at two food companies.

2.2. Hygienic Relevant Bacteria in Drinking Water

Water has always had a substantial impact on public health. During the 20th century, public drinking water supplies have achieved great technological improvements, diminishing enormously waterborne diseases. Despite the fact that nowadays the access to safe drinking water is considered a human right, the lack of safe drinking water supplies still is a menace especially in developing countries (Ashbolt, 2004; Gleick, 2000; OECD and WHO, 2003).

Normally, harmless bacteria are present in drinking water. But, some microorganisms represent a serious risk for disease whenever present in it, being designated as pathogens. Pathogens of moderate priority include opportunistic pathogens such as *Pseudomonas aeruginosa* and *Aeromonas* sp. Opportunistic bacteria may cause disease in subjects with low immunity, may be primarily transmitted by contact or inhalation (rather than ingestion) such as *Legionella*, or may be responsible for occasional outbreaks or found exclusively in some regions.

Pathogens may enter the distribution system either through the source water or at any point within the distribution system (Rajal et al., 2010). In the network microorganisms may survive and even exhibit metabolic activity in biofilms on the surfaces of stagnant parts of piped distribution systems, domestic plumbing, reservoirs, and in plumbed-in devices as softeners and carbon filters (Bartram et al., 2004b; Schwartz et al., 2009).

Infectious agents associated to drinking water may be classified within four broad

groups: bacteria, viruses, protozoa and helminths or parasitic worms. These infectious agents derive principally from infected persons and other warm-blooded animals, and the diseases associated to these agents are primarily transmitted through human and animals excreta. Some examples of human pathogens transmitted by drinking water are listed in Table 2.1 together with a summary on their degree of pathogenicity, mode of transmission, infective dose, reservoir and other relevant sources, and persistence in water and/or water drinking supplies.

Dose-response is an important issue, it is going to vary depending on the pathogen and on the host and it is also affected by many factors (OECD and WHO, 2003; Szewzyk et al., 2000). Pathogenic bacteria and parasites normally lose viability and the ability to infect after leaving their host. Therefore, most of the microorganisms are not expected to stay infectious in water, and some will disappear over time since they are unable to multiply in these conditions. But, some conditions can promote regrowth of bacteria in distribution systems. Re-growth of bacteria in drinking distribution systems can affect the water quality ranging from taste and odor characteristics to true health threats as re-growth of pathogens. The principal determinants of regrowth are temperature, nutrients availability, residence time of water in the distribution system, physical-chemical characteristics of pipeline materials, and lack of residual disinfectant (LeChevallier et al., 1991; Niquette et al., 2001; Obst and Schwartz, 2007). Some species of *Pseudomonas*, *Aeromonas* and *Serratia* may even multiply in drinking water. It is important to notice that waterborne bacteria, in contrast to viruses, parasites and prions, are capable of multiplying rapidly when introduced to foodstuffs. This increases their inoculum's potential enormously and makes even initially low and non-infectious doses of bacterial pathogens a hazard in food production (Casani and Knøchel, 2002).

In this work, some hygienic relevant bacteria were specifically monitored in water of food companies. A short description of these bacteria and of their hygienic relevance in drinking water is named below.

Listeria monocytogenes: are Gram positive bacteria that can cause human and animal life-threatening infections. Immunocompromised people, pregnant women, old individuals and neonates present a high risk for listeriosis. *Listeria* are unlikely to grow in low nutrient conditions as drinking water, but their incidence increases in water exposed to animal and human activity, as polluted water and sewage/sludge due to their high nutrient condition. It is known that *Listeria* has to tolerate various external stresses to survive in the environment. For example, they can survive freezing temperatures during winter and extreme outdoor heat in summer in river

water and sludge, while in food processing environments they can survive the exposure to alkaline detergents and sanitizers (Kathariou, 2003). Listeriosis outbreaks have been associated with the consumption of ready-to-eat foods, especially meat and dairy products, being uncooked or processed food the most common cause of infection (Kathariou, 2002). *L. monocytogenes* readily adheres to food processing surfaces as benches, machineries, and floors, subsequently growing in biofilms with increased resistance to adverse conditions. Apparently this bacterium is extremely agile in its response to stress situations as extreme pH, temperature and osmotic media (Chmielewski and Frank, 2003; Kathariou, 2003; Møretrø and Langsrud, 2004; Pan et al., 2006). *L. monocytogenes* can form biofilms on stainless steel, plastic, and polycarbonate surfaces, and can coexist with *Salmonella* and other pathogens (Chmielewski and Frank, 2003).

***Mycobacterium avium* subsp. *paratuberculosis*:** are facultative anaerobic acid - alcohol resistant obligate zoonotic pathogens that cause Johne's disease, a chronic intestinal infection in ruminants (Pavlik et al., 2000). Animals with paratuberculosis shed viable *Mycobacterium avium* subsp. *paratuberculosis* (MAP) especially in their milk, feces and semen (O'Brien et al., 2006). This microorganism has also been implicated to cause similar type of enteritis in humans called Crohn's disease (Pickup et al., 2005). Recently it has been postulated that MAP has an occult antigen which besides Crohn's disease could as well be thought to trigger type-1 diabetes mellitus (Rani et al., 2010). It is probable that under the presence of certain concentrations, time and duration of exposition to bacterial triggers, as the use of contaminated baby food (Hruska et al., 2005) and water during the first weeks after birth, could provoke autoimmune and autoinflammatory diseases which would appear many years later, though this theory has not yet been straightforward experimentally proved. The natural reservoirs of these bacteria are wild animal populations, being manure from infected animals the most common contamination source (Pavlik et al., 2000). The most likely vehicles of transmission of MAP from animals to humans are milk and dairy products, beef, and water (Grant, 2006). MAP has been described in the past years as a new emergent foodborne pathogen. These have been detected in pasteurized milk, powdered infant milk and dairy products (Ayele et al., 2005; Hruska et al., 2005; Ikonomopoulos et al., 2005; Khare et al., 2004). Pickup et al. (2005) described that this microorganism remained culturable in lake water microcosmos for 632 days and persisted up to 841 days. MAP have been found in drinking water distribution systems (Vaerewijck et al., 2005). The relevance of MAP in drinking water is due to its high capacity

of surviving heat and chlorination treatments (Hruska et al., 2005). Vicuña-Reyes et al. (2008) recently described that *Mycobacterium avium* could be controlled by treating the water with chlorine dioxide providing a sufficient contact time. But the authors did not specifically test MAP.

***Campylobacter jejuni*:** are Gram negative spiral-shaped bacteria normally found in warm-blooded animals. *C. jejuni* and *C. coli* have been described as the most common *Campylobacter* species implicated in human disease and are generally regarded as the most common bacterial cause of gastroenteritis worldwide. In developed and developing countries, they cause more cases of diarrhea than, for example, foodborne *Salmonella*. In developing countries, *Campylobacter* infections in children under the age of two years are especially frequent, sometimes resulting in death. In almost all developed countries, the incidence of human *Campylobacter* infections has been steadily increasing for several years. The reasons for this are unknown. Disease-causing bacteria generally get into people via contaminated food, often undercooked or poorly handled poultry, although contact with contaminated drinking water or ice, livestock, or household pets can also cause disease (WHO, 2000). Contaminated drinking-water supplies have also been identified as a source of outbreaks, as a consequence of unchlorinated or inadequately chlorinated surface water supplies and fecal contamination of water storage reservoirs by wild birds (WHO, 2008). Federighi et al. (1998) described that some strains of *C. jejuni* became coccoids when they entered the VBNC state in aging microcosm-water cell suspensions while other strains remained spiral shaped after 30 days of starvation. *C. jejuni* has been found in chicken samples, surface and ground water, and milk (Yang et al., 2003). This microorganism does not resist many ambient conditions, but they can be present in food by cross-contamination.

***Enterococcus* spp.:** are Gram positive facultative anaerobic non spore forming cocci. These bacteria are important nosocomial pathogens; they can cause many clinical infections in immunocompromised individuals. Enterococci are natural habitants of human and animal gastrointestinal tract. Due to this and to their tolerance to environmental conditions as extreme temperatures, pH, desiccation and high NaCl concentration, they are traditionally used as more persistent hygiene indicators in drinking water.

***Salmonella* spp.:** are Gram negative non spore forming motile enterobacteria. They are found worldwide in warm and cold blooded animals and also in nonliving habitats. Eggs and poultry are the most common sources of infection, though ingestion

of contaminated water, milk, milk products, beef, fruit, vegetables, and dairy products are also common sources. These bacteria are the typhoid fever, paratyphoid fever and salmonellosis causing agent. Some studies have shown that *Salmonella* can attach and form biofilms on surfaces found in food processing plants, including plastic, cement, and stainless steel; while others found that sanitation with 150 ppm chlorine was not sufficient to remove a *Salmonella* biofilm from stainless steel (Chmielewski and Frank, 2003). In 2008 a *Salmonella* outbreak, linked to tap water, alarmed the Alamosa's population. Berg (2008) stated that the water supply was not previously chlorinated due to the belief that the aquifer was deep enough to be considered safe from microbial contamination.

***Escherichia coli*:** are Gram negative facultative anaerobic non spore forming motile rod bacteria, which form part of the normal flora of intestinal gut of humans and other warm blooded animals. Most *E. coli* strains are harmless and can benefit their hosts by producing vitamin K or by preventing the establishment of pathogenic bacteria, but some strains (e.g. serotype O157:H7) can cause serious food poisoning in humans. A study in Pakistan, indicated a high incidence of *E. coli* in biofilms and water samples at commercial poultry farms (Ahmad et al., 2008). *E. coli* has been detected on pipe surfaces and coupons in European drinking water distribution networks where some of the cells were metabolically active but were often not detected due to the limitations of traditionally used culture - based methods, indicating that biofilms should be considered as a reservoir that should be investigated further in order to evaluate the risk for human health (Juhna et al., 2007a; Li et al., 2006). The presence of *E. coli* in water distribution networks depend on many environmental factors, including pipe material, temperature (Silhan et al., 2006), disinfectant type and dose (Momba et al., 1998; Winter et al., 2008), presence of predators (Sibille et al., 1998), amount of corrosion products (Camper et al., 1996), iron, oxygen concentration (Roslev et al., 2004), and water saturation (Juhna et al., 2007b).

***Pseudomonas aeruginosa*:** are Gram negative motile rod shaped bacteria. This bacterium is an opportunistic pathogen that presents a high nosocomial incidence. It can be found in water and soil or surfaces that are in contact with water or soil. Potable water, especially high-purity water systems, are nutrient-limited environments, but even nutrient concentrations too low to be measured are sufficient to permit *P. aeruginosa* growth and reproduction (Kayser et al., 1975). It has been described that *P. aeruginosa* growing in distilled water was markedly more resistant to acetic acid, glutaraldehyde, chlorine dioxide, and a quaternary ammonium com-

pound than cells cultured on medium with high nutrient concentrations (USEPA, 1992). This bacterium has the capacity of forming biofilms (Schwartz et al., 2007). Its occurrence in drinking water has been described as probably related more to its ability to colonize biofilms in plumbing fixtures (Bressler et al., 2009). This bacterium is tolerant to a wide variety of conditions, including temperature, high NaCl concentrations, weak antiseptics, and many commonly used antibiotics (Whiteley et al., 2001).

Table 2.1.: Examples of potential pathogenic bacteria in drinking water and their significance in water and drinking water supplies (Casani and Knøchel, 2002).

	Pathogenicity	**Transmission**	**Infective dose**	**Reservoir and other relevant sources**	**Persistence in water or drinking water supplies**[b]
Gram negative bacteria					
Campylobacter jejuni and *C.coli*	Pathogen	Ingestion	Low-moderate	A,F,W,E	Moderate
Pathogenic *E.coli*	Pathogen	Ingestion	High[c]	H,A,F,W[d]	Moderate[e]
Salmonella typhi	Pathogen	Ingestion	High	H	Moderate
Other *Salmonella spp.*	Pathogen	Ingestion	High	H,A,F	Long
Shigella spp.	Pathogen	Ingestion	Low-moderate	H,F	Short
Vibrio cholerae	Pathogen	Ingestion	High	H,F,W[f]	Short
Yersinia enterocolitica	Pathogen	Ingestion	High	H,A,F,W,E	Long
Pseudomonas aeruginosa	Opportunistic	Contact or inhalation	High	H,W,E	May multiply
Aeromonas spp.	Opportunistic	Contact, inhalation or ingestion	Moderate-high	W,E	May multiply
Flavobacterium spp.	Opportunistic	Contact or inhalation	?[g]	A,W,E	NA[h]
Acinetobacter spp.	Opportunistic	Contact or inhalation	High	H,E,W	NA
Klebsiella spp.	Opportunistic	Contact or inhalation	?	H,A,F	Long
Serratia spp.	Opportunistic	Contact or inhalation	?	E,W	May multiply
Legionella spp.	Opportunistic	Contact or inhalation	Low	W[f],E	May multiply
Gram positive bacteria					
Listeria spp.	Opportunistic	Ingestion	High	H,A,F,E	Long
Bacillus spp.	Opportunistic	Ingestion	High	H,A,W,E	NA
Clostridium perfringens	Opportunistic	Ingestion	High	H,A,F,W,E	Long
Coryneforme sp.	Opportunistic	Contact or inhalation	NA	H,A,W,E	NA
Mycobacterium	Opportunistic	Contact or inhalation	Low-moderate	H,A,W,E	May multiply

a H: Human; A: Animal; F: Feces or intestinal tract; W: Water; E: Environment

b Detection period for infective stage: short, up to 1 week; moderate, 1week-1month; long, over 1 month

c Low for verotoxigenic *E. coli*

d Polluted water

e Long for E.coli O157:H7

f Nutrient-rich waters

g Uncertain

h No data available

2.3. Biofilms

Biofilms can be defined simply and broadly as structured communities of microorganisms enclosed in an extracellular polymeric substance (EPS) that are attached to a surface (see Figure 2.1). Although biofilm formation has been a recognized and scientifically documented aspect of microbial physiology for approximately 100 years, this process at a molecular level is just beginning to be understood. A concerted effort to study microbial biofilms began only 4 decades ago, and these studies serve as an excellent model system for the study of microbial development (O'Toole et al., 2000).

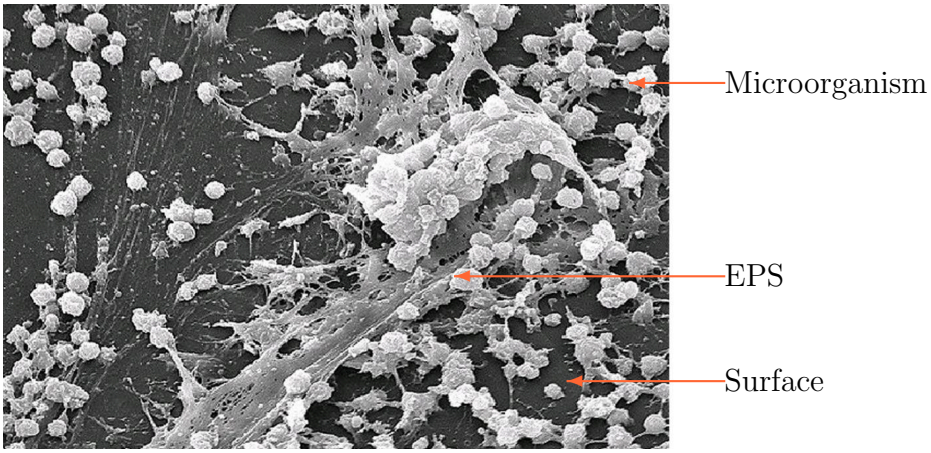


Figure 2.1.: Example of biofilm. Scanning electron micrograph of a *Staphylococcus* biofilm (PHIL-CDC, 2010).

Biofilms are ubiquitous; they can be in aquatic and industrial water systems as well as in large number of environments and industrial devices relevant for public health (Donlan and Costerton, 2002). Bacteria seem to initiate biofilms development in response to specific environmental stresses. Environmental stress can be defined as external factors that can adversely affect bacterial welfare, leading to a decreased growth rate, or in more extreme cases, to inhibition and/or death of individual cells or of the whole population. Examples of such bacteriostatic or bactericidal stresses include extreme temperatures or pH, extreme osmotic pressure, low nutrient concentrations, and the presence of toxic or inhibitory substances (McMahon et al., 2007). To form biofilms, bacteria have to start a complex genetic program to switch from planktonic to sessile lifestyle. This seems to start with the determination of their cell density by a process called quorum sensing, triggered by small water soluble molecules called autoinducers (Abraham, 2006). Recently, the initial attachment of bacteria was studied by Harmsen et al. (2010). They investigated

the role of extracellular DNA (eDNA) during biofilm formation and indicated that high molecular weight DNA is required for initial adhesion and early biofilm formation.

Water treatment processes are capable of reducing heterotrophic microorganisms to less than 10 colony forming units (CFU) per ml, although it has been reported that water from most American waterworks typically contain higher numbers (LeChevallier et al., 1991). Some viable organisms remaining in water could be able to multiply if nutrients are available (LeChevallier et al., 1991), especially in waters that are above 15 °C , and may lead to the formation of biofilms on internal surfaces (Payment and Robertson, 2004). Drinking water distribution systems have been described like an enormous heterogeneous reactor in which the different zones behave almost independently, especially regarding the density and diversity of bacterial populations (Leclerc, 2003). A 99 % of all the bacteria present in potable water are provided by biofilms (O'Toole et al., 2000). Biofilms in drinking water pipe networks can be responsible for a wide range of water quality and operational problems. Biofilms contribute to loss of distribution system disinfectant residuals, increased bacterial levels, reduction of dissolved oxygen, taste and odor changes, red or black water problems due to iron or sulphate-reducing bacteria, microbial influenced corrosion, hydraulic roughness and reduced material life (LeChevallier, 2003).

Horizontal gene transfer related to antibiotic resistance within a biofilm has also been reported (Levy and Miller, 1989). For example, vancomycin - resistant enterococci, methicillin - resistent staphylococci, and ß - lactam - resistant enterobacteria have been found in hospital wastewater biofilms and in other environmental biofilms (Schwartz et al., 2003b).

Studies of microbial resistance to treatment and disinfection have demonstrated that the microbial surface structure and composition, and the nature of the genome are a key for the determination of the transmission potential of waterborne emerging pathogens (Nwachcuku and Gerba, 2004).

Biofilms are important with respect to the survival and growth of microorganisms in the food industry. Microorganisms growing in biofilms are protected against cleaning and disinfection and are difficult to eradicate (Chmielewski and Frank, 2003; Harmsen et al., 2010; Møretrø and Langsrud, 2004). Hence, if biofilms are formed in drinking water pipelines within a food company this could be a potential high risk for the food quality and therefore for the consumers.

2.4. Pathogen Detection: Traditional Culture - Dependent Methods

Standard plate count is a procedure that provides a standardized mean of the density of heterotrophic bacteria in samples. This is an empirical measurement since organisms occur singly, in pairs, clusters, or packets, and no single growth medium or set of physical and chemical conditions can satisfy the physiological requirements of all organisms in a sample (Madigan et al., 2003).

Heterotrophic plate count (HPC) tests do not distinguish between pathogenic and non - pathogenic microorganisms, and they account only for a small undefined portion of organisms that are present in the sample (Bartram et al., 2004a). Although standardized methods have been formalized there is no universal HPC measurement. HPC tests involve a wide variety of test conditions that lead to a wide range of quantitative and qualitative results (Bartram et al., 2004b). This method mainly consists in a sterile Petri dish that contains a growth medium. The bacteria able to grow on the medium depend on the nutrients added to the agar, incubation time and incubation temperatures. The test itself does not specify the organisms that are detected. A wide spectrum of agar media is commercially available, going from non - specific media, where a great variety of bacteria are capable to grow (e.g. R2A), to specific media where only target species can grow.

Some of the most important characteristics of this counting technique are: (i) the detection of only viable culturable bacteria, (ii) some bacteria are killed due to oxidative stress that occurs upon plating (Cuny et al., 2007), and (iii) a longer time is needed for the results (3 or more days).

There are two main methods of direct plate counting: spread plate method and pour plate method, these methods are represented in Figure 2.2.

The spread plate count method consists of evenly spreading the diluted sample over an agar plate. When using this method, a volume higher than 0.1 ml of the diluted sample should not be used since the agar will not be able to absorb the excess. Using this method, colonies that form on the surface of the agar can be counted.

When the pour plate method is used, a diluted sample is pipetted into a sterile Petri plate, and then melted agar is poured in and mixed with the sample. Using this method, bacteria present in a larger volume of the diluted sample can be counted (0.1 - 1.0 ml sample). This method yields colonies formed throughout the agar and not only on the surface. Caution must be taken with this method to ensure that the organism to be

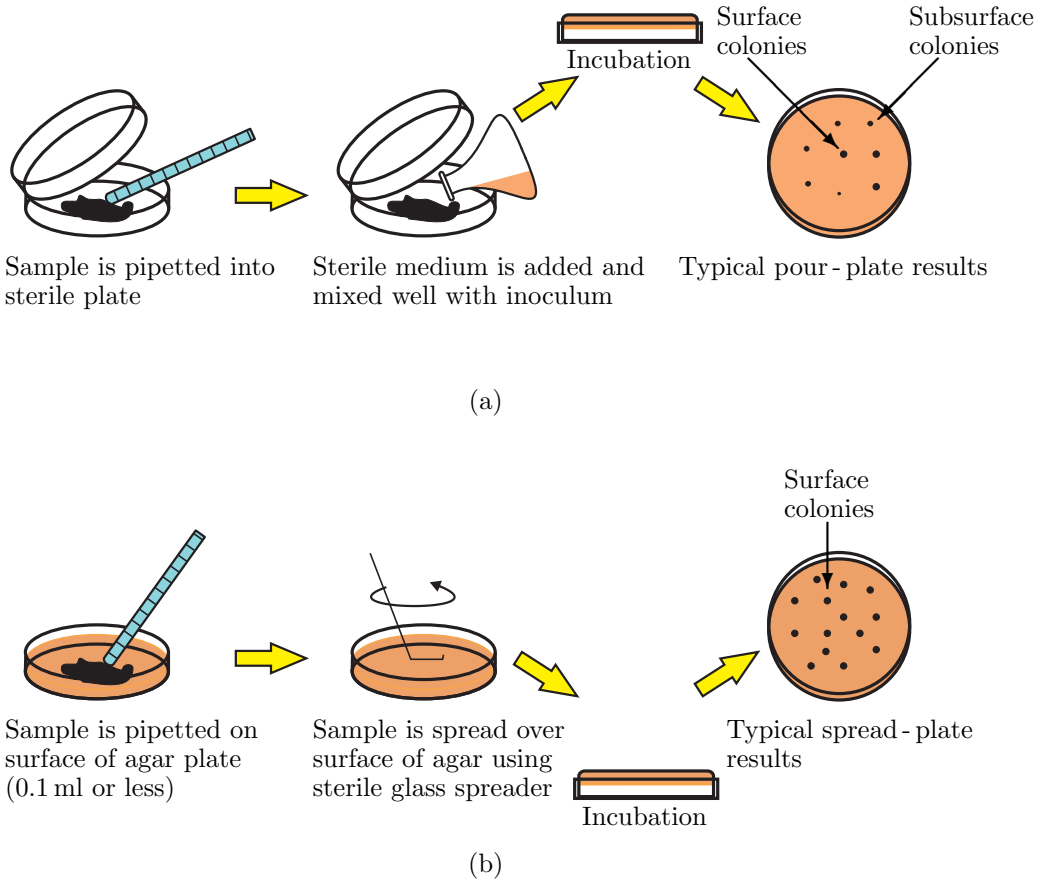


Figure 2.2.: Spread plate method (a), and pour plate method (b).

counted can withstand the temperatures associated with the melted agar (Madigan et al., 2003).

Membrane filtration - heterotrophic plate count (MF - HPC) is the world wide standard method used to determine heterotrophic bacteria present in water. This method is a kind of modified spread plate count, which consists in filtering a known amount of water sample, and setting the filter on the agar. Drinking Water Directive 98/83/EC (EU Council decision, 1998) of the European Union establishes that indicator microorganisms should be routinely monitored in drinking water in order to control microbial water quality of public distribution systems. This directive stipulates that no *E. coli*, enterococci, and coliform bacteria should be present in 100 ml drinking water of public distribution systems. According to the German water regulations (TrinkwV 2001, 2001), the number of heterotrophic bacteria determined by MF - HPC should not be higher than 100 CFU/100 ml when water leaves the tap of the consumer. If waterworks notice an abrupt or continuous

increase of this parameter they should report it to the authorities.

2.5. Pathogen Detection: Culture - Independent Techniques

Besides monitoring studies based on regulations, a tendency of using new culture - independent methods instead of culture - dependent techniques for the detection of pathogens has been lately observed. This is principally due to the ability of culture - independent methods to overcome problems associated with selective cultivation and isolation of bacteria from natural samples. The lack of knowledge of the real conditions under which most bacteria grow in their natural habitats makes it difficult to develop media for cultivation. Some additional reasons of this trend are due to the specificity and sensibility of the first ones, and their reduced analysis time (Ercolini, 2004).

The culture - independent techniques used in this work to detect pathogens are mainly based on the polymerase chain reaction (PCR). DNA sequencing was used as verification method.

Polymerase chain reaction was developed by Kary B. Mullis in 1985. From there on, many variations of the basic PCR technique have been developed. Mainly, this revolutionary method is used to make numerous copies of a specific DNA segment, meeting the sensitivity needed for the subsequent DNA analyses. For this, a denatured strand of DNA is incubated with a DNA polymerase, deoxyribonucleotide triphosphate (dNTPs), and two oligonucleotide primers whose sequences flank the DNA sequence of interest, directing the synthesis of new complementary DNA strands. Hot Start DNA polymerases are enzymes that need high temperatures to be activated. When these are used, an initialization step has to be carried out too.

A PCR cycle mainly consists of three steps:

1. *Denaturation*: the two strands of the parent DNA molecule are separated by heating the solution.
2. *Annealing*: the solution is abruptly cooled to allow each primer to hybridize the correspondingly 3' end of the single stranded DNA (ssDNA) strands.
3. *Elongation*: the solution is heated to the optimal temperature of the DNA polymerase, and then this enzyme elongates both primers in the direction of the target

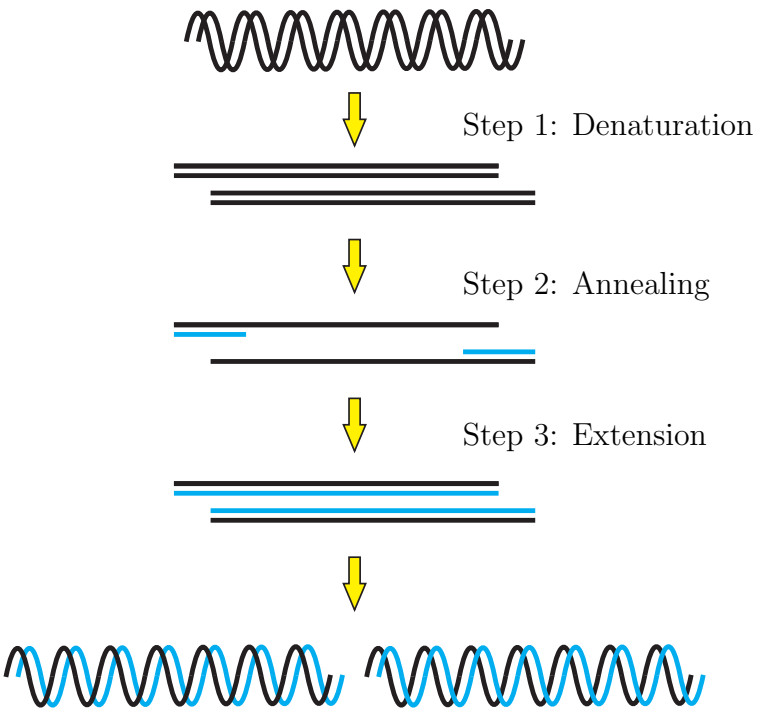


Figure 2.3.: One PCR cycle.

sequence (5' → 3').

These three steps constitute one cycle of the PCR and can be carried out repetitively just by changing the temperature of the reaction mixture (see Figure 2.3). The temperatures used and the time of each cycle depend on parameters as the DNA polymerase used, the concentration of divalent ions and dNTPs, and the melting temperatures of the primers.

Normally, an additional final elongation step is done to ensure a full extension of DNA.

Multiple cycles of this process allow a small amount of DNA molecules to be amplified in an exponential manner (see Figure 2.4), following Equation 2.1, where n represents the number of cycles.

$$\text{Number of copies} = 2^{n+1}. \tag{2.1}$$

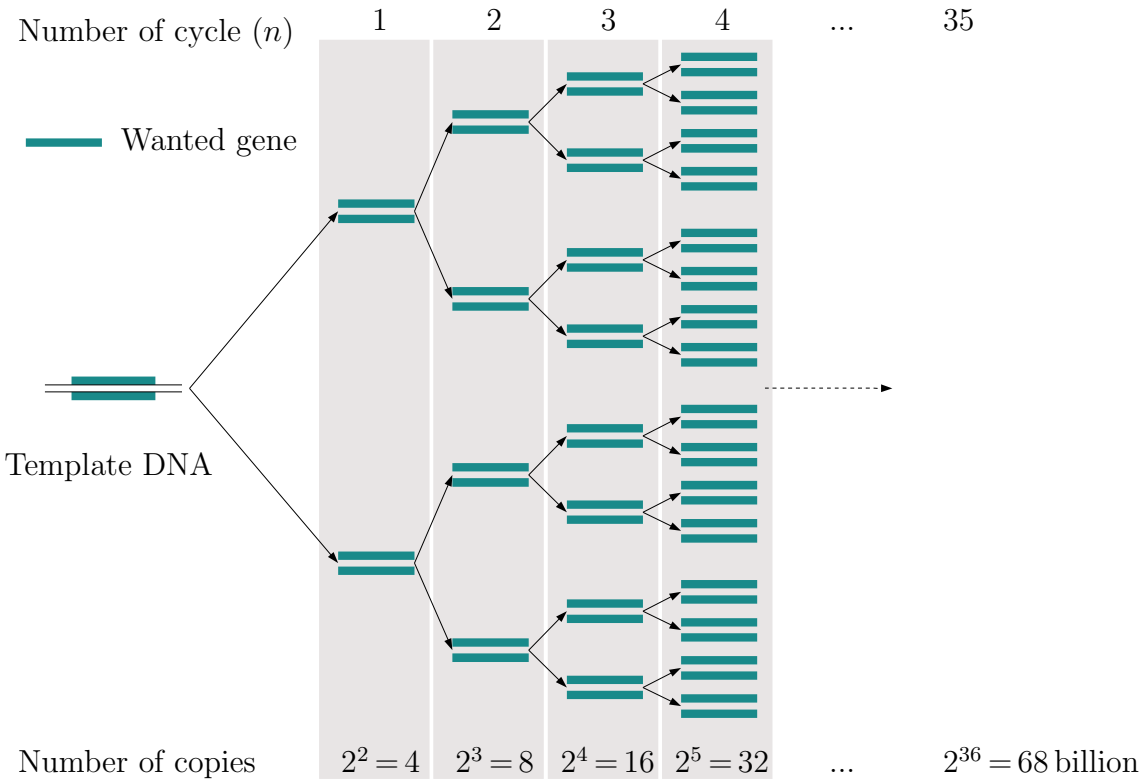


Figure 2.4.: Exponential amplification of DNA by PCR.

2.5.1. Conventional Polymerase Chain Reaction

The conventional PCR used in this work, is an end point procedure, where HotStarTaq DNA Polymerase utilizes a chemically - mediated hot - start to completely inactivate the polymerase until the initial heat activation step is done. This PCR consists of the already named basic elements and follows the typical PCR steps described above. Depending on the sought bacteria, specific genes are targeted, and different temperature profiles are used for the PCR.

Conventional PCR is commonly carried out in reaction volumes of $10 - 200\,\mu$l in small thin walled reaction tubes in a thermal cycler that quickly heat and cool the reaction tubes. PCR products are usually run by electrophoresis on agarose gels containing ethidium bromide as DNA dye, in order to verify their sizes and amounts. Qualitative or semi - quantitative measurements of templates can be achieved with this method (see Figure 2.5).

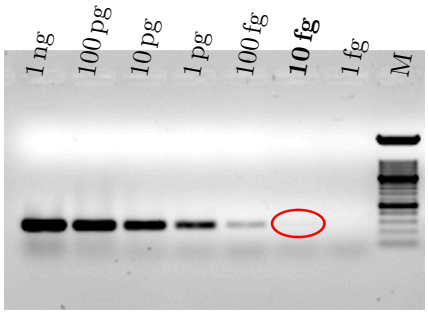


Figure 2.5.: PCR products of a serial dilution of *P. aeruginosa* genomic DNA.

2.5.2. Seminested Polymerase Chain Reaction

This is a variation of PCR which increases the specificity of DNA amplification, by reducing background due to non - specific DNA amplification. For this, three primers are used in two successive PCRs. In the first reaction, one pair of primers is used to generate DNA products, which may still consist of non - specifically amplified DNA fragments. Then, the product is used to do a second PCR with a set of primers whose binding sites are completely or partially different from the primers used in the first reaction (see Figure 2.6). Seminested PCR is often more successful than conventional PCR in specifically amplifying long DNA fragments, but it requires more detailed knowledge of the target sequences.

