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**Development and evaluation of a PCR protocol  
to detect *Escherichia coli*  
in drinking water samples**

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*für Mama*

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## Abbreviations

<i>16Sr DNA</i>	gene sequence on the DNA coding for the 16S ribosomal RNA
°fH	French degree of total hardness ( $1^{\circ}\text{fH} = 10 \text{ mmol} (\text{Ca}^{2+} + \text{Mg}^{2+})/\text{L}$ )
bp	base pair
cfu	colony forming units
Ct	cycle number of reaching threshold value
DMPC	dimethylpyrocarbonate
EHEC	enterohaemorrhagic Escherichia coli
EMBL	Europe's primary nucleotide sequence database (at the European Molecular Biology Laboratory)
FN	false negatives
FP	false positives
HACCP	hazard analysis and critical control points
ISO	International Organization for Standardization
kb	= 1000 bp
LB	Luria Bertani medium
<i>mip</i>	gene encoding for the macrophage invasive potentiator protein
MPN	most probable number
NTC	no-template-control
OD <sub>546</sub>	optical density at 546 nm wave length
PBS	phosphate buffered saline
PC agar	plate count agar
PCR	polymerase chain reaction
Rn	fluorescence signal monitored by ABI Prism 7000 Sequence detection (Applied Biosystems)
RT-PCR	real-time PCR
sg	sero-group
TBX	tryptone bile agar TBX with the chromogenic substrate 5-bromine-4-chlorine-3-indolyl-β-D-glucuronide
TET	Tris-EDTA-Triton-X-100 buffer
TN	true negatives
TP	true positives
TSA	tryptic soy agar
TSB	tryptic soy broth
<i>uidA</i>	gene encoding for β-D-glucuronidase
UV	ultra violet light radiation
VBNC	viable but not culturable

## Summary

For decades, drinking water has been monitored for its hygienic quality by methods that detect the growth of bacteria. Such culture methods were standardised. Their routine use over extended times proved to effectively advise against waterborne threats to human health. However, the culture methods need a certain time of incubation for the target bacteria to multiply. It takes at least 24 h until results are available. With the development of molecular methods, their possible application in drinking water quality analysis were discussed and investigated. Time saving was the main advantage attributed to these modern techniques. The polymerase chain reaction (PCR) was seen as one of the most promising techniques. It apparently had the potential to shorten the analysis time while maintaining the same low detection limit as the standardised culture method.

In this thesis a published PCR protocol was explored for its applicability to detect the faecal indicator bacterium *Escherichia coli* in drinking water samples. The PCR protocol was thought to improve handling, speed, cost effectiveness and to eventually replace routinely used standard culture methods. To avoid time-consuming procedures, a pre-cultivation step before the PCR was omitted. Instead, a DNase digestion step was introduced into the protocol to avoid false positive results caused by free DNA of dead bacteria. Nested PCR with two subsequent rounds allowed achieving a low detection limit. A sequence of the *E.coli uidA* gene served as specific target. This gene encodes for the enzyme  $\beta$ -D-glucuronidase. The activity of the  $\beta$ -D-glucuronidase is also used for identification in the standard culture method. Water samples were analysed in parallel with the two methods. The culture method served as reference for the evaluation of the performance of the PCR protocol. Water samples from the environment were collected during routine drinking water controls. Their origin ranged from raw source water before treatment to finished drinking water. The variety of origins gave an idea on the application range of the PCR protocol and its robustness. With defined, artificially spiked samples specific questions were addressed, as for example, the detection of non-culturable *E. coli* cells present in water samples.

The detection limit with the PCR protocol was almost as low as with the culture method. As compared to the culture method the performance values “sensitivity” and “specificity” for the PCR protocol were not satisfactory. The sensitivity suffered due to false negative results probably mostly caused by inhibitory substances in environmental samples. The specificity

decreased because of false positive results mostly caused by non-culturable *E. coli* cells that were still detected with the PCR protocol. Such interference could be shown to occur in an experiment where drinking water was used, spiked with *E. coli* cells. This suspension was incubated at low temperature up to several weeks.

The PCR protocol including sample preparation was adapted to analyse also the Gram-negative *Legionella*. The detection of *Legionella spp.* and *L. pneumophila*, was also possible with the PCR protocol and primers that targeted the 16S rDNA and the *mip* gene.

Quantification of the initial *E. coli* DNA concentration in the sample was possible by real-time PCR. Requirements for the quantification are (i) no pre-cultivation step during sample preparation and (ii) no substances in the sample that could inhibit the PCR. The cycle number in the first PCR round was adapted for more precise quantification. The DNase digestion step in course of the sample preparation of the PCR protocol was also developed in this work. It was found to be best working with a buffer (pH 7) that contained 5 mM of Mg<sup>2+</sup>. However, the ionic strength was found to be too low for fast grown *E. coli* cells that were transferred directly from a rich batch culture medium to drinking water and subsequently to the DNase incubation buffer. These cells often led to false negative PCR results probably because the cells were damaged by the osmotic shock in such a way that the DNase could penetrate the cells and digest their DNA. Thus, the following PCR could not completely amplify the target sequence. PCR inhibiting substances in environmental samples could partly be eliminated by filtration of the water samples. The dissolved components passing through the filter did not – or just barely – influence the subsequent reactions. Particles such as clays trapped on the filter together with bacteria were assumed to cause false negative results in the PCR protocol.

Based on the work done for this thesis, the PCR protocol evaluated cannot replace satisfactorily the standard culture method. Although the PCR protocol was designed to be simple in handling and cheap, it remained by far more expensive and laborious as compared to the culture method. But more so, the results obtained with the two methods in parallel differed much too often. For routine monitoring of drinking water productions and distribution systems, cheap online methods are more adequate. They comprise the measurement of turbidity, conductivity, flow-rate etc. Coupling them to alarm-systems which indicate abnormalities allows quick reactions to prevent catastrophes. Methods based on the specific detection of DNA and/or RNA, have their advantage for special requirements such as typing or tracing contami-

nation sources. Molecular methods in drinking water analysis have up to now mostly been used in case studies or specific research projects but not in routine quality control.

## **Zusammenfassung**

Seit Jahrzehnten wird Trinkwasser mit Hilfe von Kultivierungsmethoden überwacht, mit welchen sich vermehrende Bakterien detektiert werden. Diese Kultivierungsmethoden wurden standardisiert. Ihre Anwendung hat sich über einen langen Zeitraum als erfolgreich erwiesen, die Gefahr von Wasser-vermittelten Krankheiten anzuzeigen. Die Kultivierungsmethoden brauchen jedoch relativ lange Inkubationszeiten, mindestens 24 Stunden bis ein Resultat verfügbar wird. Im Zuge der Entwicklung von molekularen Methoden wurde vermutet, dass es hierfür neue Einsatzmöglichkeiten in der Qualitätsanalyse von Trinkwasser geben könnte. Insbesondere der Vorteil der schnellen Analyse wurde diesen modernen Methoden zugeschrieben. Die Polymerase-Kettenreaktion (PCR) ist eine viel diskutierte und viel versprechende Technik. Sie scheint das Potential zu haben, die Analysezeit zu verkürzen und trotzdem die gleich tiefe Nachweisgrenze zu haben wie die standardisierte Kultivierungsmethode.

In dieser Dissertationsarbeit wurde ein publiziertes Protokoll übernommen und dessen Möglichkeit untersucht, in Trinkwasserproben das Fäkal-Indikator-Bakterium *Escherichia coli* zu detektieren. Das PCR-Protokoll sollte dahingehend optimiert werden, dass es einfach in der Anwendung, schnell und kostengünstig wird, und es sollte schliesslich die Standard-Kultivierungsmethode ersetzen können. Um die kurze Analysezeit beizubehalten, wurde auf einen Vorkultivierungsschritt verzichtet. Stattdessen wurde ein DNase-Verdau-Schritt eingeführt, um falsch-positive Resultate aufgrund freier DNA von toten Bakterien zu vermeiden. Der eigentliche Nachweis wurde mit einer „nested PCR“, mit zwei aufeinander folgenden Amplifizierungs-Runden durchgeführt. So konnte eine tiefe Nachweisgrenze erreicht werden. Eine Sequenz des *uidA*-Gens konnte zur spezifischen Detektion von *E. coli* eingesetzt werden. Das *uidA*-Gen kodiert für die  $\beta$ -D-Glucuronidase, das Enzym, auf dessen Nachweis die Identifikation von *E. coli* in der Standard-Kultivierungs-Methode basiert. Mit beiden Methoden wurden parallel jeweils Wasserproben analysiert, und die Kultivierungsmethode wurde als Referenz festgelegt, um das PCR-Protokoll zu evaluieren. Umweltproben wurden während routinemässigen Trinkwasser-Kontrollen gesammelt und reichten von Rohwasser aus Quellen bis zum aufbereiteten Trinkwasser. Die Vielfalt an Umweltproben gab Auskunft über den möglichen Einsatzbereich der PCR-Methode und über ihre Robustheit. Mit definierten, künst-

lich kontaminierten Wasserproben konnten spezielle Fragen untersucht werden. Beispielsweise interessierte das Resultat der PCR-Methode im Falle der Anwesenheit nicht-kultivierbarer *E. coli* Zellen.

Die Bestimmungsgrenze des PCR-Protokolls war fast so tief wie mit der Kultivierungsmethode. Verglichen mit der Kultivierungsmethode als Referenz waren Leistungsindizes der Sensitivität und Spezifität für das PCR-Protokoll nicht zufrieden stellend. Die Sensitivität wurde geschwächt durch falsch negative Resultate, verursacht durch hemmende Substanzen in den Umweltproben. Die Spezifität war tief wegen falsch positiver Resultate, hervorgerufen durch nicht kultivierbare *E. coli* Zellen in der Wasserprobe, die mit der PCR trotzdem detektiert wurden. Letzteres Phänomen wurde bestätigt in einem Experiment, bei dem Trinkwasser mit *E. coli* Zellen versetzt und über längere Zeit bei tiefer Temperatur aufbewahrt wurde.

Die PCR-Methode mit ihrer Probenaufbereitung konnte auch auf andere Gram-negative Bakterien übertragen werden, die im Wasser von Interesse sind, wie die Gattung *Legionella*. Dazu mussten neue Primer hergestellt werden, die auf Sequenzen der 16S rDNA und des *mip*-Gens passen. Die Detektion von *Legionella spp.* bzw. *L. pneumophila* war so möglich mit dem angepassten PCR-Protokoll.

Die anfängliche *E. coli*-DNA-Konzentration in der Wasserprobe konnte mit Real-Time-PCR quantifiziert werden. Dazu waren folgende Voraussetzungen wichtig: (i) die Probenaufbereitung durfte keinen Vorkultivierungsschritt enthalten und (ii) es durften keine PCR-hemmende Substanzen in der Wasserprobe vorhanden sein. Um die Quantifizierung zu verbessern müsste die Zyklen-Zahl in der ersten Amplifizierungsrunde noch angepasst werden. Der DNase-Verdau-Schritt im Verlauf der Probenaufbereitung im PCR-Protokoll wurde neu entwickelt. Am besten bewährte sich ein Puffer (pH 7) mit einer  $Mg^{2+}$ -Konzentration von 5 mM. Für schnell wachsende *E. coli* Zellen, die direkt von einem reichen Nährstoffmedium in Trinkwasser und danach in den DNase-Inkubationspuffer transferiert wurden, erwies sich die Ionenstärke jedoch als zu gering. Diese Zellen ergaben oft ein falsch negatives PCR-Resultat. Möglich ist, dass die Zellen durch den osmotischen Schock beschädigt wurden, so dass die DNase die zell-eigene DNA verdauen konnte. Die PCR konnte danach die Zielsequenz nicht mehr vollständig amplifizieren. Das Problem der hemmenden Substanzen in Umweltwasserproben konnte zum Teil gelöst werden, indem die Probe filtriert wurde. Gelöste Substanzen wurden durch den Filter gewaschen und beeinflussten die folgenden Reaktionen

entweder nicht mehr, oder nur noch schwach. Vermutlich verursachten Ton- und mit organischem Material beladene Partikel, die zusammen mit den Bakterien auf dem Filter zurückgehalten wurden u.U. falsch negative Resultate.

Das hier entwickelte und evaluierte PCR-Protokoll kann nicht als geeignet betrachtet werden, die standardisierte Kulturmethode zu ersetzen. Die Resultate der beiden Methoden, die von den Parallel-Untersuchungen stammten, stimmten zu wenig überein. Obwohl die PCR-Methode als einfach in der Handhabung und als möglichst kostengünstig entwickelt wurde, ist sie immer noch wesentlich teurer und arbeitsaufwändiger. Für die stichprobenmässige Überwachung und die Bestätigung der einwandfreien Funktionstüchtigkeit der Wasseraufbereitung und der Verteilung sind kostengünstige Methoden erwünscht. Parameter der online Überwachung (Trübung, Leitfähigkeit, Durchflussmengen, etc.), die an Alarmsysteme gekoppelt sind, können schneller Anomalien feststellen, Reaktionen einleiten und somit Katastrophen vorbeugen. Methoden, die auf der spezifischen Detektion von Sequenzen der DNA und/oder RNA beruhen, haben ihre Vorteile bei speziellen Fragestellungen, wie bei der Typisierung („Fingerprint“) und der eindeutigen Zuordnung einer Kontaminationsquelle. In der Praxis der Trinkwasserkontrolle werden bislang molekulare Methoden nur in Fallstudien oder bei speziellen Forschungsprojekten angewandt.

## 1. General introduction

### 1.1 History of drinking water purification technology

An abundant freshwater supply is a basic necessity for the development of human civilization. Historically, borders of rivers and lakes were favourite places to settle and to build cities. The presence of large fresh water resources not only provided enough water for drinking and cleaning, but also contributed to higher mobility. In many civilisations rivers and streams play the role of lifeline, along which prosperous agriculture, trade and life can freely develop.

Historical documents lead us to assume that in ancient times the main subject of water technology was to develop remarkably complex hydraulic systems for water distribution. Water was probably only marginally treated to remove components hazardous to human health. The Greek physician Hippocrates (460-377 BC) stated that water contributes much to health. He focused more on selecting the healthiest water source, rather than purifying less desirable sources.

In the 1<sup>st</sup> to 3<sup>rd</sup> century AD, the Romans were responsible for the first extensive aqueduct system in the area of present day Switzerland. They piped pristine water from far away sources to water supplies in settlements. In Augusta Raurica (now: Augst, Switzerland) the city's drinking water distribution system was under pressure and consisted of tubes made from lead, wood and clay. Generally no major treatment was provided (Illi, 2003), other than the incidental mild disinfection effect of sunlight on water in open aqueducts.

As in other scientific areas, little progress was made in the Middle Ages toward an understanding of water treatment and its importance to public health. Excavations let conclude that some convents continued to apply the Roman skills of using tubes to distribute drinking water. However, large part of the population in the area of today's Switzerland was drawing and carrying drinking water from wells and fountains.

In the 17th century, the British philosopher and scientist Sir Francis Bacon described thousands of experiments on water purification methods, including percolation, filtration, boiling, distillation, and coagulation. In 1684, the Dutch naturalist Antony van Leeuwenhoek published descriptions and sketches of "little living animalcules", common forms of bacteria viewed with a simple microscope that he had self constructed. It took the scientific commu-

nity another 200 years to make the connection between these "animalcules", treatment and purity of water, and public health.

During the last 4 decades of the 19th century, many cities in Switzerland built central water distribution systems with pipes under pressure. Planning and construction only considered the water distribution to households. The fear of fire was the driving force behind the distribution system. Not much attention was paid yet to water treatment as a means of producing hygienically safe drinking water. At first there was no or little awareness of the danger of water contaminated by microbes. Clear and odourless water was considered healthy for consumption. For instance in Zurich and Lausanne untreated lake water was pumped into the distribution system and delivered to numerous households without concern for its purity. This facilitated outbreaks of waterborne diseases within entire cities (Illi, 2003).

In the 1870's, Dr. Robert Koch demonstrated that microorganisms existing in water supplies are able to cause diseases (Koch, 1893). From this time on, developments in water treatment rapidly increased. Soon it was recognised that filtration is able to remove undesirable particles and deadly bacteria, as those communities that utilized it, had fewer outbreaks of typhoid. Around 1910, the detection of the use of chlorine as a cheap and effective disinfecting agent further decreased the number of waterborne outbreaks (Medema *et al.*, 2003; Madigan *et al.*, 2003).

## 1.2 Indicator organisms

While the connection between faecal contamination of drinking water and diseases like cholera was recognised, it remained a very difficult task to analyse the occurrence of these dangerous pathogens directly in drinking water. The concentration of the pathogens was very low and the isolation method was complicated. The idea of indicator organisms was proximate.

In 1886, Theodor Escherich, a pioneer paediatrician in Munich, described the abundant occurrence of the bacterium „Bacterium coli“ (*Escherichia coli*, *E. coli*) in high numbers in intestines of children (Escherich, 1886). These findings were confirmed and extended to generally all intestines of warm blooded animals. Numerous studies were published on the survival and occurrence of the “Bacterium coli” in the environment (cited in Sulzer, 1913). In the early 1910, the principle of “Bacterium coli” as indicator organism for faecally contaminated drinking water was already widely discussed. In France, England and the USA the detection of the

“Bacterium coli” was one of the main tasks in hygienic assessment of drinking water. In Germany, opinions still were divided: Some researchers doubted the importance of the analytical finding of “Bacterium coli” in drinking water (Sulzer, 1913).

The varieties of microbial health threats as well as low concentration of specific pathogens make it difficult and expensive to directly monitor for possible pathogens in drinking water. Therefore, the concept to use methods indicating microbial contamination of faecal origin is a feasible and good solution to alert against health risks. Criteria for a good indicator parameter were recently established as follows (Medema *et al.*, 2003):

- The indicator should be absent in unpolluted water and present when the source of pathogenic microorganisms of concern is present.
- The indicator should not multiply in the environment.
- The indicator should be present in greater numbers than the pathogenic microorganisms.
- The indicator should respond to natural environmental conditions and water treatment processes in a manner similar to the pathogens of concern.
- The indicator should be easy to isolate, to identify, and to enumerate.
- The test should be inexpensive thereby permitting numerous samples to be taken.
- The indicator should not be pathogenic microorganism (to minimise the health risk to analysts).

Medema *et al.* (2003) also explain the differentiation between an “index” that describes the charge of a water source with faecal contamination and an “indicator” that should estimate a treatment efficiency for a certain group of microbial pathogens. However, as technical drinking water treatment is not a central topic in this study, it is considered, in order to simplify, to only use the “old” term “indicator”. It designates microbial parameters that indicate faecal contamination in water samples.

*E. coli* is the classical and most widely used indicator organism that fulfils the criteria listed above. It indicates risk of infection by pathogenic Gram-negative bacteria of intestinal origin such as *Salmonella*, *Vibrio cholerae* and pathogenic *E. coli* strains. In the last quarter of the

20<sup>th</sup> century, other indicator organisms were proposed. Enterococci have been studied as Gram-positive analogy to *E. coli* for faecal contamination. Other indicators are used for special investigation, such as treatment efficiency: *Clostridium perfringens* or sulphite-reducing clostridia are spore-forming bacteria that are in the state as spores much more resistant to disinfecting agents than *E. coli* and Enterococci. *C. perfringens* occurs commonly in human and animal faeces. In the environment, in fresh water and sediments, spores of clostridia persist over long periods of time. This behaviour is similar to the persistence of the oocysts of pathogenic *Cryptosporidium*. *C. perfringens* was therefore proposed as indicator organism for *Cryptosporidium* oocysts (Payment *et al.*, 1993). However, between the occurrences of the protozoan pathogens and presence *C. perfringens* no clear correlation was found in surface water samples (Hörman *et al.*, 2004). The parameter is recommended to apply on a voluntarily basis, to gain information for treatment efficiency, if the raw water consists of, or is strongly influenced by surface water (Standing Committee of Analysts, 2002).

F-specific RNA bacteriophages and somatic coliphages were investigated for their potential to indicate faecal pollution as well as transportation and treatment fates of possibly present human viruses. For their identification, standard protocols are available (ISO 10705-1, 1995; ISO 10705-4, 2001; ISO 10705-2, 2003). Bacteriophages were used as tracers to indicate natural or artificial filtration processes (Payment *et al.*, 1993; Auckenthaler *et al.*, 2002). However, there are reports (reviewed in Leclerc *et al.*, (2000)) that indicate low specificity of F-specific bacteriophages to *E. coli*. Only 3% of humans also have F-specific bacteriophages associated with their *E. coli*, which causes low probability of occurrence of this indicator in case of faecal contamination. In case of somatic coliphages, reliable correlation with the occurrence of enteric viruses proved to be low (Leclerc *et al.*, 2000; Harwood *et al.*, 2005).

### 1.3 Emerging waterborne infectious diseases and direct detection of pathogens

Emerging waterborne infectious diseases can be defined as those water related infections waterborne that have recently appeared, or have existed but are increasing in incidence or geographic range. Factors causing the emerging waterborne infections comprise natural microbial evolution (in case of pathogenic *E. coli* strains), man-made ecological changes (e.g. international travel and trade, demographic changes and new technologies of water processing) and last but not least improved diagnostic technologies that allow identifying a causative agent of disease.