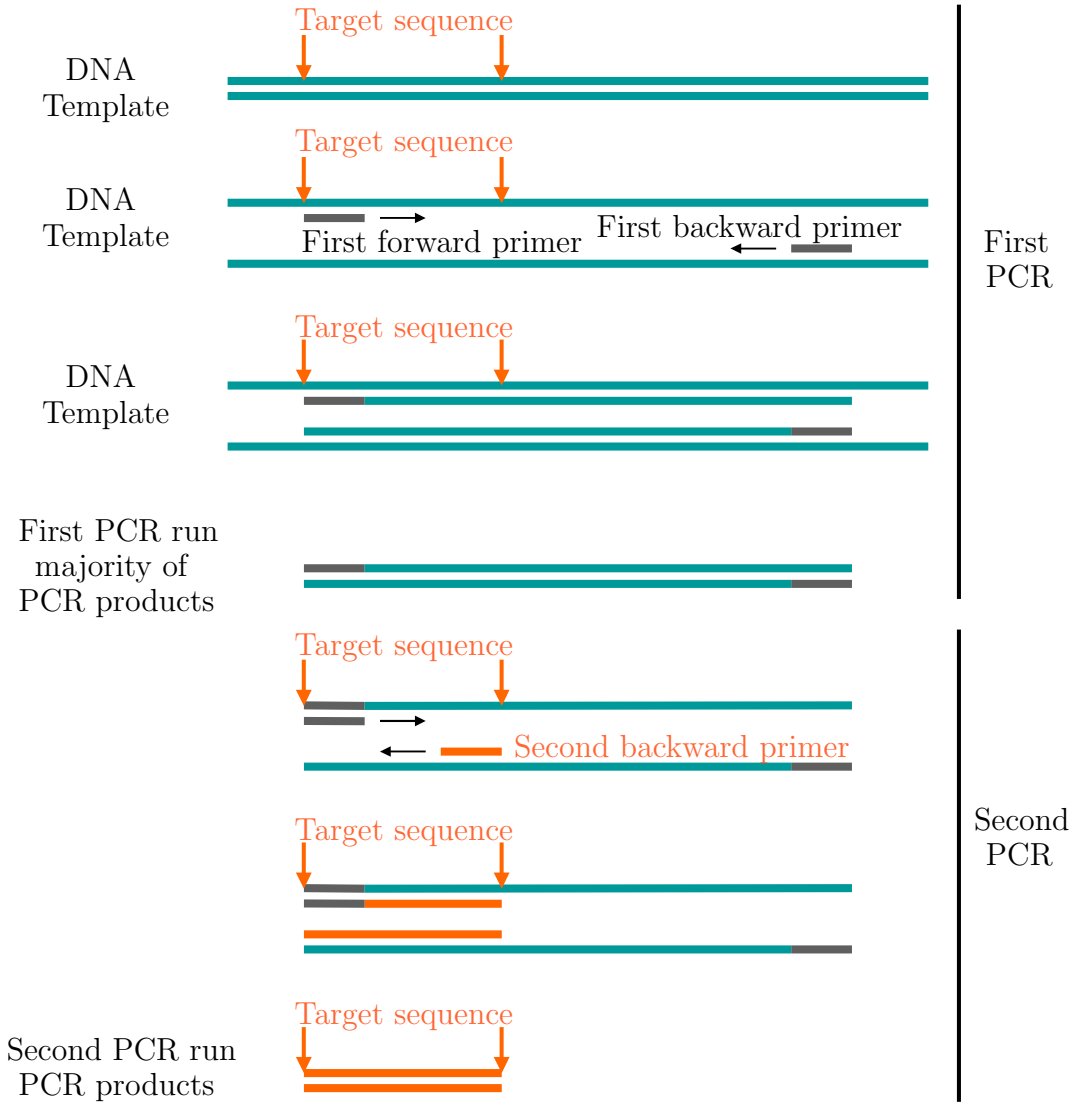


Figure 2.6.: Principle of a seminested PCR.

2.5.3. Quantitative Polymerase Chain Reaction

Quantitative PCR methods measure the amount of amplified DNA in real time, therefore it can also be named as real time PCR. There are three general methods used for quantitative assays:

a) *SYBR Green I technique*: SYBR Green I fluorescence is enormously increased upon binding to double - stranded DNA. During the extension phase, more and more SYBR Green I will bind to the PCR product, resulting in an increased fluorescence. Consequently, during each subsequent PCR cycle more fluorescence signal will be detected.

b) *Hydrolysis probe technique*: (e.g. TaqMan®) the hydrolysis probe is conjugated with a quencher fluorochrome, which absorbs the fluorescence of the reporter fluorochrome as long as the probe is intact. However, upon amplification of the target sequence, the hydrolysis probe is displaced and subsequently hydrolyzed by the Taq polymerase. This results in the separation of the reporter and quencher fluorochrome and consequently the fluorescence of the reporter fluorochrome becomes detectable. During each consecutive PCR cycle this fluorescence will further increase because of the progressive and exponential accumulation of free reporter fluorochromes.

c) *Hybridization probes technique*: in this technique one probe is labeled with a donor fluorochrome at the 3' end and a second probe is labeled with an acceptor fluorochrome. When the two fluorochromes are in close vicinity (i.e. within 1 - 5 nucleotides), the emitted light of the donor fluorochrome will excite the acceptor fluorochrome. This results in the emission of fluorescence, which subsequently can be detected during the annealing phase and first part of the extension phase of the PCR reaction. After each subsequent PCR cycle more hybridization probes can anneal, resulting in higher fluorescence signals.

A common factor from these quantification assays is that during the run the instrument records the fluorescence emission. Then, the software processes the raw fluorescence data. Based on the (background) fluorescence intensity detected during the first three to 15 PCR cycles, a threshold is determined. The cycle threshold (C_T) is defined as the PCR cycle at which the fluorescence exceeds the threshold for the first time. The C_T value will be directly proportional to the amount of target sequence present in the sample. The increase in fluorescence, on the y - axis, is indicated as ΔR_n (Figure 2.8.a). The data obtained here are used to prepare the standard curve (Figure 2.8.b). The slope of the

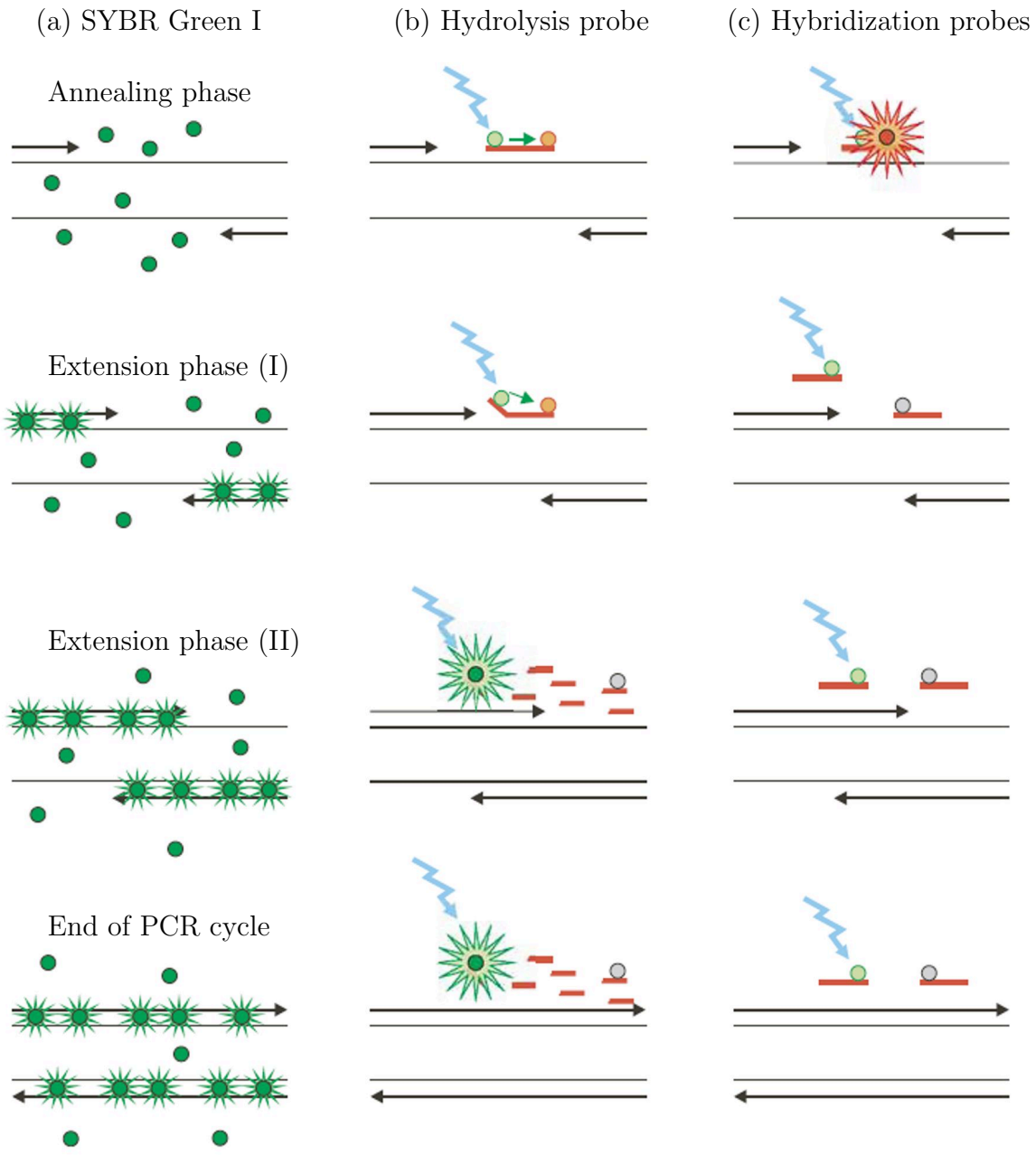


Figure 2.7.: Principles of quantitative PCR techniques (van der Velden et al., 2003).

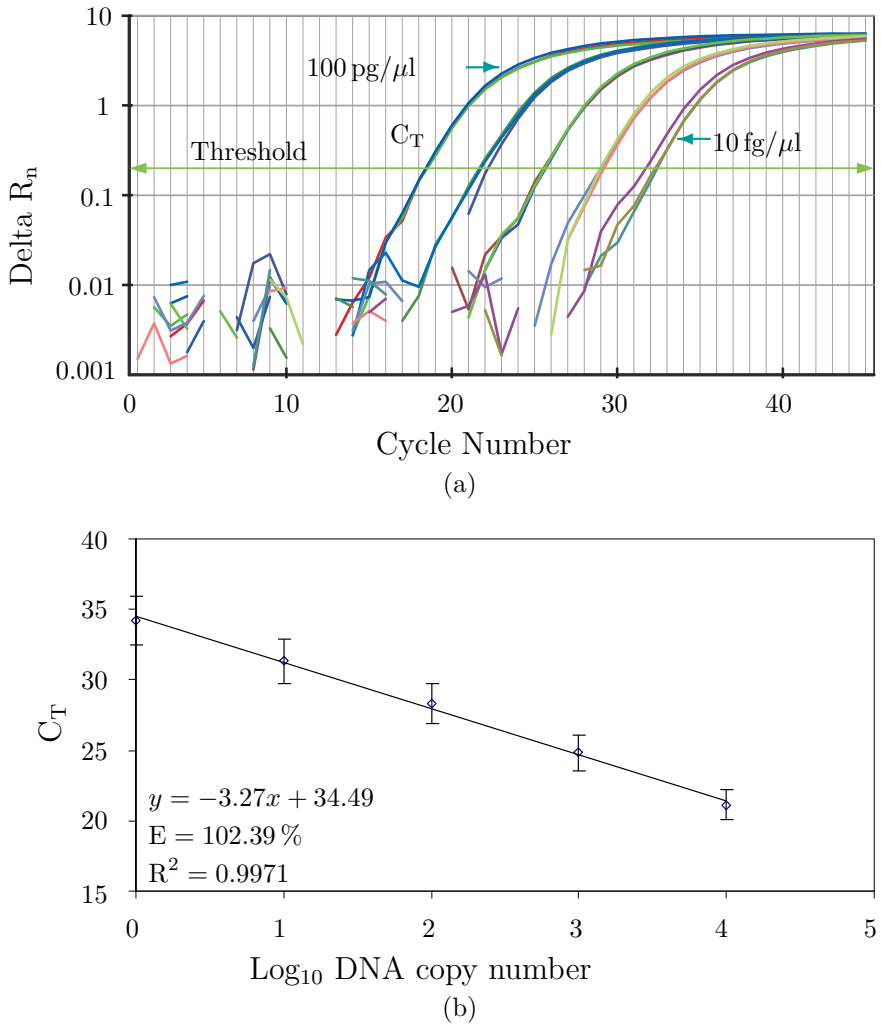


Figure 2.8.: Quantitative PCR plots. (a) Amplification plot of several 10-fold dilutions of enterococci genomic DNA. (b) Standard curve prepared from the data in (a).

standard curve is close to the theoretical slope of -3.3. Unknown samples run in the same assay can be plotted in the standard curve, and based on their C_T value the amount of template DNA can be calculated.

2.6. Bacterial Population Analysis

The genetic diversity within a microbial community from a specific environment can be determined by genetic fingerprinting techniques without previous cultivation steps. Polymerase chain reaction followed by denaturing gradient gel electrophoresis (PCR - DGGE) was introduced into microbial ecology in the early nineties by Muyzer et al. (1993). This method is actually a well established tool for microbial diversity studies. Some studies indicated the use of these techniques for the analyses of drinking water bacterial populations (Eichler et al., 2006; Revetta et al., 2010), though no studies have been published about the use of these techniques to compare the bacterial stability of water within a food company. Additionally, the bacterial species from the analyzed samples can be identified by purifying and sequencing the bands in the denaturing gradient gel electrophoresis (DGGE) profile.

2.6.1. PCR - DGGE

PCR - DGGE is based on the separation of PCR amplicons of the same size but with different sequences. The use of universal primers allows any microbial community to be analyzed; although in ecosystems with a high diversity only the dominant microbiota will be visualized. In order to focus on specific subpopulations, group - specific PCR primers can be used. In most PCR - DGGE applications on bacteria, universal or specific primers are targeting the 16S rDNA gene. These fragments can be electrophoretically separated based on their differential denaturation profile (see Figure 2.9). In the acrylamide gels, the denaturing conditions are provided by formamide and urea. In a DGGE gel, double - stranded DNA (dsDNA) fragments are subjected to an increasing denaturing environment and partially melt in discrete regions called “melting domains”. The melting temperature of these domains is sequence specific. Once the fragments are partially melted, their mobility in the acrylamide gel reduces.

Therefore, DNA fragments of the same size but with different base pair compositions will show a different pattern (Ercolini, 2004; Muyzer et al., 1993). An optimal resolution is obtained when molecules do not completely denature. The addition of a 30 to 40 bp

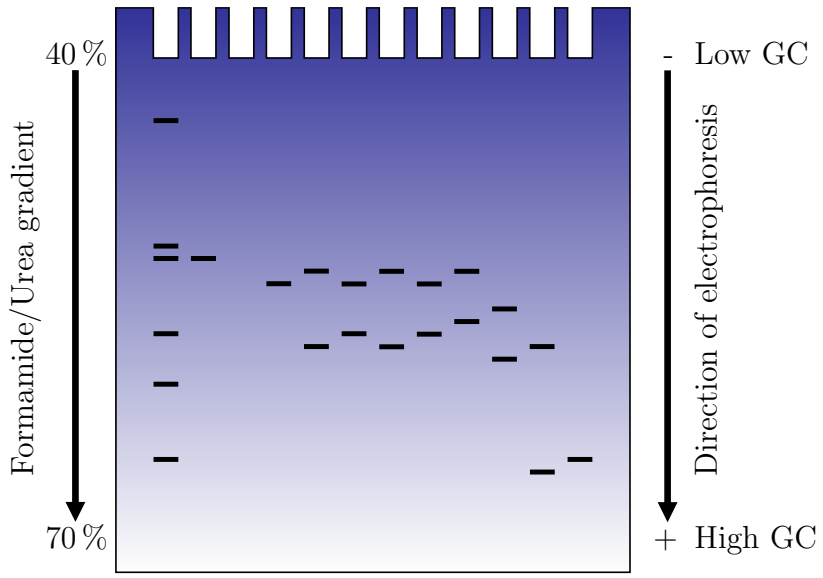


Figure 2.9.: Principle of DGGE.

GC - clamp to one of the PCR primers insures that the fragment of DNA will remain partially double - stranded and that the region screened is in the lowest melting domain (Sheffield et al., 1989). Following gel electrophoresis and gel staining, DGGE gels are captured digitally and are further analyzed by computer software packages. The final result is a specific fingerprint of the sample that can be compared with the fingerprint of other samples run in the same gel.

The analysis and comparison of the autochthonous bacterial population of water for the identification of possible critical control points according to the HACCP concept at different food production points where water is involved, is a new application of the PCR - DGGE technique.

2.6.2. Sequencing of DNA

Recently, some new DNA sequencing methods together with their advantages and disadvantages have been described (Hert et al., 2008; Marziali and Akeson, 2001). But actually the Sanger (or dideoxy) method (Sanger et al., 1977) and the Maxam - Gilbert (chemical cleavage) method (Maxam and Gilbert, 1980) developed in the late 1970's are the most commonly used techniques; being the first, the one that was used in the present work.

With the advent of PCR and automation the Sanger method has been proven to be technically simple and it is able to accurately determine the sequence of long stretches of DNA, including some entire genes. Since its discovery, the method has undergone many improvements regarding labeling technology, chemistry and instrumentation, nevertheless, the base protocol remains essentially unchanged.

The Sanger method takes advantage of the ability of the DNA polymerase to incorporate analogues of nucleotide bases by using dideoxyribonucleotide triphosphate (ddNTPs) as substrate. When a dideoxynucleotide is incorporated at the 3´ end of a growing chain, chain elongation is terminated selectively at adenin (A), cytosine (C), guanine (G), or thymine (T) because the ddNTPs lack a 3´-hydroxyl group. Typically, the automated sequencing method is only accurate for sequences up to a maximum of about 700-800 base-pairs in length (Voet and Voet, 1995).

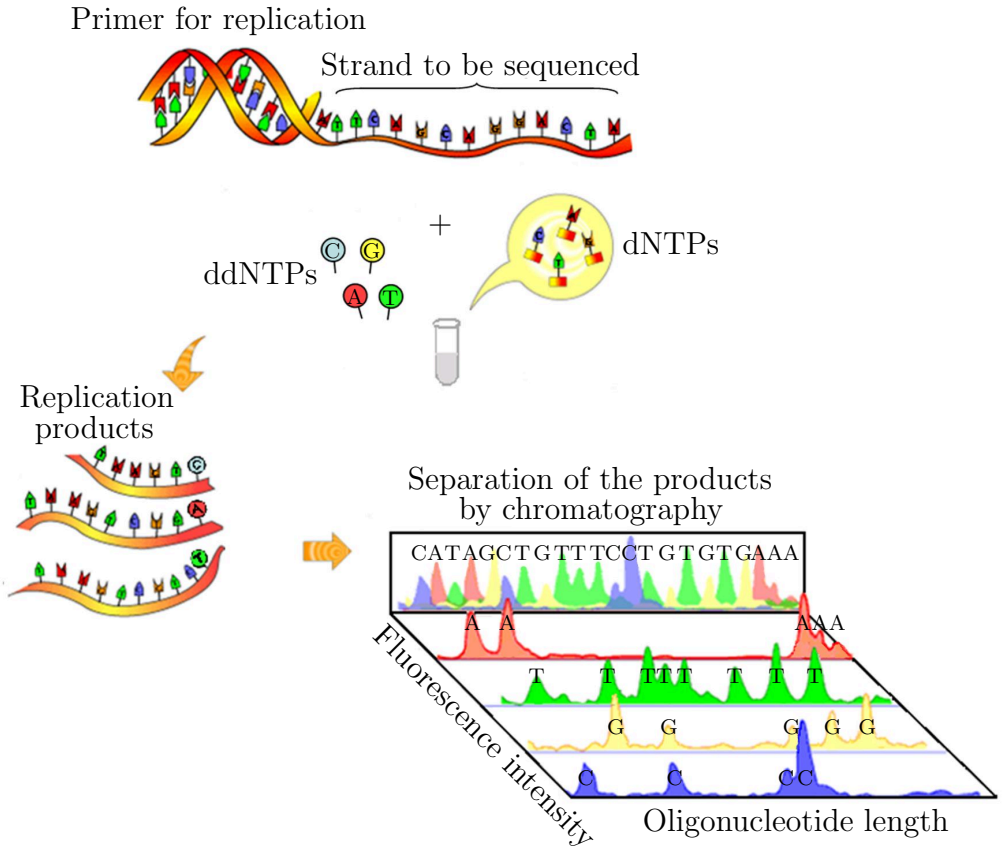


Figure 2.10.: Principle of DNA sequencing.

In the present work, four different fluorescent dyes were used to label the ddNTPs, which were added sequentially to the primer through a cycle sequencing reaction (see

Figure 2.10). This method is suitable for sequencing of ssDNA or dsDNA templates, PCR products, and large templates. It takes place in a single tube reaction for each primer, producing a series of molecules of different length, each one terminated and labeled at a different base. Reaction products can then be run in an automated sequencer to obtain the final sequence. The automated sequencer is based on the ability of capillary electrophoresis to separate the resulting DNA products under denaturing conditions, according to their size. This denaturing effect of urea, of the polymer during the filling of the capillary is added. The glass capillary is loaded with the DNA molecules to be separated by voltage, and the DNA moves along the stress field through the capillary. The DNA is separated according to interactions with the polymer and the capillary size. The fluorescence of each fragment is detected using a laser beam and the information is collected by a computer which generates chromatograms showing peaks for each color, from which the template DNA sequence can be determined.

Bacteria identification can be achieved by comparing the nucleic acid sequences with GenBank sequences using different software (e.g. BLAST program).

2.7. Live/Dead Differentiation

A very important task for many microbiology applications is the accurate determination of live, dead, and total bacteria in a sample. Bacterial viability has been traditionally taken as synonymous of the ability of live bacteria to form colonies on solid growth medium and to multiply in liquid nutrient broths. These traditional culture - dependent methods are time - consuming, can work poorly with slow - growing bacteria or with viable but non - culturable organisms, and they do not provide real - time results or timely information needed in applications such as industrial food manufacturing (Alsharif and Godfrey, 2002).

DNA - and RNA - based methods have been commonly used for the detection and characterization of bacteria in research laboratories. ribonucleic acid (RNA) - based methods have been suggested to study the active microbial fraction in environmental matrices (Revetta et al., 2010). Intracellular RNA is rapidly degraded in stressed cells and is more unstable outside of the cell than DNA. This method seems to work well, but high amounts of water should be analyzed due to the low amount of bacterial RNA present in drinking water.

It has been demonstrated that DNA - based studies may not provide accurate informa-

tion about live/active members of natural microbial assemblages since DNA may persist for long periods in the environment after cell death (Keer and Birch, 2003). Nevertheless, DNA - based methods have been chosen for the analysis of drinking water bacterial populations, due to its stability, due to its rapidity, and because it could be a more reliable detection parameter as culturable bacteria via cultivation methods.

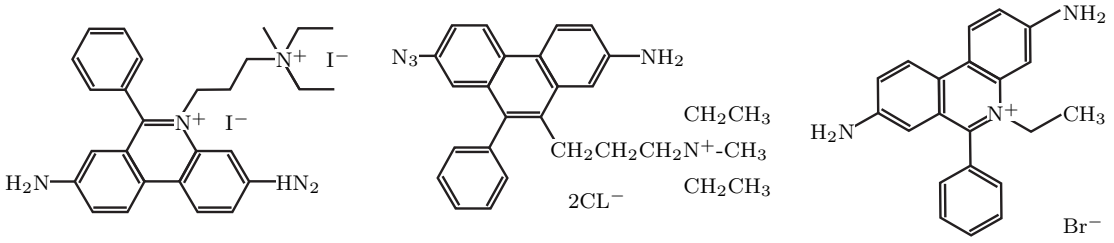
Lately, different assays have been developed to distinguish DNA coming from live cells (Darzynkiewicz et al., 1992; Nocker et al., 2006; Nogva et al., 2000, 2003).

In order to use DNA - based methods a treatment of the samples should be done to distinguish live cells - DNA from free - DNA, eDNA and DNA from dead cells.

The most relevant methods for this work, for live/dead differentiation, are briefly described in this section.

2.7.1. DNA Intercalating Dyes: PI, EMA, and PMA

In the past years cell viability assays have been developed and commercialized to differentiate live from dead bacteria. These assays are based in the ability of substances as e.g. propidium iodide (PI), ethidium monoazide (EMA), and propidium monoazide (PMA) (see Figure 2.11), to selectively enter into dead bacterial cells with compromised membrane integrity but not into live cells with intact cell membranes/cell walls (Delgado-Viscogliosi et al., 2009; Nocker and Camper, 2009; Nocker et al., 2006; Nogva et al., 2003). Once inside the cells, they are able to intercalate the nucleic acids with a high affinity. The presence of an azide group allows the crosslinking of the dye to the DNA by exposure to strong visible light.



(a) (b) (c)

Figure 2.11.: Chemical structure of: (a) propidium iodide, (b) propidium monoazide, and (c) ethidium monoazide bromide.

The light leads to the formation of a highly reactive nitrene radical, which can react

with any organic molecule in its proximity including the bound DNA. This modification strongly inhibits the PCR amplification of the extracted DNA. At the same time when the crosslinking occurs, the light promotes the reaction between unbound excess dye with water molecules. The resulting hydroxylamine is no longer reactive; hence, the DNA from cells with intact membranes is not modified in the DNA extraction procedure (see Figure 2.12).

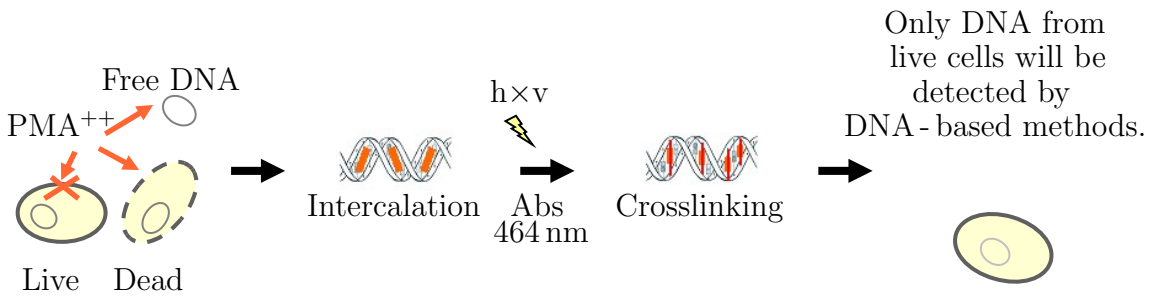


Figure 2.12.: Principle of action of PMA.

Nocker et al. (2006) provided evidence over a better ability of PMA than EMA for this technique, due to the higher charge of PMA, explaining that the general application of EMA is hampered by the fact that it can also penetrate live cells of some bacterial species. This theory was also sustained by Flekna et al. (2007), when they tried to differentiate live and dead *C. jejuni* and *L. monocytogenes*.

2.7.2. DNase I

Deoxyribonuclease I (DNase I) is an endonuclease that non - specifically cleaves, single and double stranded DNA.

DNase I properties have already been used in the early 90's in the field of cancer research. Darzynkiewicz et al. (1992) described the use of trypsin and DNase I for the differentiation of live and dead cancerous cells in an experiment carried out to differentiate and characterize cell death, apoptosis and necrosis by flow citometry.

Nogva et al. (2000) used DNase I to determine the reduction of the PCR signal generated by dead *Campylobacter* in a food matrix. The results indicated relatively good discrimination between exposed DNA from dead *C. jejuni* and protected DNA from living bacteria. No further investigations about the use of this enzyme were done.

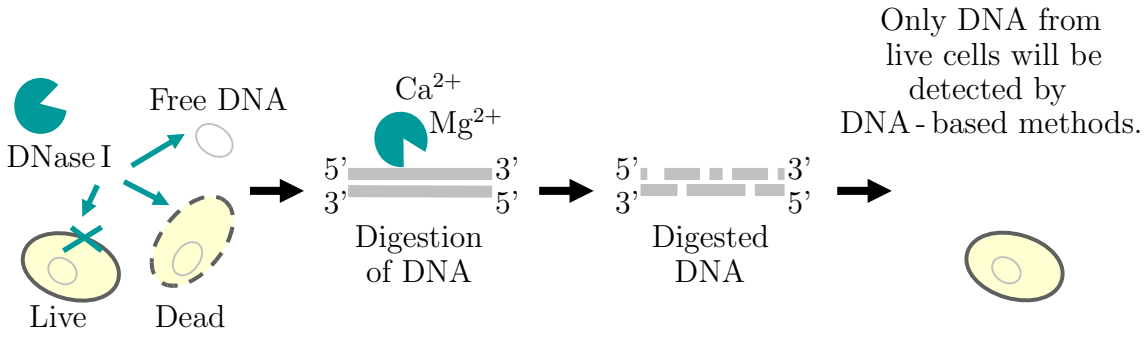


Figure 2.13.: Principle of action of DNase I in the presence of Ca^{2+} and Mg^{2+} ions.

This enzyme has been principally used in the molecular biology field for the removal of bacterial genomic DNA contamination in samples, for further RNA analyses (Wang et al., 2002).

DNase I hydrolyzes phosphodiester bonds adjacent to pyrimidine nucleotides producing mono- and oligodeoxyribonucleotides with 5'-phosphate and 3'-OH groups. The enzyme has an optimal pH of 6.5-8, a molecular mass of 30-40 Da (Kishi et al., 2001), and its activity strictly depends on Ca^{2+} and is activated by Mg^{2+} or Mn^{2+} ions:

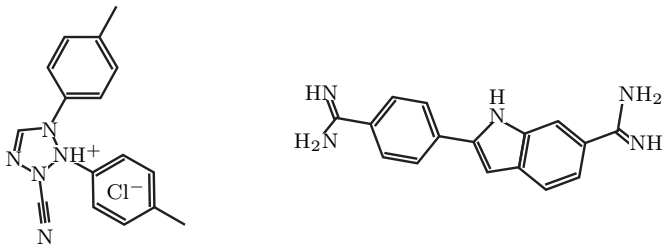
- *In the presence of* Mg^{2+}: DNase I cleaves each strand of dsDNA independently, in a statistically random fashion (see Figure 2.13).
- *In the presence of* Mn^{2+}: the enzyme cleaves both DNA strands at approximately the same site, producing DNA fragments with blunt ends or with one or two nucleotide overhangs (Sambrook and Russell, 2001).

Theoretically, if a sample containing free DNA, eDNA, live and dead cells is exposed to DNase I, nucleic acids from living cells would by protected from the action of the enzyme due to its intact cell membrane. In dead cells this barrier is compromised and the nucleic acids are thus exposed to the action of the enzyme. Hypothetically, after the DNase I treatment only DNA from live cells will be present in the sample (Nogva et al., 2000).

2.7.3. Staining Techniques

In the past years different cell staining techniques have been used in order to differentiate live and dead cells of a sample (Bhupathiraju et al., 1999; Cappelier et al., 1997; Morató et al., 2004).

CTC(see Figure 2.14.a) is a tetrazolium salt that forms a red fluorescent intracellular insoluble precipitate (formazan) when it is biologically reduced by components of the electron transport system and/or dehydrogenases of metabolically active bacteria. CTC can be used in conjunction with counterstaining fluorescent dyes as DAPI.

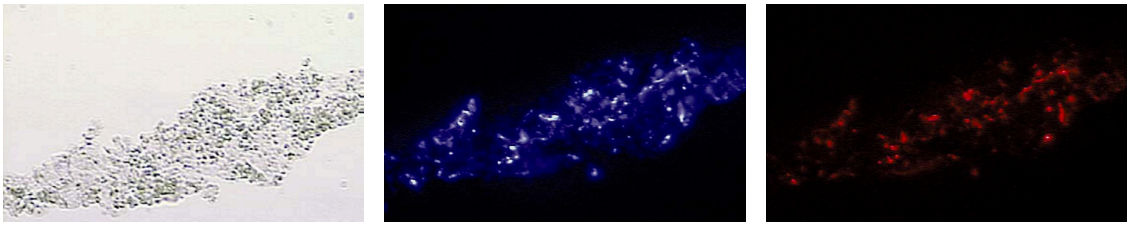


(a) (b)

Figure 2.14.: Chemical structure of CTC (a) and DAPI (b).

The blue - fluorescent DAPI nucleic acid stain (see Figure 2.14.b) preferentially stains dsDNA; apparently it binds to AT clusters in the minor groove of DNA (Kubista et al., 1987). A 20-fold fluorescence enhancement is produced when DAPI is in presence of dsDNA, this might be due to the displacement of water molecules from DAPI and from the minor groove (Barcellona et al., 1990). DAPI is generally used as a counterstain, its blue fluorescence stands out in vivid contrast to green, yellow, or red fluorescent probes of other structures (see Figure 2.15). This substance penetrates intact cell membranes intercalating dsDNA as described before (Cappelier et al., 1997).

In the present work the CTC/DAPI staining was used. CTC stained cells indicated the metabolically active bacteria, and DAPI stained bacteria indicated the total mass of bacteria.



(a) (b) (c)

Figure 2.15.: Staining with DAPI and CTC (Objective 100x): (a), Bright field, (b), DAPI, (c), CTC. (Morató et al., 2004).

3. Materials and Methods

3.1. Equipments and Materials

The following equipment and materials were commonly used and therefore are not listed individually for each method.

Equipment:

- Autoclave steam-sterilizer (Varioklav, Dreiech, Germany)
- Microwave (Bosch, Stuttgart, Germany)
- Lumi-Imager T1TM (Roche Diagnostics, Mannheim, Germany)
- pH-Meter 766 Calimatic (Knick, Berlin, Germany)
- Thermomixer compact (Eppendorf, Hamburg, Germany)
- Vortex mixer (Bibby Sterilin, Staffs, United Kingdom)
- Centrifuge Biofuge pico (Thermo Fischer Scientific, Dreiech, Germany)
- Analytical Balance LC 220 S (Sartorius, Göttingen, Germany)
- Balance BL 3100 (Sartorius, Göttingen, Germany)
- Incubators
- Vacuum pump

Materials:

- Centrifuge tubes; 15 ml and 50 ml (Sarstedt)
- Tweezers (VWR)
- Research® pipettes and respective tips, 10 μl, 100 μl, 1000 μl, 5 ml and 10 ml (Eppendorf, Hamburg, Germany)

- Centrifuge tubes; 0.5 ml, 1.5 ml and 2 ml (Eppendorf, Hamburg, Germany)
- PCR-Centrifuge tubes; 0.2 ml (Eppendorf, Hamburg, Germany)
- Duran-bottles 250 ml, 500 ml, 1000 ml, 2000 ml and 5000 ml (Schott Engineering, Mainz, Germany)
- Nitrile gloves (Ansell health care, Belgium)
- Latex gloves
- Petri plates (90 mm diameter; Greiner, Nürtingen Germany)

3.2. Bacteria

The following reference bacteria were used:

- *Enterococcus faecium* German collection of microorganisms and cell cultures (DSMZ) 20477 (Braunschweig, Germany)
- *Enterococcus faecalis* DSMZ 2981 (Braunschweig, Germany)
- *Salmonella enterica* DSMZ 9274 (Braunschweig, Germany)
- *Campylobacter jejuni* DSMZ 4688 (Braunschweig, Germany)
- *Mycobacterium avium* subsp *paratuberculosis* DSM 44133 (Braunschweig, Germany)
- *Listeria monocytogenes* American type culture collection (ATCC) 19112 (Rockville, MD.USA)
- *Pseudomonas aeruginosa* DSMZ 1117 (Braunschweig, Germany)
- *Escherichia coli* DSMZ 1103 (Braunschweig, Germany)
- *Staphylococcus aureus* ATCC 29213 (Rockville, MD.USA)

Reference strains were stored in 25 % glycerin at -80 °C until use.

3.3. Cultivation Methods and Extraction of Genomic DNA

- Middlebrook 7H10 agar (Difco™, BD, Le Pont de Claix, France)
- Middlebrook OADC growth supplement (BBL™, BD, Maryland, USA)
- Mycobactine J (Synbiotics Europe, Lyon, France)
- Harrold´s egg yolk agar slants with Mycobactine J and amphotericin B, nalidixic acid and vancomycin (ANV) (BD, Le Pont de Claix, France)
- Campylosel agar (bioMérieux, Nürtingen, Germany)
- Columbia agar (bioMérieux, Nürtingen, Germany)
- Chromocult Enterococci agar (Merck, Darmstadt, Germany)
- Slanletz-Bartley agar (Oxoid, Hampshire, England)
- Tryptic soy broth (TSB; BD, Le Pont de Claix, France)
- Nutrient broth (Merck, Darmstadt, Germany)
- Selective *Salmonella* ÖNÖZ agar (Merck, Darmstadt, Germany)
- Brain heart infusion (BHI) (Merck, Darmstadt, Germany)
- Reasoner's 2A agar (R2A) (Merck, Darmstadt, Germany)
- Anaerobic bags
- Microanaerobic atmosphere bags; GENbag microaer (bioMérieux, Nürtingen, Germany)
- Capnophilic atmosphere bags; BD GasPak™ EZ (BD, Le Pont de Claix, France)
- Anaerobic atmosphere indicator; GENbag anaer (bioMérieux, Nürtingen, Germany)
- Capnophilic atmosphere indicator; CO_2 indicator (BD, Le Pont de Claix, France)
- Shaker Unimax 2010 (Heidolph) with incubation chamber Certomat® H (Braun Biotech International)
- PrepMan® Ultra Sample preparation (Applied Biosystems, Darmstadt, Germany)
- Qiagen genomic-tip 500/G (Qiagen, Hilden, Germany)

DNA of *Listeria monocytogenes* was provided by the Max Rubner Institute in Karlsruhe, Germany. *Mycobacterium avium* subsp. *paratuberculosis* was grown in two different media: Middlebrook 7H10 agar with Middlebrook OADC growth supplement and Mycobactine J, and Harold's egg yolk agar slants with Mycobactine J and ANV at 37 °C for 1 month. *Campylobacter jejuni* was plated on Campylosel agar and Columbia agar and incubated at 37 °C for 48 h. *Enterococcus faecium* and *Enterococcus faecalis* were plated on Chromocult Enterococci agar and Slanletz-Bartley agar and were incubated at 37 °C for 48 h. *Escherichia coli* and *Pseudomonas aeruginosa* were grown in trypticase soya broth and nutrient broth at 37 °C for 24 h. *Salmonella enterica* was grown in selective agar *Salmonella* at 37 °C for 48 h. Single colonies of each strain were transferred to rich nutrient media, i.e. tryptic soy broth or BHI. Cells were harvested by centrifugation at 5000 rpm for 5 min and supernatant decant off. Reference strains were stored in 25 % glycerin at −80 °C until use.

Genomic DNA was extracted in order to carry out standard curves and to determine the detection limits of the quantitative PCR assays, and was used as positive control of PCR assays. Total genomic DNA was purified from each bacterium starting with a colony or a cell suspension of the isolate. DNA was purified using PrepMan® Ultra Sample preparation or using Qiagen genomic-tip 500/G in accordance with the manufacturer's guidelines. Concentration of each purified DNA template was determined by spectrophotometry (NanoDrop 1000, peqlab, Erlangen, Germany). Genomic DNA aliquots were stored at −20 °C until use.

3.4. Plating Methods

- Agar media named in Section 3.3
- Mac Conkey agar (Merck, Darmstadt, Germany)
- Lactose TTC agar (Merck, Darmstadt, Germany)
- Cetrimide agar (Merck, Darmstadt, Germany)
- Mixed cellulose ester membrane filters; 0.2 μm pore size, 20 mm diameter (ME; Whatman, Dassel, Germany)
- Filtration device

The number of viable culturable bacteria in the water samples was quantified by plating methods. 100 ml water sample was filtered, as indicated by most of the drinking water guidelines, placed on each specific agar, and subjected to the required cultivation conditions. Enterococci, *Campylobacter jejuni*, and *Salmonella* sp. were cultivated using the same agar media as described above. *Escherichia coli* were grown in two different media, Mac Conkey agar and Lactose TTC agar, at 37 °C for 48 h. *Pseudomonas aeruginosa* were grown on Cetrimide agar at 37 °C for 48 h. Heterotrophic bacteria were cultivated using R2A at 20 °C for 48 h.

3.5. Sampling Procedures

3.5.1. Sampling at Food Companies

Water sampling

- 2000 ml and 5000 ml Duran glass flasks (Schott Engineering, Mainz, Germany)
- Mixed cellulose ester membrane filters; 0.2 μm pore size, 20 mm diameter (ME; Whatman, Dassel, Germany)
- Centrifuge tubes; 2 ml (Eppendorf, Hamburg, Germany)

Water samples were aseptically taken at each selected sampling point using sterile flasks and were cool-transported as fast as possible for laboratory analysis. If the water was not processed at the same day it was kept at 4 °C. The water samples were used later for culture - dependent and/or - independent techniques. In the case of the first, 100 ml of the samples were filtered for each chosen agar media (Table 3.1). For culture - independent techniques planktonic bacteria from water samples were concentrated by filtration using 0.2 μm mixed cellulose ester membrane filters. The processed samples were frozen in case of transportation. The bacteria on the filter were then resuspended by thorough vortexing in an aliquot of the sampled water, the filter was thrown away. Due to the low number of bacteria expected in drinking water samples, cells in the suspension were disrupted by the commonly used freezing-thaw method (Muldrew and McGann, 1994) and kept at −20 °C until use.

Table 3.1.: Agar media and cultivation conditions of culture - dependent techniques.

Bacteria	**Agar media**	**Cultivation conditions**
Enterococcus faecium	Chromocult Enterococci agar / Slanletz - Bartley agar	37 °C for 48 h in aerobiosis
Salmonella enterica	Selective *Salmonella* ÖNÖZ agar	37 °C for 24 h in aerobiosis
Campylobacter jejuni	Campylosel agar / Columbia agar	37 °C for 48 h in microaerobiosis
M. avium subsp. *paratuberculosis*	Middlebrook 7H10 agar with Middlebrook OADC growth supplement and Mycobactine J / Harold's egg yolk agar slants with Mycobactine J and ANV	37 °C for 1 month in capnophilic atmosphere
Listeria monocytogenes	-	-
Pseudomonas aeruginosa	Cetrimide agar	37 °C for 48 h in aerobiosis
Escherichia coli	Mac Conkey agar / Lactose TTC agar	37 °C for 48 h in aerobiosis

Pipeline biofilm sampling

- Cotton swabs
- Sterile LiChroSolv PCR Water (Merck, Darmstadt, Germany)
- Centrifuge tubes; 2 ml (Eppendorf, Hamburg, Germany)

Biofilm samples of the pipelines were aseptically taken using cotton swabs. These were kept in 1 ml sterile water and were cool-transported as fast as possible for laboratory analysis. Cells were resuspended by mixing thoroughly and were finally removed from the swab by centrifugation. Cells in sample were disrupted by the already named freezing-thaw method (Muldrew and McGann, 1994), and kept at −20 °C until use.

3.5.2. Sampling at Waterworks

Biofilm samples were aseptically taken from each pipeline material and transported in sample water in cool conditions to the laboratory. If the samples were not processed at the same day they were kept at 4 °C. A cell scraper (PE Blade, PS Handle, 23 cm^2, Nalgene Nunc International) was used to remove the biofilm of each slide. Eight slides were scraped in 2.5 ml sterile water for DNA-based methods. Cells in the suspension were disrupted by the commonly used freezing-thaw method (Muldrew and McGann, 1994) and kept at −20 °C until use.

One slide was directly dyed in CTC solution for CTC/DAPI staining (see Section 3.15).

3.6. Polymerase Chain Reaction (PCR)

Different PCR primers targeting specific DNA regions were used. Primer specificity was determined with softsequence alignments using BLAST software and NCBI data.

A final 25 μl PCR reaction mixture contained 2,5 Unit (U) HotStar Taq-DNA polymerase (Qiagen, Hilden, Germany), 10 pmol of each primer (refer to Table 3.2), 10 x PCR buffer, 200 mM dNTPs (Amsham Bioscience) and 1-10 μl template. A GeneAmp PCR System 9700 (Applied Biosystems, Darmstadt, Germany) was used for the amplification. A reference strain was always amplified as positive control and sterile H2O as negative control. The temperature profile started always with 95 °C for 15 min, then the temperature and time of the PCR cycles varied with the primer sets (see Table 3.2), and

a final elongation step at 72 °C for 7 min was done. Aliquots of 10 µl PCR product were run by electrophoresis on 1-2 % agarose gel, depending on the product size, to verify their sizes and amounts.

3.7. Quantitative PCR

TaqMan primers and carboxyfluorescein (FAM)/Carboxytetramethylrhodamine (TAMRA) probes were provided by Sigma Aldrich Chemie (Taufkirchen, Germany) and Biomers.net (Ulm, Germany). Sequences are listed in Table 3.3. Quantitative PCR was accomplished by amplifying aliquots of 1-10 µl template in 25 µl reaction volumes containing 300 nM of each primer, 200 nM FAM/TAMRA-labeled probe, and 12.5 µl TaqMan Universal Master Mix (Applied Biosystems). Duplicates or triplicates of each sample were run. Sterile water was used as no template control (NTC). The temperature profile was standardized for all detection systems and comprised 2 min at 50 °C, 10 min at 95 °C, 45 cycles of 15 s at 95 °C and 1 min at 60 °C. Results were analyzed with the ABI Prism 7000 SDS software 1.1 (Applied Biosystems).

Table 3.2.: Primers used for PCR and seminested PCR assays for specific detection of pathogens.

Primers	Sequences (5'→ 3')	Microorganism	Gene	Gene Function	Amplification temperature profile			Product size (bp)	Literature Source
InlA1F InlA2R	CCTAGCAGGTCTAACCGCAC TCGCTAATTTGGTTATGCCC	*Listeria monocytogenes*	*inlA*	Internalin	94 °C 54 °C 72 °C	1:00 min 1:00 min 1:00 min	x 36	255	Jaradat et al. (2002)
Ecst784F Enc854R	AGAAATTCCAAACGAACTTG CAGTGCTCTACCTCCATCATT	*Enterococcus* sp.	23S rDNA		94 °C 60 °C 72 °C	1:00 min 1:30 min 1:30 min	x 35	92	Frahm et al. (1998)
Pa23F Pa23Rb	TCCAAGTTTAAGGTGGTAGGCTG ACCACTTCGTCATCTAAAAGACGAC	*Pseudomonas aeruginosa*	23S rDNA		94 °C 54 °C 72 °C	0:30 min 0:30 min 0:30 min	x 30	93	Volkmann et al. (2007)
Cam1F Cam2R	GCTCAAAGTGGTTCTTATGCNATGG GCTGCGGAGTTCATTCTAAGACC	*Campylobacter jejuni*	*flaA*, *flaB*	Flagellin	95 °C 53 °C 72 °C	0:05 min 0:30 min 0:40 min	x 40	336	Waage et al. (1999)
Cam1F Cam2R	GCTCAAAGTGGTTCTTATGCNATGG AAGCAAGAAGTGTTCCAAGTTT				95 °C 53 °C 72 °C	0:05 min 0:30 min 0:40 min	x 20	180	
mycF2 mycR2	AATGACGGTTACGGAGGTGGT GCAGTAATGGTCGGCCTTACC	*Mycobacterium avium* subsp. *paratuberculosis*	IS900	Insertion sequence IS900-like transposase	95 °C 54 °C 72 °C	0:05 min 0:30 min 0:40 min	x 35	76	Cook and Britt (2007)
InvA139F InvA141R	GTGAAATAATCGCCACGTCGGGCAA TCATCGCACCGTCAAAGGAACC	*Salmonella* spp.	*invA*	Membrane spanning protein	94 °C 64 °C 72 °C	0:30 min 0:30 min 0:30 min	x 35	284	Malorny et al. (2001) and Hein et al. (2006)

Table 3.3.: Primers and probes used for quantitative PCR assays for specific detection of pathogens.

Primers and probes	**Sequences (5'→3')**	**Microorganism**	**Gene**	**Gene function**	**Product size (bp)**	**Literature source**
hlyQF	CATGGCACCACCAGCATCT	*Listeria monocytogenes*	*hly*	Hemolysin	64	Rodríguez-Lázaro et al. (2004)
hlyQR	ATCCGCGTGTTTCTTTTCGA					
hlyQP	FAM-CGCCTGCAAGTCCTAAGACGCCA-TAMRA					
Ecst784F	AGAAATTCCAAACGAACTTG	*Enterococcus* sp.	23S rDNA		92	Frahm et al. (1998)
Enc854R	CAGTGCTCTACCTCCATCATT					
Gpl813TQ	FAM-TGGTTCTCTCCGAAATAGCTTTAGGGCTA-TAMRA					
Pa23F	TCCAAGTTTAAGGTGGTAGGCTG	*Pseudomonas aeruginosa*	23S rDNA		93	Volkmann et al. (2007)
Pa23Rb	ACCACTTCGTCATCTAAAAGACGAC					
Pa23P	FAM-AGGTAAATCCGGGGTTTCAAGGCC-TAMRA					
VS1F	ATTAGGTCTTAATACTAAAGATCAGCAAGGT	*Campylobacter jejuni*	16S rDNA VS	Variable sequence	115	This work*
VS1R	CGTCCTTTGTCTTATGGTTTGAATT					
VS1P	FAM-TGGCGTATTTGATGAATGTTT-TAMRA					
mycF2	AATGACGGTTACGGAGGTGGT	*Mycobacterium avium* subsp. *paratuberculosis*	IS900	Insertion sequence IS900-like transposase	76	Cook and Britt (2007)
mycR2	GCAGTAATGGTCGGCCTTACC					
mycP	FAM-TCCACGCCCGCCCAGACAGGTTG-TAMRA					
InvA139 F	GTGAAATAATCGCCACGTCGGGCAA	*Salmonella* spp.	invA	Membrane spanning protein	284	Malorny et al. (2001) and Hein et al. (2006)
InvA141 R	TCATCGCACCGTCAAAGGAACC					
InvAP	FAM-TTATTGGCGATAGCCTGGCGGTGGGTTTTGTG-TAMRA					
ECOuidAF	GTGTGATATCTACCCGCTTCGC	*Escherichia coli*	*uidA*	Glucuronidase	87	Frahm and Obst (2003)
ECOuidAR	AGAACGGTTTGTGGTTAATCAGGA					
ECOuidAP	FAM-TCGGCATCCGGTCAGTGGCAGT-TAMRA					

*designed by Dr. H. Volkmann.

3.8. DGGE

The eubacterial ribosomal primer systems targeting 16S ribosomal DNA (rDNA) (described in Table 3.4) were subsequently used for the DGGE analyses. Forward primers were modified by adding a GC clamp at the 5´ end for subsequent DGGE analysis. The primers GC27F/517R and GC341F/907R were used to obtain 490 base pair (bp) and 566 bp PCR products, respectively. 25 μl PCR final reaction mixture contained 2.5 U HotStar Taq-DNA polymerase (Qiagen, Hilden, Germany), 10 pmol of each primer, 10 x PCR buffer, 200 mM dNTPs (Amsham Biosciences), and 10 μl template. A GeneAmp PCR System 9700 (Applied Biosystems) was used for the amplification. DGGE analysis of PCR products was performed by means of the D-Code-System (BioRad Laboratories GmbH, Munich, Germany) using polyacrylamide gels containing a 40-70 % denaturing gradient of formamide-urea. DGGE gels were run in 1 x buffer solution containing tris base, acetic acid and EDTA (TAE) buffer (40 mM tris (hydroxymethyl) aminomethane (Tris base), 20 mM acetate, 1 mM Ethylenediaminetetraacetic acid (EDTA)) at 70 V and 60 °C for 16 h. The gels were stained with SYBR® Gold (Invitrogen, Karlsruhe, Germany). The stained gels were immediately analyzed using the Lumi-Imager Working Station (Roche Diagnostics, Mannheim, Germany). DGGE fingerprints were scored manually by the presence or absence of DNA bands. Pattern similarities were calculated using the Sørensen similarity index (Q_s):

$$Q_s = 2j(a+b)^{-1} \tag{3.1}$$

where j is the number of bands common to samples A and B, and a and b are the total numbers of bands in sample A and B, respectively. This index ranges from 0 (no common bands) to 1 (100 % similarity of band patterns) (Murray et al., 1996).

For the determination of population shifts within the downstream drinking water facilities at a food company, the main entrance point of public conditioned drinking water at the food company facilities was always used as reference.

To have a deeper knowledge about the DNA present in the samples, intensively stained bands were excised from DGGE (see point 3.9) for DNA sequencing.

Table 3.4.: Eubacterial ribosomal primer systems targeting 16S rDNA.

Primers	Sequences (5'→ 3')	Amplification temperature profile			Product size (bp)	Literature Source
GC27F 517R	GC-CAGAGTTTGATCCTGGCTCAG ATTACCGCGGCTGCTGG	95 °C 94 °C 54 °C 72 °C	15:00 min 1:00 min 1:00 min 1:00 min	 x 36	490	Emtiazi et al. (2004); Muyzer et al. (1993)
GC314F 907R	GC-CTACGGGAGGCAGCAG CCGTCAATTCTTTGAGTTT	95 °C 94 °C 60 °C 72 °C	15:00 min 1:00 min 1:30 min 1:30 min	 x 35	566	Green and Minz (2005)

3.9. Isolation and Preparation of DNA from DGGE Gels for DNA Sequencing

- Scalpel (VWR)
- Ultraviolet (UV)-Table (Fröbel Labortechnik)
- UV protection glasses
- Thermo Mixer (Eppendorf)
- Sterile LiChroSolv PCR Water (Merck, Darmstadt, Germany)
- ExoSAP-It® for PCR Product Clean-Up (usb, Staufen, Germany)

DNA bands separated by DGGE were visualized by exposition to UV light. Each band was carefully cut using a sterile scalpel. Each band was placed in a 1.5 ml reaction tube with 15 μl sterile water and were then incubated overnight at room temperature. 1 μl of this suspension was used as template for PCR (see section 3.8). The purity of bands can be verified by running these PCR products newly on a DGGE. If the bands are not pure the DNA bands should be cut again until a pure band is seen on the DGGE. If they are pure the subsequent purification can be done. For this, the PCR products were purified using the ExoSAP-It® kit, and this was used as template for the sequencing reaction.

3.10. Sequencing Reaction

The sequence reaction was carried out using the BigDye® Terminator v1.1 Cycle Sequencing Kit. A final volume of 10 µl contained: 2 µl premix, 5 pM forward or backward primer, and 3-10 µg DNA depending on the size of the DNA to be sequenced.

The temperature profile used for the sequencing reaction is described in Table 3.5:

Table 3.5.: Temperature profile of sequencing reaction.

5:00 min	96 °C	
0:10 min	96 °C	
0:05 min	58 °C	25 cycles
1:00 min	72 °C	

Subsequently, a purification of the sequencing product to remove excess ddNTPs was done using the DyeEx 2.0 Spin kit or precipitating the DNA with ethanol as indicated in Section 3.13. When using the DyeEx 2.0 Spin kit, the products were pipetted onto the gel matrix in a spin-column, and were then centrifuged at 2800 rpm for 3 min, following the instructions of the kit.

3.11. DNA Precipitation with Ethanol

- 3 M Sodium acetate solution (Sigma)
- 100 % Ethanol (Roth)
- Sterile LiChroSolv PCR Water (Merck, Darmstadt, Germany)

DNA was precipitated to remove salts and other impurities using ethanol. The DNA solution was mixed with 0.1 volume of 3 M sodium acetate (pH 5.4) and three volumes of ethanol 100 % (−20 °C), and left 1.5 h at −20 °C for DNA precipitation. This was then centrifuged at 13300 rpm for 30 min at 4 °C. The supernatant was discarded and the pellet was resuspended in sterile water.

3.12. DNA Sequencing

- BigDye® Terminator v 1.1 Cycle Sequencing Kit (Applied Biosystems)
- Hi-Di-Formamide
- Glass capillary (47 cm × 50 μm, Applied Biosystems)
- ABI PRISM® Genetic Analyser 310 (Applied Biosystems)
- Sterile LiChroSolv PCR Water (Merck, Darmstadt, Germany)
- DyeEx 2.0 Spin Kit (Qiagen)
- Polymer POP4 (Applied Biosystems)
- Sequencing Analysis Software (Applied Biosystems)

Once the sequencing product is pure, 6 μl of this DNA were added to 9 μl Hi-Di formamide and this was finally loaded in the ABI PRISM® Genetic Analyzer 310, and run under the conditions described in Table 3.6.

Table 3.6.: Conditions of sequencer.

Modul	P4rapidSeqE.md4
Injection time	10 s
Electrophoresis voltage	20 V
EP voltage	15 kV
Heat plate temperature	50 °C

Finally, the DNA sequences were analyzed using the Sequencing Analysis software and compared with known sequences of the NCBI database (http://www.ncbi.nlm.nih.gov/BLAST)

3.13. PMA Treatment

- Propidium monoazide (20 mM in 20 % DSMO, Biotium)
- Dimethylsulfoxide (DMSO; Sigma, Munich, Germany)
- Mμlti® - Safety reaction tubes (1.5 ml, CarlRoth)

- Halogen light source (650 W, GE Commercial Lighting Products)
- Qiagen Genomic - Mini tip 20/G or Midi tip 100/G kit (Qiagen, Hilden, Germany)

PMA was dissolved in 20 % DMSO to create a stock solution of 20 mM, and stored at −20 °C in the dark until use. PMA stock solution was added to the template to reach a final concentration of 20 μM. Following an incubation period of 15 min in the dark with occasional thorough mixing, samples were laid then horizontally on ice and were light exposed for 8 min, using a 650 W halogen light source. Samples were placed about 20 cm from the light source. Placing the samples horizontally on ice should avoid excessive heating and might optimize light exposure by reflection. A light exposure time >120 s is essential to guarantee efficient binding of PMA to DNA and at the same time to achieve efficient inactivation of free PMA that did not bind to DNA. This is important in order to make sure that no active PMA remains in solution, which could bind to DNA originating from viable cells after the cell lysis step (Nocker et al., 2006). Occasional mixing of the samples makes sure that every single part receives good light exposure. After photo - induced crosslinking, cells were pelleted at 8000 rpm for 5 min, then the pellet was resuspended in sterile water and centrifuged again to wash out the PMA. The supernatant was thrown away and the DNA contained in the pellet was isolated using the Qiagen Genomic - Mini tip 20/G or the Midi tip 100/G kit in accordance with the manufacturer's guidelines.

3.14. DNase I Treatment

- DNase I (Fermentas, St. Leon - Rot, Germany)
- 10 x reaction buffer with $MgCl_2$ (Fermentas, St. Leon - Rot, Germany)

Samples were firstly exposed to DNase I in the presence of a buffer for a determinate time. Then the DNase I was inactivated. The concentrations of DNase I, the concentrations and type of buffer used, and the DNase I inactivators varied depending on the experiment, being described in more details in the corresponding section.

3.15. CTC/DAPI: Metabolic - active/Total DNA Staining

- 5 - cyano - 2,3 - ditoryl tetrazolium chloride (CTC)(Polysciences inc., Eppelheim, Germany)
- 4' - 6 - diamidino - 2 - phenylindole (DAPI) (AppliChem, Darmstadt, Germany)
- Shaker Unimax 2010 (Heidolph)
- Polycarbonate filter; 0.2 μm pore size, 20 mm diameter (Whatman, Dassel, Germany).
- Anti - fading agent AF1: Glycerol and PBS (Citifluor Ltd., London)
- Axioplan epifluorescence microscope (Zeiss, Germany)
- Mercury short ARC photo optic lamp HBO® 50 W/AC (OSRAM, Augsburg, Germany)

Metabolic active bacteria were counted using the CTC method, while total cells were counted by staining DNA with DAPI.

CTC was applied directly to the sample at an end concentration of 4 mM. After 4 hours of incubation at 22 °C in darkness it was filtered using a 0.2 μm polycarbonate filter. In the case of staining of biofilms from slides, these were scrapped in 2 ml of this CTC solution and then filtered. DAPI staining was done directly, applying 1 ml DAPI solution (1 μg/ml) on the filter for 5 min in darkness, for counterstaining purposes. Finally, the stain was removed by filtration and the filter was air - dried and fixed on a glass slide. A drop anti - fading agent (Citifluor) was used to fix the coverslip to the glass slide. The stained cells were counted using an epifluorescence microscope equipped with a 50 W light source, to examine the filters at a magnification of 1000x. Observations were performed with a fluorescence light fitted with a BP365/FT395/LP397 blue filter for DAPI and a BP546/FT580/LP590 red filter for CTC, allowing simultaneous visualization of both dyes. Counting was carried out randomly on the basis of 10 microscopic fields per filter. For each sample, three filters were counted. Metabolic active cells, showing CTC formazan crystals, and total cell counts, with staining by DAPI (i.e., viable and non - viable), were determined. Results were expressed as the number of corresponding bacteria per milliliter of the original sample, and percentages of metabolic active bacteria relative to total cell counts were determined. Experiments were conducted in triplicate.

4. Results

The potential role of water in foodborne disease and in the distribution of pathogens to various types of processed commodities is well known. As already described in Section 2.2, some pathogens have been shown to survive in water for long time periods. The water - food route is therefore an essential point for monitoring the occurrence and preventing the spread of pathogens.

Molecular-biology techniques were first optimized in this work to detect pathogens. Later, a strategy where these techniques were applied was developed to detect possible water - derived critical control points at two food companies. A questionnaire was done for the food companies (see Appendix A) to collect more information about their drinking water sources, and the conditioning and distribution of drinking water during food processing. This questionnaire and the work - together with the companies were essential to have a better understanding of laboratory results and to apply when necessary hygiene measures. Regarding food safety, it is believed that only live bacteria are important for the food industry. Therefore, further experiments were done in order to detect only live bacteria in water samples. These experiments were also applied with drinking water biofilm samples of a waterworks.

A German dairy company and a Spanish dry cured ham company were chosen for this investigation. Even though cheeses have been characterized as safe for consumption they have been implicated in foodborne outbreaks associated with severe symptoms and high fatality rate. The foodborne pathogens in raw milk are generally originated in the farm environment. In dairy plants the pathogens may enter via contaminated raw milk, colonize the dairy plant environment and consequently contaminate dairy products. An important source of contamination during the handling and processing might be the workers as well (Blackburn and McClure, 2002; Kousta et al., 2010; Zottola and Smith, 1991). Some foodborne outbreaks have also been associated with dry cured ham (Baver-Cid et al., 2010). Dry cured ham shows a low water activity (a_w, usually lower than 0.92) and high salt concentration (higher than 4 %). In case of a potential contamination, these intrinsic characteristics hardly support the growth of pathogens but may allow

their survival. *Listeria monocytogenes* constitutes the major concern of dry cured ham, since it is a wide-spread environmental microorganism and it is difficult to eradicate from the product environment. The main cause of contamination with pathogens in these kinds of companies has been associated with cross-contaminations, therefore a safe drinking water source also has to be guaranteed.

The German dairy company was founded in 1930 as a cooperative. In 1949 the cooperative was converted into a GmbH. It has approximately 70 employees. The dairy relies on 330 farmers of the Allgäu-Bodensee region supplying 55 millions kg milk annually of which 8 % are produced according to organic (Bioland/Demeter) guidelines. It is well known for its cheese specialities which include feta cheese, camembert and semi-soft cheeses. The pipeline material was stainless steal, and the water provided by the waterworks was groundwater. Some hoses were used in the production. The microbiological control of the drinking water was done externally and internally every 6 months.

The Spanish dry cured ham company is a family company that was established in 1898. Currently, they have approximately 120 employees. It produces high quality dry cured hams and dry cured loins. It produces long ripened ($>$ 20 months) hams from three different pork breeds: Jamón Serrano from white pigs, Jamón Ibérico from Iberian pigs and Jamón Mangalica from a traditional Hungarian pig. This company has an annual production of approximately 500.000 hams of which 30 % are exported world wide. The drinking water samples were taken from the production line at the building constructed in 1987. The pipelines and connexions were 20 years old. The water provided by the waterworks was chlorinated groundwater. Some hoses were used in the production. The microbiological control of the drinking water was done externally every 4 months.

The drinking water samples from both food companies were taken at points where water could be a possible source of food contamination, i.e. where the water was directly used in the production of food, where it was used for cleaning and rinsing of machines which had direct contact with the food products, and where it was used to wash the hands of employees.

The techniques applied in the strategy developed to detect possible water-derived critical control points at both food companies detected viable culturable bacteria (plating techniques) and total DNA (DNA-based techniques), but were not capable to determine the total live bacteria fraction. Some culture-independent methods were tested and optimized in this work to detect total live bacteria (i.e. viable culturable and viable but non-culturable bacteria) in water samples. And were finally applied to test drinking water biofilm samples of a German waterworks.

4.1. Sensitivity Tests of Pathogen Detection Methods

The DNA of the reference bacteria was used to determine the sensitivity of the different specific detection systems instead of bacterial cultures. For this, serial 10-fold dilutions of the DNA from the reference strains were applied as PCR template.

The amounts of bacteria corresponding to the DNA used for measuring standard parameters were calculated from their genome lengths (Süß et al., 2006). This calculation was based on the assumption of the average weight of a base pair (bp) as 650 Daltons. This means that one mole of a bp weighs 650 g. Using the Avogadro number 6.022×10^{23} molecules/mol, the number of bp molecules in one gram can be calculated as:

$$\frac{1\,\text{mol}}{650\,\text{g}} \times 6.022 \times 10^{23}\,\frac{\text{bp molecules}}{\text{mol}} = 9.26 \times 10^{20}\,\frac{\text{bp molecules}}{\text{g}}. \tag{4.1}$$

And the molecular weight (Mw) of any dsDNA template (i.e. 1 genome) can be estimated by dividing the genome length (in bp) by $9,26 \times 10^{20}$ bp molecules/g, as follows:

$$Mw\,\text{genome}\,[\text{g}] = \frac{\text{genome length}\,[\text{bp}]}{9.26 \times 10^{20}\,[\text{bp/g}]}. \tag{4.2}$$

This result can be expressed in fg by multiplying by 10^{15} or in ng by multiplying by 10^9. The genome lengths (Fogel et al., 1999) and the calculated weight of the genomes (in fg) of the bacteria used in the present work are shown in Table 4.1.

Finally, the number of bacteria or number of copies of template present in 1 μl sample can be estimated by multiplying by 10^9 for conversion to ng and then multiplying by the DNA concentration (in ng/μl). The formula used was:

$$\begin{aligned} \text{Bacteria per } \mu\text{l} &= \frac{\text{DNA conc.}\,[\text{ng}/\mu\text{l}]}{Mw\,\text{genome}\,[\text{g}] \times 10^9[\text{ng/g}]}; \\ &= \frac{\text{DNA conc.}\,[\text{ng}/\mu\text{l}] \times 9.26 \times 10^{20}}{\text{genome length}\,[\text{bp}] \times 10^9[\text{ng/g}]}\,\frac{[\text{bp}]}{\text{g}}. \end{aligned} \tag{4.3}$$

To calculate the final amounts of bacteria in the samples, the initial volume and the respective concentration rate of each sample were considered.

Table 4.1.: Genome lengths and weight of bacteria.