The direct analysis of pathogens in drinking water is laborious and expensive. Microbial agents that might cause infections in humans, generally occur in very low concentrations and represent rare events. It would be helpful to obtain analysis data over extended time-points for routine quality control or treatment process monitoring. Cost effective and fast tests for indicator organisms are more useful in these cases. During normal operation of the water-supply systems, it is sufficient to verify, if faecal contamination is detectable or not. It is not necessary to know exactly which pathogen hazard might be present (or absent in the normal case). Special investigations, epidemiological studies, and emergency cases of a waterborne disease outbreak require the direct and specific detection of pathogens. Below some possible pathogens that could be transmitted along the path of the drinking water route are selected and cases are described, which imply the direct detection of specific pathogens. Some peculiarities of the detection procedure are included.

a) *Salmonella* and *Campylobacter*

One of the most frequent food-borne pathogens are *Salmonella* bacteria, which cause symptoms from mild diarrhoea to severe typhoid fever depending on strain specificities. Generally, all *Salmonella* species are potentially pathogenic. The hosts of *Salmonella* are intestines of humans and domestic animals such as swine, poultry, and cattle. Also wild animals, birds, rodents, and even cold-blooded reptilians can be reservoirs of salmonellae. The gram negative, facultative anaerobe rods are closely related to the genus *Escherichia*. Characteristic physiological properties of *Salmonella* are their ability to produce H<sub>2</sub>S on triple-sugar-iron-Agar, and they are usually urease negative. The detection and isolation of *Salmonella* from water samples normally comprises a non-selective pre-enrichment step, followed by a more selective enrichment. Subsequently the liquid culture is plated onto selective plates, incubated and the colonies further analysed using diagnostic tests for the special physiological properties of *Salmonella*. For differentiation of *Salmonella* from other closely related enteric bacteria, miniaturized test arrays that are interpreted with the aid of computer analysis are often applied (Madigan *et al.*, 2003).

*Campylobacter* is another Gram-negative intestinal bacterium that is supposed to have similar transmission routes in drinking water like *Salmonella*. Poultry and pork are animal hosts of concern. Probably *Campylobacter* are more frequently transmitted with contaminated meet

than with polluted drinking water. *Campylobacter* are microaerophiles and difficult to isolate and cultivate. Hence, their description and recognition as agent of gastrointestinal disease was only established in 1971/72 (Percival *et al.*, 2004). The common and standardised isolation procedure from drinking water comprises concentration by filtration and pre-enrichment in Preston broth that contains lysed blood, trimethoprim, rifampicin, polymyxin B and cycloheximid. The enriched sample is plated onto agar media according to Karmali (Ettel *et al.*, 2000). *Campylobacter* rods show characteristic features under microscopic examination: they are twisted and motile. Suspected colonies can be further analysed with commercially available, miniaturised arrays of physiological tests, analogous to the identification of *Salmonella*. Swiss legislation (*ordinance of hygiene* (Hygieneverordnung (HyV), 1995)) comprehends a value of limit for *Salmonella* spp. as well as for *Campylobacter* spp.: these bacteria may "not be detected in 5 L of drinking water". If the values are surpassed (i.e. *Salmonella* is detected) the water has to be declared as hazardous to health.

b) *Pathogenic E. coli strains*

The enterohaemorrhagic *E. coli* (EHEC) can cause severe diarrhoea. EHEC can be transmitted by contaminated water in cases of bad hygienic conditions, which might also occur in Europe. In a case study in Switzerland EHEC was found in water after heavy rain fall in correlation with high concentration of the faecal indicator *E. coli* (Auckenthaler *et al.*, 2002).

Strain O157:H7 can be differentiated by using culture methods. For the identification of other EHEC strains the verotoxin genes VT1 and VT2 have to be analysed with PCR (Böttger *et al.*, 2005).

c) *Legionella*

The Gram negative bacterium *Legionella* is the agent of Legionellosis. The symptoms range from mild fever ("Pontiac" fever) to severe pneumonia (Legionnaires' disease). The infection does not occur via the faecal-oral route as with other waterborne pathogens but by inhalation of water-droplets in aerosols. *Legionella* are present in many waters, probably also in clean drinking water, but usually at very low concentrations. However, *Legionella* survive and multiply in stagnant water of temperatures between 20 and 45°C. In technical installations like warm water systems, cooling towers and thermal bathing water, *Legionella* can be found quite

frequently. Having evolved to parasitic bacteria with amoebal and ciliated protozoa as hosts, legionellae are also able to infect human macrophages. *Legionella pneumophila* serogroup 1 is considered the most important species to cause Legionnaires disease. Immuno-compromised people face a much higher risk to acquire infections of *Legionella* (Hoffman *et al.*, 1999). Legionnaires' diseases have been registered as single cases or outbreaks. The world's largest outbreak was recently, in July 2001 in Murcia, Spain where in a short time span around 450 confirmed cases were recorded (García-Fulgueiras *et al.*, 2003).

*Legionella* is considered to bear risk for infection to humans, if it occurs in technical water systems in high concentration. Therefore for risk assessment purposes, it suffices to analyse smaller volumes of water from 1 ml to 1 L to obtain information on the presence of *Legionella*. In this case, it is not imperative to replace the direct detection of *Legionella* by an indicator organism. A standardised detection and isolation protocol of *Legionella* from water samples is available (ISO 11731, 1998; ISO 1173-2, 2004). The detection method by cultivation is described in chapter 4 of this thesis.

#### d) Cryptosporidium

The Milwaukee outbreak of cryptosporidiosis in 1993 is the largest documented in the industrialised world. The protozoan pathogen *Cryptosporidium parvum* became famous, not only among microbiologists and physicians but also among politicians. On or about April 5, 1993 remarkably many workers in the city of Milwaukee (Wisconsin, USA) called in sick due to gastrointestinal illness which initiated outbreak investigations. Analysed stool samples from patients suffering from diarrhoea strongly indicated that the major cause of the outbreak was *Cryptosporidium parvum*. In the stool samples, other enteric pathogens were not measured in unusually increased amounts during this investigation period. *C. parvum* was also found in ice that was produced from the city's drinking water before and during the outbreak period. Two drinking water treatment plants produced the city's drinking water from water of Lake Michigan. Examination of the drinking water plants' records on the quality of untreated water (intake) and treated water (supplied to customers) revealed an increase in the turbidity of treated water from the southern plant. These findings pointed to the water supply as the likely source of infection. (Mac Kenzie *et al.*, 1994).

Waterborne cryptosporidiosis has received increased attention and publicity since the first documented *Cryptosporidium* outbreak in 1985 in the USA. Standard methods for the detection of *Cryptosporidium* have been published by the Environmental Protection Agency, USA (U. S. EPA Method 1622, 2001) and by the Drinking Water Inspectorate, UK (Drinking Water Inspectorate SI No. 3184, 2000). Recent method development intend to include information on the infectivity of the *Cryptosporida* oocysts (Wiedenmann *et al.*, 1996; Slifko *et al.*, 1999).

#### 1.4 Classical methods based on culture versus molecular methods

Culture methods identify the bacteria based on their phenotypic characteristics. More or less unspecific groups of microorganisms like “heterotrophic plate count” can be defined as parameter. To identify specific groups or species, identification tests are performed after the cultivation on agar plates or liquid media.

Tests based on bacterial growth on agar plates or in liquid media, are relatively cheap and simple to perform. By counting the colonies or estimating the “most probable number” (MPN) of parallel liquid cultures in dilution series, the “colony forming units” (cfu) can be enumerated. If the composition of the media and the temperature of the incubators can be controlled, the methods are robust and the results comparable in inter-laboratory trials. Due to a widespread and standardised application of the methods, a high degree of reliability and acceptability has been achieved. With the development of growth media with supplemented reagents for identification of bacterial groups or species, the handling of the methods is simplified and the analysis time shortened. A good example is the TBX (tryptone bile 5-bromo-4-chloro-3-indolyl-beta-D-glucuronide) medium for the detection of *E. coli* (ISO 16649-2, 2001). This makes the identification of *E. coli* in a water sample is possible in 20 hours.

One of the main drawbacks of the classical culture methods is the long time it takes until the bacteria have divided and grown to a visible colony on agar plates. This asks for an extended period before results are available. Such an initial time investment is especially disadvantageous in case of slow growing bacteria such as *Legionella* which needs up to 10 days of incubation to develop visible colonies on agar plates. The search for faster and more sophisticated methods was fuelled by the fact that valuable time passes before a clear result is available.

With the development of new molecular methods in the last decades, attempts have been made to establish alternative methods to the culture methods for routine analysis of bacteria in environmental samples. Molecular methods have the promising potential to be faster than culture methods. They also can be automated. The identification of the microorganisms is performed on the genotypic level which can be a disadvantage, if a certain physiological state such as viable cells should be detected. A vast palette of analytical principles of molecular methods provides possibilities for differentiated applications, also in combination with culture methods.

Molecular methods base on bio-molecule reactions in miniaturised systems. For quantitative examination, this means that the original sample must either contain a high concentration of target organisms (or target molecules) or the sample has to be highly concentrated. High concentration bears at least two problems: recovery rates normally decrease and interfering particles and agents are also concentrated.

#### 1.5 PCR – a promising technique. Experience and known problems with environmental samples

The Polymerase Chain Reaction (PCR) has been the most promising technique among molecular methods in reaching about the same low detection limit as culture methods. However, it can be performed much faster. The *in-vitro*-biochemical reaction is driven by the heat stable polymerase enzyme (Taq-polymerase) isolated from the thermophile bacterium *Thermus aquaticus* (Saiki *et al.*, 1988). In a thermocycler that varies the temperature in repeating cycles, the three subsequent steps of the reaction are continuously repeated (denaturation of double stranded DNA, annealing of the primer and extension of the remaining single stranded DNA to the double strand with help of the Taq-polymerase). The primer directed amplification of a DNA sequence of a specific (short) length can be visualised by staining the DNA and e. g., loading the sample on an agarose gel and performing gel electrophoresis.

Several applications of PCR in water analysis for faecal bacteria have been published (Bej, Mahbubani *et al.*, 1991; Fricker *et al.*, 1994; Juck *et al.*, 1996; Iqbal *et al.*, 1997). These protocols do not discriminate between living or dead organisms or even free DNA in the sample. These protocols deliver a presence/absence answer of the DNA of the target organism in the sample. Some authors proposed a pre-enrichment step in culture medium to detect only the

culturable organisms. However, this procedure prolongs the whole protocol for several hours and would hamper developments towards quantification of the initial concentration of the target organisms (Tsen *et al.*, 1998; Campbell *et al.*, 2001; Theron *et al.*, 2001; Frahm *et al.*, 2003, Heijnen *et al.* 2006). To perform a quantitative analysis with these protocols, several parallel samples of dilution steps would have to be performed and the results interpreted with the MPN concept. This would demand a large amount of samples to be analysed with PCR.

In recent studies possible target DNA from “dead” bacteria is eliminated by using of ethidium monoazide (EMA). This DNA intercalating agent was developed as live-dead staining dye. The structure of EMA allows it to penetrate only in cells with damaged cell walls and membranes. EMA covalently binds to DNA. Living cells with intact cell walls can protect their DNA from this dye. With stressed cells that were stained with EMA, the yield of amplified DNA in a subsequent PCR was much decreased (Nocker *et al.*, 2006). With this approach “dead” cells are defined as cells with damaged membranes in a way that EMA can penetrate and intercalate the DNA. This definition is not necessarily equal to the definition of culture methods, where living cells are able to multiply on a defined growth medium. Some cells with damaged walls might recover and multiply or cells with intact walls might not be able to grow on the assigned medium.

Another important and often neglected issue is the concentration of the sample. The reagents used for PCR, especially the enzyme Taq-polymerase are expensive. Therefore, only small reaction volumes can be used. As indicator bacteria are present in very low concentrations in drinking water (and pathogens might be even rarer), the samples have to be concentrated. Concentration protocols bear losses of analytes and, with the concentration of the sample, not only the target for the PCR assay will be concentrated but probably as well substances that potentially inhibit the PCR (Wilson, 1997).

Using direct PCR to detect organisms in environments, i.e. without pre-cultivation, no information is obtained on the physiological state of the organism. It is not known, if the amplified DNA belonged to a living or dead bacterium or if it was present in the sample in free form. Free DNA was shown to persist in environments like seawater (Dupray *et al.*, 1997) and seawater sediment (Deere *et al.*, 1996). In soil, extra-cellular DNA persists for up to several months or years. Clay-bound DNA is physically protected against digestion of nucleases (Demanèche *et al.*, 2001) but could perhaps respond to PCR.

There is still laborious research to be done to solve the already experienced and known difficulties of the application of PCR to environmental water samples. For routine methods in drinking water analysis a high robustness and security of correctness of the result is an absolute prerequisite.

#### *1.6 Aspects of risk analysis and quality management in drinking water supply*

In Switzerland, consumers are still fortunate to enjoy a generally high quality of drinking water. The geographically advantageous situation provides huge water reservoirs in form of ground water, lakes and glaciers. Many groundwater streams flow very slowly in the area of the "Schweizer Mittelland" in gravel sandy grounds of ancient glacio-fluvial deposits. This natural filtration is very efficient and the groundwater is mostly of excellent quality. Due to well established and strictly followed regulations of water protection, also the surface water in the natural reservoirs is of high quality. The mean composition of Swiss drinking water contains 80% groundwater from which half is spring water and half is pumped actively. Twenty percent is treated surface water (SVGW, 2003). Surface or spring water which may get into contact with sources of faecal contamination goes through one or several steps of disinfection treatment. Clean water from protected natural ground water reservoirs normally does not need further treatment. In these cases increase of microbial burden only occurs during storage (regrowth in reservoirs or scarcely used pipes) or because of breakdowns in the distribution system (e.g. massive loss of pressure, breaks of pipes).

Drinking water deserves a high level of protection. Therefore, in regulations the tolerance values of the microbial indicators after treatment, storage and distribution are strictly low. Drinking water production and distribution are regulated in the ordinance for drinking-, source- and mineral water (Verordnung des EDI über Trink-, Quell- und Mineralwasser, 2005). The microbiological requirements for finished drinking water are regulated in the ordinance of hygiene (Hygieneverordnung (HyV), 1995, Table 1.1), and in the ordinance of substances of content. The limit values of chemical substances are listed in the ordinance on substances and contaminants (Fremd- und Inhaltsstoffverordnung (FIV), 1995).

Table 1.1: Microbial quality requirements for drinking water according to the *ordinance of hygiene* (1995)

Water	Organisms	Colony forming units (cfu) / ml water sample (value of tolerance)
Untreated water at source	Aerobic, mesophilic organisms	100/1
After treatment	Aerobic, mesophilic organisms	20/1
In the distribution system	Aerobic, mesophilic organisms	300/1
drinking water for consumption	<i>E. coli</i>	not detectable/100
	Enterococci	not detectable/100

As every drinking water production and distribution has its own peculiarities, it is highly recommended to implement a tailored quality management for each system (SVGW Regelwerk – W1, 1997) or a "water safety plan" (Medema *et al.*, 2003). The quality management should include a description of the state of the infrastructure, personal and organisation of the enterprise. With the help of the method of Hazard Analysis and Critical Control Points (HACCP), possible hazard at relevant points (the critical control points (CCP)) in drinking water catchments, treatment and distribution should be identified. Attendance and surveillance of the CCPs has to be organised. Results of monitoring parameters, improvements of the system, actions of attendance have to be documented. An external party should supervise the management system.

In regular monitoring, the samples are generally taken from several points - the CCPs of the drinking water treatment and distribution system. Monitoring is also of great importance to gain experience on the qualitative and quantitative aspects of sources, treatments and drinking water system. The responsible person of the water production should know the "normal" or expected values of the parameters routinely analysed. An extraordinary change in the value of a parameter indicates malfunctioning. This asks for immediate measures. The methods to estimate the drinking water's microbiological, chemical and physical quality parameters are given in the Swiss Food Manual (Ettel *et al.*, 2000).

### 1.7 Validation of microbiological methods in drinking water monitoring

To survey the CCPs of drinking water distribution systems, microbiological methods are necessary. Of course it is of great importance that the methods reliably deliver results that lead to correct interpretation. To allow comparisons to samples of other drinking water systems, the methods have to be standardised. Before a method can become a standard method it has to be validated. Validation of a new microbiological method comprises an extended research project including collaboration of several laboratories. For the detailed procedure, appropriate experiments have to be designed and suitable statistical tests applied (Lightfoot *et al.*, 2003).

In difference to chemical validation procedures, with microbiological methods other statistics are considered. The variation of microbial contents in a water sample like 1 L is much greater, especially for methods for quantitative identification of few cells. Whereas in chemical analytics, even with low quantification limits in the ppb range, there are still around  $10^{13}$  single molecules or atoms present in a water body of 1 L. Few incidences in a long range or in our case: few microbiological target organisms in a water body can statistically be described by the Poisson distribution model. Target cells are ideally randomly distributed in a water sample. If there are other effects such as attraction or repulsion of cells additional “overdispersion” occurs. Figure 1.1 show a schematic random distribution of 30 cells in a water body. Sub-samples of one tenth of the volume can contain no or up to 7 particles. This variation is the least possible variation even if a perfect quantitative detection method would be applied (Tillett *et al.*, 1995).

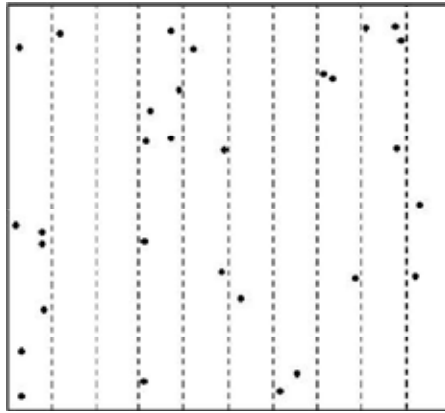


Figure 1.1: Schematic visualisation of the random (or Poisson) distribution of few target organisms in a water body (Tillett *et al.*, 1995).

Overdispersion can occur due to different factors: the properties of the target cells, the sample matrix that contains particles and other non-target organisms, the detection method properties, or the systematic inaccuracy of laboratory personal. Therefore large amounts of samples of different origin have to be tested by different laboratories, in order to gain secure information on the robustness of a method.

For several microbiological parameters, standardised methods exist which are fixed in national or international norms. Most legislation includes the possibility to use alternative methods. This gives greater flexibility to meet developments of novel techniques and knowledge on microorganisms in drinking water. However the use of an alternative method is only permitted if there is proof that the results of the alternative and the standard method lead to the same conclusion (paragraph 4 of the Swiss ordinance of hygiene, 1995). The European Drinking Water Directive contains analogous phrasing (Council Directive 98/83/EC 1998, paragraph 7, number (5) b).

There is a different approach behind the validation of an alternative method in comparison to an existing and valid standard method than behind establishing a new method. The aim is to show that under the same circumstances like the same application range (sample matrices), the same organisms are detected in the same number. The result obtained by the alternative method should lead to the same interpretation as the standard method.

Instructions on validation procedures for alternative methods exist as international standard for microbiological methods for foodstuffs in general (ISO 16140, 2003) and for water samples in particular (ISO 17994, 2004). In validation processes for alternative methods a large amount of samples is required. It is recommended to include samples from different geographical areas as well as to perform parallel analysis in different laboratories. Generally, natural samples are preferred to test the equivalency of the two methods. However, sometimes also spiked samples have to be used if the natural samples contain too few target organisms. In case of spiking, it is recommended to stress the bacteria (e.g. by cooling, starving, or slight disinfection). Stressed bacteria can show different behaviours and may grow at altered initial rates on agar plates. Alternative methods that do not base on cultivation might give other results in case of stressed bacteria.

### 1.8 Scope of the thesis

This work focuses on the applicability of PCR on drinking water monitoring. Hereby, the advantages and disadvantages of the new methods compared to the traditional cultivation methods were investigated and evaluated. The protocols and experiences available from the literature were adapted for routine use. Their strengths and potential for drinking water monitoring have been critically assessed.

The focus is on PCR to detect *E. coli* for the following reasons:

- i. *E. coli* is a worldwide accepted faecal indicator organism and a standardised cultivation method is well established.
- ii. Several publications for the detection of *E. coli* from water samples with PCR are available.
- iii. The use of marker enzyme UidA allowed the specific detection and identification of *E. coli* in drinking water samples for both, the standard method and the PCR protocol. Thus we could focus on the effects of sample preparation, physiological state of the target bacteria and parameters of the PCR protocol and their effects on the outcome of the PCR method.

### 1.9 Thesis outline

Chapter 2 describes a PCR protocol that was developed with the goal to monitor *E. coli* in drinking water samples. The method was applied to environmental samples of the routine drinking water analysis and the results were compared to the standard culture method. The performance of the PCR protocol was evaluated considering the culture method as reference.

In chapter 3, the same PCR protocol was tested in more detail for the special situation of stressed and non-culturable *E. coli* cells in drinking water. For this purpose, defined drinking water microcosms containing starved *E. coli* cells were prepared under laboratory conditions. The intention was to simulate realistic environmental conditions. The PCR based method was discussed in consideration of its potential use for "real" environmental samples. The PCR protocol was considered to be easily transposable to other bacteria in water sample. Chapter 4 describes the possible application of the PCR protocol to detect *Legionella*.