Bacteria	**Genome length (kb)**	**Genome weight (fg/bacteria)**
Enterococcus faecium	2875	3.16
Salmonella enterica	4746	5.22
Campylobacter jejuni	2067	2.30
M. avium subsp. *paratuberculosis*	5838	6.40
Listeria monocytogenes	3150	3.40
Pseudomonas aeruginosa	1637	4.00
Escherichia coli	4639	5.10
Staphylococcus aureus	2583	2.84

4.1.1. PCR and Seminested PCR

PCR assays were developed or optimized to detect hygienic relevant bacteria in drinking water. PCR primers were used to target specific virulence or taxon-specific genes. The sensitivities of the different PCR assays were obtained by running the PCR products of genomic DNA in serial dilutions on agarose gel.

An example for the determination of the detection limit of the enterococcal specific PCR system is shown in Figure 4.1. In this example, a 10-fold dilution of enterococcal genomic DNA was done. 10 μl of each dilution was amplified using the specific PCR system targeting the 16S rRNA fragment in 25 μl total reaction volume, therefrom 10 μl PCR product were run on a 1 % agarose gel. The detection limit for this specific PCR primer system was 1 fg DNA/μl. Knowing that the genome of enterococci weighs 3.16 fg (see Table 4.1) and that the start DNA concentration of the 10-fold dilutions was 10 ng/μl the detection limit can be expressed in "bacteria per μl", and it would be 0.32 bacterium/μl. Considering a 10000 times concentration rate of the original water sample from the company, the detection limit expressed as bacteria per 100 ml original water sample is 3 bacteria/100 ml.

In the present work, the biomass of the original water samples was concentrated in order to have a detection limit similar to the detection limit of the traditional plating techniques. The water samples of the first sampling period at the German dairy company were concentrated only 2000 times, and the water samples of the Spanish dry cured ham company were concentrated 3700 times. After determining the detection limits of the molecular biology detection systems, a 10000 times concentration rate was stipulated in

order to obtain detection limits similar to the traditional plating techniques.

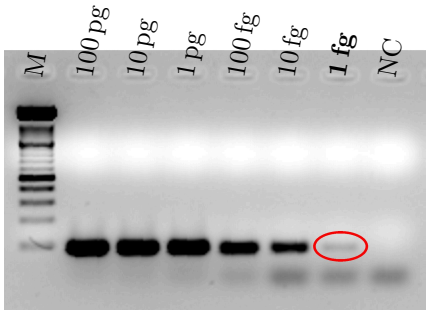


Figure 4.1.: Standard curve. PCR products of serial dilution after specific enterococcal DNA amplification. NC: negative template control, and M: 100 bp DNA marker.

The detection limits obtained by PCR (see Table 4.2) after a 10000 times concentration rate of the water samples, were still in part too high compared to the traditional cultivation methods, to be used as routine detection system. Only the system for the detection of *Enterococcus* presented a low detection limit. These detection systems could be used in case of achieving higher concentration rates of the water samples.

Table 4.2.: Detection limits of PCR and seminested PCR systems. Detection limits were calculated considering a 10000 times concentration rate of the water sample.

Bacteria	**Target gene**	**Genome length (kb)**	**Detection limit (cell/100 ml)**
Enterococcus faecium	23S rDNA	2875	3
Salmonella enterica	*invA*	4746	190
Campylobacter jejuni	*flaA* and *flaB*	2067	440
M. avium subsp. *paratuberculosis*	IS900	5838	15870
Listeria monocytogenes	*InlA*	3150	29
Pseudomonas aeruginosa	23S rDNA	1637	25

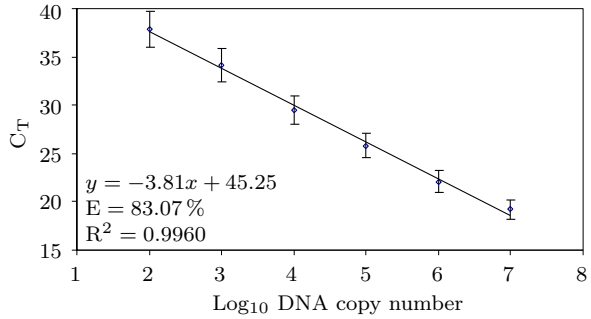
4.1.2. Quantitative PCR

qPCR assays were developed or optimized to detect and quantify hygienic relevant bacteria in drinking water. The qPCR primers and probes that were used to target specific virulence or taxon-specific genes are listed in Table 3.3. Genomic DNA dilutions were used instead of bacterial suspensions for sensitivity assays due to the retarded growth of some bacterial species, such as *Mycobacterium avium* subsp. *paratuberculosis*. The sensitivities of the qPCR assays shown in Table 4.3 were obtained when the standard curves were done, after amplifying genomic DNA serial dilutions of each target bacteria. Average C_T values were calculated from triple reactions. Considering that the DNA of the samples would be detected by qPCR in a volume of 10 μl template and that the bacteria present in this template would be concentrated 10000 times by filtration of the original water sample, the detection limits calculated for *E. faecium*, *S. enterica*, and *P. aeruginosa* were similar to those of the standard plating methods (1 cell/100 ml). The qPCR detection limits calculated for *C. jejuni*, *L. monocytogenes*, and *E. coli* were 2 to 4 cells/100 ml. In the case of MAP, the qPCR detection limit was 1090 cell/100 ml. This could be due to an improper access to the DNA of the cell due to the thicker cell wall of these bacteria.

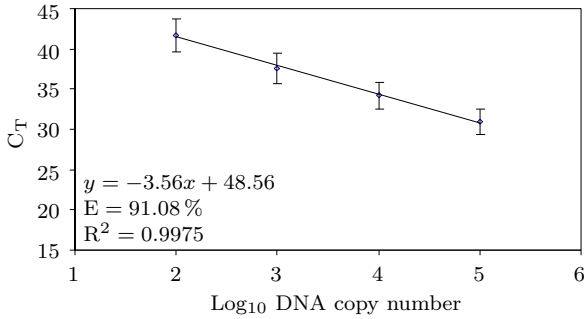
Table 4.3.: Detection limits of quantitative PCR systems. Detection limits were calculated considering a 10000 times concentration rate of the water sample.

Bacteria	**Target gene**	**Genome length (kb)**	**Detection limit (cell/100 ml)**
Enterococcus faecium	23S rDNA	2875	1
Salmonella enterica	*invA*	4746	1
Campylobacter jejuni	VS1	2067	4
M. avium subsp. *paratuberculosis*	IS900	5839	1090
Listeria monocytogenes	*hly*	3150	3
Pseudomonas aeruginosa	23S rDNA	1637	1
Escherichia coli	*uidA*	4639	2

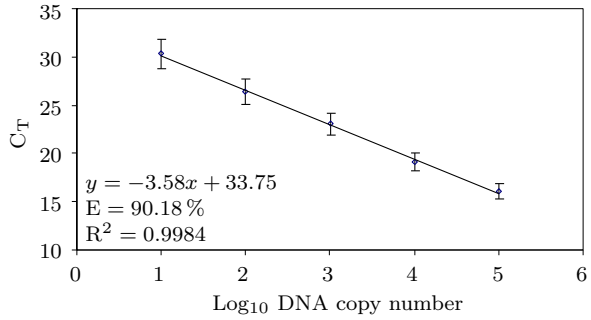
The equations of the standard detection curve of each pathogen given in Figure 4.2 were estimated by linear regression. These equations were used to determine the bacterial concentration present in the water samples from their genome lengths, as described previously. The correlation coefficients were between 0.9958 and 0.9995, indicating a high precision and a strong correlation between DNA concentrations of the template and the C_T values.



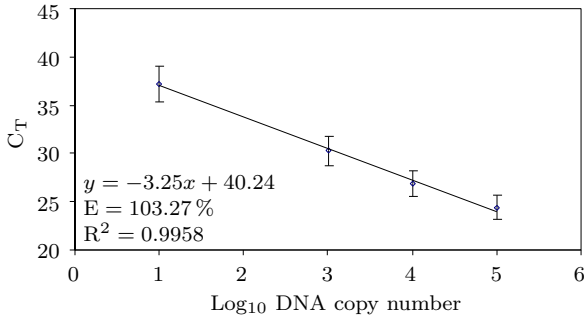
(a) *Campylobacter jejuni*



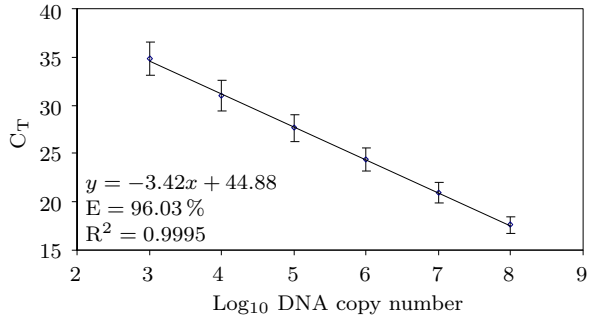
(b) *Escherichia coli*



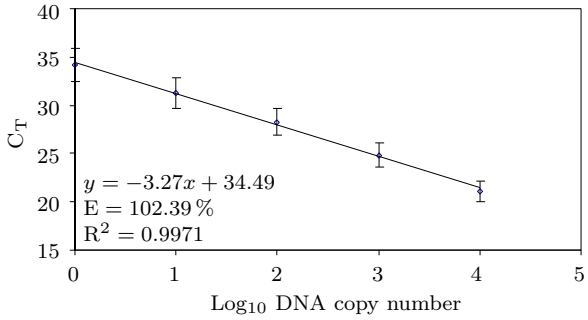
(c) *Enterococcus faecium*



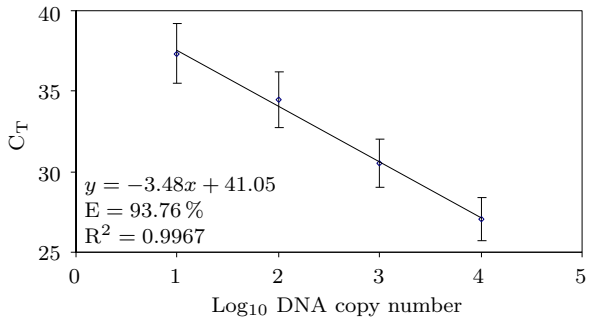
(d) *Listeria monocytogenes*



(e) *Mycobacterium avium* subsp. *paratuberculosis*



(f) *Pseudomonas aeruginosa*



(g) *Salmonella enterica*

Figure 4.2.: Quantitative PCR standard analysis curves. Serial dilutions of reference strain genomic DNA were used as template. Cycle threshold values (C_T) are plotted against log10 copies of bacterial DNA. Linear regression, PCR efficiency (E) and regression coefficients (R^2) for each bacterial detection system are shown. In parallel, sterile water was used for NTCs.

False positive results were obtained when *E. coli* DNA was amplified by qPCR using the primer system that targeted the gene which encodes the β-glucuronidase protein (*uidA*). The commonly used HotStar Taq-DNA polymerase appeared to be a contamination source of *E. coli* DNA, because this enzyme was expressed as a recombinant protein in *E. coli* (Shannon et al., 2007). In order to avoid this, the qPCR used for the detection of *E. coli* was done with the TaqMan ® Gene Expression Master Mix (Applied Biosystems). This kit uses the AmpliTaq Gold® DNA Polymerase Ultra Pure enzyme that is identical to the AmpliTaq Gold® DNA polymerase, but further purified to reduce bacterial DNA introduced from the host organism. The purification process ensures that non-specific, false-positive DNA products due to bacterial DNA contamination are minimized during PCR (protocol of AmpliTaq Gold® DNA Polymerase Ultra Pure enzyme, Applied Biosystems).

4.2. Protocol Developed for the Detection and Removal of PCR Inhibitors

Drinking water samples are supposed to have a very low amount of bacteria, therefore in order to carry out a reliable culture - independent analysis of the bacterial population and to reduce the lost of DNA from the sample, the bacteria have to be concentrated by a filtration step without any DNA-purification. One of the most important problems of a direct amplification of DNA from water samples without any DNA-purification is the possible presence of PCR inhibitors. Drinking water has different origins (e.g. groundwater, surface water), and depending on its origin substances that inhibit the action of the DNA polymerases can be present. Especially surface water has a high contact with organic matter. Tannins and other oligomeric compounds with free phenolic groups (e.g., humic acids) can be present in it. These substances can oxidize to form quinones, which covalently bond to and inactivate DNA polymerases. As a result, amplification efficiencies are reduced increasing the possibility of obtaining false negative results. Inhibition severity is directly related to the amount of tannins present in the sample Kontanis and Reed (2006). An early detection of PCR inhibitors would facilitate sample processing by conserving time, reagents, and finite DNA samples. Once the PCR inhibitors are detected a attempt to remove them can be done.

4.2.1. Detection of PCR Inhibitors

Eubacterial ribosomal primer systems targeting 16S rDNA were applied to perform the PCR. If no DNA amplification was observed after the PCR, a PCR efficiency assay was carried out. The PCR efficiency assay consisted in spiking 1 μl of a known quantity of enterococcal genomic DNA to 9 μl of each template. In parallel, the standard DNA was used exclusively. The temperature profile consisted in a treatment of 15 min at 95 °C, followed by 35 cycles of 0:30 min at 94 °C, 0:30 min at 54 °C and 1:30 min at 72 °C, and a final step of 7 min at 72 °C. Aliquots of 10 μl PCR products were subjected to electrophoresis on 1 % agarose gel to verify their sizes and estimated amounts. If no PCR inhibitors are present in the sample the intensity of the bands should be the same or higher than the band corresponding to the control DNA, if a partial inhibition is present the intesity of the bands will be lower as the control DNA, and if a total inhibition is present no PCR product will be observed (see Figure 4.3).

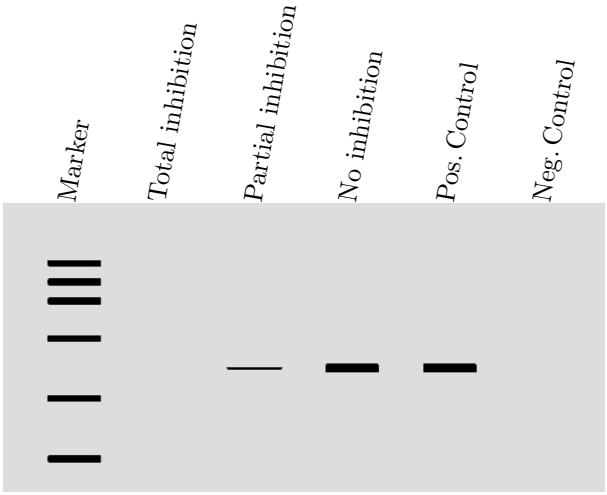


Figure 4.3.: PCR efficiency assay.

4.2.2. Removal of PCR Inhibitors

If PCR inhibitors were present, 0.5 μl sterile bovine serum albumin (BSA) (Sigma, Munich, Germany) solution (5 mg/ml) was added to the PCR reaction mix according to Kreader (1996). In case of stronger inhibitions, a polyvinylpolypyrrolidone (PVPP) (Sigma) treatment of the samples was performed according to Sutlović et al. (2007). Where, sterile PVPP was well mixed with the sample in relation 1:10 (g PVPP/ml sample), after 1 h at 37 °C it was centrifuged at 3000 rpm for 3 min. The pellet was discarded and the supernatant kept at −20 °C until use.

Once the samples had no PCR inhibitors, the battery of molecular biological assays was applied to analyze the water of each food company.

4.3. Food Industry Water Surveillance

4.3.1. Strategy Developed for Drinking Water Surveillance

A strategy was developed to monitor the bacterial water quality and stability at a German and a Spanish food company.

The identification of potentially water - derived critical control points at the food companies was achieved following the steps shown in Figure 4.4.

Selection of the sampling points together with the person responsible for quality control at the food company was of great importance. Water samples had to be taken strategically at those points, where the water could be a potential risk for food hygiene. The points that were considered, were those places where drinking water had a direct contact with food (i.e. as ingredient, washing processes) and where drinking water had an indirect contact with the food, in order to avoid cross - contaminations (i.e. hand washbasins, rinse of machines that are in contact with foodstuffs).

Drinking water is not sterile, it has an autochthonous mostly harmless bacterial population. The bacterial population present in the water within a food company is supposed to be similar. To evaluate the bacterial stability of water within the food companies, autochthonous bacterial population analyses were done. For this, first the bacteria present in the sample were concentrated by filtration, and then the presence of PCR inhibitors was tested with the PCR efficiency assay. If no inhibitors were found, PCR of the sample could be done. But, if PCR inhibitors were found, these first had to be removed in order

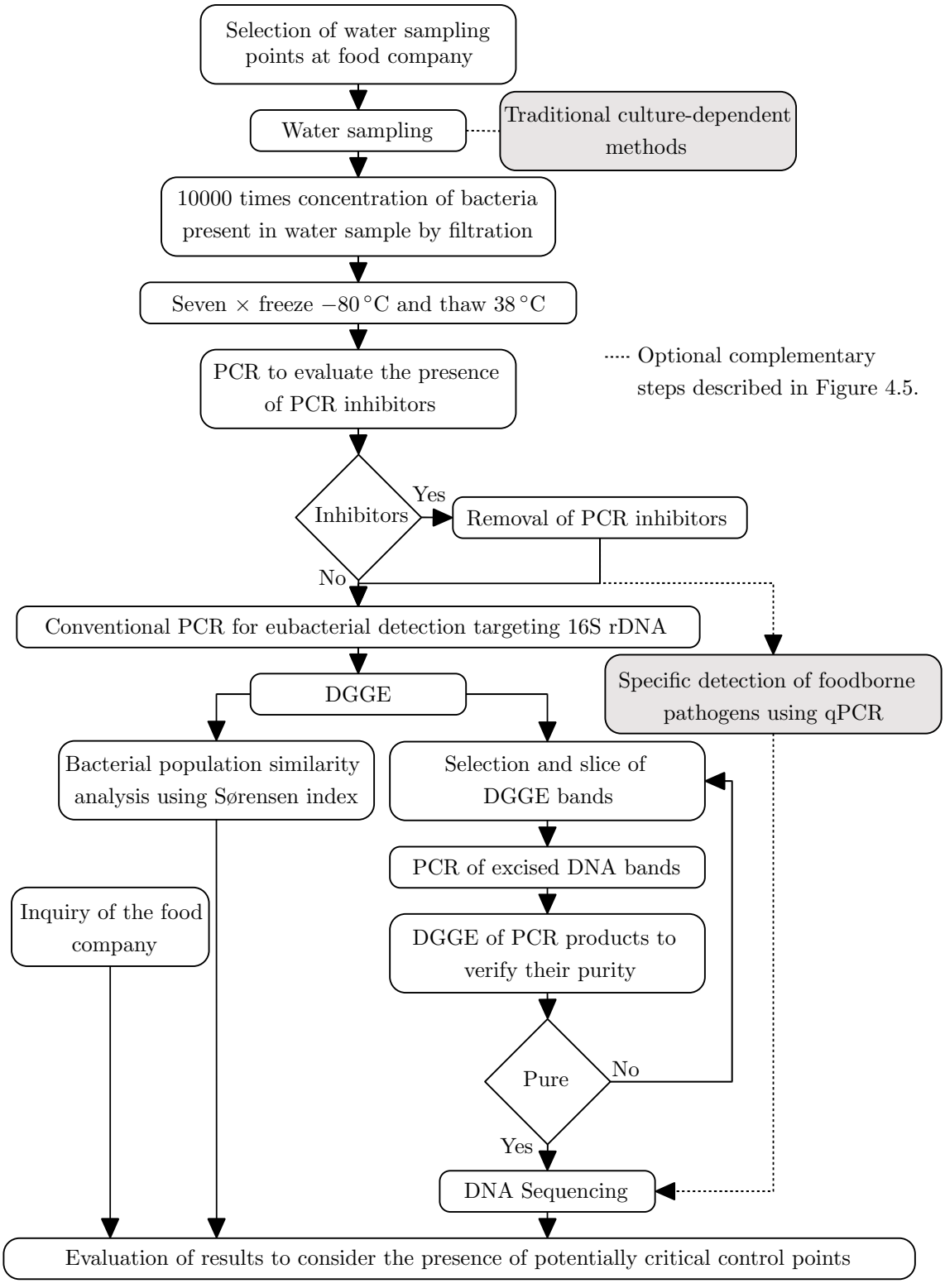


Figure 4.4.: Strategy used for the detection of possible water - derived critical control points at the food companies.

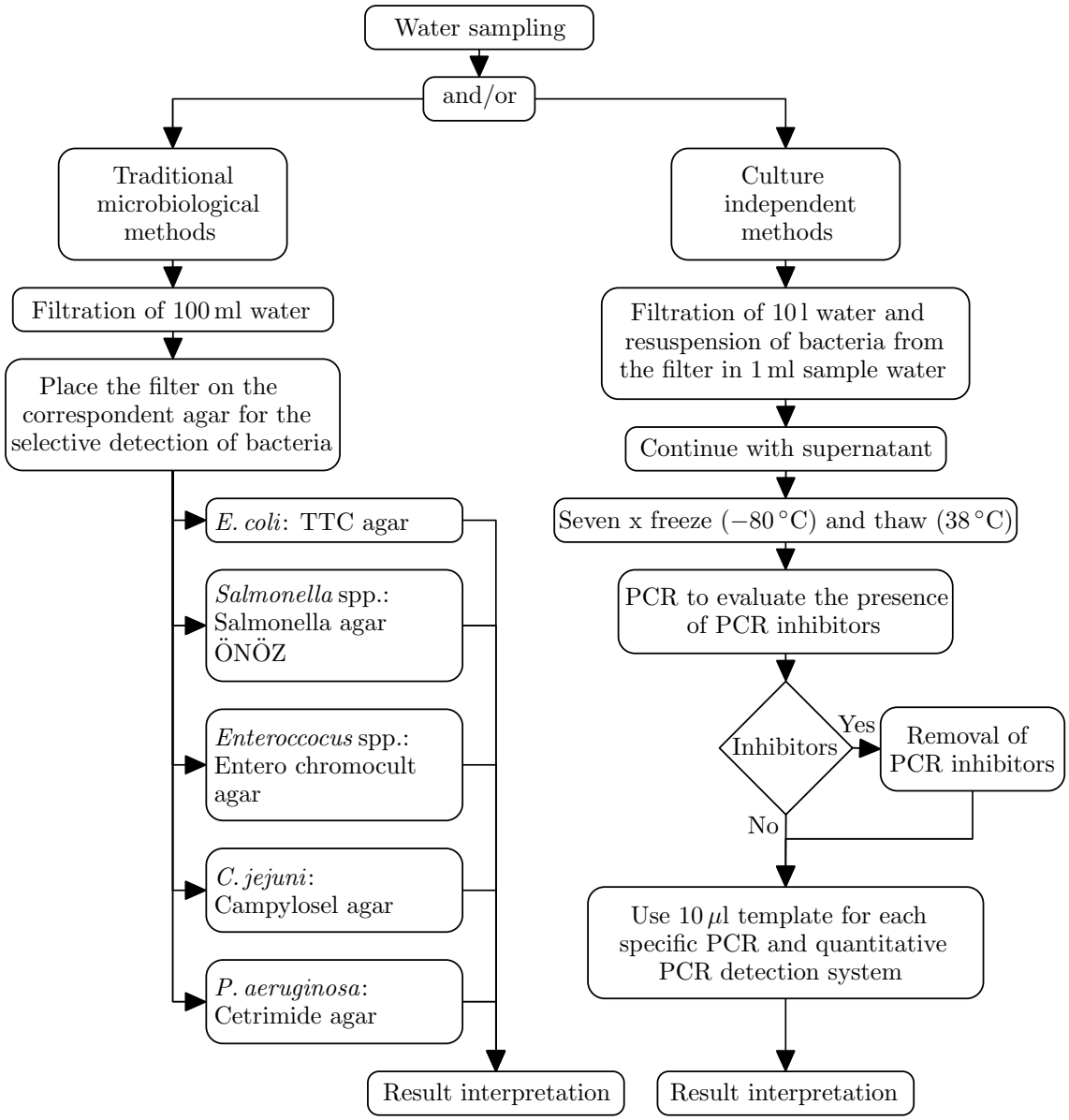


Figure 4.5.: Overview of the developed strategy used for the monitoring of pathogens or indicator organisms in drinking water.

to proceed to the PCR - DGGE assays. The PCR used for the analyses of bacterial stability targeted the 16S rDNA gene in order to amplify the DNA of eubacteria present in the sample. These PCR products were then run on DGGE gels in order to compare the DNA patterns of the water samples taken at the different points of the food companies. To have a deeper analysis of the bacterial population DNA sequencing was done.

The complementary step exposed in Figure 4.5, developed for the specific detection of pathogens via culture - dependent and - independent techniques, together with the answered questionnaire done for the food companies (see Appendix A), and with the inquiry done to the food companies, are the keys for the achievement of a general evaluation of potentially water - derived critical control points within a food company.

4.3.2. German Dairy Company Analysis

The German dairy company was supplied with conditioned groundwater exclusively and no further disinfection was performed on - site.

The first sampling point was the point where the water entered the food company, and this point was taken as reference for all the downstream bacterial population analysis.

Cross - contaminations can occur in a food company if the water used for rinsing of the room and/or machineries is not appropriate, therefore the following sampling points were chosen (see Figure 4.6):

1. Entry of public conditioned drinking water: reference.
2. Lactic acid tank: water used to rinse the tank.
3. Portioner: water used to rinse the portioner machinery.
4. Hand washbasin: water used to wash hands in salting room.
5. Maturation room: water used to clean the room.
6. Feta packaging: water used to clean room and machinery.

The pipeline system was made of stainless steel, hoses were used at sampling points 2 (lactic acid tank) and 3 (portioner), and warm water was used at points 2 (lactic acid tank) and 4 (hand washbasin).

(a) (b) (c)

(d) (e) (f)

Figure 4.6.: Sampling points of German dairy company. (a) 1. Entry of public conditioned drinking water: reference, (b) 2. Lactic acid tank: water used to rinse the tank, (c) 3. Portioner: water used to rinse the portioner machinery, (d) 4. Hand washbasin: water used to wash hands in salting room, (e) 5. Maturation room: water used to clean the room, (f) 6. Feta packaging: water used to clean room and machinery.

Table 4.4.: Conventional plating, PCR and quantitative PCR results of water samples of the German dairy company (first sampling period). Duplicates or triplicates of each sample were run.

	Sampling Point					
	1	**2**	**3**	**4**	**5**	**6**
Plating methods	Negative for all pathogens					
PCR						
Enterococcus spp.	-	+	-	-	-	-
Salmonella spp.	-	-	-	-	-	-
Campylobacter jejuni	-	-	-	-	-	-
M. avium subsp. *paratuberculosis*	-	-	-	-	-	-
Listeria monocytogenes	-	-	-	-	-	-
Pseudomonas aeruginosa	-	-	-	-	-	-
Quantitative PCR						
Enterococcus spp.	-	+*	-	-	-	-
Salmonella spp.	-	-	-	-	-	-
Campylobacter jejuni	-	-	-	-	-	-
M. avium subsp. *paratuberculosis*	-	-	-	-	-	-
Listeria monocytogenes	-	-	-	-	-	-
Pseudomonas aeruginosa	-	+*	-	-	-	-
Escherichia coli	-	-	-	-	-	-

* positive results are described in more detail in the text.

No PCR inhibition was detected after performing the PCR efficiency assay.

PCR and qPCR results of the first sampling period are shown in Table 4.4. The drinking water at the entrance point met all requirements of the German drinking water regulations. The sample from point 2 (lactic acid tank), where hoses were involved in the process, was the only sample that exhibited positive results for *P. aeruginosa* and enterococci after qPCR analysis. An average C_T value of 33.21 (see Figure 4.7) was found for *P. aeruginosa*. By transpolating this value to the standard curve, a value of 2.45 fg *P. aeruginosa* DNA per μl was obtained. Knowing that one *P. aeruginosa* bacterial cell DNA weighs 3.99 fg, that 10 μl template were used for the qPCR, and that the bacteria present in the sample were concentrated by a factor of 2000 by filtration, the calculated number of *P. aeruginosa* for this sample was 31 cells/100 ml water sample.

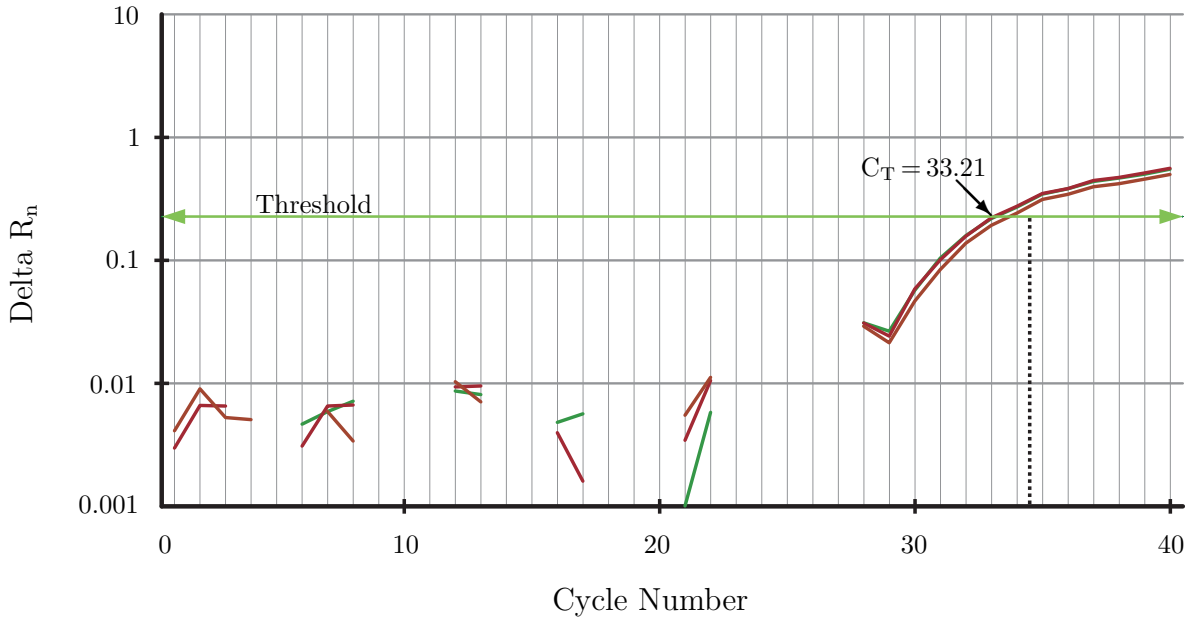


Figure 4.7.: Quantitative PCR curve: Detection of *P. aeruginosa* first sampling period of German dairy company. Detection limit (dotted line): $C_T = 34.48$.

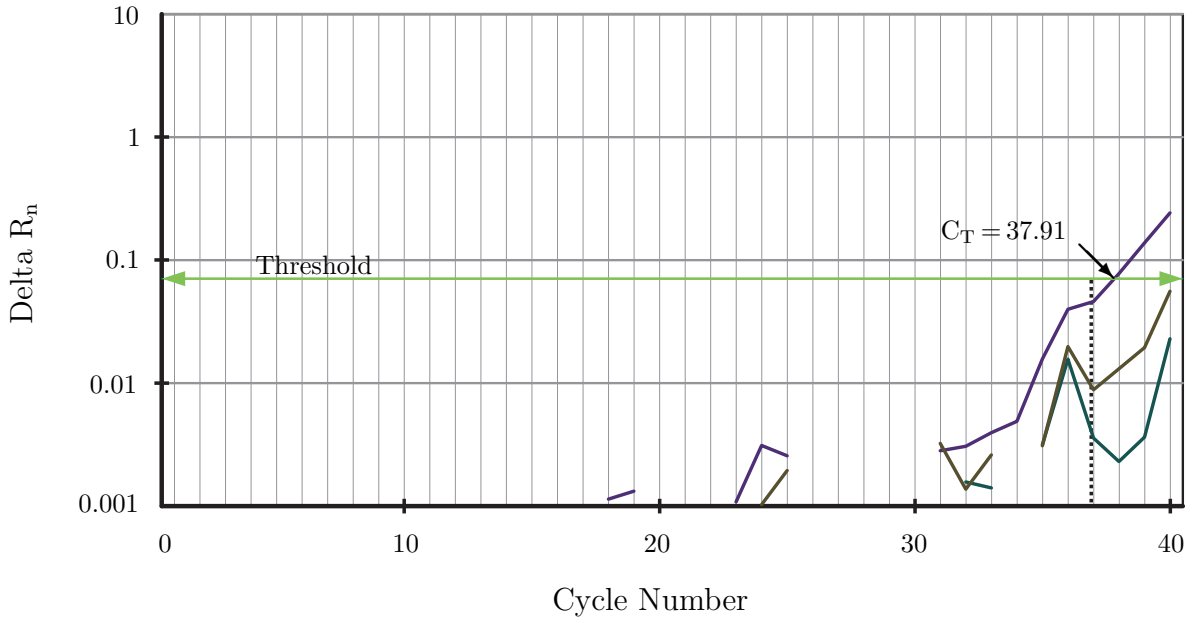


Figure 4.8.: Quantitative PCR curve: Detection of enterococci first sampling period of German dairy company. Detection limit (dotted line): $C_T = 36.94$.

One of three samples presented a positive enterococci - specific signal at this point, but the C_T value was 37.91 (see Figure 4.8), this value was not in the range of the standard curve (see Figure 4.2), being even higher as the calculated detection limit (C_T 36.94, see Table 4.3). Therefore, this C_T value was not completely reliable.

None of the other water samples taken at this company exhibited positive qPCR results for any of the specific targeted pathogens (Table 4.4).

None of the indicated pathogenic bacteria were detected after filtering 100 ml of each water sample and carrying out the plating methods on the specific selective media. In some cases, unspecific bacterial growth was observed on agar plates, but these colonies were identified as false positive isolates after sequencing of 16S ribosomal DNA.

Analysis of the autochthonous bacterial population of water samples during the first sampling period (Figure 4.9) revealed a total number of 9 DGGE DNA bands in the sampling point of the entrance of water to the company used as reference point for the Sørensen similarity indexes (Figure 4.9, lane 1). Each DNA band was assumed to represent one bacteria species.

In the subsequent samples the number of bands did not differ, or increased only slightly by 1 to 3 bands when compared to the reference sample. Previous studies revealed that Sørensen similarity indexes between 0.40 and 1 (i.e. between 40 and 100 % similarity) reflected a natural range of population diversity in a drinking water distribution system (Emtiazi et al., 2004). Hence, similarities below 40 % are discussed to indicate a population shift in the autochthonous bacterial population of drinking water systems. Only sampling point 6 (feta packaging) was found to exhibit a decreased similarity value of 30 % (see Table 4.5). All the other points presented high bacterial population similarities ranging from 44 to 60 %. Consequently, point 6 was considered a potentially critical point.

Table 4.5.: Sørensen indexes: First sampling period at German dairy company.

Reference	Q_s values for the sampling points					
point	1	2	3	4	5	6
1	1	0.56	0.40	0.53	0.42	**0.29**

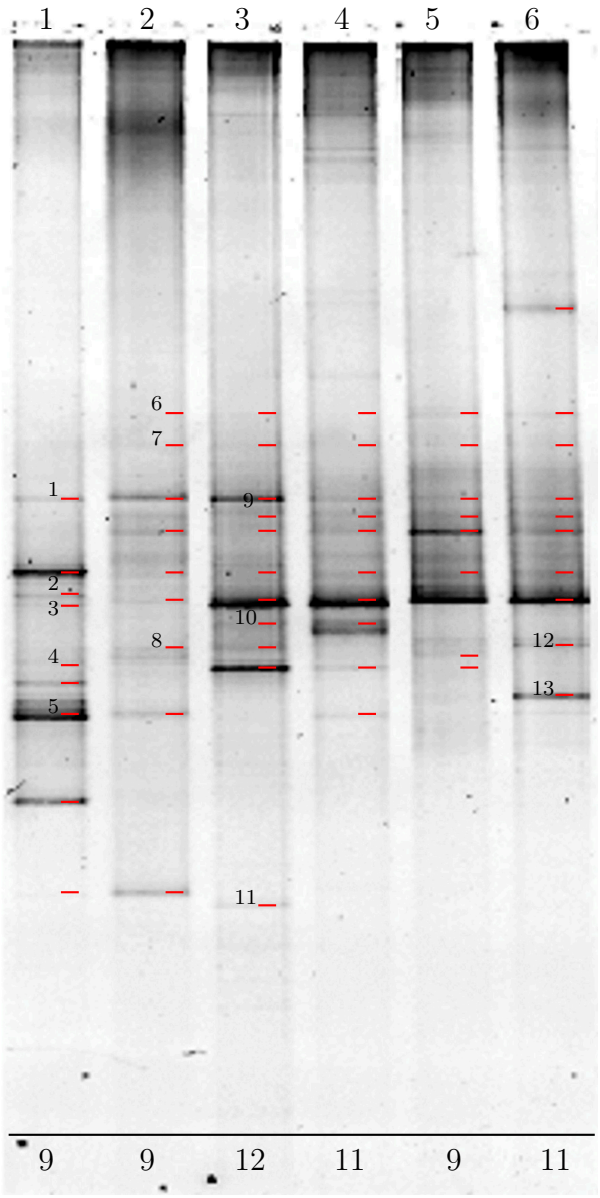


Figure 4.9.: DGGE DNA fingerprints of 16S rDNA amplicons (GC27F/517R) from the German dairy company's water samples (first sampling period). Lanes 1 to 6 correspond to the sampling points, the numbers on the gel correspond to the sequenced DNA bands (see Table 4.6), and the numbers at the bottom are the total DNA bands of the lane.

Table 4.6.: Identification of bacteria in water samples from the German dairy company (first sampling period) after sequencing the DNA bands excised from the DGGE gel shown in Figure 4.9. Numbers correspond to the respective DNA bands.

	Bacterium	**Class**	**Max. identity**	**Accession number**
1	*Rhodoferax* sp.	β - *Proteobacteria*	100 %	AY788965.1
2	*Acidovorax*	β - *Proteobacteria*	99 %	DQ153906.1
3	Uncultured bacteria	β - *Proteobacteria*	99 %	DQ409991.1
4	Uncultured bacteria	β - *Proteobacteria*	98 %	DQ664220.1
5	*Caulobacter crescentis*	α - *Proteobacteria*	98 %	AE005673.1
6	*Aquabacterium*	β - *Proteobacteria*	98 %	EF651436.1
7	*Aquabacterium*	β - *Proteobacteria*	99 %	EF651436.1
8	*Sphingomonas*	α - *Proteobacteria*	95 %	AY026948.1
9	*Acinetobacter*	γ - *Proteobacteria*	98 %	EF570077.2
10	*Aquabacterium*	β - *Proteobacteria*	88 %	EF179861.1
11	*Meiothermus*	Deinococci	94 %	AY845055.1
12	*Sphingomonas*	α - *Proteobacteria*	99 %	AY026948.1
13	*Sphingomonas*	α - *Proteobacteria*	99 %	AY026948.1

A total number of 13 bands were sliced from the DGGE gel for sequencing. Most of these bacteria were α- or β- *Proteobacteria*. None of the targeted pathogens were identified by sequencing, but some opportunistic bacteria as *Sphingomonas* and *Acinetobacter* were aligned (Table 4.6).

Although one potentially critical point was identified after analyzing the autochthonous bacterial population, no technical problems or irregular operation during food production were encountered during the evaluation.

Some hygienic recommendations, such as a more frequent exchange of hoses, were made before the second sampling period.

The second sampling period was carried out in order to use the optimized strategy and to corroborate if the practical application of hygienic recommendations had an influence in the results of the autochthonous bacterial population analysis.

During the second sampling period, higher volumes were filtered in order to achieve detection limits similar to those of the standard plating techniques (1 bacteria/100 ml water). No PCR inhibitors were found in the samples, though a higher amount of water was filtered. Monitoring of pathogens during the second sampling period did not produce any positive results, no matter whether traditional plating methods or culture-independent methods were applied.

When the bacterial populations of the water samples during the second sampling period were analyzed (see Figure 4.10), the similarity values between the different sampling points and the reference point (see Table 4.7) were between 53 % and 86 %. No sampling point presented a similarity value below 40 %.

Table 4.7.: Sørensen indexes: Second sampling period at German dairy company.

Reference point	Q_s values for the sampling points					
	1	2	3	4	5	6
1	1	0.82	0.55	0.53	0.61	0.55

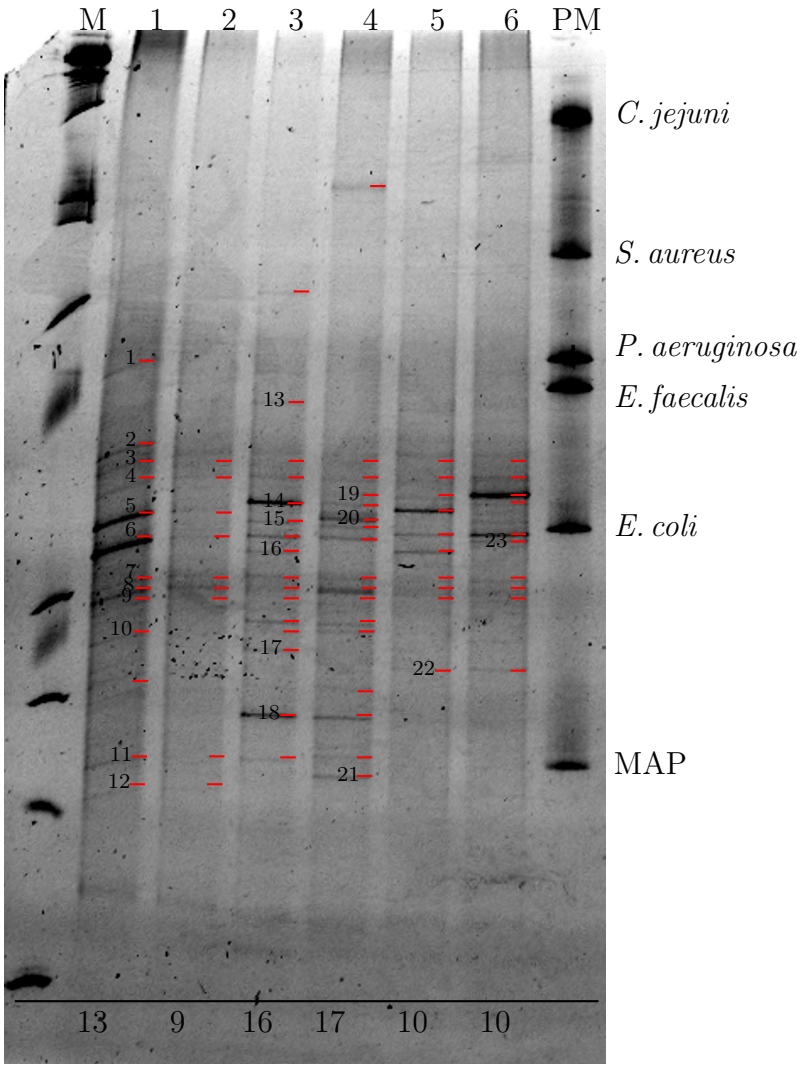


Figure 4.10.: DGGE DNA fingerprints of 16S rDNA amplicons (GC27F/517R) from the German dairy company's water samples (second sampling period). Lanes 1 to 6 correspond to the sampling points. A 100 bp DNA marker (M) and a pathogen marker were run (PM). The numbers on the gel correspond to the sequenced DNA bands (see Table 4.8), and the numbers at the bottom are the total DNA bands of the lane.

Table 4.8.: Identification of bacteria in water samples from the German dairy company (second sampling period) after sequencing DNA bands excised from DGGE gel shown in Figure 4.10. Numbers correspond to the respective DNA bands.

	Bacterium	**Class**	**Max. identity**	**Accession number**
1	Uncultured bacteria	γ - *Proteobacteria*	93 %	AB468957
2	*Acidovorax*	β - *Proteobacteria*	100 %	EF422199
3	*Brevundimonas* sp.	α - *Proteobacteria*	99 %	AM988999
4	Uncultured bacteria	β - *Proteobacteria*	98 %	AB252909
5	Uncultured bacteria	-	94 %	AF150757
6	Iron-reducing bacteria	-	99 %	FJ269043
7	Uncultured bacteria	β - *Proteobacteria*	98 %	AJ622889
8	Uncultured bacteria	δ - *Proteobacteria*	98 %	AF351212
9	Uncultured bacteria	-	81 %	FM206273
10	Uncultured bacteria	β - *Proteobacteria*	96 %	FM206220
11	Uncultured bacteria	Chloroflexi	91 %	EU374062
12	Uncultured bacteria	Chloroflexi	90 %	EU374062
13	Uncultured soil bacteria	-	85 %	AY242608
14	Uncultured *Comamonadeceae*	β - *Proteobacteria*	99 %	EU112284
15	Uncultured bacteria	γ - *Proteobacteria*	95 %	AF431351
16	*Methylibium petroleiphilum*	β - *Proteobacteria*	100 %	CP000555
17	Uncultured bacteria	γ - *Proteobacteria*	94 %	AM411939
18	*Meiothermus timidus*	Deinococci	96 %	AJ871168
19	*Sphingobium* sp.	α - *Proteobacteria*	98 %	AB461016
20	Uncultured *Sphingomonas*	α - *Proteobacteria*	96 %	EF547951
21	Uncultured bacteria	γ - *Proteobacteria*	98 %	FM209096
22	Uncultured bacteria	β - *Proteobacteria*	99 %	FJ516907

A total number of 22 bands were sliced from the DGGE gel for sequencing. Unlike the first sampling period a high proportion of uncultured bacteria were found.

The bacteria belonged mostly to the β - *Proteobacteria* class, though α -, δ -, and γ - *Proteobacteria* were also present. Most of the aligned bacteria are widely distributed in fresh water or in soil, presenting no threat for humans. Again, none of the targeted pathogens were identified by sequencing, but some opportunistic bacteria as the Sphin-

gomonadales were aligned as in the first sampling period (Table 4.8).

4.3.3. Spanish Dry Cured Ham Company Analysis

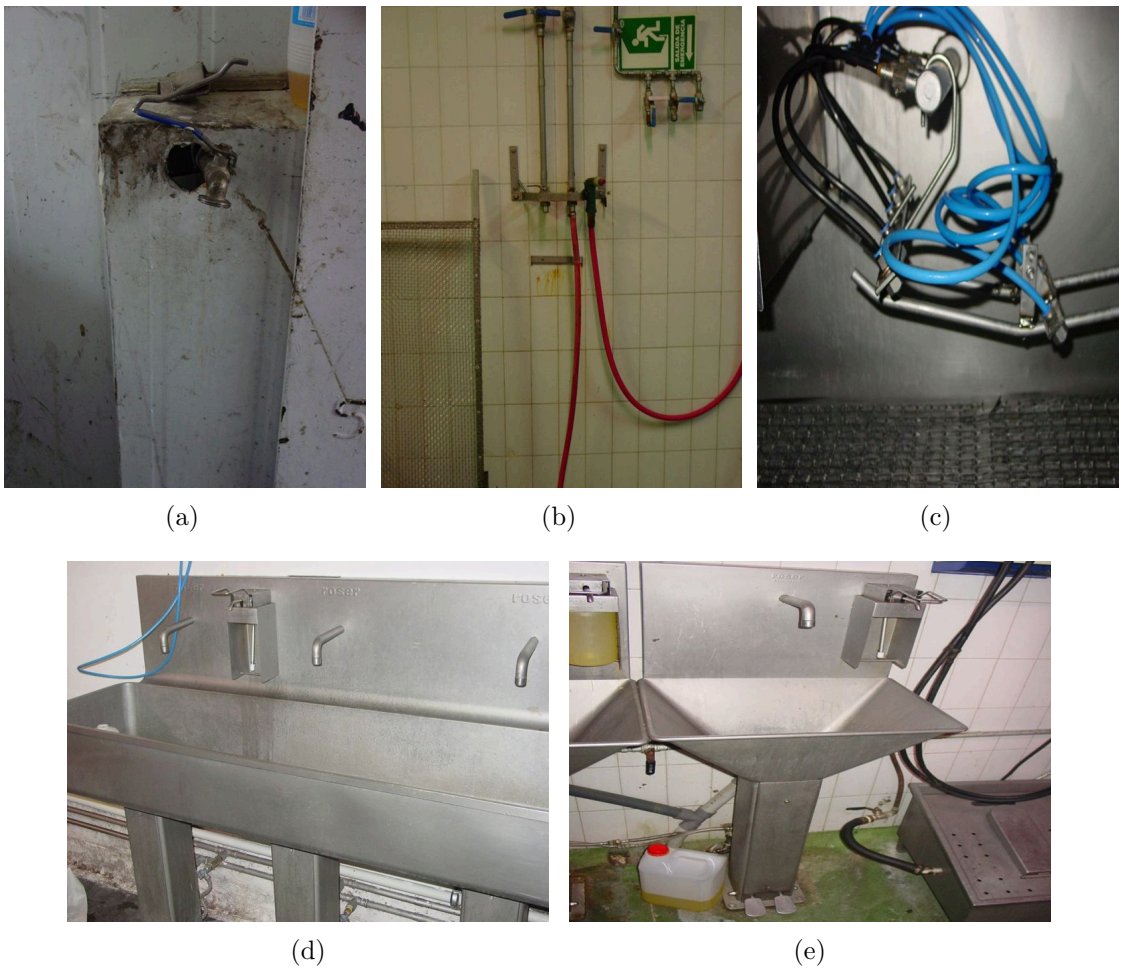
The water supplied at the dry cured ham company by the Spanish public distribution network was chlorine - treated conditioned groundwater having a residual chlorine content of 0.4 mg/l. No additional treatment was done at the company.

The first sampling point was the point where the public water entered the food company, and this point was taken as reference for all the bacterial population analysis.

Drinking water samples and biofilm samples were taken at the following sampling points at this food company (see Figure 4.11):

1. Entry of public conditioned drinking water: reference.
2. Hygienic sluice: water used to rinse the room and machinery.
3. Salt wash - off: water used to wash off the salt of the ham.
4. Hand washbasin: water used to wash hands in deboning room.
5. Hand washbasin: water used to wash hands in packaging room.

The water samples were filtered directly after the sampling procedure in Spain and the membranes were transported to Germany, where culture - independent methods exclusively were applied for their analysis. It was not possible to apply traditional plating methods due to the lack of time and equipment at the sampling place. Biofilms samples were also taken from the same places where water samples were taken. Initially, no DNA amplification was observed (see Figure 4.12 (a)). The absence of amplification product after carrying out the PCR efficiency assay indicated the presence of PCR inhibitors (Figure 4.12 (b)). BSA was used to remove PCR inhibitors with no success (results not shown). The samples were then treated with PVPP, and weak PCR products were observed (Figure 4.12 (c)). To confirm that the intensity of these bands corresponded to a low DNA concentration in the samples and not to the presence of PCR inhibitors, a PCR efficiency assay was performed again. The bands observed after this PCR efficiency assay (Figure 4.12 (d)) exhibited the same or even higher intensities than the added genomic DNA (Figure 4.12, lane P), indicating that no PCR inhibitors were present in the water samples after the PVPP treatment anymore.



(a) (b) (c)

(d) (e)

Figure 4.11.: Sampling points of Spanish dry cured ham company. (a) 1. Entry of public conditioned drinking water: reference, (b) 2. Hygienic sluice: water used to rinse the room and machinery, (c) 3. Salt wash - off: water used to wash off the salt of the ham, (d) 4. Hand washbasin: water used to wash hands in deboning room, (e) 5. Hand washbasin: water used to wash hands in packaging room.

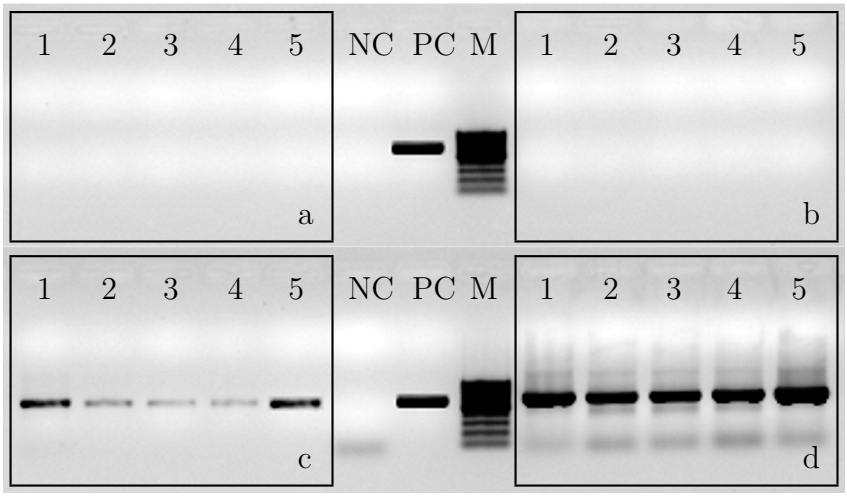


Figure 4.12.: PCR efficiency assay. Lanes 1 - 5 correspond to the Spanish dry cured ham company's water sampling points. 10 μl of the respective 16S rDNA amplicons were separated in 1 % agarose gel (amplicon size: 566 bp). Panel (a), original water templates; panel (b), original water templates spiked with enterococcal genomic DNA; panel (c), original water templates after PVPP treatment; panel (d), original water templates spiked with enterococcal genomic DNA after PVPP treatment. NC: negative template control, PC: positive control, and M: 100 bp DNA marker.

PCR and qPCR results are shown in Table 4.9. Some positive signals became obvious after *P. aeruginosa* - specific qPCR analysis (see Figure 4.13) from points 2 (salt wash - off), 3 (hand washbasin of bone removal room), 4 (hand washbasin of deboning room) and 5 (hand washbasin of packaging room). The water and the biofilm samples of point 2 (salt wash - off) presented one positive signal of a triplicate (C_T 28.00 and C_T 38.02, respectively), the C_T value of the biofilm sample corresponded to 9 bacteria/swab and the C_T value of the water sample was higher as the detection limit of the system (C_T 34.21). The other average C_T values were between 37.2 and 39.5; all these C_T values were out of the standard curve range (see Figure 4.2 and Table 4.3). In consequence, it was not possible to completely rely on these values.

One *E. coli* positive signal was detected on the biofilm sample of point 4 (hand washbasin of deboning room), one sample of the duplicate presented a C_T value of 38.02 (see Figure 4.14), this C_T value corresponded to 8 bacteria/swab.

None of the other water or water biofilm samples of this company showed positive qPCR results for any of the specific targeted pathogens (see Table 4.9).

Table 4.9.: PCR and quantitative PCR results of water and biofilm samples of the Spanish dry cured ham company. Duplicates or triplicates of each sample were run.

	Sampling point									
	1		**2**		**3**		**4**		**5**	
	W	**B**	**W**	**B**	**W**	**B**	**W**	**B**	**W**	**B**
Plating methods	Not determined									
PCR										
Enterococcus spp.	-	-	-	-	-	-	-	-	-	-
Salmonella spp.	-	-	-	-	-	-	-	-	-	-
Campylobacter jejuni	-	-	-	-	-	-	-	-	-	-
M. avium subsp. *paratuberculosis*	-	-	-	-	-	-	-	-	-	-
Listeria monocytogenes	-	-	-	-	-	-	-	-	-	-
Pseudomonas aeruginosa	-	-	-	+	-	-	-	+	-	-
Quantitative PCR										
Enterococcus spp.	-	-	-	-	-	-	-	-	-	-
Salmonella spp.	-	-	-	-	-	-	-	-	-	-
Campylobacter jejuni	-	-	-	-	-	-	-	-	-	-
M. avium subsp. *paratuberculosis*	-	-	-	-	-	-	-	-	-	-
Listeria monocytogenes	-	-	-	-	-	-	-	-	-	-
Pseudomonas aeruginosa	-	-	+*	+*	+*	-	-	+*	+*	+*
Escherichia coli	-	-	-	-	-	-	-	+*	-	-

* positive results are described in more detail in the text. W: water, B: biofilms.

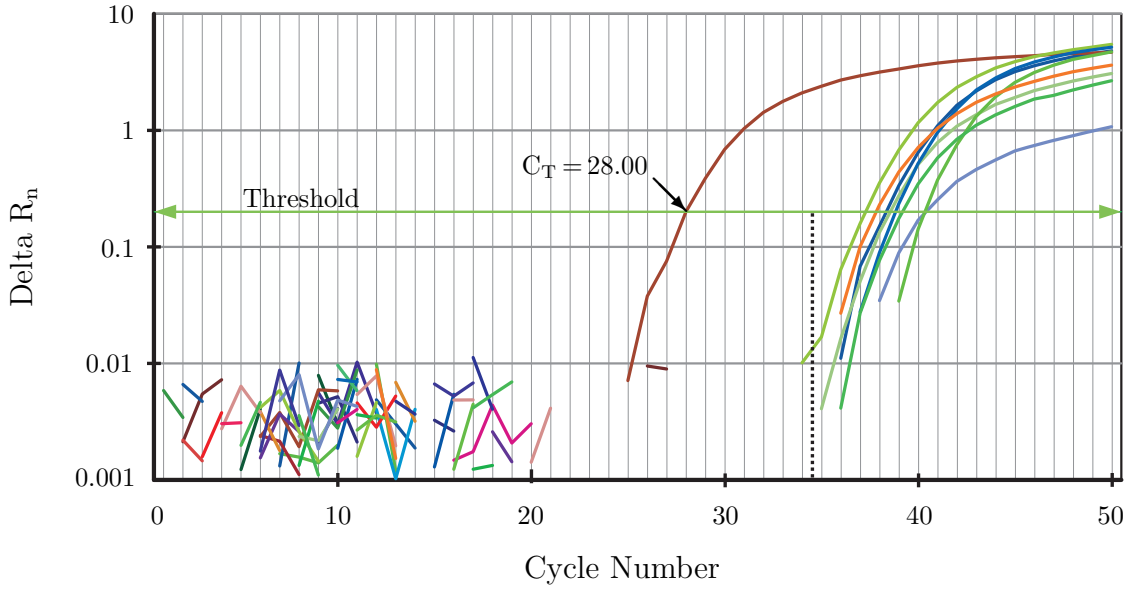


Figure 4.13.: Quantitative PCR curve: Detection of *P. aeruginosa* from water and biofilm samples from Spanish dry cured ham company. Detection limit (dotted line): $C_T = 34.48$.

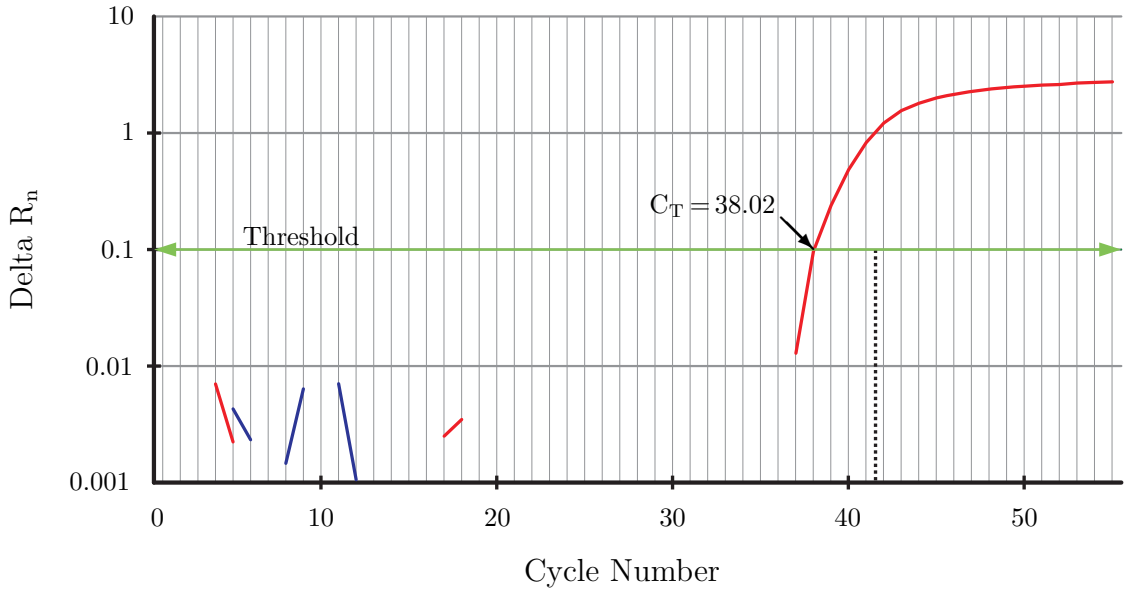


Figure 4.14.: Detection of *E. coli* from water and biofilm samples from Spanish dry cured ham company. Detection limit (dotted line): $C_T = 41.66$.

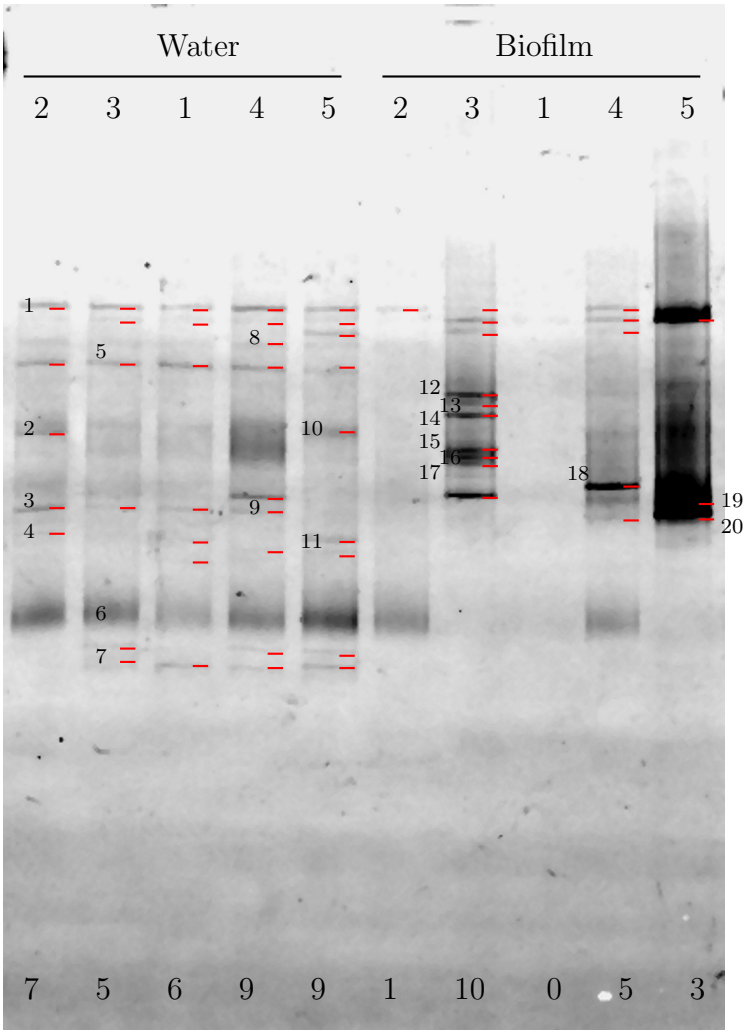


Figure 4.15.: DGGE DNA fingerprints of 16S rDNA amplicons (GC341F - 907R) from the Spanish dry cured ham company's water and biofilm samples. Lanes 1 to 5 correspond to the respective water and biofilm sampling points. The numbers on the gel correspond to the sequenced DNA bands (see Table 4.12), and the numbers at the bottom are the total DNA bands of the lane.

When the autochthonous bacterial population of the water samples of the company was analyzed (see Figure 4.15), a total number of 7 DGGE DNA bands were observed in the reference sample (Point 1). The downstream water samples exhibited 5 to 9 bands.

When the bacterial populations of the water sample of the company and the public entrance water were compared using the already described Sørensen similarity index, no significant difference was found. The similarities of the samples with the reference

sample were quite high (see Table 4.10). They ranged between 63 % and 77 %, indicating a biological stability of the analyzed water samples.

Table 4.10.: Sørensen indexes: Comparison between water samples at Spanish dry cured ham company.

Reference point	Q_s values for the sampling points				
	1	2	3	4	5
1	1	0.67	0.77	0.63	0.63

Table 4.11.: Sørensen indexes: Comparison between water and biofilm samples at Spanish dry cured ham company.

		Q_s values for the sampling points				
		Water samples				
		1	2	3	4	5
Biofilm samples	1	**n.d.**				
	2		**0.33**			
	3			**0.25**		
	4				0.43	
	5					**0.17**

When the biofilm samples were analyzed, no DNA bands were observed for the reference sample, therefore no Sørensen similarity indexes were calculated.

The Sørensen similarity index obtained for the water sample and the biofilm sample (see Table 4.11) of the same sampling points were low (between 0 % and 33 %) indicating a low similarity between the bacterial populations of water and biofilm within the same sampling point. Only the sampling point 4 (hand washbasin of deboning room) presented a similarity higher than 40 % (43 %).

Twenty DNA bands were sliced from the DGGE gel for sequencing. Most of the sequenced DNA fragments belonged to the γ - *Proteobacteria* subclass. Non - pathogenic *Bacillus* sp. and some opportunistic bacteria, as *Sphingomonas* sp., *Enterobacter* sp., and *Stenotrophomonas maltophilia* were also identified after sequencing the DNA of DGGE

bands (see Table 4.12). Most DNA bands belonged to *Pseudomonas* sp. Hence, the presence of *Pseudomonas* found by the previous PCR and qPCR was confirmed. No *E. coli* were found after sequencing the DNA bands sliced from DGGE.

Table 4.12.: Identification of bacteria in water and biofilm samples from Spanish dry cured ham company after sequencing the DNA bands excised from the DGGE gel shown in Figure 4.15. Numbers correspond to the respective DNA bands.

	Bacterium	**Class**	**Max. identity**	**Accession number**
1	Uncultured bacteria	γ - *Proteobacteria*	99 %	AY328730.1
2	Uncultured bacteria	β - *Proteobacteria*	98 %	EF651499.1
3	*Bacillus* sp.	Bacilli	100 %	FM866300.1
4	*Stenotrophomonas maltophilia*	γ - *Proteobacteria*	99 %	EU221397.1
5	*Pseudomonas* sp.	γ - *Proteobacteria*	100 %	EU815635.1
6	*Propionibacterium*	Actinobacteria	100 %	FJ222613.1
7	*Enterobacter*	γ - *Proteobacteria*	100 %	EU162036.1
8	Uncultured bacteria	γ - *Proteobacteria*	100 %	AY456980.1
9	*Xanthomonas*	γ - *Proteobacteria*	99 %	DQ213024.1
10	*Pseudomonas* sp.	γ - *Proteobacteria*	100 %	EU864269.1
11	*Stenotrophomonas maltophilia*	γ - *Proteobacteria*	99 %	EU221397.1
12	*Psychrobacter* sp.	γ - *Proteobacteria*	100 %	AM990814.1
13	*Psychrobacter* sp.	γ - *Proteobacteria*	100 %	AM990814.1
14	*Brevundimonas*	α - *Proteobacteria*	100 %	EF093132.1
15	*Pseudomonas* sp.	γ - *Proteobacteria*	100 %	AM421975.1
16	*Pseudomonas* sp.	γ - *Proteobacteria*	98 %	AM421981.1
17	*Pseudomonas* sp.	γ - *Proteobacteria*	91 %	AM886088.1
18	*Sphingomonas*	α - *Proteobacteria*	100 %	AY162145.1
19	*Pseudomonas* sp.	γ - *Proteobacteria*	100 %	EF550156.1
20	*Pseudomonas* sp.	γ - *Proteobacteria*	99 %	EU815635.1

4.4. Live/Dead Differentiation

The differentiation of the physiological stages of bacteria is a main concern for microbiologists. Drinking water regulations establish the use of traditional plating methods for the detection of pathogens in drinking water (TrinkwV 2001, 2001). These techniques detect only viable culturable bacteria. But, viable but non-culturable bacteria VBNC are of great concern for the food industry. The presence of this physiological stage of bacteria has a high hygienic relevance. They cannot be determined by traditional plating methods but are able to survive hostile conditions and are capable of re-growth when optimal growth conditions are promoted.