In chapter 5, experiments are summarised that were performed during the process of improvement of the PCR protocol. They provide contributions to a better understanding of the mechanisms of the PCR protocol and indicate draw-backs of the PCR protocol.

With concluding remarks in chapter 6 the experiences obtained in this thesis are critically reflected in the larger context of routine drinking water monitoring.

## 2. Evaluation of a nested-PCR protocol to detect *Escherichia coli* in drinking water

### Abstract

A fast and direct polymerase-chain-reaction (PCR)-based protocol was developed to specifically detect *Escherichia coli* (*E. coli*) in drinking water samples. In our PCR protocol, a DNase digestion step was introduced during the sample preparation to avoid false positive results by free bacterial DNA. Environmental and spiked samples were analysed with the nested-PCR protocol and compared in parallel to a standardised culture method based on membrane filtration and plate count. The culture method was regarded as reference to evaluate the PCR assay. The detection limit of the PCR protocol corresponded to 1-10 colony forming units (cfu), obtained by the culture method. The sensitivity of the PCR assay was higher for samples spiked with *E. coli* cells from a slowly growing chemostat culture compared to the samples containing cells from a fast growing batch culture (exponential phase). Environmental samples for drinking water production were analysed with the two methods in parallel with an agreement of 77%. The two methods disagreed mainly because the PCR assay also detected non-culturable *E. coli* cells. Furthermore, the PCR was found to be susceptible to inhibitory components in the environmental samples.

### Introduction

For several decades culture methods have been used to assess the microbiological, hygienic drinking water quality. These classical methods are widely accepted and internationally standardised, and legislation links to these methods. As they are based on growth of bacterial cells, it takes at least 24 h up to several days until a result is available. In Switzerland the use of alternative methods is accepted, if the equivalence of the two methods can be demonstrated and the interpretation of their results leads to the same conclusion.

The development of molecular methods supported the hope for better and faster alternatives to the culture methods. The polymerase chain reaction (PCR) has been reported as a promising technique concerning speed and detection of low concentrations of faecal indicator bacteria in drinking water. Several PCR-based protocols without pre-cultivation have been described (Atlas *et al.*, 1992, Bej, DiCesare *et al.*, 1991, Bej, Mahbubani *et al.*, 1991, Juck *et al.*, 1996).

However, these protocols do not discriminate between live and dead organisms or free DNA in the sample. Pre-cultivation steps before the PCR have been proposed to assure the detection of only viable cells (Campbell *et al.*, 2001, Frahm *et al.*, 2003, Heijnen *et al.*, 2006, Theron *et al.*, 2001, Waage *et al.*, 1999). However, such an additional step prolongs the analysis procedure for several hours.

The detection of microorganisms in environmental samples by PCR harbours a number of difficulties. Among the most prominent limitations are false positive results due to the presence of free nucleic acids (Dupray *et al.*, 1997) and false negative results by inhibiting substances such as humic compounds (Toze, 1999).

In this study, a PCR protocol for the detection of *Escherichia coli* in drinking water samples was further developed to reduce these problems of PCR. To separate inhibiting substances from the target bacteria, the water sample was concentrated by vacuum filtration of a 100 ml water sample (Juck *et al.*, 1996). The filter, including the collected *E. coli* cells, was subjected to a DNase-digestion step that should eliminate free DNA and avoid false positive results. The experiences obtained with the here described PCR protocol are a valuable contribution to the discussion on the application of molecular technologies for safe drinking water.

## Material and Methods

### *Strains and media*

The following bacterial strains were used in this study: *E. coli* ML30 (DSM1329), *Salmonella enterica typhimurium* (ATCC 14028), *Enterobacter aerogenes* (ATCC 13048), *Enterococcus faecalis* (ATCC 19433) and *Bacillus subtilis* (ATCC 6633).

For nutrient rich batch cultivation Luria-Bertani (LB) medium and tryptic soy broth (TSB) were used. The LB medium contained 10 g/L NaCl, 10 g/L tryptone, 5 g/L yeast extract and was adjusted to pH 7. The TSB medium was prepared according to the supplier's instruction (Biolife, Italy). For cultivation in a continuous chemostat culture, a defined phosphate buffered mineral medium was used (Wick *et al.*, 2001) with 10 mg/L (0.055 mM) glucose as sole carbon and energy source.

### *Cultivation conditions*

Batch cultures of *E. coli* were grown in LB medium at 37°C, shaken at 200 rpm, to an optical

density of 0.1 to 0.5 (OD<sub>546</sub>). Overnight cultures of *E. coli* were cultivated the same way to stationary phase. *B. subtilis* was cultivated on a shaker (200 rpm) in 10 ml TSB in test tubes at 30°C overnight. *S. typhimurium*, *E. aerogenes*, and *E. faecalis* were each grown the same way but at 37°C.

The chemostat culture of *E. coli* was operated at a dilution rate of 0.05 h<sup>-1</sup> at 37°C.

#### *DNA isolation*

Genomic DNA of 1ml of an overnight batch culture of each strain was isolated with the Easy-DNA-Kit<sup>TM</sup> (Invitrogen, Karlsruhe, Germany) according to the supplier's instruction, which yielded 0.4 µg DNA/µl or approx. 10<sup>8</sup> genomes per µl.

#### *In silico constructing and testing of primer*

The primers were designed with the help of the software Primer3 (Release 0.2) (Rozen *et al.*, 2000) using the sequence of the *uidA* gene (GenBank<sup>®</sup> accession number A00196) of *E. coli*, coding for β-D-glucuronidase. For this work an “outer” primer pair was designed to produce fragments of 1000 bp in the first PCR step. The forward primer *uidA*-F1 had the sequence 5'-ACCGTTTGTGTGAACAACGA-3'. The reverse primer *uidA*-R1 had the sequence 5'-GGCACAGCACATCAAAGAGA-3'. In the second PCR round the “inner” primer pair was used to amplify a 408 bp fragment. The sequence of the forward primer *uidA*-F2 was 5'-CTGTAACCACGCGTCTGTTG-3'. The sequence of the reverse primer *uidA*-R2 was 5'-GGGTAATGCGAGGTACGGTA-3'. The sequences of the newly designed primers were tested with FASTA3 on the Database EMBL – Prokaryotes (Pearson *et al.*, 1988) for their specificity for *E. coli*.

#### *Drinking water samples*

Water samples were collected at different water distribution systems in Switzerland during routine drinking water control or from private sources in rural and mountainous regions. The waterworks extracted water from gravel-sandy aquifers in tertiary molasse and moraines or from sources in carstic areas. Within the distribution systems water samples were taken from the raw water, after treatment steps such as UV-radiation, chlorination or ozonation, or from sampling points within the distribution net. The sampling taps were sterilised by gas flame. The water was allowed to run for 10 min or until it reached a stable temperature before it was

sampled in sterilised glass flasks. The samples were transferred to the laboratory in cooled containers and processed within 24 h.

#### *Enumeration and detection of viable cells and spiking of drinking water*

Dilution series of *E. coli* and *B. subtilis* cultures were made in phosphate buffered saline (PBS, 0.13 M NaCl, 7 mM Na<sub>2</sub>HPO<sub>4</sub>, pH 7.2). The numbers of viable cells of *E. coli* and *B. subtilis* in 100 µl were determined by pour plating in standard method plate count agar (TGY) (BD (Difco), NJ, USA) and spread plating onto tryptic soy agar (TSA, Biolife, Italy), respectively. After incubation at 30°C for at least 3 days, the colonies were counted. Water samples were spiked with aliquots from the appropriate dilution.

To detect *E. coli* in water samples by the reference culture method, water samples were concentrated by filtration through a sterile membrane filter, 0.45 µm pore size and 47 mm in diameter (HAWG047S1, Millipore, Bedford, MA). In case of spiked samples, 50 ml water was filtered and in case of natural water samples during routine drinking water control, 100 ml water was filtered. The filter with the retained *E. coli* cells was taken from the filter holder unit, placed onto a tryptic soy agar plate (Hain Diagnostica, Nehren, Germany) and incubated at 20°C, overnight to allow injured cells to recover. Then, the filter was transferred to a tryptone bile glucuronide agar TBX (Hain Diagnostica) with the chromogenic substrate 5-bromine-4-chlorine-3-indolyl-β-D-glucuronide and incubated at 44°C for 24 h. The blue-turquoise colonies were counted as *E. coli* (Ettel *et al.*, 2000).

#### *Sample preparation for PCR*

Polyvinylidene fluoride filters, 0.45 µm pore size and 13 mm in diameter (HVL P01300, Millipore, Bedford MA) were placed in epifluorescence stainless steel filter holders (Millipore, Bedford MA), previously sterilised by gas flame. The filters had been autoclaved in a buffer that contained 50 mM KCl, 20 mM Tris, 100 mM threonine, pH 8.4. This conditioning was performed to keep the protein binding capacity of the filter low. Samples of 50 ml were filtered. The filters were transferred to PCR tubes of 0.5 ml total volume, containing 103 µl of DNase incubation buffer (5 mM Mg<sup>2+</sup>, 2.5 mM SO<sub>4</sub><sup>2-</sup> and 5 mM acetate, pH 7). To digest free DNA, 36 units of DNase I, grade II (Roche) were added and the tubes were mixed by vortexing. The solution was incubated 30 min at 37°C with a short vortex-step after the first 15 min. The DNase was inactivated by heating the tubes to 98°C for 15 min. To obtain cellular DNA,

11  $\mu\text{l}$  of a 10-times concentrated lysis buffer TET (100 mM Tris, 10 mM EDTA, 10% Triton-X-100) (Goldenberger *et al.*, 1995) was added and the sample incubated again for 15 min at 98°C. The samples were processed further or stored at -20°C until use. With each sample series a positive and a negative control was processed the same way in parallel. For the positive control, water of the samples was pooled and filtered. After the heat treatment, isolated DNA of *E. coli* was added to the PCR tube (corresponding to about  $10^5$  genomes). If inhibition of PCR due to the sample matrix should occur, it would be indicated by a negative result of the positive control. For the negative control, autoclaved deionised water was filtered. A positive result of the negative control would indicate contamination of the PCR reagents with fragments of target DNA.

#### *Nested PCR*

Reagents for the PCR were added according to the recommendation of the supplier of the Taq-DNA-Polymerase (Sigma-Aldrich, Missouri, USA). The final volume for the PCR was 150  $\mu\text{l}$ . The final concentrations were: one time concentrated PCR buffer, 5 mM  $\text{Mg}^{2+}$ , 1 mg/ml bovine serum albumine (BSA, a facilitator for PCR), 0.2 mM dNTP, 0.03 units/ $\mu\text{l}$  Taq-DNA-polymerase, 0.4  $\mu\text{M}$  of each primer *uidA*-F1 and *uidA*-R1. The second step of the nested PCR was performed in a smaller volume of 20  $\mu\text{l}$  with the same concentration of the PCR reagents, except that the  $\text{Mg}^{2+}$  concentration was only 1.5 mM, and the BSA was omitted. The concentration of the primers *uidA*-F2 and *uidA*-R2 was also 0.4  $\mu\text{M}$ . One  $\mu\text{l}$  of the suspension resulting from the first step was transferred to the second step reaction mixture.

The PCR was performed in a TECHNE-Progene-thermocycler (Witec AG, Littau, Switzerland). The temperature program started with initial denaturation at 94°C for 2 min. The amplification of the first PCR step occurred during 30 cycles. Each cycle included 30 sec denaturation at 94°C, 1 min annealing at 63°C and 1 min extension at 72°C. The program closed with a final extension step at 72°C for 7 min. The second PCR step was performed principally in the same way, except that the cycle number was 25, the annealing temperature was 62°C and the extension time was 30 sec.

The PCR products were separated by gel electrophoresis according in a 2% agarose gel, stained with ethidium bromide (Sambrook *et al.*, 1989). One-Kb Plus DNA ladder (Invitrogen – Life Technologies, Basel, Switzerland) was used as marker for molecular weights.

## Results

### *Species-specificity of primers*

The FASTA3 search on the EMBL prokaryote data base revealed 100%-matches for the primers exclusively for the target sequence within the *uidA* gene of *E. coli*. Most *E. coli* strains and a few *Shigella* and *Salmonella* strains were reported to have the gene encoding the enzyme  $\beta$ -D-glucuronidase (Feng *et al.*, 1991). The primers *uidA*-F1 and *uidA*-R1 hybridised with the *E. coli* target gene *uidA*, producing a visible, single band of 1000 bp on an ethidium bromide stained agarose gel (not shown). The PCR performed with isolated DNA from other bacterial species namely *S. typhimurium*, *E. aerogenes*, and *E. faecalis* gave no amplification product of the appropriate length.

### *Results of the culture method*

Water samples analysed with the culture methods were part of routine quality control of drinking water performed according to “standard operation procedures” (SOP). The results of valid analyses with the culture method were set as reference. The results of the PCR based protocol were interpreted in relation to the result of the reference culture method.

### *Detection limit of PCR (compared to the culture method)*

Non-sterile and non-chlorinated tap water from the local drinking water distribution net (Dübendorf, Switzerland) was spiked with *E. coli* cells cultivated in the chemostat. For each dilution step, 6 parallel aliquots were analysed using the PCR protocol. At the same time, the spiked drinking water was analysed with the reference culture method. Figure 1 shows that the PCR method was able to detect *E. coli* cells in drinking water samples at low concentrations, in the range of 3 colony forming units (cfu) in 50 ml water. At this low concentration the PCR was positive in four out of 6 aliquots. With the culture method all samples were positive. On 2 of the 6 plates 2 cfu were counted and on the remaining 4 plates 3 cfu were counted per 50 ml.

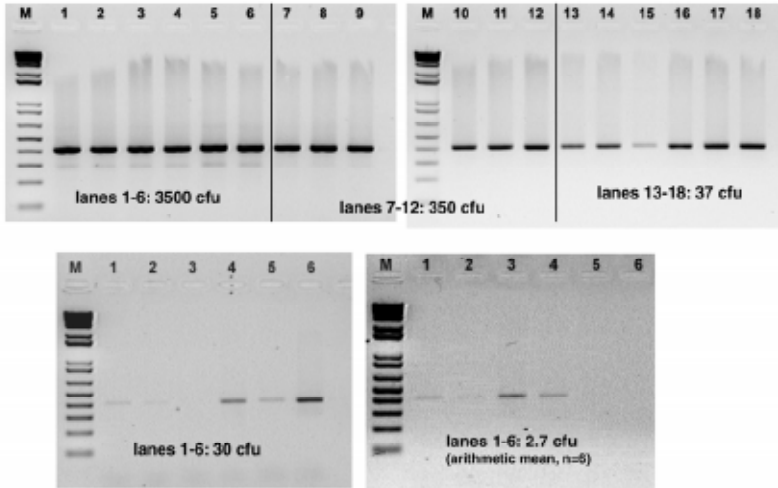


Figure 2.1: Relative detection limit: non-sterile drinking water was spiked with *E. coli* cells from the chemostat culture. For each dilution step 6 repetitive samples were processed by the PCR protocol. In parallel the cfu per 50 ml were determined by the culture method.

To compare the quantitative culture method with the qualitative PCR protocol, the result of the culture method was “down-graded” to a qualitative result in following statistical evaluations.

### Sensitivity

The sensitivity of the PCR protocol was defined by the number of the samples that are truly positive (TP) in the PCR assay divided by the number of all positive samples (the true positives (TP) + the false negatives (FN), equation 1).

$$\text{sensitivity} = \frac{TP}{TP + FN} \quad (1)$$

*E. coli* cells, originating from different growth media and culture conditions were used for spiking experiments. With cells taken from the exponential phase of a LB batch culture 104 experiments were performed. In 115 spiking experiments, the cells were taken from a very slowly growing chemostat culture. The cells from this chemostat culture were only growing at about 6 % of their maximum specific growth rate that can be achieved with this medium under the set conditions (37°C, pH 7) (Kovářová-Kovar *et al.*, 1998). In 23 of these 115 spiking

experiments the PCR method was negative (FN) whereas the culture method rendered a positive result. The sensitivity of the PCR method in this kind of spiking experiments with slow growing cells from the chemostat was 0.8. However, in spiking experiments with cells from batch cultures the sensitivity was only 0.57 (Table 2.1).

Table 2.1: Parallel analysis of spiked water samples with the PCR and the culture method. Numbers of true positive (TP) and false negatives results (FN) of the PCR protocol in relation to the culture method and the resulting sensitivity

description of sample	TP	FN	TP+FN	sensitivity
drinking water samples, spiked with <i>E. coli</i> cells from the chemostat culture	92	23	115	0.80
drinking water samples spiked with fast grown cells from the LB batch culture	59	45	104	0.57
total of spiked samples	151	68	219	0.69

To further evaluate the PCR protocol, in comparison to the reference culture method, environmental water samples were analysed in parallel. The number of TP was 10, of FN 5, which gave the sensitivity of 0.67. Among the samples which delivered false negative results by the PCR protocol, many caused clogging during filtration and/or brownish staining of the filter after the filtration process. Table 2.2 shows some examples where inhibition of the PCR was suspected due to components in the water sample. Of 17 environmental samples exhibiting  $\geq 1$  cfu/100 ml 5 were negative, as measured by the PCR assay.

Table 2.2: Natural water samples in context of drinking water production (raw, polluted and treated water): comparison between the culture method and the PCR-protocol. Examples of false negative results or partly inhibition of the PCR method. To compare the quantitative culture method with the qualitative PCR protocol, the results of the culture method were “down-graded” to qualitative results and are indicated in brackets.

Description of sample	cfu / 100 ml sample volume (“down- graded” result)	PCR result, 50 ml sample vol- ume
source with strong infiltration of manure of a close by farm; the water had a bad odour and a brownish colour	>1000 (positive)	negative
river water	>100 (positive)	positive
water pumped from the aquifer close to the river	1 (positive)	negative
raw water, source in a carstic area (water had brownish colour)	36 (positive)	very weakly positive
tap water of a distribution net in carstic region after heavy rainfall	3 (positive)	negative
turbid water sample from a farm house source in the alps (Switzerland)	5 (positive)	negative
source in a carstic area	6 (positive)	negative

### *Specificity*

The PCR methods specificity relative to the standard culture method, was characterised by the number of the samples detected truly negative (TN) by the PCR method divided by the number of all negative samples (the true negatives (TN) + the false positives (FP), equation 2).

$$specificity = \frac{TN}{TN + FP} \quad (2)$$

To avoid false positive results compared to the reference culture method, the DNase digestion step prior to PCR has been introduced in the protocol. In a control experiment, the influence of other viable bacteria on the DNase activity was tested. At least 200 vegetative *B. subtilis* cells were added to 50 ml non-sterile drinking water that had been spiked with approx. 1000 free genomes of *E. coli*. The DNase was able to fully digest the free DNA. Even in the presence of other bacteria as in the following nested-PCR no products were amplified. In parallel control experiments performed without the DNase digestion step, the *E. coli* DNA was amplified in the following nested-PCR (data not shown).

With the environmental water samples that were analysed in parallel with both methods, the number of TN was 54 and of FP 14 which gave the specificity for the PCR protocol of 0.79. The main contribution to the number of FP was probably given by water samples that contained non-culturable *E. coli* cells. These were often raw water samples of sources which delivered not very deeply filtrated ground water.

Among the environmental water samples analysed in parallel with both methods 38 were taken before and after UV treatment. Within these 19 sample pairs, 5 different combinations of results occurred. A representative example for each case is listed in Table 2.3.

Table 2.3: Environmental water samples, examples of analysis before UV treatment of the raw water and after UV treatment. To compare the quantitative culture method with the qualitative PCR protocol, the results of the culture method were “down-graded” to qualitative results and are indicated in brackets.

Description of sample	cfu / 100 ml („down- graded result)	PCR result, 50 ml sample volume	number of cases
1. source, raw water, carstic region	0 (negative)	negative	6
after UV treatment	0 (negative)	negative	
2. source, raw water, mo- raines, molasse	≥1 (positive)	positive	7
after UV treatment	0 (negative)	negative	
3. source, raw water, mo- raines, molasse	0 (negative)	positive	2
after UV treatment	0 (negative)	negative	
4. source, raw water, mo- raines, molasse	0 (negative)	positive	3
after UV treatment	0 (negative)	positive	
5. source, raw water, mo- raines, molasse	2 (positive)	negative	1
after UV treatment	0 (negative)	negative	
total cases			19

Over all 83 environmental water samples 64 samples (77%) agreed with the two methods analysed.

## Discussion

A published nested PCR protocol to detect *E. coli* in drinking water samples (Juck *et al.*, 1996) was further developed. The goal was to provide a fast and cheap PCR protocol that is able to replace the classic, standardised culture method in routine drinking water analysis.