In the first part of this work the methods used to detect bacteria in drinking water systems were exposed. The relevance of formation of biofilms in drinking water distribution networks, including pipelines of households and food companies was described. The importance of biofilms as potential habitats for all kinds of bacteria, including pathogens, was considered.

DNA-based methods were used to detect and characterize (i) bacteria present in the water at different sampling points of food companies, or (ii) bacteria that were part of biofilms formed on the pipelines. These systems detected the presence of DNA but they were not able to differentiate if the DNA was from live or dead cells or if it was from free or eDNA.

Live/dead differentiation methods have already been described (see Section 2.7). Being the PMA and the DNase I treatments, together with the staining methods the most relevant methods for this work.

DNase I kits already exist in the molecular biology field, but these kits do not face the problematic of live/dead differentiation. These kits have been commonly used to eliminate free DNA from samples for further RNA analysis.

In order to detect and characterize only live bacteria from the samples, a battery of methods is presented in this section, giving special attention to the DNase I treatment. The basis of using DNase I, is its ability to digest DNA. It has been demonstrated that 1 Unit (U) of this enzyme can completely degrade 1 μg of plasmid DNA in 10 min at 37 °C. In consequence, if live cells are exposed to DNase I together with free-DNA and dead cells with compromised cell membranes, the DNase I will digest the free-DNA and the DNA from dead cells, leaving DNA from live cells available for further DNA-based analyses.

A DNase I treatment protocol used for the detection, characterization and analysis of live populations of bacteria present in drinking water and drinking water biofilms was developed in this work and is presented in this section.

4.4.1. DNase I Method Optimization

The treatment of the samples with DNase I for the subsequent molecular biology analysis mainly consists in three steps:

1. Digestion of free DNA or DNA from dead cells with injured cell membrane.
2. Inactivation of DNase I.
3. Inactivation of DNase I inactivator.

The following experiments were carried out in order to optimize this method for the treatment of drinking water samples and biofilms from drinking water pipelines, in order to detect only the DNA from live bacteria in the samples via DNA - based methods.

Inactivation of DNase I

Three different DNase I inactivation steps were tested:

- Heat treatment
- EDTA
- Proteinase K

Heat treatment: DNase I was used to treat the samples in presence of a buffer (100 mM solution of Tris base and concentrated hydrochloric acid (Tris - HCl), 25 mM $MgCl_2$ and 1 mM $CaCl_2$) and it was inactivated with heat at 75 °C for 10 min. This procedure completely digested the DNA from free enterococcal genomic DNA suspensions but when cells were treated an overlap between the time that the DNase I needed to be inactivated and the time that the cells were killed by the increasing temperatures, could result in a false under estimation of live cells.

EDTA: Ethylenediaminetetraacetic acid (EDTA) is a chelating agent, which is able to sequester metal ions such as Ca^{2+} and Mg^{2+}. DNase I needs these ions to be active, therefore one hypothesis was to use EDTA to sequester these ions, heat the samples

to inactivate the enzyme, and then add these ions again since the DNA polymerases used for the PCR and qPCR needed their presence to be active. For this, 4 reaction tubes containing sterile water, buffer, and DNase I were incubated at 37 °C for 10 min, then EDTA was added. Two tubes were afterwards heat treated (70 °C for 10 min) and the other two were not heat treated. At the moment of carrying out the qPCR, $MgCl_2$ was added to one heat treated sample and to one sample without heat treatment. An additional tube was used as control, this tube contained sterile water without the DNase I and the buffer, and was also incubated at 37 °C for 10 min. Finally, a known amount of free enterococcal genomic DNA was added. If the DNase I was inactivated by any of these combinations, the same amount of DNA obtained for the control should have be observed. But this hypothesis did not work, low or no PCR amplification results were able to be seen after this procedure (see Figure 4.16).

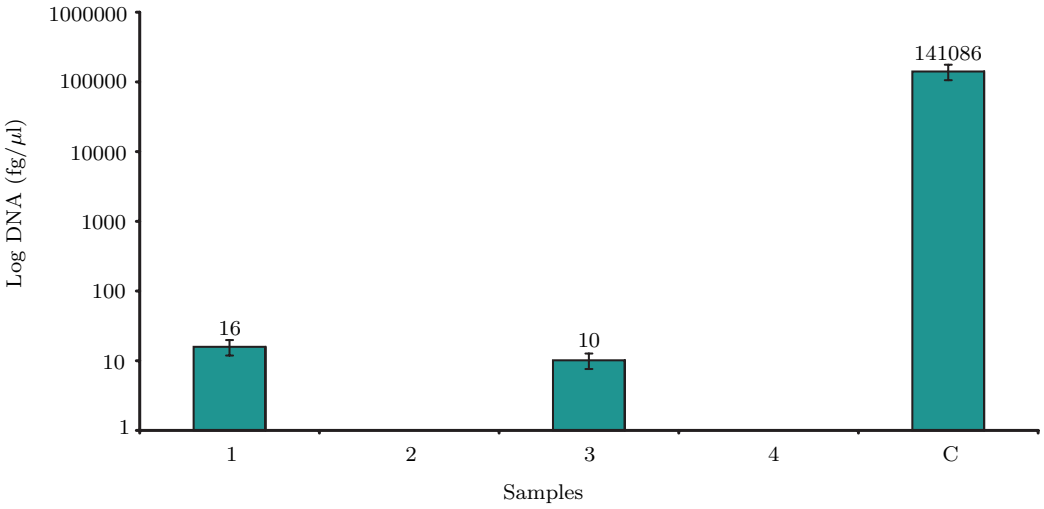


Figure 4.16.: Quantitative PCR results of DNase I inhibition assay by EDTA. qPCR results of free enterococcal genomic DNA added to sterile water samples with buffer and DNase I that were previously incubated at 37 °C for 10 min, and afterwards treated: (1) without heat treatment and without $MgCl_2$, (2) without heat treatment with $MgCl_2$, (3) with heat treatment but without $MgCl_2$, (4) with heat treatment and with $MgCl_2$; and C: control sample without DNase I or buffer.

Proteinase K: This enzyme needs the presence of Ca^{2+} to be active, the optimal pH is 8 but it is active in pHs between 4.3 and 12, and its optimal temperature is between 50 °C and 60 °C. Proteinase K is not completely inactivated by EDTA, urea, SDS, citrate, or other serinprotease inhibitors. To inactivate this enzyme, temperatures higher than 90 °C have to be used for unless 10 min. Proteinase K is an enzyme that digests proteins. Therefore, hypothetically the DNase I would also be digested by proteinase K. To verify this hypothesis the following experiment was done.

500 µl sterile water that contained a final concentration of 0.1 U DNase I/µl in the presence of buffer (final concentration: 10 mM Tris - HCl, 2.5 mM $MgCl_2$ and 0.1 mM $CaCl_2$) were incubated for 10 min at 37 °C, to imitate the conditions of the DNase I protocol used for DNA digestion. After this incubation period the DNase I inactivation step via proteinase K was done, by adding a final concentration of 0.24 µg proteinase K per µl. This mixture was incubated at 54 °C for 1 h for an optimal protein digestion. Proteinase K would also digest the DNA polymerases used for PCR and qPCR, therefore this enzyme was inactivated be heating the samples at 90 °C for 10 min. To evaluate if the DNase I was completely inactivated, 1 ng genomic enterococci DNA was spiked into the samples and then PCR (27F/517R) and Sybr green qPCR (27F/517R) were done. A positive control (PC) was done without DNase I and proteinase K, and a negative control (NC) was done as the positive control but without the addition of DNA. 10 µl and 1 µl of the spiked samples, positive (PC2) and negative (NC2) PCR controls were used as template in 25 µl final reaction mixtures for PCR and qPCR assays.

The results of the PCR, shown in Figure 4.17 indicated that apparently the proteinase K effectively inactivated the DNase I and that the whole procedure did not inhibit the PCR when 10 µl or 1 µl sample was used in a 25 µl final reaction mix.

More precise results were given by qPCR. When 10 µl were used as qPCR template a slight inhibition was observed (see Figure 4.18a). But, when 1 µl was used as template the semiquantitative results observed in the traditional PCR were verified, indicating that proteinase K can be used to inactivate DNase I (see Figure 4.18b).

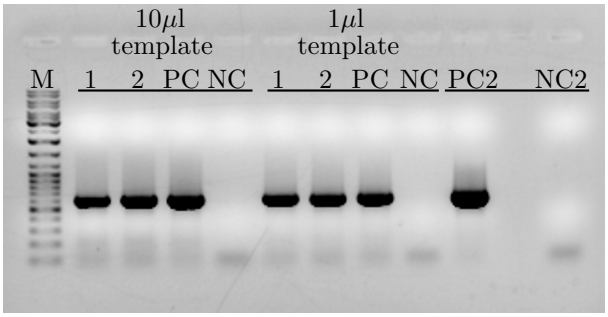


Figure 4.17.: PCR products of DNase I inhibition assay by proteinase K. 10 µl of the respective 16S rDNA amplicons separated in 1 % agarose gel (amplicon size: 566 bp). On the left side: 10 µl template was used; on the right side: 1 µl template was used. M: 100 bp DNA ladder, 1 and 2 are the duplicate of the samples containing DNase I, PC: positive control containing no DNase I or proteinase K, and NC: negative template control containing no DNase I or proteinase K, and no DNA. PC2 and NC2, are the positive and negative control of the PCR, respectively.

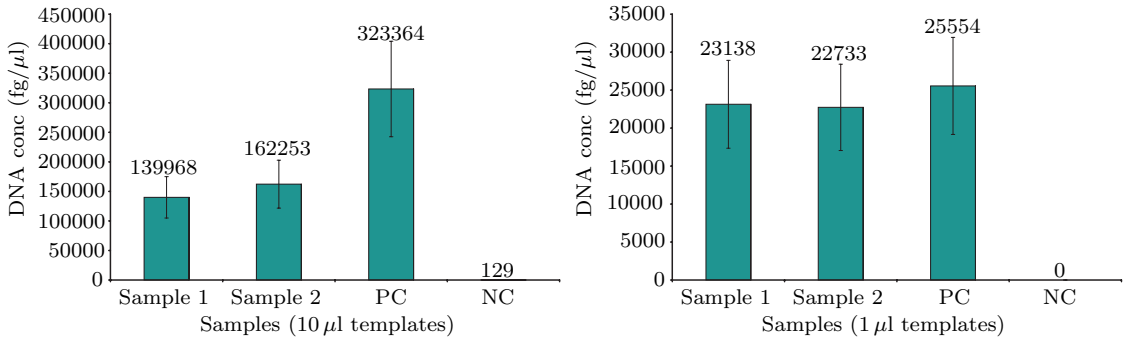


Figure 4.18.: Quantitative PCR results of DNase I inhibition assay by proteinase K. On the left side (a): when 10 µl template was used; on the right side (b): when 1 µl template was used. 1 and 2: duplicates of the samples containing DNase I, PC: positive control containing no DNase I or proteinase K, and NC: negative template control containing no DNase I or proteinase K, and no DNA.

Determination of Time Required for Complete DNA Digestion and Estimation of Optimal Reagent Concentrations

In this section, the time required by the DNase I to completely digest DNA, and the reagent concentrations necessary to achieve this were estimated.

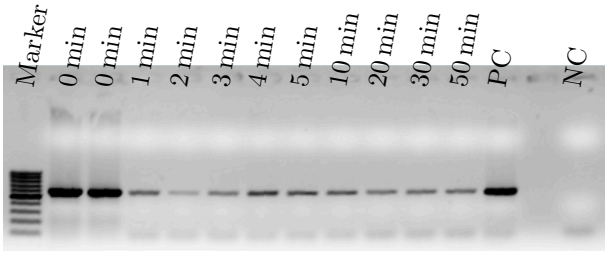
The DNase I inactivation step was achieved by addition of proteinase K, and an additional step was used to inactivate the proteinase K.

Experimental Approach 1

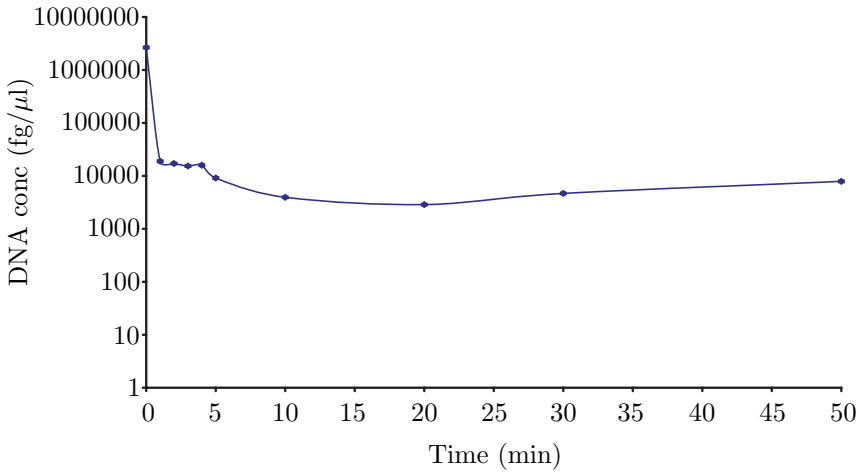
To stipulate how much time the samples had to be in presence of DNase I to be completely digested, a progress curve was done in presence of free DNA.

For this 9 ng/μl *E. faecalis* genomic DNA was digested by 0.1 U DNase I (Fermentas)/μl in the presence of the Fermentas Buffer (10 mM Tris - HCl, 2.5 mM $MgCl_2$ and 0.1 mM $CaCl_2$) at 37 °C at different time intervals (0 min, 1 min, 2 min, 3 min, 4 min, 5 min, 10 min, 20 min, 30 min, and 50 min). DNase I was inactivated after these intervals by exposing the samples to 0.24 μg proteinase per μl at 56 °C for 60 min, followed by heating the samples at 90 °C for 10 min. Then 1μl DNA was used as template for PCR and qPCR, using the eubacterial ribosomal primer system 27F/517R targeting 16S rDNA.

Figure 4.19a shows the PCR products (10 μl) run on a 1 % agarose gel. The digestion of DNA was observed already after 1 min and apparently, the amount of DNA did not decrease with the time of exposure indicating that the activity of the enzyme was limited by high concentrations of substrate resulting in a saturation of the enzyme. The qPCR results of the progress curve of the digestion of DNA by the DNase/PK treatment (see Figure 4.19b) verified the results of the PCR.



(a)



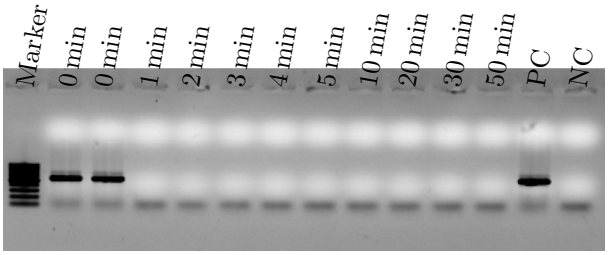
(b)

Figure 4.19.: Progress curve of digestion of free genomic enterococci DNA (9 ng DNA/μl) by DNase I (0.1 U DNase I/μl) at different time intervals: (a) PCR products: 10 μl of the respective 16S rDNA amplicons were separated in 1 % agarose gel (amplicon size: 566 bp). Marker: 100 bp DNA ladder, PC: positive control, and NC: negative template control. (b) Quantitative PCR results: DNA concentration was measured by Sybr Green quantitative PCR. Detection limit of the method: 100 fg DNA/μl.

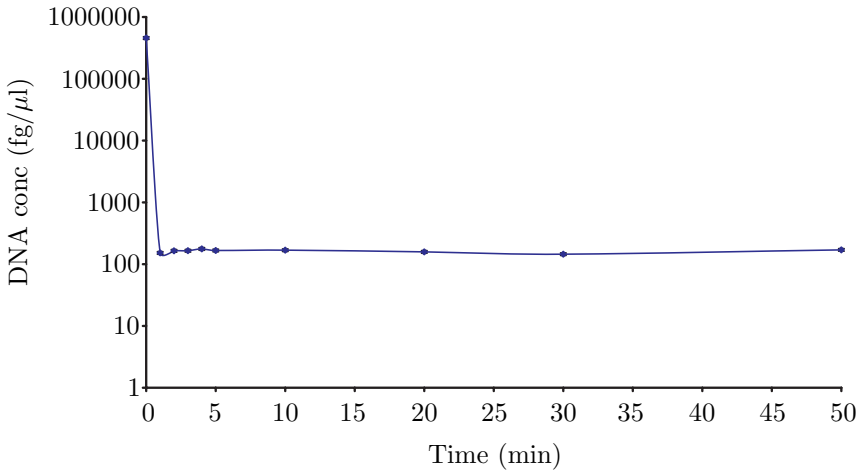
Experimental Approach 2

To confirm the theory of enzyme saturation and to prove that the complete DNA content present in the sample can be successfully removed by using higher DNase I concentrations, a second experiment was done with the same conditions but this time only 1 ng DNA/μl was exposed to 0.25 U DNase I/μl in the presence of 10 mM Tris - HCl, 2.5 mM $MgCl_2$ and 0.1 mM $CaCl_2$ at 37 °C at different time intervals. The inactivation of the DNase I was done like mentioned above.

Figure 4.20a shows the PCR products run on a 1 % agarose gel. A complete digestion of DNA was observed already after 1 min. The qPCR results of the progress curve of the digestion of DNA by DNase I (see Figure 4.20b) verified the results of the PCR, obtaining after 1 min already results that corresponded to the detection limit of the qPCR method. Therefore, the enzyme saturation theory was confirmed, hence a total DNA digestion in the samples can be achieved when a high DNase I concentration is used.



(a)



(b)

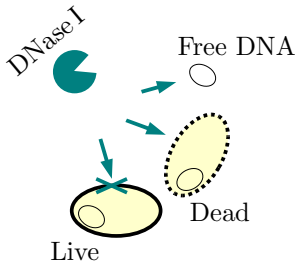
Figure 4.20.: Progress curve of digestion of free genomic enterococci DNA (9 ng DNA/μl) by DNase I (0.25 U DNase I/μl) at different time intervals: (a) PCR products: 10 μl of the respective 16S rDNA amplicons were separated in 1 % agarose gel (amplicon size: 566 bp). Marker: 100 bp DNA ladder, PC: positive control, and NC: negative template control. (b) Quantitative PCR results: DNA concentration was measured by Sybr Green quantitative PCR. Detection limit of the method: 100 fg DNA/μl.

4.4.2. DNase I/Proteinase K (DNase/PK) Protocol

With the results obtained, it can be concluded that DNase I can be successfully inactivated by proteinase K, and that DNase I can completely digest DNA in a sample when the enzyme is present at high concentrations. Knowing this, the following protocol was developed (Figure 4.21). This DNase/PK protocol was used from now on for live/dead differentiation assays.

	Final Concentration
DNA	$\leq$1 ng DNA/μl
DNase I	0.25 U/μl
Buffer with $MgCl_2$	10 mM Tris - HCl, 2.5 mM $MgCl_2$ 0.1 mM $CaCl_2$

DNA digestion by DNase I: 37 °C for 10 min



DNase I inactivation: Addition of Proteinase K (Final conc.: 0.24 μg/μl) Incubation at 54 °C for 1 h

Proteinase K inactivation: 90 °C for 10 min

Figure 4.21.: DNase/PK treatment protocol used for live/dead differentiation assays.

4.4.3. DNase/PK Treatment in Presence of Filter Membranes

The following experiments were done to demonstrate the ability of the DNase/PK treatment to digest DNA in presence of filter membranes used for biomass concentration of water samples.

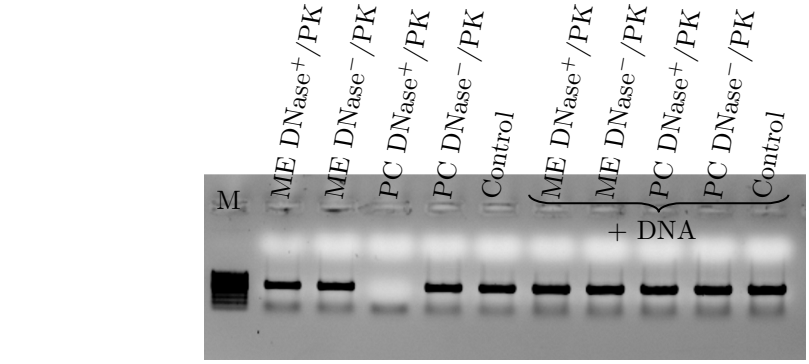
Experimental Approach

Polycarbonate (PC) and mixed cellulose ester (ME) filters are the most used filters for water analyses, therefore these were chosen for the further experiment. 1 ng/μl genomic enterococci DNA was exposed to 0.25 U DNase I/μl in the presence of the chosen filters following the DNase/PK treatment protocol exposed in Section 4.4.2, these samples were called DNase$^+$/PK. An aliquot of the same samples were exposed to the same conditions as in the DNase/PK treatment but without the addition of DNase I as control, these were called DNase$^-$/PK. In parallel, a DNase$^-$/PK control without filter was run as a reference of the total DNA added to the samples. To examine if the filters had PCR inhibitors, a PCR efficiency assay was done by adding 100 pg DNA/μl to the samples before the PCR.

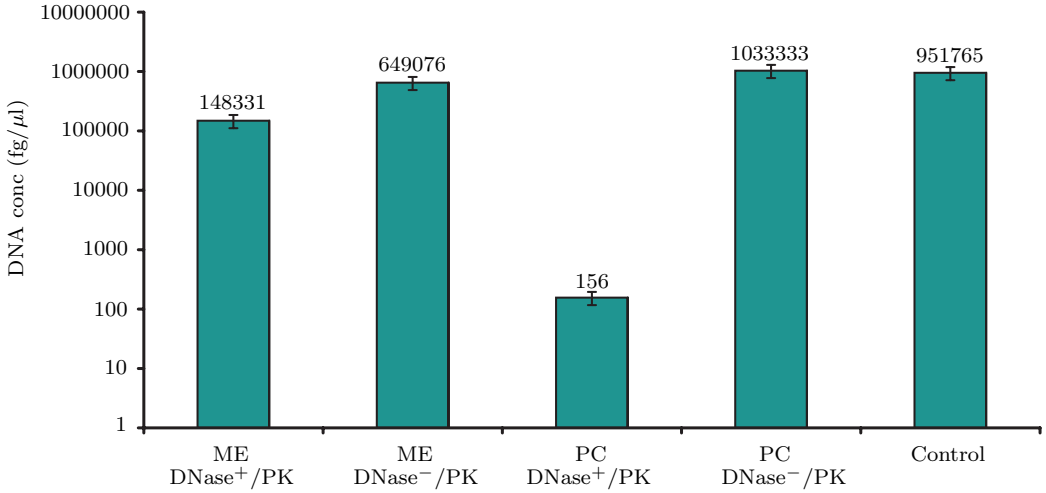
A dependence of the effectiveness of the DNase/PK treatment, within others, with the DNase I concentration, was already exposed. Consequently, in theory if the ME filter samples and the polycarbonate (PC) filter samples are exposed to higher DNase I concentrations a complete DNA digestion should be observed. To demonstrate this, an additional experiment was done by exposing 1 ng/μl genomic enterococci DNA with polycarbonate and mixed cellulose ester filters to increasing DNase I concentrations: 0.1, 0.2, and 0.3 U DNase I per μl.

Results

On the left side of Figure 4.22a in lanes 1 (ME DNase$^+$/PK) and 2 (ME DNase$^-$/PK) no DNA digestion was observed, showing that the DNase/PK treatment did not work in the presence of mixed cellulose ester filters in the concentrations and conditions of this experiment. In lane 3 (PC DNase$^+$/PK) no PCR band was observed due to an apparently complete digestion of DNA, reflecting the capacity of DNase I to act in presence of polycarbonate filters at the reaction conditions. In lane 4 (PC DNase$^-$/PK) no DNA digestion was observed demonstrating that the conditions of the DNase/PK treatment did not diminish per se in a visible way the amount of DNA of the sample when it was



(a)



(b)

Figure 4.22.: DNase I inhibition due to the presence of filter: PCR and quantitative PCR results. (a) 0.1 % Agarose gel of PCR products of DNase I inhibition due to the presence of filter. ME: Mixed cellulose ester membrane filters, 0.20 μm; PC: Polycarbonate filter; 0.2 μm; Control: DNA without DNase/PK treatment and no filter; DNase$^+$/PK: with DNase/PK treatment; DNase$^-$/PK: samples exposed to the same conditions of the DNase/PK treatment but without DNase I. On the right side the same samples of the left side are exposed but with the addition of DNA to seek PCR inhibitors. (b) qPCR results of DNase I inhibition due to the presence of filter. ME: Mixed cellulose ester membrane filters, 0.20 μm; PC: Polycarbonate filter; 0.2 μm; DNase$^+$/PK: with DNase/PK treatment; DNase$^-$/PK: samples exposed to the same conditions of the DNase/PK treatment but without DNase I; Control: DNA without DNase/PK treatment and no filter.

compared to the reference (lane 5). On the right side of Figure 4.22a the products from the PCR efficiency assay were run. Here the intensities of the DNA bands are the same or greater as the control indicating that no PCR inhibitors were present in the samples.

qPCR results (see Figure 4.22b) showed a higher DNA digestion in presence of the PC filters than in the presence of ME filters, reconfirming that polycarbonate filter membranes are more suitable as mixed cellulose ester filters for DNase/PK treatments.

When the samples were exposed to higher DNase I concentrations (see Figure 4.23), no matter which filter was used a total digestion of DNA was observed, as it was expected.

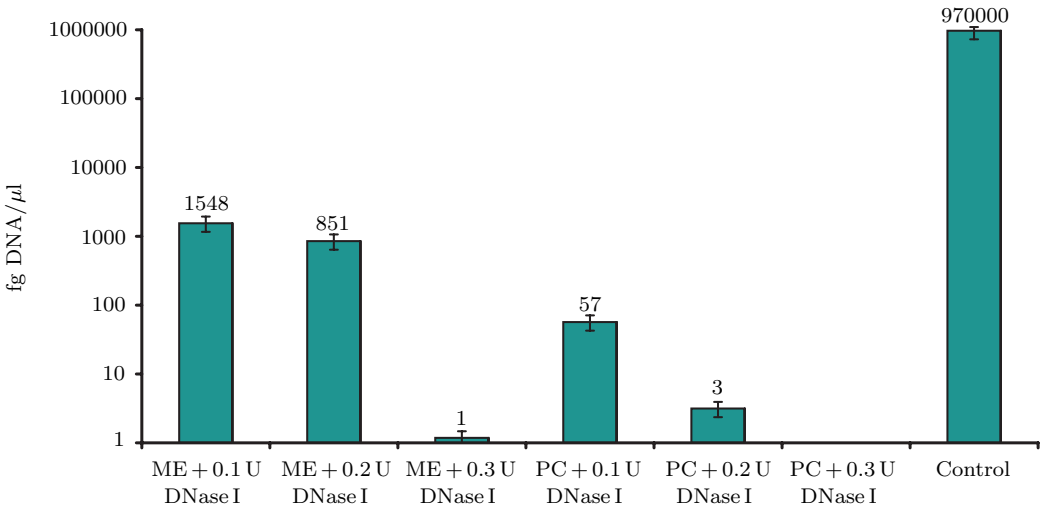


Figure 4.23.: Quantitative PCR results of DNase I inhibition due to the presence of filter with increasing amounts of DNase I. ME: Mixed cellulose ester membrane filters, 0.20 μm; PC: Polycarbonate filter, 0.2 μm; Control: DNA without DNase/PK treatment and no filter.

4.4.4. DNase /PK vs. PMA for Live/Dead Differentiation

As demonstrated before, the DNase/PK treatment was useful for digestion of free DNA present in samples. The following experiment demonstrated the ability of the DNase/PK and PMA treatments to discriminate live and dead bacteria. A general flow scheme from the experiment is shown in Figure 4.24.

Experimental Approach

The bacterial strains used for this study were Gram positive *Staphylococcus aureus*, Gram negative *Pseudomonas aeruginosa* and genomic DNA from *Salmonella enterica*. Single colonies were transferred in aseptic conditions to 50 ml tubes containing 25 ml BHI (1:4). The cultures were grown to log phase on a shaker at 30 °C. The cells were then centrifuged at 4000 rpm for 10 min, washed with 50 ml sterile water by mixing thoroughly and centrifuged again at 4000 rpm for 10 min. The bacteria were finally resuspended in 25 ml sterile water.

An aliquot of *Pseudomonas aeruginosa* cells were killed by heating at 99 °C for 15 min. Loss of viability was examined by spreading 100 μl cell suspension on R2A agar plates followed by incubation at 37 °C for 28 h. This suspension of *Pseudomonas aeruginosa* was called *SS2 dead*. The other aliquot of *Pseudomonas aeruginosa* cells was used as a reference of the culturable cells before the heating procedure, this suspension was called *SS2 live*.

Genomic DNA from *Salmonella enterica* was obtained following the procedure described in Section 3.3. This solution of 10 ng/μl genomic DNA was called *SS3 DNA*. To assure no cell viability, 100 μl were spread on R2A agar plates and incubated at 37 °C for 28 h and 72 h.

The stock suspensions were combined to obtain a work suspension (*WS1 live/dead/DNA*) as shown in Figure 4.24. Controls consisted of separate suspensions of live cells, dead cells, and DNA in the same final proportion as in the work suspension. In order to have a reference of the culturable cells of the heat treated cells before heating, a control (*C2 dead*) was done by resuspending *SS2 live* in the same final proportion as in *C1 dead*.

A 10 - fold serial dilution of *WS1 live/dead/DNA*, *C1 live* and *C2 live* was done to count the viable culturable bacteria, for this 100 μl were spread on R2A agar plates and were incubated at 37 °C for 28 h.

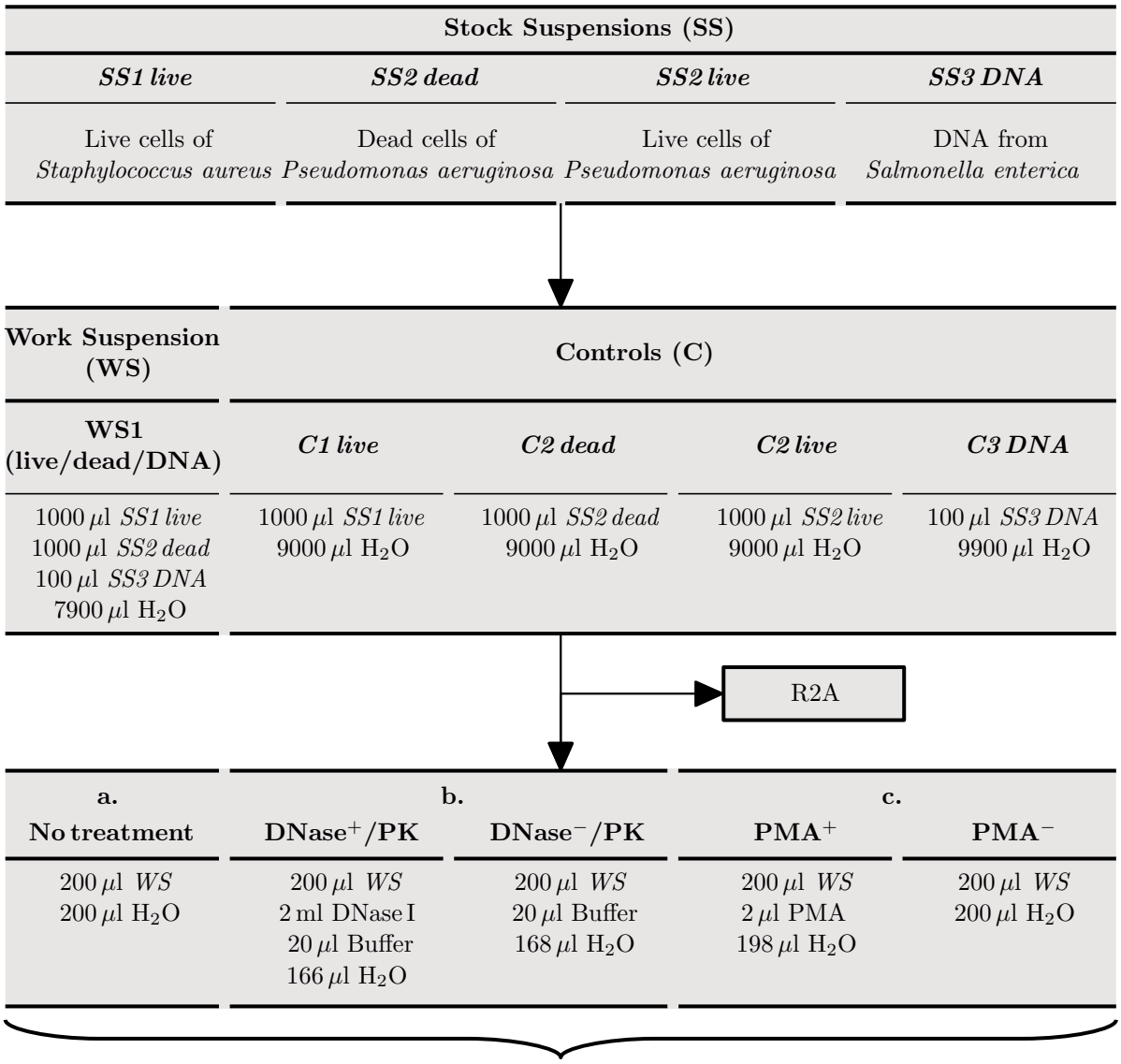


Figure 4.24.: General flow scheme of live/dead differentiation assay.

The *WS1 live/dead/DNA* and the controls were then exposed to different treatments before DNA quantification:

a) *No treatment*: The samples were diluted with sterile water in order to have the same dilution factor as the samples with DNase/PK or PMA treatment.

b) *DNase/PK treatment*: was done by exposing the work suspensions to DNase/PK following the final protocol described in Section 4.4.2. DNase$^+$/PK samples are the samples that were treated with DNase I; and DNase$^-$/PK samples are those without the addition of DNase I. All other conditions were kept constant.

c) *PMA treatment*: was done following the method described in Section 3.13 but with a small modification, the DNA of bacteria after the two wash steps was not isolated after the quantification methods in order to avoid an even larger amount of loss of valuable sample. To verify that these samples did not inhibit later DNA amplification, PCR efficiency assays were done. PMA$^+$ samples are the samples that were treated with PMA; and PMA$^-$ samples are those without the addition of PMA. All other conditions were kept constant.

The amounts of reagents used in the different procedures are exposed in Figure 4.24.

Once the samples were treated, 1 μl template was quantified by Sybr Green qPCR, using the 16S rDNA primer system 27F/517R in a 25 μl final reaction mixture as described in Chapter 3. To have some additional information about these treatments, PCR - DGGE using the eubacterial ribosomal primer systems targeting 16S rDNA (GC27F/517R) and DAPI staining were done.

The quantification of total cells (live + dead) was achieved by counting the DAPI stained cells using an epifluorescence microscope. For this, 100 μl sample was exposed to 1 ml DAPI in darkness for 5 min, then it was filtered using PC filter (Nucleopore Track - etched membranes; Whatman, Dassel, Germany), with 0.2 μm pore size and 300 mm^2 area. The stained cells were counted using an epifluorescence microscope equipped with a 50 W light source, to examine the filters at a magnification of 1000x. Observations were performed with a fluorescence light fitted with a BP365/FT395/LP397 blue filter for DAPI. Counting was carried out randomly on the basis of 10 microscopic fields per filter. For each sample, three filters were counted. Results were expressed as the number of corresponding bacteria per microliter of sample. Experiments were conducted in triplicate.

With this information the cells per μl sample were calculated:

$$\frac{\text{Cells}}{\mu\text{l}} = \frac{300\,\text{mm}^2}{0.0156\,\text{mm}^2} \times \frac{\text{average of cells counted}}{100\,\mu\text{l}} \tag{4.4}$$

Quantification Results

The results obtained after the cultivation method on R2A are shown in Table 4.13.

Table 4.13.: R2A culture results.

Suspension	**Bacterium**	**CFU**/100 μl^*	**CFU**/μl^*
WS1	*S. aureus*, *P. aeruginosa* and *S. enterica*	1.37×10^6	6.85×10^4
C1 live	*S. aureus*	1.45×10^6	7.26×10^4
C2 live	*P. aeruginosa*	6.91×10^5	3.45×10^4
SS3 DNA	*S. enterica*	0	0

*An average of the triplicate is shown.

The work suspension *WS1 live/dead/DNA* presented a similar value as its control *C1 live*, as expected. The determination of live bacteria in *C2 live* was done to have a reference of the amount of dead bacteria that were added to the *WS1 live/dead/DNA*. No bacteria grew when *SS3 DNA* was plated confirming the absence of live bacteria in the genomic DNA added to *WS1 live/dead/DNA*.

The amounts of cells of *WS1 live/dead/DNA* after the different treatments quantified by qPCR are represented in Figure 4.25. Hypothetically, the samples with 'No treatment', and with DNase$^-$/PK, and PMA$^-$ treatment should have had the same amount of bacteria. The difference obtained between the results after 'No treatment' (1.16×10^5 Bacteria/μl) and after DNase$^-$/PK (8.31×10^4 Bacteria/μl) was 3.32×10^4 Bacteria/μl, and the difference obtained between No treatment (1.16×10^5 Bacteria/μl) and PMA$^-$ (5.41×10^4 Bacteria/μl) was 6.21×10^4 Bacteria/μl. Despite, the results after DNase$^-$/PK treatment were closer as the PMA$^-$ results to the results of the sample without treatment (see Figure 4.25), a complete correlation was not observed. After DNase$^-$/PK treatment the amount of DNA was slightly lower, this could be due to a partial DNA disintegration due to the heating steps of this procedure. After the PMA$^-$ treatment the DNA amount

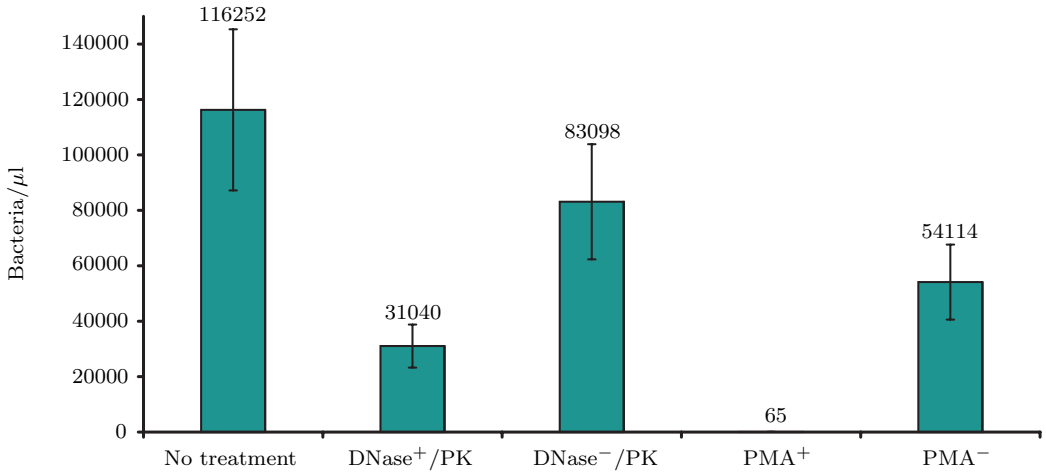


Figure 4.25.: Comparison of quantitative PCR results from the work suspension *WS1 live/dead/DNA* after different treatments for the quantification of live cells. DNase$^+$/PK: with DNase/PK treatment; DNase$^-$/PK: samples without DNase I; PMA$^+$: with PMA treatment; and PMA$^-$: samples without PMA.

was the half of the DNA present in the sample without treatment, this might be due to the wash steps of this procedure. Noticing that the treatments *per se* have an impact in the end quantification, the live cells fraction were calculated in a relative manner.

This means, the result obtained after DNase$^-$/PK or PMA$^-$ treatment was considered as the total amount of DNA (live cells + dead cells + free DNA), and the result after DNase$^+$/PK or PMA$^+$ was the live cell fraction. Therefore the live cell fraction determined by qPCR after DNase/PK and PMA treatment was 37.4% and 0.12%, respectively. Using the plate count method it was seen that the live cell fraction contained 6.84×10^4 Bacteria/μl and the dead cell fraction contained 3.45×10^4 Bacteria/μl. As consecuence the live cell fraction determined by plate count was 61.4%. In fact, the live fraction obtained by qPCR (37.4%) is almost the half as the live cell fraction obtained by plate count (61.4%).

PCR inhibitors were controlled to verify if the results obtained after the DNase/PK and the PMA treatment of *WS1 live/dead/DNA* were not lower as those obtained by the R2A cultivation method due to PCR inhibition. The PCR efficiency assay was done by

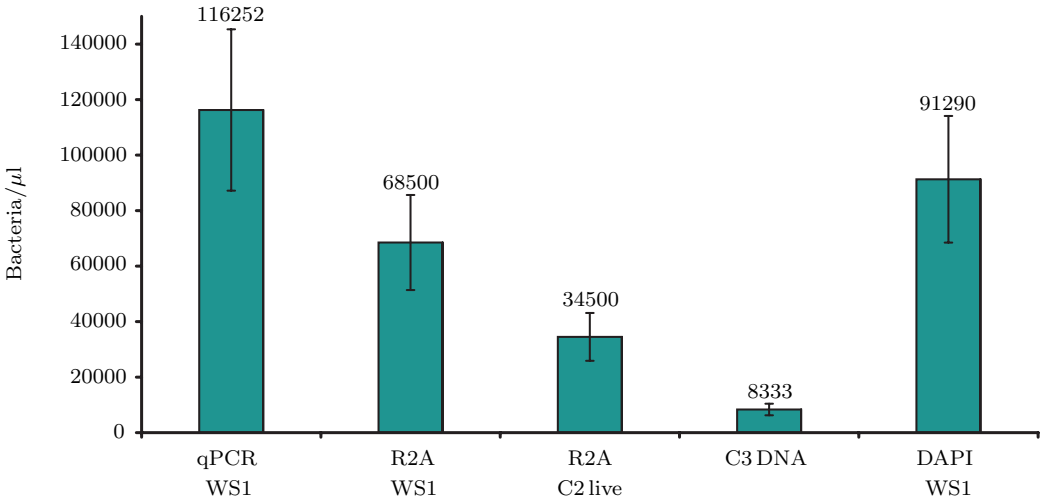


Figure 4.26.: Comparison of total bacterial amounts determined by different quantification methods. qPCR: quantitative PCR results of total DNA in the samples; R2A: quantification of culturable cells; DAPI: microscopic quantification of total cells in the samples; *WS1 live/dead/DNA*: work suspension with live and dead bacteria and free DNA; *C2 live*: live cells control suspension equivalent to the dead cells (*C2 dead*) used in the experiment; *C3 DNA*: free genomic DNA control suspension.

adding 1μl of a known amount of DNA (100 pg/μl = 2.5×10^5 bacteria/μl) to each sample after qPCR. No PCR inhibition was observed.

Different controls were done to compare the amount of bacteria in *WS1 live/dead/DNA* using different quantification methods. These results are exposed in Figure 4.26. Summing, the amount of cells from the *WS1 live/dead/DNA* sample that were cultured on R2A (6.85×10^4 Bacteria/μl), the amount of cells from *C2 live* cultured on R2A (3.45×10^5 Bacteria/μl; that represent the amount of cells in *C2 dead*), and the amount of DNA (*C3 DNA*) added to the *WS1 live/dead/DNA* sample (8.33×10^3 Bacteria/μl), a value of 1.11×10^5 bacteria/μl was obtained. This result reflects the total amount of DNA in the *WS1 live/dead/DNA* sample and its corresponding bacterial amounts. If this result is compared with the results of qPCR (1.16×10^5 bacteria/μl) and DAPI staining (9.13×10^4 bacteria/μl), the same order of magnitude was observed demonstrating a good correlation between the methods. The cells counted by DAPI staining were cocci and

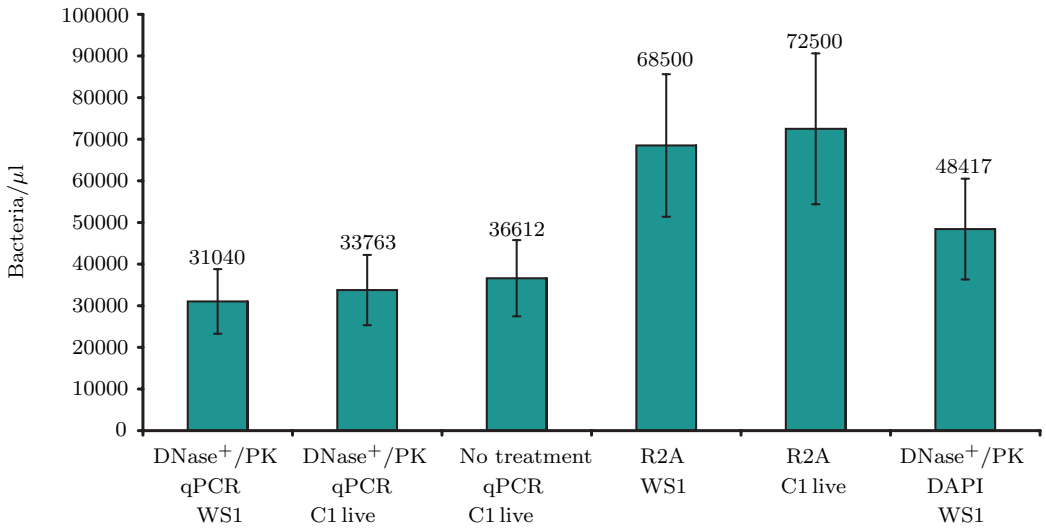


Figure 4.27.: Comparison of live bacteria determined by different quantification methods after DNase$^+$/PK treatment. qPCR: quantitative PCR results of total DNA in the samples; R2A: quantification of culturable cells; DAPI: quantification of total cells in the samples; *WS1 live/dead/DNA*: work suspension with live and dead bacteria and free DNA; *C1 live*: live cells control suspension.

rod shaped bacteria (see Figure 4.29e).

To control and compare the amount of live bacteria present in the *WS1 live/dead/DNA* and in the control sample *C1 live* after DNase$^+$/PK treatment, the quantification results after the different quantification methods are shown in Figure 4.27. The values that indicate live cells after DNase$^+$/PK treatment are shown together with the values of culturable cells and the cells counted after DAPI staining. The cells counted by DAPI staining were only cocci, no rod shaped bacteria were seen (see Figure 4.29a). With this observation it cannot be concluded that the DNase$^+$/PK treatment worked, but it gives information of the total amount of cocci present in the sample. Hypothetically, *WS1 live/dead/DNA* after DNase$^+$/PK treatment, *C1 live* after DNase$^+$/PK treatment, and *C1 live* without treatment should have had the same amount of bacteria. This was verified by the similar qPCR results that were obtained. These values and the culturable bacteria R2A of *WS1 live/dead/DNA* and *C1 live* should have been also similar, but this correlation was not optimally observed. These results were in the same order of magnitude but the results seen for qPCR (3.10×10^4 Bacteria/μl) were the half of those obtained

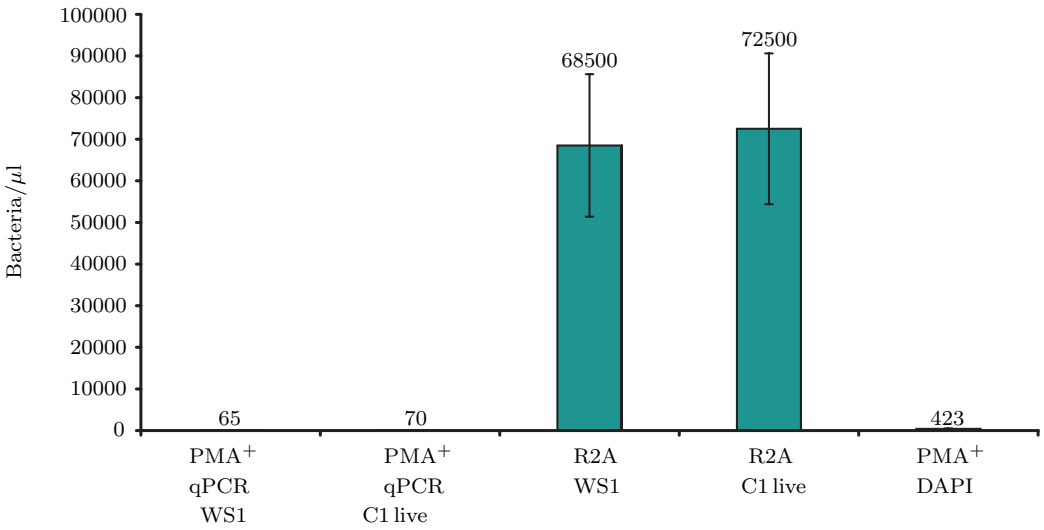


Figure 4.28.: Comparison of live bacteria determined by different quantification methods after PMA treatment. qPCR: quantitative PCR results of total DNA in the samples; R2A: quantification of culturable cells; DAPI: microscopic quantification of total cells in the samples; *WS1 live/dead/DNA*: work suspension with live and dead bacteria and free DNA; *C1 live*: live cells control suspension.

with the plating techniques (6.85×10^4 Bacteria/μl).

The quantification results obtained by the different quantification methods done to control and compare the amount of live bacteria present in the *WS1 live/dead/DNA* and in the control sample *C1 live* after PMA treatment, are shown in Figure 4.28. The values that indicate live cells after PMA$^+$ treatment are shown together with the values of culturable cells and the cells counted after DAPI staining. A clear loss of bacteria can be seen after PMA treatment, this can be due to the wash steps. When the cells were counted on the DAPI staining cocci and rod shaped bacteria were seen (see Figure 4.29c and d). The cells counted by DAPI staining were much lower as in the case of DNase/PK treatment, this could be due to a loss of bacteria during the wash steps of the PMA protocol.

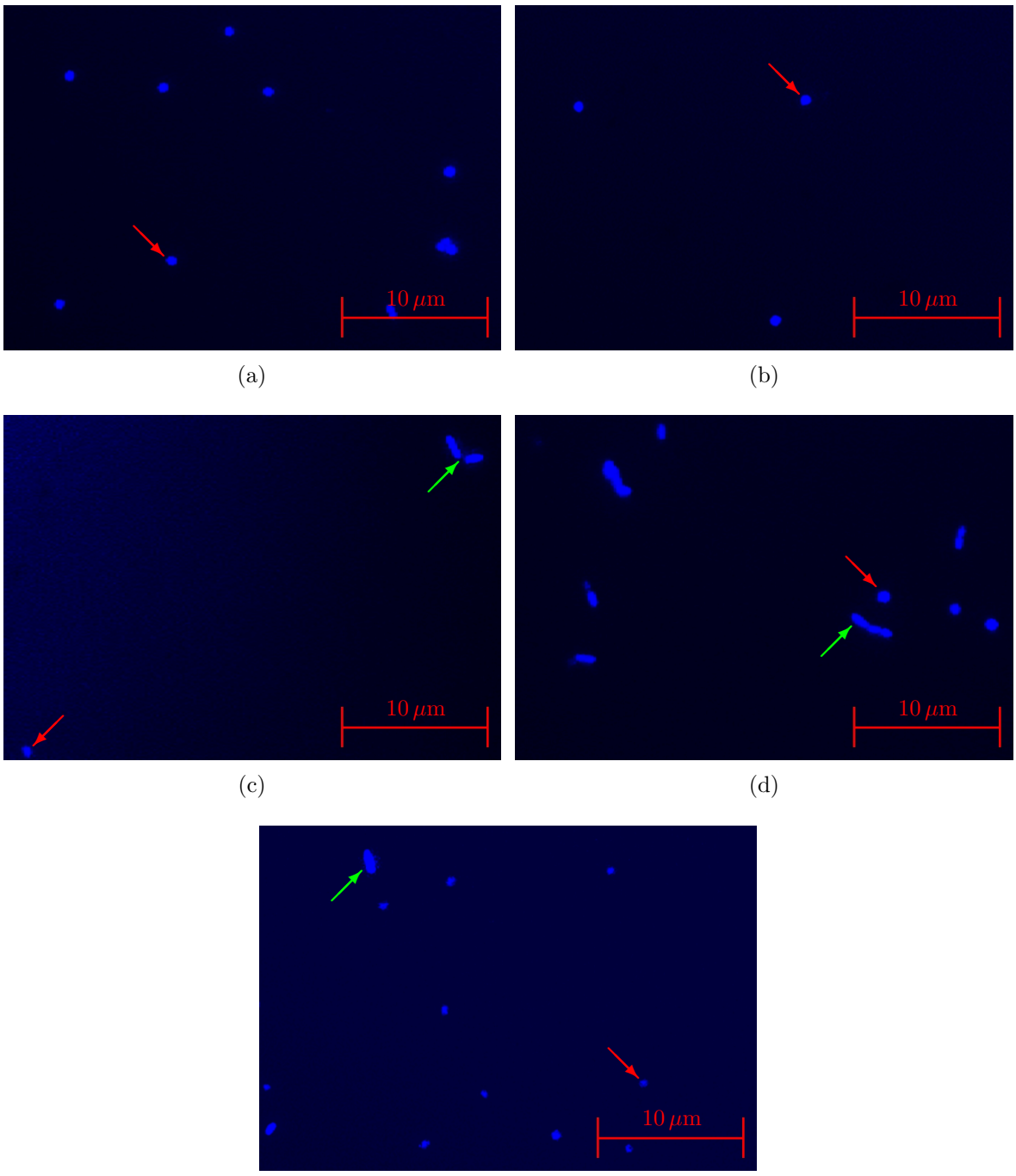
Microscopy Results

In Figure 4.29 pictures of DAPI staining of the *WS1 live/dead/DNA* sample after different treatments are shown.

During the DNase/PK treatment the samples were exposed to the protease activity of proteinase K and to several heating steps. Therefore, only dead cells were supposed to be observed after the treatment. When the DNase/PK treatment was done with addition of DNase I (see Figure 4.29a) or without DNase I (see Figure 4.29b), only cocci were observed. DAPI stains DNA present in live or dead cells. These cocci could have been present due to the higher resistance of the cell membrane of Gram positive bacteria respect to Gram negative. Using only the DAPI staining it could not be determined if the bacteria were live or dead.

During the PMA treatment, the PMA intercalates the free DNA and the DNA from injured or dead cells. During this treatment no high temperature steps and no exposure to proteases was done, therefore the observation of cocci and rod shaped bacteria was expected. When the PMA treatment was done with the addition of PMA (see Figure 4.29c) or without PMA (see Figure 4.29d) cocci and rod shaped bacteria were observed, as expected. But, a much lower amount of cells was counted, this may be due to a loss of material during the washing steps.

A sample without treatment (see Figure 4.29e) was also stained as control, here cocci and rod shaped bacteria should have been present. This sample presented cocci and rod shaped bacteria, as expected.



(a) (b) (c) (d) (e)

Figure 4.29.: DAPI pictures of the live/dead/DNA suspension (WS1) after live/dead differentiation assay. Sample with DNase/PK treatment: DNase$^+$/PK (a); sample exposed to the same conditions of the DNase/PK treatment but without DNase I: DNase$^-$/PK (b); sample with PMA treatment: PMA$^+$ (c); sample exposed to the same conditions of the PMA treatment but without PMA: PMA$^-$ (d); and sample without treatments (e). Red arrows: cocci; green arrows: rod shaped bacteria.

DNA Fingerprint Results

PCR - DGGE of the *WS1 live/dead/DNA* sample and the control samples (*C1 live*, *C2 dead* and *C3 DNA*) was done using the ribosomal GC27F/517R primers to see if the treatments worked (see Figure 4.30). In the first lane the *WS1 live/dead/DNA* sample without any treatment was run; here the three bands that corresponded to live *S. aureus*, dead *P. aeruginosa*, and to free genomic DNA of *S. enterica* were clearly observed. The second lane had the *WS1 live/dead/DNA* sample but after DNase$^+$/PK treatment, here only the band that corresponded to live *S. aureus* was seen; indicating that the DNase I digested the DNA of the dead *P. aeruginosa* and the free genomic DNA of *S. enterica*. On the third lane the *WS1 live/dead/DNA* sample that followed the same protocol of DNase/PK treatment but without the addition of DNase I was run, here again the three bands were observed as expected. The forth lane presents the *WS1 live/dead/DNA* sample after PMA$^+$ treatment, here no band was seen; this could be due to the low amount of DNA present in the samples. As it was already observed by qPCR and by DAPI staining a very low amount of bacteria were present in this sample may be due to the wash steps. Apparently, no PCR band was seen due to the high detection limit (low sensitivity) of the PCR. The fifth lane contained the *WS1 live/dead/DNA* sample that followed the same protocol of PMA treatment but without the addition of PMA, here the bands of live and dead bacteria are seen but the band corresponding to free genomic DNA was not observed, this can also be due to the loss of DNA after the wash steps and to the high detection limits of the PCR. In the last lanes the *C1 live*, *C2 dead*, and *C3 DNA* samples without treatments were run as control. And the PCR positive (PC) and negative (NC) controls were also run.

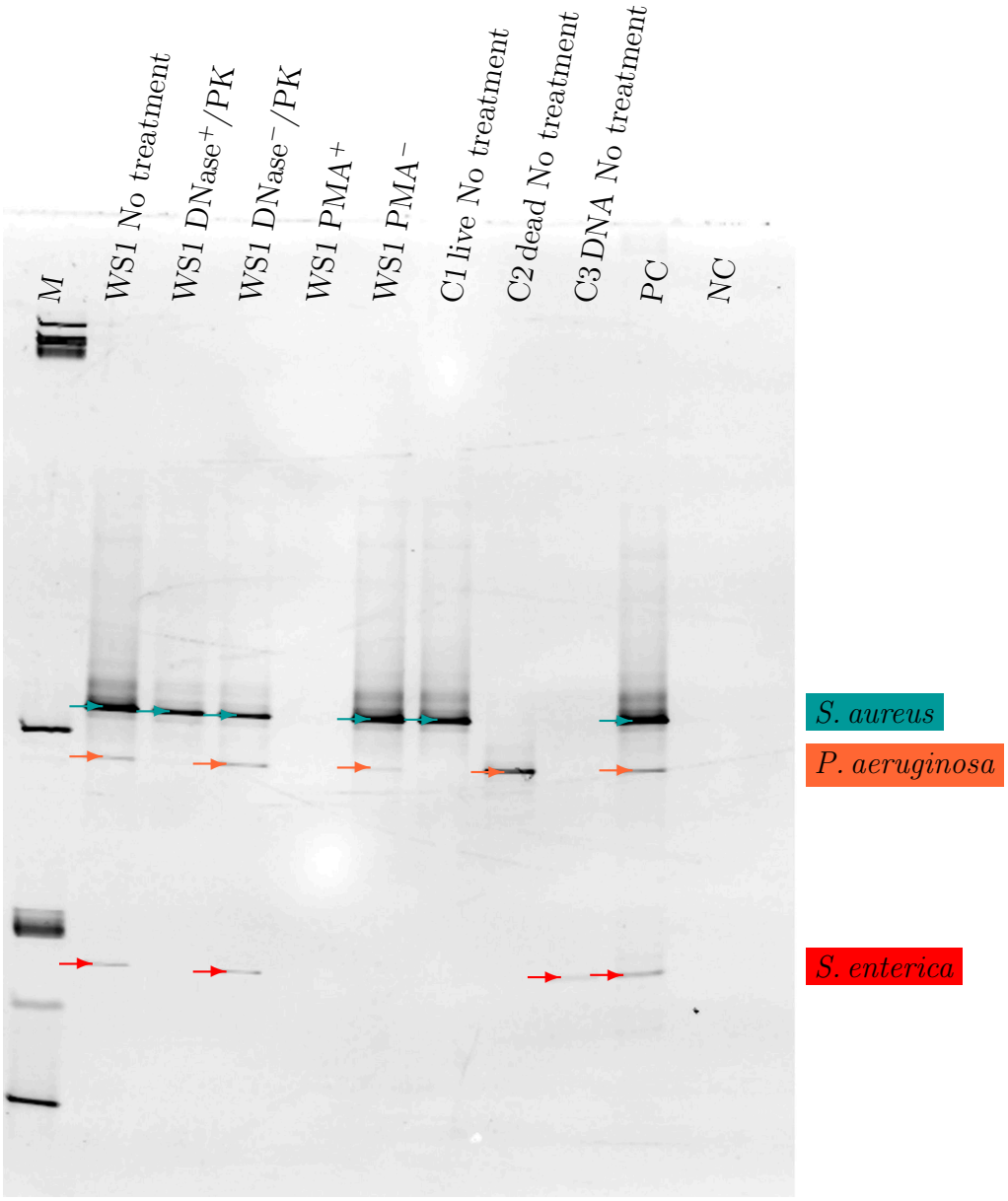


Figure 4.30.: DGGE of live/dead differentiation assay. M: 100 bp DNA marker; *WS1 live/dead/DNA*: work suspension with live and dead bacteria and free DNA; *C1 live*: live cells control suspension; *C2 dead*: dead cells control suspension; *C3 DNA*: free genomic DNA control suspension; DNase$^+$/PK: samples with DNase/PK treatment; DNase$^-$/PK: samples without DNase I; PMA$^+$: samples with PMA treatment; and PMA$^-$: samples without the addition of PMA.

4.4.5. Live/Dead Differentiation of Bacteria from Drinking Water Biofilms from Waterworks

This experiment was carried out to analyze live cells in natural drinking water biofilm samples. Quantification of bacteria and bacterial population analysis were done, by qPCR and PCR - DGGE, respectively. The toolbox used for the quantification of live bacteria consisted in conventional R2A plating technique, DNase/PK and PMA treatments before DNA - based quantification methods, and CTC/DAPI staining.

Conditioned surface water disinfected with ozone/ClO_2 flowed through a pilot scale (see Figure 4.31), built up according to DIN 50931 - 1 (Norm, 1997). It mainly consisted in 3 parallel pipelines, each was 5 m long, and had an inner diameter of 13 mm. Different pipe materials were used: stainless steel (St), copper (Cu), and polyvinyl chloride (PVC). Each pipeline had a modified Robin's device (Kalmbach et al., 1997) which contained the slides of the respective materials where the drinking water biofilms were harvested. Each slide had an area of 34 cm^2.

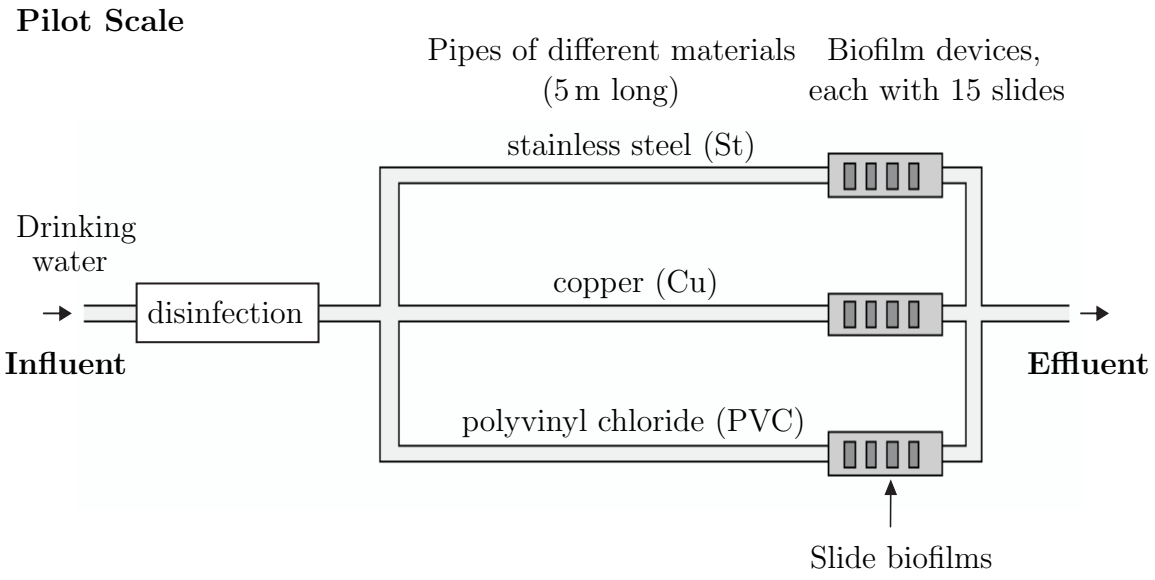


Figure 4.31.: Scheme of pilot scale.

Three months old biofilm samples were taken. Autochthonous bacterial population analysis was done by PCR - DGGE (GC27F/517R), comparing direct samples (total DNA) and samples treated with PMA or DNase/PK (DNA from live cells). 2 μl template of each sample was quantified in a 25 μl reaction mixture by Sybr Green qPCR

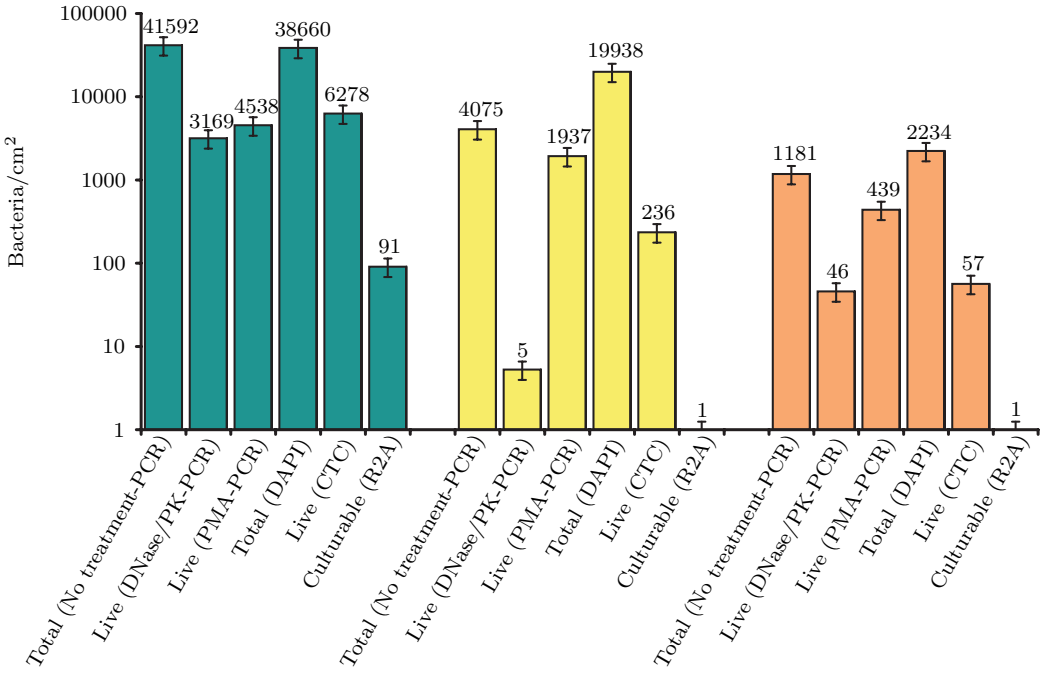


Figure 4.32.: Comparison of quantification methods of the live/dead differentiation toolbox. Green bars: stainless steel; yellow bars: copper; and orange bars: polyvinyl chloride.