As with the culture method which served as reference to evaluate the PCR protocol, the water sample was concentrated by filtration through a membrane filter with 0.45  $\mu\text{m}$  pore size. The bacteria retained on the filter were transferred together with the filter into the reaction tube. In the subsequent protocol, reagents were added, but no extraction or purification of DNA was performed, in order to avoid loss of target DNA and to keep handling economical. With the two steps of the nested PCR protocol it was possible to reach almost the same low detection limit as with the culture method (Figure 1). When the PCR product after the first step was loaded on an agarose gel, the detection limit corresponded to about 500-700 cfu (not shown). Furthermore, the second PCR step is much less exposed to possible inhibiting substances because the reaction mix from the first PCR round is 20 times diluted.

The main novel approach of this PCR protocol was the introduction of a DNase digestion step prior to PCR. With this step we intended to decrease the probability of false positive results due to free DNA of dead *E. coli* bacteria. Experiments performed with water samples spiked with isolated, free DNA, showed that the addition of DNase prior to the PCR prevented this kind of false positive results. However, there were new problems introduced with this technique. We observed that especially samples spiked with fast grown cells from the batch culture failed to generate positive results compared to the culture method. This suggests that fast grown cells from the LB batch culture might be more affected by the DNase treatment than slowly grown cells from the chemostat culture. The cells in our experiments were diluted in drinking water, concentrated and re-suspended in a buffer with 5 mM  $\text{Mg}^{2+}$ . During this procedure, the spiked cells experienced environmental stresses such as shifts of temperature and osmolarity which could have affected the fast growing cells. Additionally, fast growing cells could have been more susceptible to DNase during the first minutes of heat treatment. In a control experiment we found that DNase still kept some DNA digesting activity after it was heated for 1 min at 98°C (data not shown).

We observed that the PCR protocol often delivered false negative results when we analysed raw water samples from sources before treatment. This kind of water samples might contain substances which can inhibit the enzyme activity of Taq polymerase or reduced its efficiency (Demanèche *et al.* 2001, Wilson 1997). During concentration by filtration, inhibitory substances in solution passed through the mesh of the filter. But particles larger than 0.4  $\mu\text{m}$  present in the sample remained trapped on the filter together with the bacteria to be analysed. High concentrations of these particles might have interfered with the PCR in different ways. The composition of drinking water including inhibitory substances varies from sample to sample.

Samples of drinking water disinfected with high doses of UV radiation at 254 nm wave length caused negative results with the PCR protocol, which agreed with the results of the culture method. The UV light is absorbed by DNA, proteins in membranes and RNA, which leads to the disruption of the DNA and the cell membrane (Bolton, 1999). After disinfection treatment, the DNase digestion step is helpful and important to achieve agreement with the standard culture method.

Although free DNA was eliminated, a significant part of the samples showed a positive result by PCR but were negative with the culture method. We assume that non-culturable or even dead *E. coli* can exist for an extended time with their DNA protected against DNase digestion before they eventually undergo lysis. To increase specificity in this sense, only the time consuming measure of a pre-cultivation step seems possible. Authors performing a pre-cultivation before the PCR had a good agreement of 98% with the culture method (Frahm *et al.*, 2003).

## Conclusions

When using a direct PCR method for detection of *E. coli* in drinking water samples the following restrictions remain to be considered:

In case of fast grown *E. coli* cells present in water samples, the DNase step during the PCR protocol may damage target DNA. This situation is probably of minor importance for pristine water samples. Thus, slow growing cells are better suited for spiking experiments.

In water samples containing particles larger than 0.4  $\mu\text{m}$ , the PCR might be inhibited to some extend, and the PCR method can yield false negative results. To avoid analysis of samples

that contain inhibitory substances for the PCR, turbid samples may not be used. Alternatively, or a pre-filtration step may be introduced before sample preparation. These possibilities need to be evaluated.

In water samples containing non-culturable *E. coli* the PCR method yields a positive result. The application either excludes such samples, which is not practical for routine drinking water control, or the non-culturable cells are explicitly included in the range of the target organisms. In this case, the direct PCR protocol could be considered as a “new” method. The aim of use of the PCR protocol would be distinct from the standard culture method. Aspects such as application range and interpretation of the results would have to be reconsidered. A possible application for the direct PCR protocol could be the detection of faecal contamination of drinking water that dates back several days or weeks.

### 3. Detection of starved *Escherichia coli* cells in drinking water samples with a PCR based method

#### Abstract

A PCR-based protocol that specifically detects *Escherichia coli* (*E. coli*) in water samples was tested for the special situation of starved bacterial cells in water. With the intention to realistically simulate environmental conditions, pristine drinking water samples were spiked with *E. coli* cells at low concentrations. These "microcosms" were incubated for several weeks. At time-points ranging from days to weeks sample aliquots were analyzed with the PCR protocol. The sample preparation included concentration by membrane filtration. A DNase digestion step was inserted prior to PCR in order to digest free DNA possibly present in the sample. In parallel, these samples were also tested with the standard method for the detection of *E. coli* in drinking water based on membrane filtration, culturing and plate count. At the beginning of the incubation the 2 methods delivered comparable results. At later time-points, the *E. coli* cells became non-culturable, whereas the results obtained by PCR did not show a significant decrease of amplifiable DNA. The PCR protocol detects intact *E. coli* cells harboring a copy of the *uidA* gene regardless of the physiological state of the cell: alive, non culturable or even dead.

#### Introduction

The hygienic quality of drinking water has been routinely tested for many decades by determining several bacterial parameters. These tests typically screen for indicator organisms such as the intestinal bacterium *E. coli*. The analysis has been performed using cheap and simple methods based on cultivation. However, classical culture methods are time consuming and take up to several days until a single bacterium grows to a visible colony. With the development of new molecular methods in the last decades, attempts have been made to establish modern and faster alternatives for bacterial routine analysis. Using PCR seems to be a promising approach in reaching almost the same low detection limit as classical, culture based methods.

Several propositions for applications of PCR in water analysis for faecal bacteria have been published (Bej, Mahbubani *et al.*, 1991; Frahm and Obst, 2003; Fricker and Fricker, 1994;

Heijnen *et al.*, 2006; Iqbal *et al.*, 1997; Juck *et al.*, 1996; Tsen *et al.*, 1998). With these new protocols disadvantages towards the culture plate count technique and general questions turned up. The PCR protocols deliver a presence/absence answer of the DNA of the target organism in the sample. For a quantitative answer of these protocols, several replicate samples of dilutions steps would have to be analysed and the results interpreted with the most-probable-number (MPN) concept. This would demand a large amount of samples to analyse. Another important and often neglected issue is the concentration of the sample. The reagents used for PCR, especially the enzyme Taq-Polymerase are expensive. To make the analysis economic, small volumes should be used. As indicator bacteria are expected to be present in very low concentrations in drinking water, the samples have to be concentrated. Concentration protocols bear losses of analytes and therefore are, especially with organisms and biomolecules, difficult to keep quantitative and robust, i.e. insensitive to small changes in the protocol. If PCR is used directly and without pre-cultivation to detect organisms in environments, no information is obtained on the physiological state of the organism. The result of the protocol does not provide any information as to whether the amplified DNA belonged to a live or dead bacterium or if it was just freely present in the sample.

There is an ongoing debate on the relevance of possible physiological states that bacteria can enter (Berney *et al.*, 2006). Roszak *et al.*, (1987) proposed the expression viable but not culturable (VBNC) for the state in which bacterial cells show physiologic activity but are not able to be cultured on common nutrient media. Yet, as experienced with *Vibrio vulnificus* and seawater at different temperatures, the cells can resuscitate under appropriate environmental properties (Oliver *et al.*, 1995). By exposing enteropathogenic *E. coli* to seawater and sunlight, a VBNC state was induced. These VBNC cells were still potentially pathogenic, i.e. produced the enterotoxin that could be detected with the ganglioside-enzyme-linked immunosorbent assay and the rabbit intestinal loop assay. Due to these findings some authors consider it important to discover also bacteria in the VBNC state. Molecular methods represent the tools to discover the VBNC bacteria (Pommepuy *et al.*, 1996). In various reviews findings the supposition are summarised that bacteria in VBNC state in drinking or recreational water present harm to humans as they can resuscitate and regain infectivity (Colwell, 2000; Keer *et al.*, 2003; Oliver, 2005). However, it seems difficult to confirm the findings of resuscitation. With the “mixed culture recovery method” Bogosian *et al.* (1998) found that resuscitation oc-

curs in samples with high cell densities due to high stochastic probability that there is still a viable bacterial cell present, but hardly due to the shift from a VBNC state to viable state. With careful statistical planning of the experiments, infectivity of active but not culturable *Salmonella enterica* serovar Typhimurim on mice could not be confirmed (Smith *et al.*, 2002). As long as the mechanism on molecular level is not understood, the existence of a VBNC state should not be regarded as granted (Nyström, 2001). Since the relevance of the “VBNC” state of gram negative pathogenic bacteria in drinking water is still a matter of discussion, we regard the the standard culture method to detect *E. coli* as base for our evaluation of the here developed PCR method.

In this study, we specifically analyse the suitability of the PCR method for the detection of *E. coli* cells starved in drinking water where they were incubated for prolonged period of time. The cells originate from 3 different growth histories: (i) a chemostat culture with low dilution rate, (ii) an exponentially grown batch culture, and (iii) a high density suspension of stored *E. coli* cells in PBS. The plate count method was performed in parallel to the PCR method and served as control.

## Methods

### *Strains and media*

In this study the strains *E. coli* ML30 (DSM1329) and *Bacillus subtilis* (ATCC6633), *Salmonella typhimurium* (ATCC 14028), *Enterobacter aerogenes* (ATCC 13048) and *Enterococcus faecalis* (ATCC 19433) were used.

Luria-Bertani (LB) medium, tryptic soy broth (TSB) and a defined, phosphate buffered mineral medium were applied for cultivation of bacteria. The LB medium contained 10 g/L NaCl, 10 g/L tryptone, 5 g/L yeast extract and was adjusted to pH 7. The TSB medium was prepared according to the supplier’s instruction (Biolife, Italy). For cultivation of *E. coli* in a continuous chemostat culture, a defined mineral medium was used according to Wick *et al.*, (2001) with 10 mg/L (0.055 mM) glucose as sole carbon and energy source. Tap water used in this study was from the local drinking water distribution system and consisted of a mixture of around 1-2% spring water, ca. 48% groundwater and 50% treated lake water. It had a mean pH of 7.3 and the sum of calcium and magnesium was around 5-6 mM (Sigg and Stumm, 1994; Genossenschaft Wasserversorgung Dübendorf [online]). The tap was adjusted to a flow

of around 3 L/min. for 30 min. When the temperature remained constant (14°C), sterile 2 L Erlenmeyer flasks were filled with 1 L of pristine tap water. In this tap water no *E. coli* could be detected, as demonstrated by analysis of control samples.

### *Cultivation*

For our experiments we used *E. coli* with 3 different growth histories, namely cells from a slowly growing continuous culture in the chemostat, cells from an exponentially growing batch culture and cells that had been stored in PBS as high density cell suspension.

The *E. coli* cells in the chemostat were cultivated with mineral medium and glucose. The chemostat was operated at a dilution rate of 0.05 h<sup>-1</sup> at 37°C for 45 days.

Fast growing *E. coli* cells in the batch culture were cultivated to the optical density (OD<sub>546</sub>) of 0.4 in 10 ml LB medium in test tubes at 37°C, continuously shaken at 200 rpm.

For the third history of cells, *E. coli* cells were inoculated in LB medium at 37°C over night and shaken at 200 rpm. Twenty ml of the culture was then centrifuged at 3000 x g for 30 min. The pellet was washed, re-suspended in 2 ml phosphate buffered saline (PBS, 0.13 M NaCl, 7 mM Na<sub>2</sub>HPO<sub>4</sub>, pH 7.2) and stored at 4°C for 3 months.

*B. subtilis* was cultivated in 10 ml TSB in test tubes at 30°C overnight, shaken at 200 rpm. *S. typhimurium*, *E. aerogenes*, and *E. faecalis* were each grown in 10 ml TSB in test tubes at 37°C overnight, shaken at 200 rpm.

### *Spiking and incubation of drinking water*

Drinking water was spiked with 3 different concentrations of *E. coli* cells of 3 different "histories". In the first experiment, cells were taken from the chemostat culture, grown for 45 days; in the second experiment, cells had been grown in a batch culture to the exponential phase in LB medium; in the third experiment, cells had been stored at high density in PBS for 3 month.

For each concentration, 3 two-litres-Erlenmeyer flasks with drinking water were spiked in parallel. They served as 3 individual repetitions. These drinking water "microcosms" in the Erlenmeyer flasks were incubated at 4°C for up to 2 months and continuously shaken at 100 rpm. One microcosm was not spiked and served as negative control.

### *Detection and enumeration*

At intervals of 12 h, 24 h or several days, samples were taken from the drinking water microcosms and analyzed in parallel by the classical culture method and the PCR protocol. In each of the 2 experiments, each concentration was analyzed in triplicate. Except for the negative control only 1 analysis was performed per time-point.

For the enumeration of viable cells in bacterial cultures, dilution series in phosphate buffered saline (PBS, 0.13 M NaCl, 7 mM Na<sub>2</sub>HPO<sub>4</sub>, pH 7.2) were performed and 100 µl of each dilution was pour plated in standard method plate count agar (TGY) (BD (Difco), NJ, USA). The number of viable cells of *B. subtilis* was estimated by spread plating 100 µl of dilution steps onto tryptic soy agar (TSA, Biolife, Italy) plates.

To detect *E. coli* in water, 50 ml of sample were filtered through a sterile membrane filter, 0.45 µm pore size and 47 mm in diameter, consisting of mixed esters of cellulose (HAWG047S1, Millipore, Bedford MA). The filter was taken from the filter holder unit and placed onto a tryptic soy agar plate and incubated at 22°C for at least 4 h. Then, the filter was transferred to an *E. coli*-direct-agar (ECD agar, Biolife) with the fluorogenic substrate methyl umbelliferyl glucuronide (MUG, Biolife) and incubated again at 37°C. The enzyme β-D-glucuronidase of *E. coli* hydrolyses MUG into a fluorescing product. After excitation with UV light of 365 nm wavelength, the fluorescing colonies were counted as *E. coli*. The plate count numbers of the triplicate samples were averaged and their standard deviation was calculated.

### *Preparing the sample for PCR*

Polyvinylidene fluoride filters, 0.45 µm pore size and 13 mm in diameter (HVLP01300, Millipore, Bedford MA), had been autoclaved in a buffer containing 50 mM KCl, 20 mM Tris, 100 mM threonine, pH 8.4. The filters were placed in sterilized stainless steel filter holders (XF3001200, Millipore, Bedford MA), and 50 ml of water sample was filtered. The filters were transferred to PCR tubes, which contained 103 µl of DNase incubation buffer (5 mM Mg<sup>2+</sup>, 2.5 mM SO<sub>4</sub><sup>2-</sup> and 5 mM acetate, pH 7). The tubes were placed in ice, 36 units of DNase I, grade II (Roche) added and the solution mixed by vortexing. Two times 15 min incubation at 37°C followed, with a short vortex-step after the first 15 min. The DNase was inactivated by heating the tubes to 98°C for 15 min. To make the DNA in the whole cells acces-

sible to the PCR, a tenth of the actual liquid volume in the tube of a lysis buffer TET (10 mM Tris, 1 mM EDTA, 1% (v/v) Triton-X-100) (Goldenberger *et al.*, 1995) was added. After mixing, the solution was heated to 98°C for 15 min. The so treated samples were collected and stored at -20°C until use for the PCR.

#### *First PCR round*

Reagents for the PCR were added according to the recommendation of the supplier of the Taq-DNA-Polymerase (M1865 (isolated from *Thermus aquaticus*) Promega, Madison WI, USA) and the according PCR buffer. The final concentrations were: one time concentrated PCR buffer, 5 mM Mg<sup>2+</sup>, 1 mg/ml BSA, 0.2 mM dNTP, 0.03 units/μl Taq-DNA-polymerase, 0.4 μM forward and reverse primer. The primer targeted the *uidA* gene of *E. coli*. The forward primer (*uidA*-F1) had the sequence 5'-ACCGTTGTGTGAACAACGA-3'. The reverse primer (*uidA*-R1) had the sequence 5'-GGCACAGCACATCAAAGAGA-3'. The cycling was performed in a TECHNE-Progene-thermocycler (Duxford Cambridge, England). The first round started with 2 min initial denaturation at 94°C, followed by 30 cycles of 30 sec at 94°C, 1 min annealing at 63°C and 1 min extension at 72°C and closed with a final extension step at 72°C for 7 min.

#### *Second PCR round*

For the second round real-time-PCR was used. One μl of the product from the first PCR-round was transferred into a well of 96-well-plate for PCR (Abgene, Surrey, UK). Each well contained the reagents for a real-time SYBR Green PCR, which had been prepared according to the suppliers instructions (Applied Biosystems, Foster City CA, USA). For each primer the concentration was 0.9 μM. The forward primer (*uidA*-F3) had the sequence 5'-TTACGTGGCAAAGGATTCGAT-3' and the reverse primer (*uidA*-R3) the sequence 5'-GCCCAATCCAGTCCATTA-3'. The real-time-PCR round was performed on an ABI Prism 7000 Sequence Detection System (Applied Biosystems) applying the standard amplification profile. The data were analyzed with the ABI Prism 7000 SDS software version 1.0 choosing the analysis settings as recommended by the user manual. The threshold was set on a level which was not reached by the no-template-controls (NTC) within 40 cycles. The numeric result of the positive PCR was a so-called Ct-value: the cycle number of the fluorescence signal reaching the threshold. The mean and the standard deviation of the Ct-values for

the 3 repetitions were calculated with Excel (Microsoft) and the latter was marked with a T-bar in Fig. 1 and 2 and for standard deviations consisting only of 2 repetitions, simple lines were drawn. In these experiments, in 1 PCR reaction the fluorescence did not reach the threshold value within 40 cycles. The averages were plotted against the time-points in days of incubation and against a reversed y-axis with the Ct-values.

#### *DAPI staining and microscopy*

To 135  $\mu$ l of a LB batch culture of *E. coli* in the exponential phase ( $OD_{546} = 0.17$ ), 35  $\mu$ l of the TET-buffer was added and the cells were heat-treated for 30 minutes at 98°C. From the non-treated cells from the original batch culture and from the heat-treated cells 2  $\mu$ l were dropped into an examination well of a slide (10.0230.01, slides with 10 wells of 7 mm diameter, Huber, Reinach, Switzerland) and dried at 45-50°C. The DNA stain DAPI (4'6-diamidino-2-phenylindole) was added (5  $\mu$ l of a 0.1% solution), and the slides were incubated at 4°C overnight. After carefully rinsing the slides, the samples were mounted in Citifluor™ (CITIFLOUR LTD, London UK) and were examined with a fluorescence microscope (Olympus, Japan) connected to a CCD-camera.

#### *DNA isolation*

Genomic DNA of 1 ml of the overnight culture of *E. coli* cells was isolated with the Easy-DNA-Kit™ (Invitrogen, Karlsruhe, Germany) according to the suppliers instruction, which yielded 0.4  $\mu$ g DNA/ $\mu$ l or approx. 108 genomes per  $\mu$ l.

The genomic DNA of the cultures of *S. typhimurium*, *E. aerogenes* and *E. faecalis* were isolated in the same way.

#### *Designing and testing of primers*

The primers were designed with the help of the software Primer3 (release 0.2; Rozen *et al.* (2000) [<http://www-genome.wi.mit.edu/cgi-bin/primer/primer3.cgi>]) based on the sequence of the *uidA* gene of *E. coli*. The outer primer pair was constructed to amplify products of 1000 bp. The primer pair for the real time PCR was chosen within the amplified sequence of the primer pair of the first round using the software package Primer Express Version 2.0 (Applied Biosystems). The sequences of the newly designed primer were tested with FASTA3 on the Database EMBL – Prokaryotes (Pearson *et al.*, 1988) for their specificity for *E. coli*.

## Results

### *Survival of E. coli in drinking water measured by the culture method*

*E. coli* were spiked to drinking water. They originated from cultures with 3 different histories: slowly cultivated cells of a chemostat culture, fast growing cells of a batch culture in nutrient rich LB medium and cells stored in PBS at high density. From each history, 3 different initial concentrations of cells were spiked. They are indicated in Figures 3.1, 3.2, 3.3 and 3.4 as "high-", "middle-" and "low-cell-number". In all experiments the number of  $\beta$ -D-glucuronidase positive colonies per 50 ml decreased over the time of incubation in drinking water. The reduction rate seemed to be dependent on the "*E. coli*-history". With the *E. coli* cells from the chemostat culture a 90% reduction was observed after 6 days of incubation (Figure 3.1, upper row). With fast grown cells from the rich LB medium, the same reduction was achieved after 18 h (Figure 3.3 upper row). With the cells stored in PBS, the number of colony forming units was reduced to 10% after one and a half day (Figure 3.4 upper row).

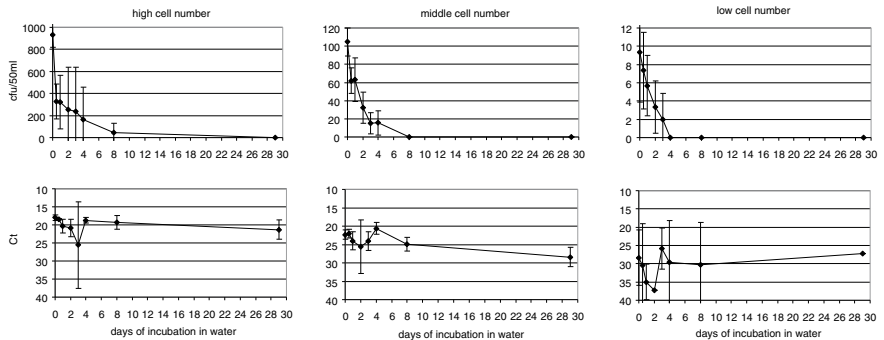


Figure 3.1: Drinking water was spiked with *E. coli* cells grown in a chemostat culture at 3 different initial concentrations (indicated with "high cell number", middle and "low cell number"). At different time-points up to 29 days samples of 50 ml were analyzed with the culture method (upper row) and the PCR method (lower row). The Ct values of the 2<sup>nd</sup> PCR round with real-time PCR indicate the cycle number where the fluorescence detection reaches a certain threshold. The Ct values were plotted in reverse order on the y-axis. The experiments were performed generally in triplicate and the t-bars indicate the standard deviation of each time-point measurement.

While the number of culturable *E. coli* cells decreased over time,  $\beta$ -Glucuronidase negative cells increasingly formed colonies. As the drinking water in the incubation experiment had

not been sterilised, the autochthonous bacterial flora multiplied during incubation at 4°C and full aeration. In all late time-point samples, colonies of other Gram-negative bacteria overgrew the filters during incubation on the agar plates.

*Quality of PCR result visualised by gel electrophoresis compared to real-time-PCR*

For all experiments and samples, the tubes with the amplified DNA from the first PCR round were stored at -20°C. This DNA fragments were also used for the "classical" second round PCR (detailed method description in chapter 2), where the amplified DNA fragments were visualised in an ethidium bromide stained agarose gel with the characteristic band pattern. Figure 3.2 shows the inverted image of the agarose gel compared to its corresponding graph of Ct values obtained from the real-time-PCR. The example was chosen from the experiment of microcosms that had been spiked with exponentially grown *E. coli* cells. The incubation time was 17 h. The figure shows the results of all 10 microcosms obtained with the PCR protocol at this time point. Qualitatively, the brighter bands in the gel correspond to lower Ct values (to make it optically better comparable the scale was reversed in figure 3.2).

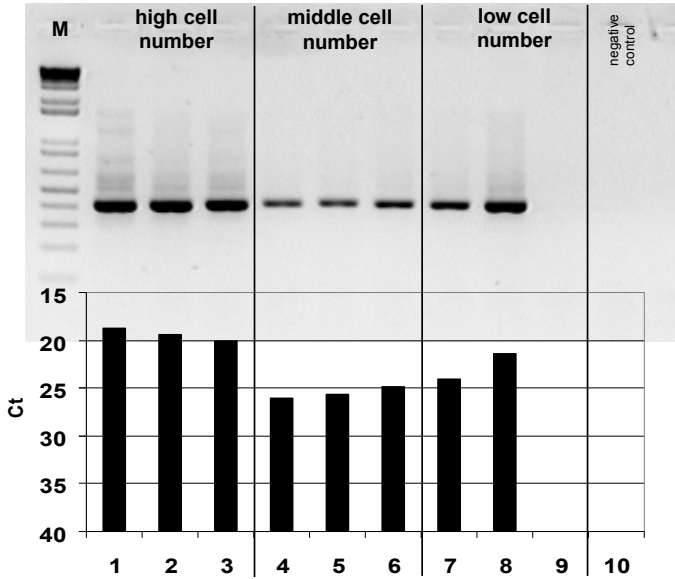


Figure 3.2: The second PCR round products (408 bp in size) of the nested PCR are visualised in an ethidium-bromide stained agarose gel (inverted picture) in the upper part of the figure. In the line “M” at the left the “one-Kb Plus DNA ladder” (Invitrogen – Life Technologies, Basel, Switzerland) is loaded as marker. In the lower part of the figure the corresponding Ct values obtained by real-time-PCR (reversed scale of the y-axis) are indicated. As example for this visualisation the PCR products were taken from the experiment with exponentially grown *E. coli* cells that were incubated in drinking water for 17 h.

#### *Detection of E. coli in drinking water by PCR*

At time point 0, the result of the PCR protocol was comparable to the number of cfu. In the experiments with the low cell number the PCR method also returned a Ct value below 40. This indicates that the detection limit of the PCR method is in the range of 1 to 10 cells per 50 ml water sample.

The results obtained by the PCR protocol showed no evident decrease over time in all microcosms. At time-points between 5 and 10 days of incubation some reactions failed in efficient production of the target DNA sequence. In case of only 2 Ct values of the three replicate microcosms that were larger than the threshold value, the cross bar of the standard deviation line is omitted. In case of only 1 Ct value larger than the threshold, the standard deviation is omitted.

ted. When all Ct values of the three repetitional samples were lower than the threshold value, no data point is given in figures 3.1, 3.3 and 3.4, lower rows.

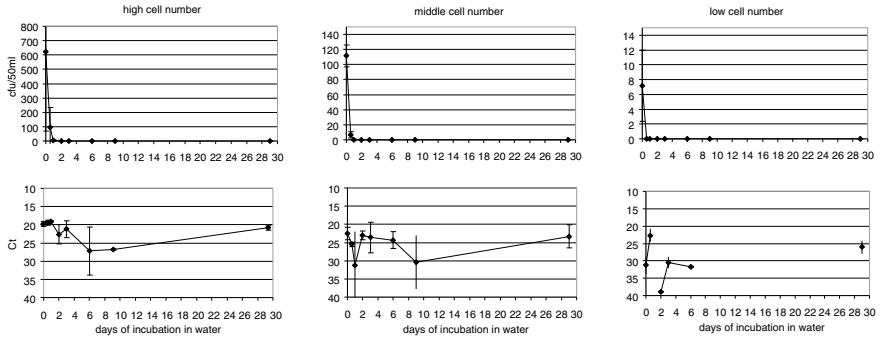


Figure 3.3: Drinking water was spiked with *E. coli* cells obtained from an exponentially grown batch culture in LB medium and analysed with the culture and the PCR method, analogous to Figure 3.1. Standard deviations with a simple bar (missing cross bar) consist of only 2 values larger than the detection limit and values without standard deviation of only 1 value. In the graph with low cell number, at some time points in all 3 replicates none of the PCR reactions reached the threshold. These datapoints are not given in the graph.

In the experiment with the cells stored in PBS, the fraction of non-culturable cells was many times larger than the fraction of the culturable cells. Therefore, the Ct values were markedly lower as compared to the other two experiments with cells from the chemostat and exponentially grown cultures (Figure 3.4).

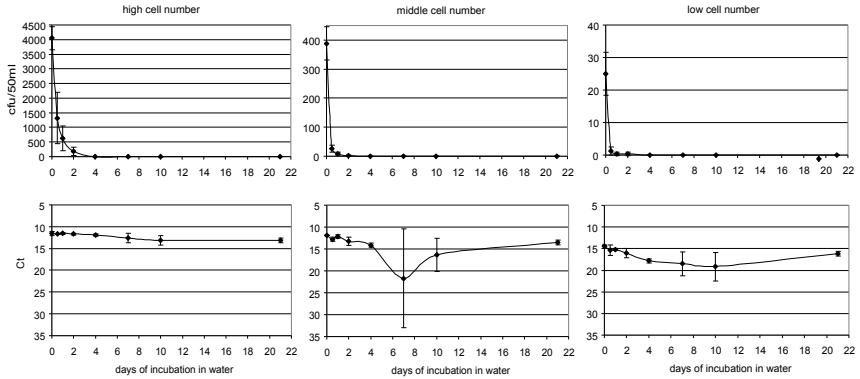


Figure 3.4: Drinking water was spiked with *E. coli* cells obtained from a high density cell suspension stored in PBS for 3 months and analysed with the culture and the PCR method, analogous to Figure 3.1.

In the experiments with cells from the batch culture, another sample was taken after 2 months of incubation in drinking water. No significant change occurred in this period (data not shown).

#### *DNase activity in the presence of Bacillus subtilis*

To assure that DNase also digests and eliminates free DNA in presence of other bacteria, living *B. subtilis* cells were added to 50 ml non-sterile drinking water to a concentration of 290 *B. subtilis* cells/ml. 16 microcosms were prepared in parallel. 6 microcosms consisted of a suspension of heat treated *E. coli* cells (30 minutes at 98°C) in drinking water. The final concentration of treated *E. coli* cells was  $10^7$  per 50 ml water sample. To 6 others, isolated DNA from *E. coli* was added to a final concentration of about  $10^8$  *E. coli* genomes per 50 ml water sample. Four microcosms served as *E. coli*-negative controls. All experimental microcosms were processed according to the PCR protocol. Three repetitions of the microcosm with *E. coli* remnants were exposed to DNase prior to PCR and for the other 3 repetitions the DNase step was omitted. Figure 3.5 shows that the DNase step is effective, especially if free, isolated DNA has to be digested. With DNA from the heat treated *E. coli* cells, the difference is less clear (wide standard deviation). The signals obtained from the negative controls with and without DNase were below the threshold level (not shown).

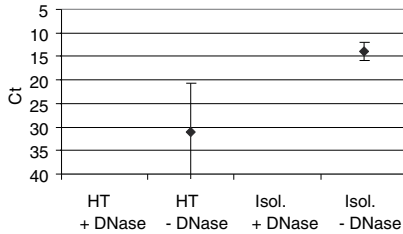


Figure 3.5: Performance of the DNase digestion step previous to the PCR to eliminate free DNA in presence of other living bacteria (*B. subtilis*). Free DNA from heat treated *E. coli* (HT) and isolated DNA (isol.) was spiked to drinking water containing its indigenous microflora and furthermore *B. subtilis* cells from a batch culture. The 50 ml-samples were analyzed with the PCR protocol. To evaluate the effect of the DNase digestion step, in the control samples this step was omitted (" - DNase"). The samples performed with the "normal" PCR protocol are indicated with "+ DNase". All experiments were performed in triplicate.

#### *DAPI staining and examination of heat-treated cells by microscopy*

During the PCR protocol the bacterial cells undergo a series of treatments before the amplification step. In order to obtain more information to what extent these procedures might affect the integrity of the cell envelope, *E. coli* cells were boiled in TET buffer and stained with DAPI for microscopy. The cells remained in the original shape and size like the non-treated cells (Figure 3.6). However, the heat treated cells formed larger aggregates, probably as a result of the detergent present. In agreement with our findings other groups report that boiled (Murphy *et al.*, 2001), non-culturable (Ericsson *et al.*, 2000) and dead (Saby *et al.*, 1997) *E. coli* become visible as whole cells under the fluorescent microscope after treatment with nucleic acid stains.

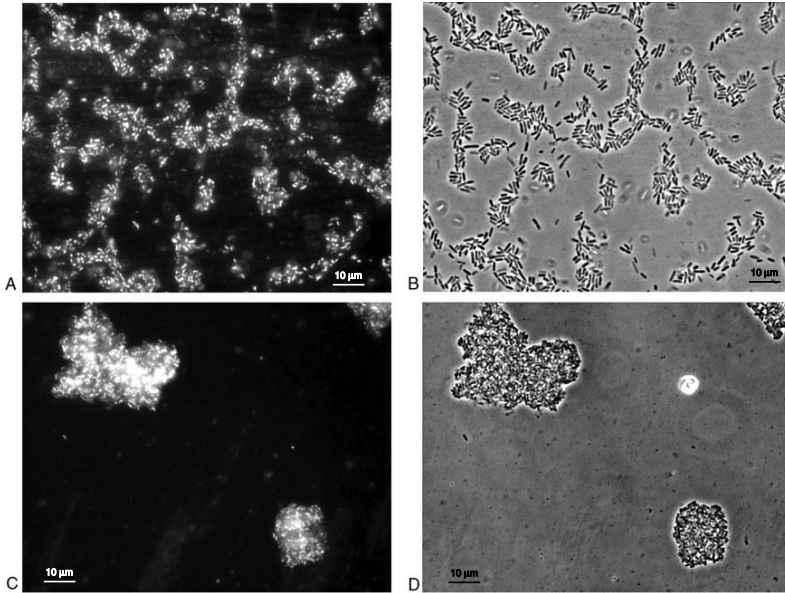


Figure 3.6: DAPI stained *E. coli* cells from an exponentially grown batch culture in LB medium examined under the microscope with UV excitation (A) and phase contrast (B). The same *E. coli* culture was lysed with the TET-buffer (see Materials and Methods) previous to DAPI staining and examination with UV excitation (C) and phase contrast (D).

### *Specificity of primers*

The FASTA3 search on the EMBL prokaryote data base revealed no other 100%-matches for the first round primers than the target sequence on the gene coding for  $\beta$ -D-glucuronidase in *E. coli*. One target sequence was found on a published sequence of *Shigella flexneri* which matched the reverse primer *uidA*-R3 of the second round. The primers for the first round were tested on isolated DNA of *Salmonella typhimurium*, *Enterobacter aerogenes* and *Enterococcus faecium*. With the non-*E.-coli* target DNA, the PCR remained negative throughout (not shown).

### **Discussion**

Real-time PCR in place of a second PCR round and analysis by gel electrophoresis gave slightly more quantifiable results than bands on an agarose gel. However, absolute quantifica-

tion in relation to an external standard curve was not possible in our case and the results obtained have to be regarded as semi-quantitative. With all the second round PCRs performed with the "classical" PCR and the real-time PCR, the results were comparable and reproducible as demonstrated in the selected example shown in Figure 3.2. We assume that the second PCR round is efficient, reproducible and bears no further interference like inhibition of the Taq polymerase. Thus, the quality of the final results depends mainly on the performance of the first PCR round and/or problems during sample preparation before the PCR analysis.

To exploit the useful potential for quantification of the real-time PCR technique, the pre-amplification in the first PCR run might be optimized by reducing the cycle number (see chapter 5.1). Attention would have to be paid to not increase the detection limit with this measure.

In the experiment with the exponentially grown cells from the batch culture, some PCRs of the triplicate were negative (indicated with simple bar standard deviations in Figure 3.3). Possible explanations for this observation may be: (i) The cells were not homogeneously distributed in the Erlenmeyer flasks. 50 ml aliquots may therefore not contain the necessary amount of target cells. If this point was the only reason, with high probability, also the results should have generated a relatively wide standard deviation at the beginning of the experiment. (ii) Due to incomplete cells lysis during heat treatment the bacterial DNA did not become entirely accessible to Taq-DNA-Polymerase. (iii) Cell envelopes or proteins protected the DNA, thus, DNA became unavailable for PCR. (iv) Other substances were present in the water sample, which inhibited the Taq-DNA-Polymerase (a possible reason when different environmental samples are examined). However, we used the same type of medium throughout all experiments. It is extremely unlikely that at some time-points inhibitory substances are present and at others not. Point (ii) and to a lesser extent point (iii) are the probable causes for the observed variability. This leads to the conclusion that at concentrations close to the detection limit, our direct PCR protocol is less robust than the culture method which it was compared to.

The cell suspension in PBS (third "history") was rather "ill-defined". *E. coli* had been grown in LB medium to the stationary phase, was then collected by centrifugation and re-suspended in a 10 times smaller volume of PBS than the initial LB culture. Subsequently the suspension was stored in the refrigerator (without shaking) for 3 months. The development of the number

and/or the state of the cells in the suspension was not monitored. Before diluting the cell suspension in drinking water, the number of viable cells had been estimated by plating dilution series on agar plates with subsequent cultivation. Then, the microcosms were spiked with an estimated amount of around 4000 cfu/50 mL. The amount of DNA in the samples that could be amplified was very high compared to the other two “history” experiments with cells from the chemostat and from the batch culture with exponentially grown cells. The viable cells originating from the cell suspension in PBS showed a “normal” behaviour in the drinking water microcosms. Their number of colony forming units decreased relatively quickly. The decrease was not as fast as in the experiments with exponentially grown cells but faster than in the experiments with slowly grown cells from the chemostat. Previous studies on the survival of *E. coli* or other enteric bacteria used at least 35 times higher cell densities than we used in this study. For example, Kerr *et al.* (1999) observed a slower decrease of *E. coli* cells in mineral water with a high cell number ( $10^6$  /ml) as compared to a 1000 times lower initial cell density. With our low cell densities, we did not notice any concentration effect. However, we noticed that the reduction rate, which was derived from the curve obtained by the culture method (Figure 3.1 to 3.3 and 3.4 upper lane), was dependent on the history of the spiked cells. Similar history dependent results were reported also by others. García-Lara *et al.* (1993) tested two groups of *E. coli*, one adapted to seawater, the other not. After being exposed to seawater, the non-adapted cells became faster non-culturable than the adapted cells. By staining the intracellular DNA with acridine orange no significant difference could be seen between the two *E. coli*-groups. Dupray and Derrien (1995) incubated enteric bacteria in tryptic soy broth (TSB), wastewater and treated wastewater prior to inoculation into seawater. Survival was found to be better when the cells had previously spent 24 h to 48 h in raw and treated wastewater.

During incubation, autochthonous bacteria grew in all our experiments. This is a common phenomenon for non-sterile and stored ground water (Leclerc *et al.*, 2002). We obtained no evidence that the autochthonous microflora had an influence on the amount of *E. coli* DNA. One could have speculated that the other living organisms would digest remnants of dead *E. coli* cells. But not even the added pancreatic DNase I was able to significantly reduce *E. coli* DNA. It can therefore be excluded that starved and/or dead cells would lyse and release their cytoplasmic content into the surrounding environment, as suggested in former studies

(Bogosian *et al.*, 1998). We assume that most of the cells remained intact and kept their chromosomal DNA protected within the cell envelope. The DNA of the cells incubated in drinking water is maybe protected against DNase digestion by proteins, also when the cells are in the non-culturable or even dead state.

The data from the PCR method differed from the standard culture method for *E. coli* incubated for a long time in drinking water. While with prolonged incubation in drinking water the cells become non-culturable, their genome is still detectable even with a DNase digestion step prior to PCR. The connection of non-culturable bacterial indicators and possible harm in drinking water is still matter of discussion. Since the PCR method has the potential to detect contamination of drinking water with *E. coli* dating back for several days or weeks, it may provide quality information that is of importance particularly for natural resources. The PCR protocol (including all the sample preparation) proved to reach almost the same low detection limit as the culture method. It was possible to keep the handling steps of sample preparation for the direct PCR method at a minimum. The PCR method could be adapted (by primer design) to specifically detect other gram negative bacteria of interest in water samples such as *Legionella*.

#### 4. Direct PCR method to detect *Legionella* in water samples

##### Abstract

The presence of pathogenic *Legionella* strains in man made water systems is a concern of the industrialised, modern society. The standard detection method based on growth on agar plates takes up to 10 days because *Legionella* cells grow relatively slowly. Therefore a faster alternative method would be desirable. Here, the PCR based method to detect *Escherichia coli* in drinking water described in chapters 2 and 3, was adapted to detect *Legionella spp.* and *L. pneumophila* in water. Primers for the PCR were chosen from literature and partially re-designed. The PCR method to detect *L. pneumophila* is specific in comparison to the standard culture method but not very sensitive. For the detection of *Legionella spp.* the PCR method is neither very specific nor sensitive. This is probably due to divergence of the detectors, i.e. the specific sequence on the 16S rDNA in the PCR and the phenotypic classification in the culture method.

##### Introduction

Warm and stagnant water systems became common components of our civilised infrastructure. As a result, Legionnaires disease emerges among modern societies, especially when numbers of immune compromised people increase. In most of the industrialised countries, the agent of Legionnaires disease, the gram negative bacterium *Legionella* is monitored in the those water systems which provide a potential infections risk (hot water systems for shower, whirlpool, cooling towers, irrigation installations, etc.).

The *International Organization for Standardization* (ISO) published a standard method to detect *Legionella* in water samples (ISO 11731, 1998) and a variation of this method for purified water with low back ground flora and the expectation of low *Legionella* concentration (ISO 11731-2, 2004). The samples are pre-treated and the cultivation is performed with and without selective supplement to enhance the chance of obtaining the highest possible number of *Legionella* colonies. The incubation takes up to 10 days. The colonies are further analysed with confirmation tests. As *Legionella* grows relatively slowly on agar plates, several researchers developed faster, alternative detection methods based on polymerase chain reaction (PCR). Target sites were the genes for 16S rRNA (Miyamoto *et al.*, 1997), 5S rRNA (Matsiota-

Bernard *et al.*, 1994) and the *mip* gene (Wellinghausen *et al.*, 2001). Mip (macrophage infectivity potentiator) is a surface protein and represents a virulence factor that is necessary for optimal intracellular survival of *Legionella* in macrophages and protozoan hosts (Heuner *et al.*, 2002). Different quantitative PCR methods have been developed and are commercialised as test kits with more or less elaborated validations on environmental water samples (Yaradou *et al.*, 2007). The European Working Group for Legionella Infections (EWGLI, <http://www.ewgli.org>) focuses the various efforts in research on *Legionella* infection, especially in the area of epidemiological studies and travel associated legionellosis and outbreak investigations. In the context of the EWGLI project, a multilocus sequence typing scheme was standardised and validated (Gaia *et al.*, 2005). It allows comparing isolated *Legionella* strains from patients with those of a suspected source. The majority of the Legionellosis infections are caused by *L. pneumophila* serogroup 1.