(27F/517R). PCR efficiency assays were also performed with these samples. Additionally, metabolic active bacteria were counted using CTC staining, total amount of bacteria were determined by DAPI staining, and viable culturable bacteria were determined by traditional culturing methods using R2A plates.

The results of the bacterial population analysis and the results of the quantification methods that provide an overview of the different physiological states of bacteria: live cells, total amount of cells, and culturable cells, are presented in Figure 4.32.

Considering that the drinking water that flowed through the pilot scale was the same, a difference between the amounts of bacteria was observed depending on the analyzed material (Figure 4.32). Biofilms of stainless steel pipes appeared to have a higher amount of bacteria than copper and PVC, the materials where the biofilms grew seemed to play an important role in biofilm formation as already Niquette et al. (2001) and Schwartz et al. (1998) described.

When stainless steel pipe biofilms were analyzed, the percentual results of live bacteria obtained after DNase/PK-qPCR and PMA-qPCR with respect to the total amount of bacteria (No treatment) were 7.6 and 10.9%, respectively. The percentage of live cells

obtained by CTC staining with respect to the total amount of cells determined by DAPI staining was 16.2%. The percentage of live cells after CTC staining was slightly higher as the percentages obtained after DNase/PK-qPCR and PMA-qPCR. Despite this, a relative good correlation of live bacteria were found after comparing these percentages of live bacteria with respect to total bacteria after the different methods.

This was not the case of copper and PVC biofilms. In the case of copper a great difference between the methods was observed. The percentages of live bacteria obtained by the DNase/PK-qPCR method (0.1%) and by CTC staining (1.2%) were much lower than those of the PMA-qPCR method (47.5%). The lower blocking of DNA from dead bacteria by the PMA treatment could be caused in theory by a formation of a kind of complex between copper and the nitrene radical generated from PMA, further studies should be done in order to affirm this hypothesis.

In the case of PVC a good correlation among the live cell fraction was observed between samples after DNase/PK-qPCR method (3.9%) and samples stained with CTC (2.5%), while the result obtained after PMA-qPCR method (37.2%) was one order of magnitude higher.

In general it can be affirmed that, as assumed, the **total amount of DNA** in a sample is not the total amount of live bacteria in drinking water biofilm samples. This assumption was confirmed in this experiment due to the presence of higher amounts of total bacteria DNA than DNA from live bacteria, in all samples. Another hypothesis that was confirmed in this experiment was that the **culturable fraction of bacteria** present in a sample is not the total fraction of live bacteria in a sample. As it can be seen in Figure 4.32 the amounts of live bacteria were much higher than the amounts of culturable bacteria.

A determination of population shifts of the bacteria present in the samples was done in order to evaluate the efficiency of the treatments to eliminate the DNA from dead cells, in order to analyze only the live bacterial population. For this, 2 μl from the samples without treatment, with DNase/PK treatment, and with PMA treatment were used as template for the PCR in a 50 μl final volume, and 15 μl were run on the DGGE.

In the DNA fingerprints (Figure 4.33) some shifts were observed between the number of DNA bands of the sample without treatment and the samples with the different treatments. In the case of stainless steel and copper pipeline biofilms a total amount of 10 and 8 DNA bands, respectively, were observed on the DGGE gel of the samples without treatment. Therefrom, 30 and 50% respectively, of the DNA bands were missing

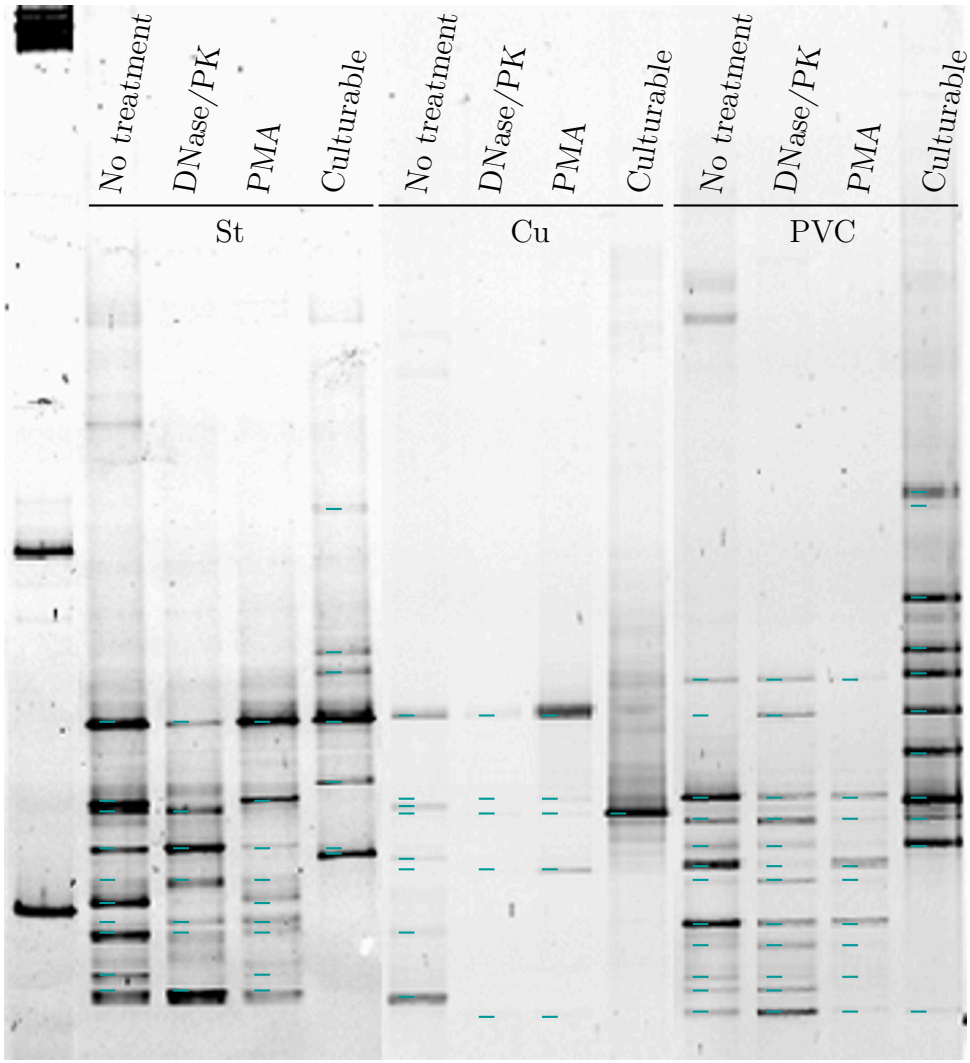


Figure 4.33.: DGGE DNA fingerprints from biofilm samples of waterworks after DNase/PK and PMA treatments.

after DNase/PK treatment. After PMA treatment the stainless steel pipeline biofilm presented 10% less DNA bands as the sample with 'No treatment', the copper pipeline biofilm presented the same bands obtained after DNase/PK treatment (i.e. 50% less bands as without treatment), and the PVC pipeline biofilms had 15% less bands as without treatment. It also was observed that some weak bands present in the sample without treatments were more intense after DNase/PK and PMA treatment. The lanes from Figure 4.33 named "culturable" corresponded to the isolated DNA of the bacteria that grew on the R2A plates. In the case of culturable cells of all the materials new bands and absence of other bands were observed, giving a very high shift when compared to the bands of the sample without treatments. This could be due to the partial selectivity given by the culture conditions of R2A.

5. Summary of Results

Culture - independent techniques were established to quantify different hygienic relevant bacteria in drinking water. A strategy based on these culture - independent methods was developed to look for possible water - derived critical control points in the production lines at two food companies. Finally, culture - independent techniques were optimized and applied to discriminate live bacteria from dead bacteria in drinking water and drinking water pipeline biofilm samples.

Sensitivity Tests of PCR and qPCR Detection Systems

Sensitivity tests were performed for the different PCR and qPCR systems used in the present work for the specific detection of hygienic relevant bacteria in drinking water of food companies.

The conventional PCR systems tested in the present work had too high detection limits to be used as reliable pathogen detection methods. Only the specific system for detection of *Enterococcus* had similar detection limits to those of the conventional plating methods (i.e. 1 bacteria/100 ml water sample).

The quantitative PCR systems seem to be a more reliable option for the specific detection of pathogens. A perfect standard curve would have a correlation coefficient of 1.0000. The correlation coefficients obtained in the present work (between 0.9958 and 0.9995) showed a high precision of the assays and a strong correlation between template DNA concentrations and C_T values. The high PCR efficiencies seen for the qPCR assays and the high correlation efficiencies indicated that they were appropriate for quantitative measurements. In the case of *Mycobacterium avium* subsp. *paratuberculosis* (MAP), the standard curve reflected a high correlation coefficient, but the calculated detection limit minimized the application of this assay.

The use of the TaqMan® Gene Expression Master Mix, using a ultra pure DNA poly-

merase for the determination of *E. coli* was successful for the amplification of *E. coli*-DNA, showing no background amplification, decreasing the possibility of false positive results due to DNA polymerase impurities.

The detection limits obtained for the specific qPCR systems were lower as those from PCR and seminested PCR, and therefore more reliable. But, with an appropriate filtration volume of the water samples, all these culture - independent methods could be applied to detect pathogens in drinking water.

Analysis of Drinking Water at Food Companies

After the first sampling period at the German dairy company, the bacterial population was analyzed using DGGE. Some opportunistic bacteria as enterococci, *P. aeruginosa*, *Sphingomonas* and *Acinetobacter* were aligned. Although point 6 (water used to clean room and machinery where feta cheese was packed) was found to be a potentially critical point after the population similarity analysis, no technical problems or irregular operation during food production were encountered during the evaluation. Nevertheless, due to presence of this possible critical control point and to the presence of DNA of enterococci and *P. aeruginosa*, some hygienic recommendations, such as a more frequent exchange of hoses, were made before the second sampling period. No pathogens were detected by using the specific PCR detection systems.

A second sampling period was organized at the German dairy company to corroborate if the practical application of hygienic recommendations had an influence in the results of the autochthonous bacterial population analysis. For this, the optimized strategy where higher water volumes were filtered was used to achieve detection limits similar to those indicated by the drinking water guidelines after standard plating techniques (i.e. 1 bacteria/100 ml water). No shifts were observed anymore after autochthonous bacterial population analysis. No PCR inhibitors were found in the samples, despite a higher volume of water was filtered. Monitoring of pathogens during the second sampling period did not produce any pathogen - positive results, no matter whether traditional plating methods or culture - independent methods were applied. Most bacteria aligned after sequencing the DGGE bands were non pathogenic bacteria of water. This proofs that the autochthonous bacterial population analysis can be used to monitor the bacterial stability of the water used within a food company to detect possible critical control points.

Water samples used in food production at a Spanish dry cured ham company and also

biofilm samples of the drinking water distribution network at this company were analyzed. PCR inhibitors were found after carrying out the PCR efficiency assay. The inhibition did not disappear when BSA was used, but was successfully removed by treating the sample with PVPP. After analyzing the stability of the autochthonous bacterial population of the water samples of this company, a quite high similarity was found between the water samples within the food company. Nevertheless, when pathogens were monitored in the samples, the DNA of some pathogenic species as *P. aeruginosa* and *E. coli* were found. This makes obvious that the autochthonous bacterial population analysis is not enough to determine possible water - derived critical control points, and that an additional specific determination of pathogens at the sampling points is a good option for the evaluation of the chosen sampling points.

Although some positive pathogenic bacteria results were seen after the use of pathogen specific culture - independent methods, it was not possible to distinguish the origin of DNA (i.e. extracellular DNA or DNA from viable, VBNC, injured, or dead cells).

Culture - independent techniques were optimized in the present work for the quantification of different hygienic relevant bacteria in drinking water at food companies. A strategy based on these techniques was developed to look for possible water - derived critical control points in the production lines at food companies.

The questionnaire (see Appendix A) and the work - together with the companies was essential for a better determination of possible water - derived critical control points.

Live/Dead Differentiation Assays

DNA - based methods were used to detect and characterize (i) bacteria present in the water at different sampling points of food companies, or (ii) bacteria that were part of biofilms formed on the drinking water pipelines at the food companies. These methods detected the presence of DNA but they were not able to differentiate if the DNA was from live or dead cells or if it was from free or extracellular DNA (eDNA).

In order to detect and characterize only live bacteria from the samples, different methods were presented, giving special attention to the DNase I treatment.

The reaction conditions, as buffer composition, DNase I concentration, time of exposure to DNase I, and inactivation of DNase I, were optimized for the DNase I treatment in the present work. Inactivation of DNase I was a key issue for the subsequent PCR pathogen

detection procedure, therefore the DNase I method was called DNase/PK treatment.

Drinking water has a very low amount of bacteria, hence the bacteria present in it have to be concentrated by a filtration step. Therefore, once the protocol was optimized, it was tested in the presence of different filter membranes commonly used for this purpose. Filters used for the concentration of biomass of water samples could inhibit the DNase I. Nevertheless, when higher concentrations of DNase I were used ($\geq 0.3\,\mathrm{U}/\,\mu\mathrm{l}$) a complete digestion of free DNA in the samples was observed. Polycarbonate filters had the lowest DNase I inhibition rate observed among the examined filters. Depending on the filter used, higher concentrations of DNase I should be added to assure a complete digestion of free DNA in the samples.

After optimizing the DNase/PK treatment a protocol was developed in order to detect only DNA coming from live cells. This technique was also compared with other methods as the PMA treatment. The ability of the DNase/PK treatment prior DNA - based methods to determine exclusively live cells in water samples that contained also dead bacteria and free DNA was demonstrated in the present work. The use of PMA was not so optimal for live cell determination in water samples apparently due to the loss of material during the wash steps of the PMA treatment protocol. qPCR, PCR - DGGE, and DAPI staining were useful tools for the analysis of the samples of this experiment.

Once it was demonstrated that the Deoxyribonuclease I/Proteinase K (DNase/PK) treatment was an adequate method to determine DNA coming from live cells, this method together with the PMA treatment, the conventional R2A plating technique, and with the CTC/DAPI staining were used for the analysis of natural drinking water biofilm samples. This toolbox was established for the differentiation of the biological states of bacteria (total/live/culturable), and was used for the quantification and for the population analysis of the bacteria present in natural drinking water biofilm samples of a waterworks. When the analysis of the autochthonous bacterial population of the samples was done some shifts in the patterns were observed. The shifts observed in the DNA patterns after DGGE analysis, demonstrated: (i) the applicability of PMA and DNase/PK treatment in natural biofilm investigation; (ii) detection of DNA from dead bacteria and eDNA was blocked by treatment with PMA or DNase/PK; and (iii) DNase/PK treatment demonstrated a clearer effect on live/dead differentiation.

As final conclusion, the DNase/PK procedure was successfully used to quantify and analyze live bacteria in water and biofilm samples, by treating the samples before the DNA was amplified by DNA - based detection techniques.

The live/dead differentiation toolbox used for the analysis of natural drinking water biofilms was useful for the quantification of total/live/culturable bacteria and for the analysis of the bacterial population present in the samples.

6. Discussion

Molecular biology techniques have been used for several years for the examination of water for multiple purposes (Frahm et al., 1998; Frahm and Obst, 2003; Grobe et al., 2001; Schwartz et al., 1998, 2003a). The present work was focused on the testing and optimization of culture - independent techniques to monitor the bacterial drinking water quality at food companies. Some case studies were described in this work to verify the applicability of these techniques.

Nowadays, drinking water is ranked as food in most industrialized countries, and is considered indeed the most important food. Therefore, the strictest quality and safety standards have been set. Strict demands for the absence of pathogens are significant for the classical pathogens. The Drinking Water Directive 98/83/EC (EU Council decision, 1998) of the European Union and the World Health Organization guidelines (WHO, 2008), state that drinking water can contain pathogenic microorganisms only in such low numbers that the risk for acquiring waterborne infection is below an accepted limit. The Drinking Water Directive 98/83/EC (EU Council decision, 1998) also states that indicator microorganisms should be routinely monitored in drinking water in order to control microbial water quality of public distribution systems. The German Drinking Water Ordinance (TrinkwV 2001, 2001) and the Spanish Drinking Water Guidelines (Real Decreto 140/2003, 2003) based on the above EU directive stipulate that no *E. coli*, enterococci, and coliform bacteria should be present in 100 ml drinking water of public distribution systems. The standard detection method described in these guidelines is the conventional plating on defined media. This standard method is based on the identification of indicator bacteria, but it has some disadvantages, like no direct identification of the pathogen, a lack of correlation to many waterborne pathogens, and only viable culturable bacteria would be determined. It has been described that bacterial indicators, as *E. coli* and enterococci can enter rapidly in the viable but non - culturable (VBNC) state after being released in freshwater (del Mar Lleò et al., 2005; Huq et al., 2000). In the past years the microbiology community has commonly accepted that culture - dependent methods do not reflect the different physiological states of bacteria that influence their

culturability (Oliver, 2000). The discovery of new emerging pathogens and new insights into the microbiology of drinking water require more elaborated norms (Szewzyk et al., 2000).

Consequently, besides the prescribed standard culture - dependent methods, culture - independent methods were applied as an alternative approach to monitor the most important foodborne pathogens in drinking water. DNA fingerprinting was used in the present work to characterize the autochthonous bacterial population of drinking water at the food companies, in order to control their microbiological quality and stability. Nowadays, the use of molecular biology methods in routine drinking water surveillance is still limited, as these new methods have not yet been accepted by the authorities. According to the EU guidelines (EU Council decision, 1998), such methods can be used for the monitoring of indicator bacteria only when it can be demonstrated that the results obtained are at least as reliable as those produced by the specified methods. Hence, the detection limits of the assays play a critical role for bacterial quantification in drinking water samples. The detection limits of the qPCR systems used in the present work were not always optimal to reach the parameters established by the water authorities, especially those obtained for the detection of *Mycobacterium avium* subsp. *paratuberculosis*. In order to reach detection limits of at least one bacterium per 100 ml without an additional enrichment step, a protocol with higher sample filtration volumes was developed. In the case of *M. avium* subsp. *paratuberculosis*, even higher bacterial concentration rates should be achieved. Ultrafiltration has been lately used to concentrate large amounts of water (Rajal et al., 2007), but for routine analysis of water this method could be too expensive and more time consuming as the regular membrane filtration.

In both food company case studies, no pathogenic bacteria were cultivated from the water samples using standard plating methods. However, some positive results were obtained when culture - independent techniques were used. This could be due to the higher sensitivity of PCR that leads to a greater number of positive results in comparison to conventional plating methods, which was also described by Sachse and Frey (2003). It is also known that culture - independent techniques based on the analysis of the DNA present in the samples cannot distinguish among viable, VBNC, injured, and dead cells. VBNC or injured bacteria are alive and metabolically active but do not grow on the routine bacteriological media (Oliver, 2000). False negative results might be obtained when traditional plating methods are used. About 60 bacterial species have been already described to enter the VBNC state. Among these are some relevant foodborne pathogens, e.g. enterococci, *Campylobacter jejuni*, *Salmonella* spp., *Helicobacter pylori*, *Klebsiella* spp., *Listeria monocytogenes*, and *Escherichia coli* (including EHEC) (Oliver,

2005b). Therefore, the detection of bacteria, including VBNC bacteria, in drinking water from food companies is essential to ensure the microbiological safety of food.

Although positive DNA - based results do not reflect the presence of exclusively live bacteria, they give hints of possible irregular operations that might support the transfer of pathogen targets. What also might be considered is the presence of eDNA. In the past years the function of eDNA has been studied. It has been reported that this kind of DNA has an active role in biofilm formation (Whitchurch et al., 2002). Studies about biofilm formation of *L. monocytogenes* strains, have also lately demonstrated that eDNA could be the only central component of the biofilm matrix and that it was a substantial key for both initial attachment and early biofilm formation (Harmsen et al., 2010). Therefore, to prevent biofilm formation in drinking water distribution systems, the presence of eDNA could also be considered as an alert of possible foodborne pathogen's presence.

Another critical topic that should be considered when using molecular biology techniques is the possible presence of PCR inhibitors. Organic substances like humic acids and other PCR inhibitors are often present in surface waters (Wilson, 1997). Such substances were found in the water samples taken at the Spanish dry cured ham company. The PCR inhibition was not removed by BSA treatment, but the use of PVPP as mentioned by Sutlović et al. (2007) and Gusbeth et al. (2009) successfully removed the PCR inhibitors in this work.

Characterization of the bacterial populations of water samples was an innovative approach applied in this work to demonstrate the biological stability of water in an industrial process. Previous studies revealed that Sørensen similarity indexes between 0.40 and 1 (i.e. between 40 and 100% similarity) reflected a natural range of population diversity in a drinking water distribution system (Emtiazi et al., 2004). Hence, similarities below 40% are discussed to indicate a population shift, suggesting that something is anyhow affecting the microbiological population of water between the compared samples (e.g. pipeline rupture, water stagnation, pipeline corrosion, etc.). Only one point at the German dairy company (feta cheese packaging) had a lower similarity when compared to the reference point, indicating that something was affecting the natural microbiological population of water. Considering the information collected with the questionnaire and discussing these results with the company, some hygienic recommendations, such as a more frequent exchange of hoses were made before an additional sampling period. The similarity values among the different sampling points and the reference point observed during the second sampling period after implementing the hygienic recommendations were high; this demonstrated that the PCR - DGGE method was adequate for the evaluation

of drinking water bacterial stability from food companies. Some opportunistic bacteria as *Sphingomonas*, *Acinetobacter*, *Enterobacter*, and *Stenotrophomonas maltophilia* were found when the DNA present in the water or biofilm samples at the food companies were sequenced. Sphingomonads have been described as non - life - threatening bacteria. *Acinetobacter* (specially *Acinetobacter baumanii*) have been actively implicated in nosocomial infections (Kuo et al., 2007). But, these bacteria have not been described as foodborne pathogens. Despite this, *Acinetobacter calcoaceticus* has been found to play a bridging function in drinking water biofilm formation (Chaves Simões et al., 2008), therefore its presence might be of interest if biofilm formation wants to be prevented.

The quality of the supplied drinking water is of significant importance for a good hygienic practice in downstream process lines. Therefore, information from raw water quality is needed in concern of potential contaminations with hygienically relevant bacteria and also with respect to the level of organic carbon (WHO, 2004b). Groundwater and surface water are frequently conditioned in Germany and many other countries. Usually, groundwater is supposed to have a better biological quality than surface water, but some waterborne diseases have also been transmitted by contaminated groundwater (Craun, 1985; Ritter et al., 2002; Scandura and Sobsey, 1997). Data about the drinking water conditioning at the waterworks is essential for the estimation of the biological stability of the drinking water during its distribution. Disinfection measures are mostly important to inactivate microorganisms. Depending on the drinking water character, sustainability of the disinfection measure is impaired. Chemical (chlorine, chlorine dioxide, ozone) disinfection and UV irradiation are the most frequently used disinfection techniques at European waterworks. It has been demonstrated that these treatments have various disinfection efficiencies (WHO, 2004b). Some hygienically relevant bacteria, such as *Pseudomonas* spp., *Helicobacter pylori*, and *Legionella pneumophila* are well - known to have a high capability to survive in chlorinated water and to form biofilms (Giao et al., 2008, 2009; Grobe et al., 2001; Leclerc et al., 2002). It was demonstrated recently that a specific DNA dark repair mechanism of *P. aeruginosa* was induced at UV exposures of $400\,\mathrm{J/m^2}$, which corresponds to the German standard for UV disinfection (Jungfer et al., 2007).

It is important to control drinking water facilities from food companies to avoid irregular operations (i.e. inadequate pipeline or connection materials, water stagnation, softening, pipe corrosion, etc.) that might influence bacterial growth or re - growth (WHO, 2004b, 2006, 2008). Furthermore, irregular operations may result in an increased biofilm formation. Biofilms are potential habitats of all kinds of bacteria, including pathogens (Emtiazi et al., 2004; Juhna et al., 2007a; Lehtola et al., 2004; Schwartz et al., 1998,

2003a) and may be responsible for contaminations of bulk water systems (September et al., 2007). Old pipes in combination with increased water hardness values may result in pipe incrustations that are also known to support undesired biofilm formation (WHO, 2004b, 2006). This might be the reason for the presence of *P. aeruginosa* at the Spanish company, where the pipelines were 20 years old. The use of accessory facilities like hoses for cleaning processes could be responsible for cross - contaminations during food production. Such hoses should be exchanged regularly, especially when warm water is used, since warm water systems support the growth of hygienically relevant bacteria, such as *E. coli*, *Pseudomonas aeruginosa*, *Aeromonas* sp., *Legionella* spp. (Leclerc et al., 2002; Legnani et al., 1999).

The extended investigations of the two food companies demonstrated that they met the drinking water standards. The culture - independent techniques used could not distinguish among viable, viable but non - culturable, injured, and dead cells. Still, such techniques were used to identify critical control points in all stages of food production where water was involved, and they were able to give more hints about the possible presence of pathogens that were not detected by traditional culture methods.

The importance of distinguishing viable from dead bacteria is gaining importance. Many studies have been done about using messenger ribonucleic acid (mRNA) as bacterial viability marker, assuming that its half - life is very short. But some studies demonstrated an unexpected durability of mRNA in dead microbial microorganisms, indicating that the mRNA decay depends on factors such as the mRNA target, target species or inactivating methods (Kobayashi et al., 2009). RNA - based methods are technically rather complicated and take more time than DNA - based methods, limiting its routine application. As already mentioned, DNA - based methods detect total amounts of DNA without distinguishing if it comes from live or dead cells or if it is eDNA, but if they are combined with methods that destroy anyhow the DNA that does not belong to viable cells with intact cell membranes, a closer determination and characterization of the viable cell fraction of the samples could be achieved.

The combination of PMA treatment with qPCR has been used to detect viable cells in the past years in order to limit false - positive PCR results (Hein et al., 2007; Kralik et al., 2010; Nocker et al., 2007a). PMA mainly acts intercalating free DNA and DNA from cells with compromised cell membranes (Nocker et al., 2006). DNase I was used in the present work following a similar idea. The DNA exposed to this enzyme is digested and only the DNA of cells with intact cell membranes will be later detected.

Darzynkiewicz et al. (1992) and Nogva et al. (2000) were pioneers in using DNase I

to digest free DNA or DNA coming from dead cells. The methods that they used were modified in the present work, paying special attention to the inactivation step of DNase I by means of proteinase K. This modification was necessary to satisfy the experimental conditions needed for the investigation of drinking water and drinking water biofilm samples.

Though it has been described that PMA - DNA - based methods can be an effective strategy for the determination of live cells in complex matrices (Lee and Levin, 2009; Nocker et al., 2007b; Rieder et al., 2008), the experiments done in the present work demonstrated that the combination DNase/PK - DNA - based methods is better than the combination PMA - DNA - based methods in the case of detection, identification and characterization of bacterial populations present in drinking water. This can be due to a more homogeneous effect of the DNase I, and to the absence of wash steps in the procedure.

Some important factors should be considered after using PMA and DNase/PK treatments for the analysis of live bacterial populations of drinking water after UV light exposure: (i) bacterial DNA repair (Jungfer et al., 2007), (ii) UV light affects cell viability by inducing DNA damage without directly affecting membrane permeability, (iii) use of PCR - based methods for direct detection of DNA damage and repair during UV disinfection (Süß et al., 2009). In order to use PCR - based methods to distinguish live and dead cells, the natural DNA - repair potential of the bacteria has to be considered. After waiting a certain time, bacteria would have enough time to repair their DNA, and cell membranes of dead cells would have enough time to be no further intact. Therefore, if PMA and DNase/PK treatments are carried out after this time only the live cells with repaired DNA and intact cell membranes would be detected by PCR - based methods. But here it also should be considered that the total procedure time could take several days, precluding this procedure as a monitoring method (Süß et al., 2009).

For a final evaluation of the quantification results it should be considered that the values obtained after qPCR, epifluorescent microscopy, and plating techniques, will not be the same. This statement was recurrently observed in the experiments of the present work where toolboxes were applied to detect and characterize the different physiological stages of bacteria present in the water and drinking water biofilm samples. qPCR provides the number of genomes per volume of water of a specific bacteria, and some bacteria can contain more than one genome per cell depending on their growth rate. Epifluorescence microscopy (e.g. CTC and/or DAPI staining) provides the values of the cells that are able to be counted in the microscopic fields, but aggregates of cells can be present in the sample. Additionally, when CTC is used it should be considered that only bacteria that

present high metabolic activities can be detected (Créach et al., 2003). And as already mentioned, plating techniques provide exclusively information of bacteria that are able to grow in the exposed cultivation conditions.

Surprising amounts of eDNA can be found in biofilms (Flemming et al., 2007; Nielsen et al., 1997). Therefore, the successful blocking of eDNA and DNA coming from dead cells by the DNase/PK - DNA - based method was an important achievement for the investigation of the live bacterial population fraction present in drinking water biofilm samples.

Summarizing, many water bacteria may be difficult to cultivate or may enter the VBNC state and are alive but not culturable at all. Many human pathogens have been described to enter the VBNC state (Oliver, 2005a) and to resuscitate later, becoming potential pathogens if present in drinking water (McKay, 1992). The results from standard water examinations usually dramatically underestimate the number and diversity of microorganisms present in drinking water (Colwell and Grimes, 2000; McKay, 1992). On the counterpart, DNA - based methods are able to determine total DNA in a sample including DNA from live and dead cells and free DNA, overestimating the number and diversity of microorganisms present in drinking water. If these methods are combined with treatments as DNase/PK or PMA, and no inhibitors are present, they are able to determine the viable cell fraction of a sample.

The battery of methods used in the present work were very useful for the examination of the different physiological stages of bacteria in drinking water and drinking water biofilms. But, as Szewzyk et al. (2000) already stated there still are many open questions to be answered: (i) is it really necessary to know which bacterial species are hidden among the high percentage of uncultured bacterial cells in a water sample? (ii) is it enough to determine indicator bacteria and the number of pathogens on selective growth media? An additional question could also be: in case that finally an ideal method is developed to determine viable cells in a sample, would that be enough to determine if a sample has a health risk, or is the presence of eDNA and DNA from dead cells also a menace?

As a consequence of the discovery of VBNC cells, new emerging pathogens, and bacterial adaptations to new environments and stress situations it should be accepted that no drinking water can be guaranteed pathogen - free. Therefore, a risk assessment based on epidemiological and microbiological data should be a key issue for the supply of safe drinking water.

7. Conclusions and Outlook

Culture - independent methods based on the analysis of DNA were optimized and applied for the detection and characterization of bacteria in drinking water and in drinking water biofilms.

The strategy developed in this work was suitable to look for possible water - derived critical control points in the production lines at a German dairy company and at a Spanish dry cured ham company. Demonstrating the applicability of molecular biology techniques for food industry water surveillance.

The different physiological stages of bacteria present in drinking water and in drinking water biofilms were successfully determined by the molecular biological methods optimized in this work. Especially the DNase/PK treatment protocol was capable to quantify and analyze exclusively DNA coming from live cells.

Future developments could be focused on the optimization of biomass concentration of samples, as e.g. reusable hollow - fiber ultrafilters (Morales-Morales et al., 2003; Rajal et al., 2007) in order to obtain higher sensitivities of the detection systems.

In the first part of the present work, the use of culture - independent methods, in special qPCR, demonstrated the ability of these methods to detect low amounts of specific bacteria in a sample. In the future, the combination of DNase/PK treatment - qPCR should also be tested with low concentrations of pathogens to determine if this method can be used for the determination of low amounts of bacteria in drinking water.

Technical improvements are needed to try to overcome the biases of the molecular biological techniques and to develop optimal conditions of analysis capable of providing complete and reliable information on microbial communities.

Consequently, a combination of the toolbox used to determine the cells at different physiological stages (specially the viable cell fraction) and the already described strategy developed for the determination of possible water - derived critical control points at food companies could be applied to evaluate the water quality at food companies and

also at other companies where water could be a hygienic risk (i.e. biomedical industry, pharmaceutic industry, cosmetic industry, waterworks, etc.).

The application of this combination would also be interesting for the specific detection of pathogens from clinical samples, but it should be further validated.

A. Questionnaire

Questionnaire for Food Companies Concerning Drinking Water Distribution

Origin and Processing of Drinking Water

1. Which waterworks supply you with drinking water?
2. Which types of raw water are used by the waterworks and how is the raw water processed?
3. Which disinfection measures are taken in drinking water processing?

Drinking Water Distribution

4. Does your company carry out a secondary treatment of the drinking water (e.g. additional disinfection, softening, etc.)?
5. Which materials were used for the drinking water pipelines and how old are the pipelines and connections?
6. Did you use several materials and in which order?
7. Did you renew your drinking water pipelines while using the building?
8. Did you detect any damage of the water pipelines in the last years (e.g. pipe ruptures, corrosion, etc.)?
9. Is the microbiological control of your drinking water carried out internally or externally? At which intervals?
10. Do you have a current version of a plan of all drinking water pipelines and flow

directions of the drinking water in your production buildings?

11. What do you think is critical to drinking water hygiene?

Additional Aspects of Drinking Water Distribution

12. Which hose materials are possibly connected to the water pipelines and used for cleaning or food processing?
13. Are you able to provide information on your water consumption and allocate the amounts of water consumed to the production lines?
14. Do you also use warm water in production?
15. How is the warm water prepared and fed into the production line?
16. Do you spray or atomize water during production?
17. Is an emulsion prepared during production?

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