As experienced in chapters 2 and 3 with the detection of *Escherichia coli* in water, a direct PCR protocol cannot simply replace a standard culture method. Different stress factors for the target bacteria and different sample matrices can interfere with one or the other method and lead to different results. This chapter describes experiences with the same PCR protocol for the direct detection of bacteria in water samples without pre-cultivation, adapted to detect *Legionella*.

## Material and methods

### *Strains and media*

*Legionella* strains were isolated from environmental water samples on selective agar plates during the detection procedure according to the ISO standard (ISO 11731, 1998). Further identification was performed with a latex agglutination test for the identification of *L. pneumophila* serogroup 1, *L. pneumophila* serogroup 2-14 and *Legionella spp.* including selected strains of medical relevance (*Legionella* latex test DR800, Oxoid, UK). The isolated strains are listed in table 4.2.

The selective medium to isolate *Legionella* is described in detail in the ISO standard. A basic and a selective medium were used. The basic medium was the buffered charcoal yeast extract agar medium (BCYE, Oxoid). The selective BCYE medium was supplemented with glycine and the antibiotics polymyxin, vancomycin and cycloheximide (GVPC, supplement SR152,

Oxoid).

#### *Isolation of DNA*

Presumptive *Legionella* strains were isolated on BCYE plates. Single colonies were suspended in 100 µl of sterile, deionised water. Isolation of DNA was performed with the Easy-DNA™ Kit (Invitrogen-LifeTechnologies, Basel, Switzerland)

#### *Isolation and detection of Legionella by culture method (ISO 11731, 1998)*

Water samples were centrifuged at 6000 x g for 10 minutes. The sediment was re-suspended in a tenth of the original volume. Two selective treatments were performed: heat treatment and acid-treatment. During the heat treatment, the concentrated sample was incubated at 50°C for 30 minutes. The acid treatment comprised incubation at pH 2.2 for 5 minutes at room temperature. Therefore, a tenth volume of a solution containing 0.27 M H<sup>+</sup>, 1.73 M K<sup>+</sup> and 2 M Cl<sup>-</sup> (pH 2.2, adjusted with KOH) was added.

Of the untreated, concentrated sample, 100 µl was plated onto a GVPC plate (BCYE agar (Oxoid) with added GVPC selective supplement, SR152, Oxoid). The treated parts of the sample were each plated onto a BCYE and on a GVPC agar plate (5 plates per sample). The plates were incubated at 37°C for 10 days with examination of the plates every second day. For confirmation, the colonies were tested for growth on BCYE and sheep blood agar plates (Milian). Further typing was performed with the *Legionella* latex test (Oxoid) for the *L. pneumophila* serogroup 1 and serogroups 2-14 and seven other *Legionella* species. Identified *Legionella*-colonies were counted as colony forming units per ml (cfu/ml).

#### *Detection of Legionella by the nested PCR protocol*

Two ml of water sample were concentrated by centrifugation at 6000 x g for 10 min. One ml of the supernatant was discharged and the sample centrifuged again at the same conditions as before. Another 990 µl was removed and the remaining 10µl were homogenised and transferred to the PCR tube containing 93 µl of DNase incubation buffer (5 mM Mg<sup>2+</sup>, 2.5 mM SO<sub>4</sub><sup>2-</sup> and 5 mM acetate, pH 7).

To examine a water sample of 100 ml, the sample was concentrated by filtration. Polyvinylidene fluoride filters with pore size of 0.45 µm, 13 mm in diameter (HVLP01300, Millipore, Bedford MA) and autoclaved in purified water, were placed in a gas flame sterilised

stainless steel filter holder (Millipore, Bedford MA). The water sample was filtrated through this filtration device. The filters were removed and transferred to PCR tubes containing 103  $\mu$ l of DNase incubation buffer and were further treated the same way as the samples concentrated by centrifugation.

Thirty-six units of DNase I, grade II (Roche) were added and the tubes vortexed. Two times 15 minutes incubation at 37°C followed, with a short vortex-step after the first 15 minutes. The DNase was inactivated by heating the tubes to 98°C for 15 minutes. To make the DNA completely available for the PCR, a tenth of the volume in the tube of a lysis-buffer (10 mM Tris, 1 mM EDTA, 1% Triton-X-100) was added and another incubation step at 98°C for 15 minutes was performed. Reagents for the PCR were added according to the recommendation of the supplier of the Taq-DNA-Polymerase (Sigma-Aldrich, Missouri, USA). For the first PCR round the final concentration of  $Mg^{2+}$  was 5 mM, 1 mg/ml BSA, 0.2 mM dNTP each base, 0.03 Units/ $\mu$ l Taq-DNA-polymerase, 0.4  $\mu$ M forward and reverse primer. A tenth of the final volume of the 10-times concentrated PCR-buffer was added. The second round was performed in a volume of 20  $\mu$ l with the same concentration of the PCR reagents, except, that the  $Mg^{2+}$  concentration was only 1.5 mM, the BSA was omitted and 1  $\mu$ l of the suspension resulting from the first run was transferred to the second run reaction. The cycling was performed in a TECHNE-Progene-thermocycler (Witec AG, Littau, Switzerland). The first run started with 2 minutes initial denaturation at 94°C, followed by 25 cycles of 30 seconds at 94°C, 1 minute annealing temperature according to the primer pair (table 4.1) and 1 minute extension at 72°C and closed with a final extension step at 72°C for 7 minutes. The second run was performed accordingly, except that the annealing temperature was 62°C and the extension time in the cycles was only 30 seconds. The PCR products were loaded on a 2% agarose gel, stained with ethidium bromide. One kb Plus DNA ladder (Invitrogen) was used as molecular weight marker.

Table 4.1: Primer used for PCR

primer	sequence (5'-3')	length of the amplicon	annealing temperature	source
<i>mip</i> , forward, 1. run	tcattagctacagacaagga-taagttg	581 bp	55 °C	Primer3
<i>mip</i> , reverse, 1. run	gtttcatttgggccaataggt			
<i>mip</i> , forward, 2. run	catgcaagacgctatgagtg	403 bp	60°C	(Casaulta Aebischer <i>et al.</i> , 1999)
<i>mip</i> reverse, 2. run	caagttgatccagctggcat			
16S, forward, 1. run	aagattagcctgcgtccgat	654 bp	63°C	(Miyamoto <i>et al.</i> , 1997)
16S, reverse, 1. and 2.run	gtcaacttatcgcgtttgct			
16S, forward, 2. run	gagggttgataggttaagagc	430 bp	62°C	

### *In silico* design and testing of primer

The primers used were as described elsewhere (Miyamoto *et al.*, 1997; Casaulta Aebischer *et al.*, 1999) or were designed with the help of the software Primer3 (Release 0.2, Rozen *et al.*, 2000) using the *mip* gene of *L. pneumophila*, GeneBank accession number S42595 (table 4.3).

All primers targeting the *mip* gene were tested with the BLAST algorithm (Altschul *et al.*, 1997) of the NCBI website on there available databases with the limitation to Bacteria and the parameter set for shorter, nearly exact matches (BLASTN 2.2.16, 2003, <http://www.ncbi.nlm.nih.gov/BLAST/>).

## Results

### *Specificity of primers tested on DNA of Legionella strains, isolated from environmental samples*

Presumptive *Legionella* spp. colonies that were isolated from environmental samples, were subcultured and the DNA was extracted and purified. Table 4.2 shows an overview of the iso-

lated *Legionella* strains.

Table 4.2. *Legionella* strains isolated from environmental samples

strain number	identification	origin of water sample
239 A	<i>Legionella spp.</i> <sup>a</sup>	treated rainwater for irrigation (ozonisation, sand filtration, UV-desinfection)
240 A	no <i>Legionella</i> strain <sup>a</sup>	treated rainwater for irrigation
RII A1	<i>L. pneumophila, sg 1</i> <sup>b</sup>	test material for ring trial
RII B1	<i>L. pneumophila, sg 2-14</i> <sup>b</sup>	test material for ring trial
644 D	<i>Legionella spp.</i> <sup>a</sup>	treated bathing water (ozonisation, sand filtration)
654 A	<i>L. pneumophila, sg 2-14</i> <sup>b</sup>	warm water of shower
654 B	<i>L. pneumophila, sg 2-14</i> <sup>b</sup>	warm water of shower
655 A	<i>L. pneumophila, sg 2-14</i> <sup>b</sup>	warm water of shower
656 A	<i>L. pneumophila, sg 1</i> <sup>b</sup>	warm water of shower
656 B	<i>L. pneumophila, sg 1</i> <sup>b</sup>	warm water of shower
657 D	<i>L. pneumophila, sg 1</i> <sup>b</sup>	warm water of tab
685 B	<i>Legionella spp.</i> <sup>a</sup>	warm water of shower

<sup>a</sup> Isolated strains from environmental samples were classified according to the standard detection procedure (ISO 11731, 1998) based on the ability or failure of growth on sheep blood agar without L-cystein.

<sup>b</sup> Further identification of *Legionella* strains with the *Legionella* latex test kit (Oxoid)

The extracted DNA was subjected to PCR in order to test the specificity of the primers used in this study. Eight out of the 12 strains produced positive PCRs resulting in bands of the expected size as shown in an ethidium-bromide stained agarose gel in Figure 4.1. However, additional unspecific bands were detected. They are marked as "unspec." in figure 4.1. The four negative strains 239 A, 644 D and 685 B had been classified according to the standard method as *Legionella spp.* based on physiological properties (failure of growth without L-cystein).

There was no positive agglutination with the *Legionella* latex test with these strains. The PCR was negative with the *16SrDNA*- and the *mip*-primer. The two bands in the lane of sample 685 B of the first round of the *16SrDNA*-PCR and of sample 644 A of the second round are unspecific.

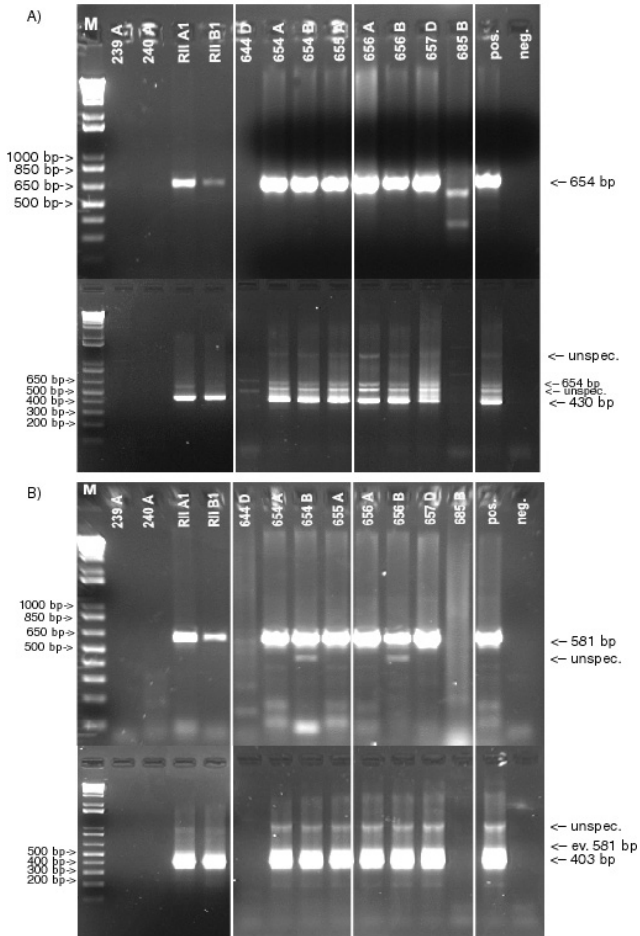


Figure 4.1: Ethidium-bromide stained agarose gel with PCR results of isolated DNA of the strains 239 A to 685 B (listed in table 4.2). Panel A) PCR with the primer set targeting the *16SrDNA*, upper part: first PCR round, lower part: second PCR round. Panel B) PCR with the primer set for the *mip* gene (analogously). The bands of the marker are explained with arrows on the left side of the panel and the bands of the PCR products on the right side. M = 1 kb Plus DNA marker; unspec. = unspecific band; pos. = positive control; neg. = negative control.

*Specificity of primers targeting the mip gene tested in silico*

In table 4.3 the BLAST hits can be inferred in detail. The best score was reached with the primer *mipF1*: 52 hits of 100% agreement with the sequence of the *mip* gene of *L. pneumophila*. Also three 100% hits with other *Legionella* (*L. worsleiensis*, *L. fairfieldensis* and *Tatlockia micdadei*) were obtained. Two sequences stored in the database of *L. pneumophila* had one mismatch compared to the sequence of the primer *mipF1* (*L. pneumophila* sg 5, accession number AF022319 and *L. pneumophila* strain ATCC 33216, accession number AF095229). The primer *mip R1* produced twenty eight 100% hits with *L. pneumophila* strains and also 2 with *Tatlockia micdadei* and an *Amoeba proteus* symbiotic bacterium. The stored sequences of the *mip* genes of *L. pneumophila* in the database did not provide for all hits obtained with the primer *mipF1* a corresponding hit for the primer R1. The inner primers F2 and R2 produced also 52 and 51 hits, respectively. Whereas the BLAST result with the primer F2 showed about the same quality of hits as the primer F1, the inner reverse primer R2 seemed to have a mismatch in 11 of the 51 hits. The missing corresponding sequences in table 4.3 had more than one mismatch and were therefore not hit by the BLAST routine with the set parameters.

Table 4.3: BLAST alignments, NCBI website (BLASTN 2.2.16, 2003)

Accession number	strain	mip F1	mip R1	mip F2	mip R2
AF095230	L. pneumophila strain V. Yu 713	tcattagctacagacaggataagttg 102	gttcattgggccaatagg 640	catgaagaagcctatagttg 202	caagttgaccagctggat 960
AF095228	L. pneumophila strain ATCC 33154	76 tcattagctacagacaggataagttg 102	656 gttcattgggccaatagg 636	183 catgaagaagcctatagttg 202	584 caagttgaccagctggat 565
AF095227	L. pneumophila strain ATCC 33155	76 tcattagctacagacaggataagttg 102	656 gttcattgggccaatagg 636	183 catgaagaagcctatagttg 202	584 caagttgaccagctggat 565
AF095226	L. pneumophila strain ATCC 33215	76 tcattagctacagacaggataagttg 102	656 gttcattgggccaatagg 636	183 catgaagaagcctatagttg 202	584 caagttgaccagctggat 565
AF095225	L. pneumophila strain ATCC 43290	76 tcattagctacagacaggataagttg 102	656 gttcattgggccaatagg 636	183 catgaagaagcctatagttg 202	584 caagttgaccagctggat 565
AF095224	L. pneumophila strain ATCC 43703	76 tcattagctacagacaggataagttg 102	656 gttcattgggccaatagg 636	183 catgaagaagcctatagttg 202	584 caagttgaccagctggat 565
AF095223	L. pneumophila strain V. Yu 1242	76 tcattagctacagacaggataagttg 102	656 gttcattgggccaatagg 636	183 catgaagaagcctatagttg 202	584 caagttgaccagctggat 565
AF095222	L. pneumophila strain ATCC 43823	76 tcattagctacagacaggataagttg 102	656 gttcattgggccaatagg 636	183 catgaagaagcctatagttg 202	584 caagttgaccagctggat 565
AF095221	L. pneumophila strain Edelstein F1480	76 tcattagctacagacaggataagttg 102	656 gttcattgggccaatagg 636	183 catgaagaagcctatagttg 202	584 caagttgaccagctggat 565
AF095220	L. pneumophila strain CDC F920	76 tcattagctacagacaggataagttg 102	656 gttcattgggccaatagg 636	183 catgaagaagcctatagttg 202	581 ...gttaccagctggat 565
AF095219	L. pneumophila strain ATCC 35096	76 tcattagctacagacaggataagttg 102	656 gttcattgggccaatagg 636	183 catgaagaagcctatagttg 202	581 ...gttaccagctggat 565
AF095218	L. pneumophila strain CDC F1053	76 tcattagctacagacaggataagttg 102	656 gttcattgggccaatagg 636	183 catgaagaagcctatagttg 202	584 caagttgaccagctggat 565
AF095217	L. pneumophila strain ATCC 33823	76 tcattagctacagacaggataagttg 102	656 gttcattgggccaatagg 636	183 catgaagaagcctatagttg 202	584 caagttgaccagctggat 565
AF095216	L. pneumophila strain ATCC 43130	76 tcattagctacagacaggataagttg 102	656 gttcattgggccaatagg 636	183 catgaagaagcctatagttg 202	584 caagttgaccagctggat 565
AF095215	L. pneumophila strain CDC F918	76 tcattagctacagacaggataagttg 102	656 gttcattgggccaatagg 636	183 catgaagaagcctatagttg 202	584 caagttgaccagctggat 565
AF023173	L. pneumophila	60 tcattagctacagacaggataagttg 86	167 catgaagaagcctatagttg 186		
AF047750	L. pneumophila sg 16	60 tcattagctacagacaggataagttg 86	167 catgaagaagcctatagttg 186	568 caagttgaccagctggat 549	
AF023336	L. pneumophila sg 1 strain ATCC43108	60 tcattagctacagacaggataagttg 86	167 catgaagaagcctatagttg 186	568 caagttgaccagctggat 549	
AF023335	L. pneumophila sg 1 strain ATCC43109	60 tcattagctacagacaggataagttg 86	167 catgaagaagcctatagttg 186	568 caagttgaccagctggat 549	
AF023334	L. pneumophila sg 1 strain ATCC43107	57 tcattagctacagacaggataagttg 83	163 catgaagaagcctatagttg 183	565 caagttgaccagctggat 546	
AF023333	L. pneumophila sg 1 strain ATCC43110	52 tcattagctacagacaggataagttg 78	159 catgaagaagcctatagttg 178	560 caagttgaccagctggat 541	
AF023332	L. pneumophila sg 1 strain ATCC33153	60 tcattagctacagacaggataagttg 86	167 catgaagaagcctatagttg 186	568 caagttgaccagctggat 549	
AF023331	L. pneumophila sg 1 strain ATCC43113	57 tcattagctacagacaggataagttg 83	164 catgaagaagcctatagttg 183	565 caagttgaccagctggat 546	
AF023330	L. pneumophila sg 1 strain ATCC43106	60 tcattagctacagacaggataagttg 86	167 catgaagaagcctatagttg 186	568 caagttgaccagctggat 549	
AF023329	L. pneumophila sg 1 strain ATCC43111	60 tcattagctacagacaggataagttg 86	167 catgaagaagcctatagttg 186	568 caagttgaccagctggat 549	
AF023328	L. pneumophila sg 14	60 tcattagctacagacaggataagttg 86	167 catgaagaagcctatagttg 186	568 caagttgaccagctggat 549	
AF023327	L. pneumophila sg 13	60 tcattagctacagacaggataagttg 86	167 catgaagaagcctatagttg 186	568 caagttgaccagctggat 549	
AF023326	L. pneumophila sg 12	60 tcattagctacagacaggataagttg 86	167 catgaagaagcctatagttg 186	568 caagttgaccagctggat 549	
AF023325	L. pneumophila sg 11	60 tcattagctacagacaggataagttg 86	167 catgaagaagcctatagttg 186	568 caagttgaccagctggat 549	
AF023324	L. pneumophila sg 10	60 tcattagctacagacaggataagttg 86	167 catgaagaagcctatagttg 186	568 caagttgaccagctggat 549	
AF023323	L. pneumophila sg 9	60 tcattagctacagacaggataagttg 86	167 catgaagaagcctatagttg 186	568 caagttgaccagctggat 549	
AF023322	L. pneumophila sg 8	60 tcattagctacagacaggataagttg 86	167 catgaagaagcctatagttg 186	565 ...gttaccagctggat 549	
AF023321	L. pneumophila sg 7	60 tcattagctacagacaggataagttg 86	167 catgaagaagcctatagttg 186	568 caagttgaccagctggat 549	
AF023320	L. pneumophila sg 6	60 tcattagctacagacaggataagttg 86	167 catgaagaagcctatagttg 186	568 caagttgaccagctggat 549	
AF023318	L. pneumophila sg 4	60 tcattagctacagacaggataagttg 86	167 catgaagaagcctatagttg 186		
AF023317	L. pneumophila sg 3	44 tcattagctacagacaggataagttg 70	151 catgaagaagcctatagttg 170	552 caagttgaccagctggat 533	
AF023316	L. pneumophila sg 2	60 tcattagctacagacaggataagttg 86	167 catgaagaagcctatagttg 186	568 caagttgaccagctggat 549	
AJ496273	L. pneumophila sg 8	76 tcattagctacagacaggataagttg 102	656 gttcattgggccaatagg 636	183 catgaagaagcctatagttg 202	581 ...gttaccagctggat 565
AJ496272	L. pneumophila sg 8	76 tcattagctacagacaggataagttg 102	656 gttcattgggccaatagg 636	183 catgaagaagcctatagttg 202	581 ...gttaccagctggat 565
AJ496271	L. pneumophila sg 6	76 tcattagctacagacaggataagttg 102	656 gttcattgggccaatagg 636	183 catgaagaagcctatagttg 202	584 caagttgaccagctggat 565
AJ496270	L. pneumophila sg 6	76 tcattagctacagacaggataagttg 102	656 gttcattgggccaatagg 636	183 catgaagaagcctatagttg 202	584 caagttgaccagctggat 565
AJ496269	L. pneumophila sg 1	76 tcattagctacagacaggataagttg 102	656 gttcattgggccaatagg 636	183 catgaagaagcctatagttg 202	584 caagttgaccagctggat 565
AJ496268	L. pneumophila sg 1	76 tcattagctacagacaggataagttg 102	653 gttcattgggccaatagg 633	183 catgaagaagcctatagttg 202	584 caagttgaccagctggat 565
AJ496267	L. pneumophila sg 8	76 tcattagctacagacaggataagttg 102	656 gttcattgggccaatagg 636	183 catgaagaagcctatagttg 202	581 ...gttaccagctggat 565
AJ496266	L. pneumophila sg 6	76 tcattagctacagacaggataagttg 102	656 gttcattgggccaatagg 636	183 catgaagaagcctatagttg 202	584 caagttgaccagctggat 565
AJ496265	L. pneumophila sg 1	76 tcattagctacagacaggataagttg 102	656 gttcattgggccaatagg 636	183 catgaagaagcctatagttg 202	584 caagttgaccagctggat 565
ST2442	mip-macrophage infectivity potentiator L. pneumophila	76 tcattagctacagacaggataagttg 102	656 gttcattgggccaatagg 636	183 catgaagaagcctatagttg 202	584 caagttgaccagctggat 565
S42595	mip-macrophage infectivity potentiator L. pneumophila, Philadelphia 1	76 tcattagctacagacaggataagttg 102	656 gttcattgggccaatagg 636	183 catgaagaagcctatagttg 202	584 caagttgaccagctggat 565
U60164	L. worshiensis	62 tcattagctacagacaggataagttg 88		169 catgaagaagcctatagttg 188	570 caagttgaccagctggat 551
U60163	L. fairfieldensis	62 tcattagctacagacaggataagttg 88		169 catgaagaagcctatagttg 188	570 caagttgaccagctggat 551
AJ496274	Tatlockia micadeci	76 tcattagctacagacaggataagttg 102	656 gttcattgggccaatagg 636	183 catgaagaagcctatagttg 202	584 caagttgaccagctggat 565
AJ496381	L. pneumophila sg 6	86 tcattagctacagacaggataagttg 112	666 gttcattgggccaatagg 646	193 catgaagaagcctatagttg 212	594 caagttgaccagctggat 575
AF095229	L. pneumophila strain ATCC 33216	76 tcattagctacagacaggataagttg 102	656 gttcattgggccaatagg 636		584 caagttgaccagctggat 565
AF023319	L. pneumophila sg 5	60 tcattagctacagacaggataagttg 86			568 caagttgaccagctggat 549
U92206	L. gratiana	562 ...gtctacagacaggataagttg 581			
AF108212	Amoeba proteus symbiotic bacterium		766 gttcattgggccaatagg 746		
U92216	L. quateniensis		1012 gttcattgggccaatagg 992		
U92223	L. steigerwaltii		982 gttcattgggccaatagg 963		
U92227	L. brunensis		1051 gttcattgggccaatagg 1032		979 caagttgaccagctggat 960

The specificity of the primer to detect *Legionella spp.* targeting the 16SrDNA was tested by Miyamoto et al. (1997): With 39 *Legionella* strains tested, the primers produced a positive PCR result and with 17 non-*Legionella* strains a negative result.

#### Environmental samples

Environmental water samples from different hot water systems, or technical water systems for air conditioning were analysed with the culture method and the PCR protocol in parallel. With the culture reference method, 1ml was normally examined. The samples were concentrated 10 fold. Without testing and estimating the greater bias and/or different recovery, the PCR method was allowed to be less "sensitive", i.e. the examination volume for the PCR method was 2 ml, not 1 ml as with the culture reference method. The results of the environmental samples are summarised in table 4.4 for *Legionella spp.* and the PCR with primer targeting the 16SrDNA and in table 4.5 for *L. pneumophila* and the PCR targeting the *mip* gene.

Table 4.4: Environmental samples tested for *Legionella spp.*

<i>Legionella spp.</i> , 49 samples analysed		number of samples analysed with the reference colony count method (1ml sample volume)	
		$\geq 2$	$\leq 1$
PCR result, after analysing 2 ml sample volume	+	11 (true positives, TP)	11 (false positives, FP)
	-	4 (false negatives, FN)	23 (true negatives, TN)

Table 4.5: Environmental samples tested for *L. pneumophila*

		number of samples analysed with the reference colony count method (1ml sample volume)	
		$\geq 2$	$\leq 1$
PCR result, after analysing 2 ml sample volume	+	8 TP	3 FP
	-	3 FN	35 TN

The sensitivity and specificity for the PCR was calculated taking the results of the culture method as reference.

For the PCR protocol to detect *Legionella spp.* the sensitivity ( $\frac{TP}{TP + FN}$ ) was 0.73. The

specificity ( $\frac{TN}{TN + FP}$ ) was 0.67.

For the PCR protocol to detect *L. pneumophila* the sensitivity was 0.72 and the specificity was 0.92.

The filtration technique to concentrate a large volume water samples for PCR detection of *E. coli* was also applied to the analysis of *Legionella*. Figure 4.2 shows the result of a dilution series of an environmental sample. The original sample was diluted with sterile deionised water. For each dilution sample, 100 ml was filtrated and further processed in the PCR protocol. Water from this sampling point was analysed with the culture method earlier several times. Therefore the original water sample was known to contain around 30 cfu of *L. pneumophila/ml*.

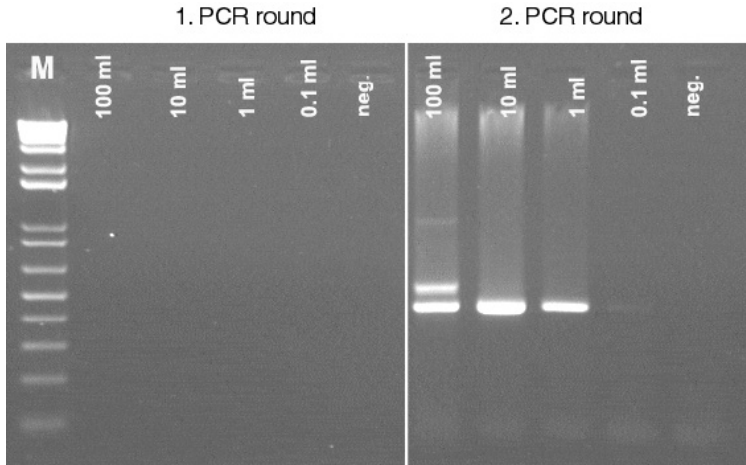


Figure 4.2: Ethidium-bromide stained agarose gel with the PCR results of a dilution series of a water sample containing *L. pneumophila*. After the first PCR round no bands can be detected (lanes 2-6 from the left). After the second round (lanes 7-10) a decrease in band-intensity can be seen corresponding to the decreasing amount of original sample analysed. The culture method detected around 30 cfu/ml. M = 1 kb Plus DNA marker; neg. = negative control.

## Discussion

Analysing environmental samples and comparing the PCR protocol with the according standard culture procedure, a relatively high value for the specificity was reached for *L. pneumophila*. The primer target sequence on the *mip* gene and the phenotypic identification via the antibody agglutination of outer membrane proteins are probably congruent detectors. With the analysed environmental samples and the analysis of *Legionella* we do not face the same problem as with *E. coli* described in chapter 3: Non-culturable *Legionella* cells seem not to be present, causing disagreement between the culture method and the corresponding PCR protocol. The *in-silico* testing of the *mip* primers revealed that they are not completely complementary to all sequences of *mip* gene *L. pneumophila* stored in the databases accessible for the BLAST routine analysis. Especially for the here designed primer *mipR1*, improvement is probably possible in design (e.g. using "wobbles") and the PCR conditions (primer concentrations, annealing temperature).

The specificity value for the PCR protocols to detect *Legionella spp.* is much lower than for

*L. pneumophila*. In comparison to the culture method the PCR protocol often delivered false positive results in the *16SrDNA*-PCR. As the two PCRs were performed in parallel, it is unlikely that problems such as contamination would occur exclusively in the samples of the 16S rDNA-PCR. The negative controls were generally negative. Sometimes unspecific bands of another length appeared in the gel. This experience indicates that it is probably more difficult to find genus specific targets for PCR primers on the *16SrDNA* than on the *mip* gene. Due to a lack of reference material, in our study, we had to rely on strains isolated from the environment and identified by the standard culture method. However, biases in the correct identification of *Legionella spp.* by failure of growth on L-cystein lacking media and cross-reactivity of antigens in serotyping methods can not be excluded (Steinert *et al.*, 2002). Reviewed in (Maiwald *et al.*, 1998), a *Francisella*, *Bordetella* and a *Nocardia* strain were reported to also require cysteine for growth.

The amplification products of the *16SrDNA* was in general weaker than those of the *mip* gene when the two PCRs were performed in parallel with assumingly the same initial amount of DNA. This finding contrasts the considerations of Miyamoto *et al.* (Miyamoto *et al.*, 1997), that there are several targets for *16SrDNA* in a chromosome. The primers targeting the *16SrDNA* might not have a 100% complement within all *Legionella* strains or in several strains the binding place is not well accessible. The first possible explanation was confirmed by a BLAST run performed in the same way as described for the *mip* primers. The sequences retrieved from databases contained more often mismatches and hits for non-*Legionella* strains (data not shown).

The sensitivity values were low and in the same range for *Legionella spp.* and *L. pneumophila*. This indicates that "outer" conditions caused false negative results, like PCR inhibitors in the sample, inefficent concentration or loss of DNA during sample preparation.

The filtration technique used to concentrate *E. coli* for PCR is also applicable for the detection of *Legionella* as shown with the experiment in figure 4.2. The treatment of the sample with DNase before the lysis and the PCR could also have been a cause for the low sensitivity of the PCR. However, we assume that the influence of DNase on the physiological state of the cell is less pronounced with *Legionella* than with exponentially growing *E. coli* (chapter 2). *Legionella* grow generally very slowly. With untreated water samples, the DNase step can probably be omitted without any influence on the outcome.

### Conclusion and outlooks

The genus of *Legionella* includes many species of environmental bacteria. Probably not all species are potentially pathogenic. The species *L. pneumophila* is most often isolated from infected patients. Therefore the presence of *L. pneumophila* indicates potentially higher risk than the presence of not further identified *Legionella* species. The results of the genus-wide detection of *Legionella* are considered in the risk assessment of high risk exposition as in hospitals and nursing homes. For the less critical institutions the detection of *L. pneumophila* (species level) indicates elevated risk (BAG, 2007, <http://www.bag.admin.ch/themen/medizin/00682/00684/01084/index.html?lang=de>).

The detection of DNA from dead cells and unknown inhibitors of the polymerase probably remain a possible problem. However, in this study too few samples have been analysed in parallel with the two methods to gain profound information on the extension of these possible problems. Investigations that use heat or other disinfection to stress or inactivate *Legionella* are needed to obtain clarification on this point.

Molecular methods increasingly play an important role in outbreak investigation and detailed typing of *Legionella* strains. However, the use of molecular methods for primary detection of *Legionella* in water samples is not (yet) widely applied. If molecular methods are applied it is in combination with pre-cultivation.

## 5. Supplementary experiments for method developments

The main goal was the development of a PCR based protocol which is suitable to specifically detect *E. coli* (or other bacteria) in water samples. Additional experiments were performed in order to answer specific questions on mechanisms or to find and evaluate possible improvements. These experiences contribute to a better understanding of the mechanisms and point to draw-backs of the PCR protocol.

### 5.1 How quantitative is the second PCR round with real-time-PCR?

#### Introduction

The standard culture method to detect *E. coli* in drinking water is a quantitative method delivering a number of colony forming units (cfu). In contrast, the PCR protocol is qualitative, providing only the two possible answers "positive" or "negative". However, to visualise the PCR products, they are loaded on an ethidium-bromide stained agarose gel, irradiated with UV light. The thickness and brightness of the bands render the impression of a semi-quantitative result. For this indirect approach to transform the pattern of the agarose gel into numbers, image-analysis tools were used (Romanowski *et al.*, 1993). A newer, more direct technique that is now widely applied is the real-time-quantitative PCR method.

With this technique the amplification process can be monitored. A UV light excitation beam penetrates the reaction tube. In the tube, the DNA is exponentially amplified and a dye staining the double stranded DNA or a dye-system indicates the amount of amplified DNA. The emitted fluorescence of the dye is registered by a CCD camera, transferred to a computer and transformed into a numeric value (Rn). With each cycle the amplified DNA is (theoretically) doubled if the reaction is efficient at 100%. The numeric value of the fluorescence can be plotted against the cycle number and an exponential curve of DNA amplification evolves. From the experiment described in chapter 3 where we incubated exponentially grown *E. coli* cells in drinking water over several weeks, each of the 96 reactions in the second PCR round represent a line in Figure 5.1.1.

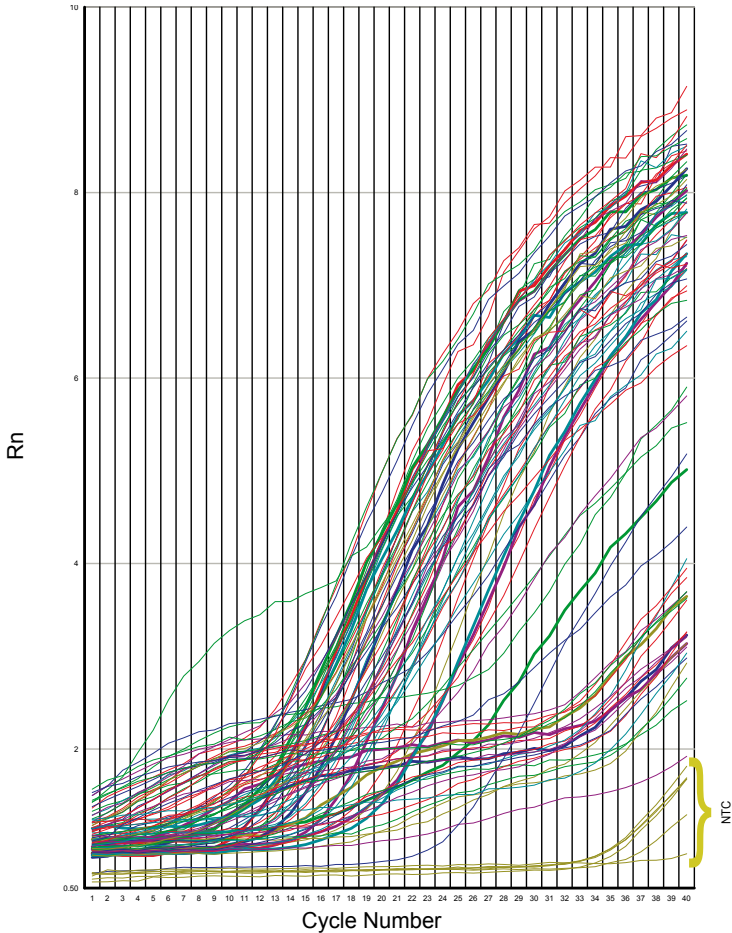


Figure 5.1.1: Second PCR round with real time PCR of the experiment where exponentially grown *E. coli* cells were spiked to drinking water and incubated for several weeks (described in chapter 3). The fluorescence of each PCR cycle monitored by the ABI Prism 7000 Sequence Detection System (Applied Biosystems) is plotted against the cycle number. Each of the 96 PCRs represent a curve in the plot (the no-template-controls (NTC) included).

From the "lag phase" of these exponential curves in the first few cycles, a section for a baseline calculation is (manually) fixed. The average from the fluorescence signal of the baseline section is calculated and regarded as background. The curves in Figure 5.1.1 let explain the

general pattern of a PCR reaction: after the DNA concentration was high enough to surpass the background signal, the amplification becomes expressed as exponential phase. In this phase the amplification should be (almost) 100 % efficient, i.e. the amount of DNA is doubled during every cycle. Towards the end of the exponential phase limitation of either primer, dNTP and/or enzyme activity cause flattening of the curve. With each real-time PCR run, one or several no-template-controls (NTC) should be performed. They indicate potential contamination and help to set the threshold value of fluorescence. The line of the threshold value should not be reached by the curves of the NTCs during all 40 cycles and it should cross the amplification curves of the samples in their exponential phases. In the example shown in Figure 5.1.1, the threshold value was set at 2.2. Then, Ct values are derived from the graphs: The Ct value is the (theoretical) cycle number at which the fluorescence of one reaction reaches the threshold value. The lower the Ct value, the earlier the fluorescence of the stained DNA reached the threshold, the more DNA was initially present in the sample.

This new PCR technique is very attractive not only because quantification of the initial target concentration is possible, but also because the laborious preparation and loading of the agarose gel can be omitted. This saves time and reduces the risk of contamination of laboratory rooms and equipment with PCR products. The enthusiasm for this new possibility is expressed in publications about molecular methods in environmental samples of the last 5 years where in case of PCR mainly real-time PCR is applied. This is also the case if the quantification of the initial target concentration is of minor importance. Frahm *et al.* (2003) for example used the real-time-PCR technique although they did a pre-culture, which distorts the initial target concentration and makes quantification impossible. Others performed quantification experiments with isolated DNA of *E. coli* cells compared to plate counts (Ludwig *et al.*, 2000). These findings can only be used for development of practical routine analysis if the extraction of the DNA is also quantitative and robust.

With our nested PCR protocol, a very low detection limit was reached comparable to the culture method (chapter 2). With this technique the quality of the final PCR result depends largely on the efficiency of the first PCR round. A quantification of the initial target concentration would be possible in the nested PCR, if (i) the first round stops cycling in the "exponential phase" of the amplification curve and (ii) the amplification during the first round is efficient, i.e. no inhibition of the reaction occurs. With this criteria fulfilled an estimation of

the initial target concentration is possible on the basis of a standard curve.

This chapter shows the experiment to generate a standard curve for quantification of the PCR protocol, and discusses the points that have to be considered if a nested PCR protocol with a quantitative second step is developed.

## Methods

*E. coli* strain, growth media, drinking water microcosms and the PCR techniques cited in this chapter are described in detail in chapter 2 and 3.

### *Standard curve for the PCR protocol*

*E. coli* strain ML30 was grown in LB medium to an OD<sub>546</sub> of 0.17. A dilution series in DNase incubation buffer (used in the PCR protocol and described in chapter 2) with 10-times-dilution steps were performed. From each dilution step up to the 10<sup>-5</sup> dilution, 2 µl were transferred to a PCR-tube and the nested PCR was performed according to its protocol. For each dilution step 3 separate tubes were processed as repetitive samples.

As a deviation from the nested PCR protocol, parts of the samples were only amplified during 17 and 20 PCR cycles in the first PCR round, which were compared to the 30 cycles of the standard protocol.

The dilution series of *E. coli* cells was also plated in plate count agar (Biolife, Italy) for exact quantification of the cell number, expressed in colony forming units (cfu).

## Results

### *External standard curve for a quantitative PCR protocol*

The PCR protocol including sample preparation was presumed to be quantitative and linear for a certain range of target cell concentration. On this starting position, we performed experiments to establish an external standard curve for quantification of the PCR protocol.

In the ideal case the whole nested PCR would be nearly 100% efficient and the first PCR round would end in its exponential phase. In this case, in every cycle the target DNA would be doubled which can be formulated as in equation (1):

$$X_n = X_m \cdot 2^{n-m} \quad (1a) \quad \text{or} \quad \frac{X_n}{X_m} = 2^{n-m} \quad (1b)$$

where:  $X_n$  = number of target molecules at cycle  $n$

$X_m$  = number of target molecules at cycle  $m$

For a 10-time-dilution series of the target DNA (whole cells in our case) we can write:

$$X_n = 10 \cdot X_m \quad \text{and divide the equation with } X_m: \quad \frac{X_n}{X_m} = 10.$$

Replacing the left part of equation (1b), we get:

$$10 = 2^{n-m}.$$

This can be resolved for  $m-n$ , which represents the cycle number to reach a 10-fold increase of the target DNA in the PCR:

$$\log_2(10) = n - m = 3.32$$

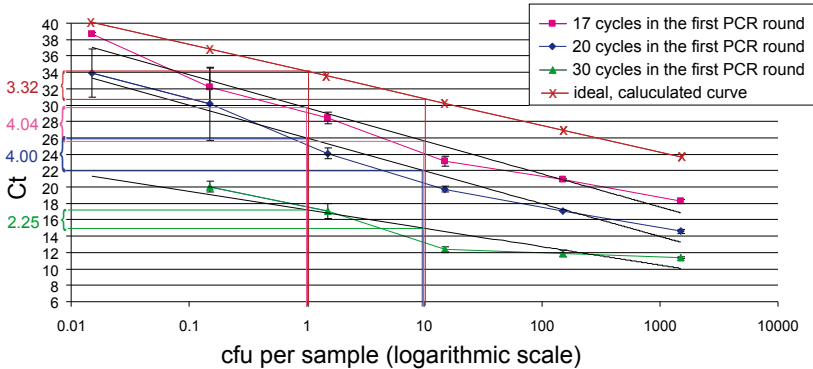


Figure 5.1.2: Standard curves of 10-times dilution series with 3 different cycle numbers in the first PCR round of the nested PCR. The ideal, calculated standard curve indicates the situation, if the first PCR round is linear over the whole range of initial cell concentrations and the second PCR round is 100% efficient.

Figure 5.1.2 shows 10-fold dilution series of *E. coli* cells on the logarithmic x-axis, plotted against their Ct values (The y-axis of the Ct values is not reversed). The slope would be  $-3.3$  for the ideal, calculated standard curve representing 100% efficiency of the PCR. A slope

lower than  $-3.3$  (as in the PCR with 17 and 20 cycles in the first round), indicates that the PCR is less efficient than 100%. In the case of the nested PCR with 30 cycles in the first PCR round, the slope is  $-2.2$ . This "more-than-100%-efficiency" indicates in this context that the first PCR round with its 30 cycles reached the phase after exponential amplification. Limitations of reagents in the reaction tubes occurred and caused decrease in amplification efficiency. This was to an unknown extent more expressed in those tubes with high initial target DNA. The first PCR round with 17 or 20 cycles probably stops in the exponential phase of the amplification. The slopes calculated with all points in the graph in figure 5.1.2 were  $-4.04$  and  $-4.00$  respectively. The points of the lowest cfu should theoretically have been undetermined as they should only contain a target with the probability of 1.5%. Probably the cfu determination underestimated the true presence of target DNA. If these points were excluded from the calculation of the slope, they became  $-3.5$  for the experiments with 17 cycles in the first round and  $-3.8$  with 20 cycles. These slopes are close to the ideal calculated one.

### Discussion

The second step of the nested PCR is much less endangered to inhibition problems than the first round. For the second round possible inhibitory substances are 20 times diluted. With the *uidA* gene as target sequence, it was easy to choose the PCR primer (as recommended by the supplier of the real-time-PCR system). The results presented in this chapter indicate that the sample preparation and the first PCR round harbour the biases of the protocol. This implies that quantification by an external standard curve only becomes possible, if a separate standard curve is performed with every sample matrix and batch of samples. The cells used for spiking should then be possibly in the same physiological state as the cells to be detected and quantified. The nested PCR protocol has – like the standard culture method – an upper limit for quantification. If the target concentration is very high, the first PCR round could end in the limiting phase after exponential amplification, which would impact good interpolation from the standard curve.

The different standard curves in Figure 5.1.2 lead to the conclusion that a low cycle number (from 17 to 20) in the first PCR run would make quantification more precise.

## 5.2 Mg<sup>2+</sup>-content in DNase-incubation buffer

### Introduction

In chapter 2 we found that slowly grown *E. coli* cells used to spike drinking water were better detected with the PCR method than fast, exponentially grown cells. We concluded that the slowly growing cells were more resistant to osmotic stress than the fast growing cells in the batch culture at the maximum specific growth rate. Furthermore, as it was inferred in literature (Azam *et al.*, 1999, Martinez *et al.*, 1997), slowly growing carbon-starved cells harbour Dps, a non-specifically DNA-binding protein, that probably protects the DNA against digestion.) The commercially available pancreatic DNase I uses bivalent cations as activator (as noted in the suppliers instructions). A working concentration of Mg<sup>2+</sup> of 5 mM is recommended. Therefore we designed our DNase incubation buffer with 5 mM Mg<sup>2+</sup>, 2.5 mM SO<sub>4</sub><sup>2-</sup> and 5 mM acetate. In the PCR protocol *E. coli* cells were concentrated by filtration and resuspended in the DNase incubation buffer. Especially for exponentially grown cells used for experimental spiking, the osmotic difference between the nutrient rich LB medium and the incubation buffer was probably stressing.

In this chapter we investigate whether the sensitivity of the *E. coli* cell toward DNase is influenced by the Mg<sup>2+</sup>-concentration.

### Methods

#### *Strain and media*

*E. coli* strain ML30 (DSM1329), was grown in Luria-Bertani (LB) medium at 37°C continuously shaken at 200 rpm to an optical density of 0.1 to 0.5 (exponential phase).

The same DNase incubation buffer as in chapter 2 was used (5 mM Mg<sup>2+</sup>, 2.5mM SO<sub>4</sub><sup>2-</sup> and 5mM acetate, pH7). In addition, 3 new buffers were prepared accordingly with the higher Mg<sup>2+</sup> concentrations of 20, 50 and 500 mM.

Standard method plate count agar (TGY) (BD (Difco), NJ, USA) and sterile Petri dishes were used to enumerate viable bacteria.

#### *Test setup*

PCR tubes containing 30 µl of DNase incubation buffer with a certain Mg concentration were

prepared. The PCR tubes representing the controls without Mg incubation buffer were provided with 30  $\mu\text{l}$  of PBS (0.13 M NaCl, 7 mM  $\text{Na}_2\text{HPO}_4$ , pH 7.2).

Exponentially grown *E. coli* cells were diluted in PBS and 2  $\mu\text{l}$  of the different dilution steps were given to the PCR tubes with the incubation buffer. The final number of *E. coli* cells ranged from 1 to 2400 colony forming units (cfu) per 30  $\mu\text{l}$  per tube. Two  $\mu\text{l}$  of DNase was added to half of the PCR tubes and all the tubes were subsequently incubated at 37°C for 30 min. After incubation, the contents of the PCR tubes was transferred to 10 ml liquid and 48°C warm PC agar in glass test tubes. The test tube was shortly and vigorously vortexed and immediately plated into Petri dishes. The plates were incubated for at least 3 days at 30°C and the (cfu) were counted. All experiments were performed in triplicate.

#### *Mathematical transformation and statistics*

The cfu obtained from the control samples without DNase were normalised to 1 and the relative difference of the according test tubes was calculated. This result was subtracted by 1. Arithmetic mean and standard deviation was calculated from the 3 replicate experiments.

#### **Results**

For each incubation buffer the number of colony forming units with different concentrations and from replicate samples were summated and compared to the reference test tubes without DNase treatment. In figure 5.2.1 the result is plotted as normalised to the reference samples. The values below 0 indicate inhibition of the DNase treatment to the subsequent growth of *E. coli* cells on plate and values  $> 0$  would indicate enhancement in cfu due to the DNase treatment. The samples with the incubation medium 5 mM  $\text{Mg}^{2+}$  showed significantly reduced growth of the *E. coli* cells on plates. With the other incubation media no significant reduction or enhancement was observed.

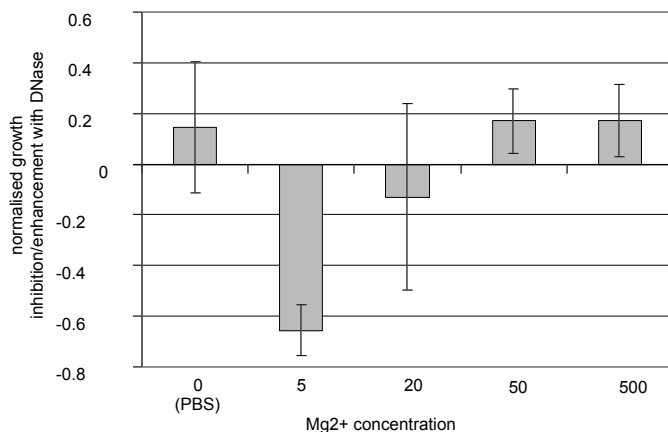


Figure 5.2.1: Normalised difference in number of colony forming units of the DNase treated cells in reference to the non-treated cells. The arithmetic mean and standard deviation of three replicate experiments were calculated.

## Discussion

The samples which were incubated in PBS (with no added  $Mg^{2+}$ ), showed no difference in growth between the DNase treated cells and their references. This lets assume that the sensitivity to DNase is caused by a general osmotic shock of the *E. coli* cell rather than specific lack of  $Mg^{2+}$ . The PBS solution contained optimised salt conditions for bacteria like *E. coli*. The 5 mM Mg-incubation buffer was the lowest in cationic strength that was used in these experiments.

The fact that we observed a reduction also with PCR for the fast grown cells incubated in a buffer with 5 mM Mg and DNase (chapter 2), supports the theory that the cell membrane is damaged to an extent, that DNase can penetrate the cell wall and digest the DNA. The cell and DNA is damaged that far that the cell is not any more able to grow to a colony and amplification of DNA by PCR is not any more possible.

The DNase activity in PBS and higher  $Mg^{2+}$ -concentrations was not tested. However, the supplier recommends PBS as reconstitution solution for DNase and the bivalent cations as "activator". Inhibition of the DNase by too high  $Mg^{2+}$  concentration seems unlikely.

This experiment supported the assumption that fast grown *E. coli* cells are more susceptible to the DNase step during the PCR protocol. To increase the  $Mg^{2+}$  concentration in the DNase

incubation buffer would maybe ease this problem. However, probably a next difficulty would be gained in the following PCR. For the Taq-Polymerase an ideal  $Mg^{2+}$  concentration between 1.0 and 3.5 mM is recommended.

### 5.3 Use of DMPC to degrade DNase after the digestion step

#### Introduction

In chapter 2 it is described that we observed a higher susceptibility of exponentially and fast growing *E. coli* cells to DNase treatment. We searched for a possibility to solve this problem by inactivating the DNase after the digestion step and before the heat treatment of the remaining intact cells. An idea was to use diethyl pyrocarbonate (DEPC) which is effective as nuclease inhibitor. More recently also diethyl pyrocarbonate (DMPC) is used for the same purpose, mainly to produce RNase free solutions for the work with RNA.

With the here described experiment we wanted to investigate the following questions:

- i. Can the addition of DMPC really inactivate the DNase I after the completed job and what would be the optimal concentration of DMPC for the PCR protocol?
- ii. Is a complete inactivation of DNase possible with the heat treatment of the PCR protocol?

#### Methods

##### *Test setup to investigate the optimum concentration for DNase inhibition*

A dilution series in DNase incubation buffer (chapter 2: 5mM  $Mg^{2+}$ , 2.5mM  $SO_4^{2-}$  and 5mM acetate, pH7) with the following concentrations of diethyl pyrocarbonate (DMPC) (Sigma) was prepared: 10%, 1% and 0.1%.

Thirty  $\mu$ l DNase incubation buffer was given to reaction tubes. To 4 tubes 36 units DNase was added according to the PCR protocol described in chapter 2. To one tube DMPC was added to a final concentration of 1%, to a second tube to 0.1% and to a third tube to 0.01%, one tube was processed without addition of DMPC. A fifth tube was used as control without addition of DNase and DMPC (incubation buffer only). All the tubes were incubated for 15

min at 37°C. During this incubation the DMPC was supposed to inactivate the DNase.

DNA (from a former PCR product of the *uidA* of *E. coli*) was added to each tube to a final concentration of around 1 µg per µl. The tubes were again incubated for 15 min at 37°C to test the remaining DNase activity. Thereafter, the tubes were heated to 98°C for 30 min with a vortex-step after 15 min. A second round PCR was performed according to the protocol described in chapter 2 and the PCR products loaded onto an ethidium bromide stained agarose gel and separated by gel electrophoresis.

*Test setup to investigate the inhibitory potential of added DMPC to a subsequent PCR step*

Thirty µl DNase incubation buffer was given to reaction tubes. DNA (from a former PCR product of the *uidA* of *E. coli*) was added to each tube to a final concentration of around 5 µg per µl. The same dilution series of final DMPC concentration was performed as in the test setup described above: The first tube was left without DMPC, to the second tube DMPC was added to a final concentration of 1%, to the third tube of 0.1% and to a fourth tube to 0.01%. The fifth tube was used as control without addition of DNase and DMPC. All the tubes were incubated at 98°C with the intention to decompose the DMPC. DNase was added in the same amount to all tubes except to the control tube, and the tubes were incubated for 15 min at 37°C. Heating followed at 98°C for 15 min, to inactivate the DNase. A second round PCR was performed according to the protocol described in chapter 2 and the PCR products loaded on an ethidium bromide stained agarose gel.

## **Results and Discussion**

In both experiments a second round PCR had to be performed to intensify the effects. Without PCR, only the controls gave a weak band on the gel (not shown).

If the application of DMPC would have inhibited the DNase, bands of amplified DNA should be visible at least in the lanes with 1% and maybe also with 0.1% DMPC. (The working solution recommended by the supplier is 0.1%.)

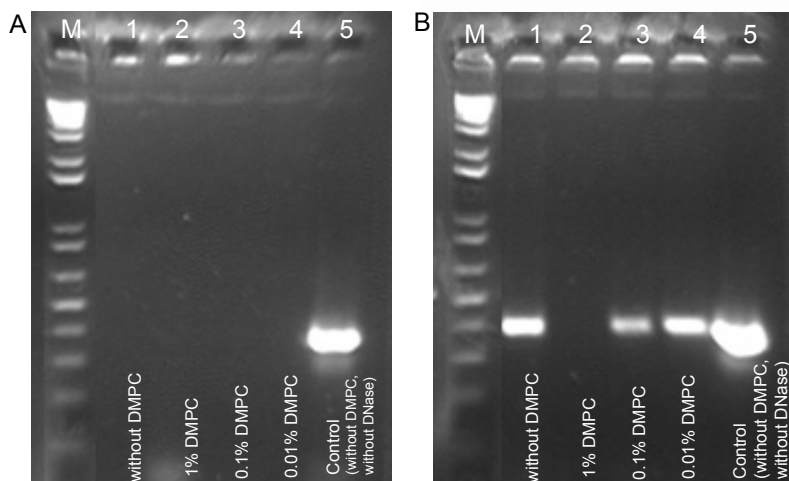


Figure 5.3.1 Ethidium bromide stained agarose gel of PCR after the experiments to evaluate the use of DMPC to inactivate DNase after its digestion step. Panel A: Results of the first test setup: different DMPC concentrations were added during DNase digestion. Panel B: same dilution series with heat-decomposed DMPC added prior to the DNase treatment and subsequent PCR. In lines 5 of the experiments the positive controls without DNase and DMPC treatment were loaded.

However, in figure 5.3.1 panel A, only the control without DNase showed some presence of DNA. That could be interpreted that none of the used concentrations of DMPC was able to inhibit the DNase. DNase was able to digest all the DNA available in the tubes no matter if DMPC was present at the same time (lanes 2-4) or not (lane 1). The other explanation was that the presence of DMPC would inhibit the subsequent PCR.

In the second experiment (figure 5.3.1 panel B) more initial "test" DNA was used than in the first experiment, which is manifested in the thicker, slightly horse-shoe shaped band of the control (lane 5). In this experimental setup the added DMPC was decomposed by heating at 98°C right in the beginning and before the DNase digestion step. Theoretically the DNase should not have been inhibited after this treatment. As the band in line 1 of panel B without DMPC is weaker than the control band in line 5 without any addition of DNase and DMPC, there can be assumed that some DNase digestion took place. In the lane with the highest

DMPC concentration no band was visible after PCR. Only a weak band appeared in the lane where the sample with 0.1% DMPC had been loaded. That strongly indicates that the subsequent PCR was inhibited by increasing amounts of added DMPC.

As no noticeable inhibition of DNase with DMPC was found, and in addition with the use of DMPC an inhibition of the PCR was experienced, the introduction of a DMPC treatment step to the PCR protocol was not further investigated.

## 5.4 Addition of lysozyme for improved sample preparation

### Introduction

In the incubation experiments in chapter 3, some time-points showed decreased PCR amplification. This inhibited amplification in the incubation experiment was manifested in wide standard deviations or no amplification at all at later time-points between 5 and 10 days (experiment with exponentially grown cells, and the experiment with the PBS stored cells). This effect may stem from a changing murein structure during different growth phases of *E. coli* (Signoretto *et al.*, 2002). During the heat-treatment process the membrane probably becomes damaged but the major part of the cells wall stays intact, consisting – after the heat and detergent treatment – mainly of the murein sacculus. For the enzyme Taq-DNA-Polymerase (94kDa) to amplify the target sequence of the chromosome, it has to enter the cell envelope. Vazquez-Laslop *et al.* (2001) obtained results which suggested that the murein layer of *E. coli* serve as 100-kDa cut-off sieve. The holes would be just large enough for the Taq-DNA-Polymerase to penetrate. Signoretto *et al.* (2002) found that *E. coli* cells incubated in lake water at 4°C (they call them VBNC-cells) alter their composition in the peptidoglycan, which becomes more cross-linked and richer in covalently bound lipoprotein. The glycan strand length undergoes a marked reduction. If the cell wall (e. g. the murein layer) becomes more stable and may become a mesh with smaller holes, and as a consequence may partially prevent the DNA-Taq-Polymerase to enter the cell. In later stages of our incubation experiments, the cells were probably in a highly stressed stage or even dead. The murein sacculus may exhibits damages and holes in these stages, which facilitated the Taq polymerase to penetrate the cell wall. This would explain why at later time points of our experiments the PCR method

rendered more consistently positive results with small standard deviations.

In this chapter it was tested if lysozyme could degrade the murein sacculus in a way that the Taq-polymerase would have better access to the DNA in the cell. The experiments show what problems arise if high amounts of another specific enzyme is introduced into the PCR protocol. The experiments gave no additional information to support the above described hypothesis.

## Methods

### *Strain and cultivation*

Strain *E. coli* ML30 was cultivated in LB medium (described in chapter 2) over night. Two ml of this overnight culture was centrifuged (10 min. at 6000 x g) and washed with PBS (described in chapter 2). This procedure was repeated. Subsequently the pellet was re-suspended in 1 ml of PBS. The suspension of bacteria was stored for 2 weeks until use.

### *Working solutions*

DNase incubation buffer, DNase, PBS and TET buffer for heat lysis were used for these experiments. Their compositions are described in chapter 2.

Lysozyme (107255, Roche, Switzerland) was dissolved in water and sterile filtrated to produce a working solution of 20 mg/ml.

Proteinase K solution (15.6 mg/ml) in 10 mM Tris-HCl, pH 7.5 (1413783, Roche, Switzerland) was diluted 10 times in PCR buffer (0.1 M Tris-HCl, 0.5 M KCl, pH 9.1) to produce a working solution of 1.5 mg/ml.

### *Experimental setups*

#### Introduction of a lysozyme treatment step

A dilution series of the PBS stored *E. coli* cells in DNase incubation buffer was performed in 10 time steps. Of each dilution step one sample was used for treatment with lysozyme and another one was processed in parallel as control without lysozyme treatment. Two  $\mu$ l of each dilution step was given to 34  $\mu$ l of DNase incubation buffer. Two  $\mu$ l of DNase solution was added and the digestion of free DNA was performed for 30 min at 37°C. The samples were heated to 98°C for 15 min. After cooling of the samples, 3.8  $\mu$ l of the TET buffer was added and 2  $\mu$ l of the lysozyme solution was added to each sample of the test dilution series. The

samples were incubated at room temperature for 15 min. The samples were centrifuged for 10 min at 6000 x g, and from the supernatant 2 µl were used for PCR. The second round real-time PCR according to the protocol described in chapter 3 was applied.

#### Lysozyme treatment and subsequent DNA separation with Quiagen Genomic Tips

A tenfold dilution series was performed from the PBS-stored cell suspension. For each dilution step from undiluted to the 10<sup>-2</sup> dilution, 4 samples were processed in parallel. From the cell suspension and its dilutions, 35 µl were added to 600 µl of DNase incubation buffer. Two control samples were processed without addition of cell suspension. For each sample 324 units of DNase were added (according to the PCR protocol described in chapter 2), and the samples were incubated at 37°C for 30 min to digest free DNA. After the digestion step the samples were heated at 98°C for 15 min. The lysis buffer TET was added (67 µl per sample) and the samples were again heated to 98°C for 15 min. Two of the 4 samples in parallel were placed on ice and to the other two, 67 µl of the lysozyme working solution was added. The samples with lysozyme were incubated at room temperature for 15 min. Thereafter, the entire volumes of all the samples were placed onto Quiagen Genomic Tips (Quiagen, Basel, Switzerland) to separate free DNA from proteins that could inhibit the following PCR. For this procedure the instructions of the user manual for the Quiagen Genomic Tips was followed. The recovered DNA from each sample was re-suspended in 10 µl water. The recovered DNA was amplified using the second round real-time PCR as described in chapter 3.

#### Degradation of lysozyme with proteinase K (without DNA isolation)

A tenfold dilution series of the PBS stored *E. coli* cells in DNase incubation buffer was made. From each dilution step one sample was used for treatment with lysozyme and another one was processed in parallel as control without lysozyme treatment. Two µl of each dilution step was given to 34 µl of DNase incubation buffer. Two µl of DNase solution was added and the digestion of free DNA was performed for 30 min at 37°C. The samples were heated to 98°C for 15 min. After cooling, 3.8 µl of the TET buffer was added and 2 µl of the lysozyme solution was given to each sample of the test dilution series. The samples were incubated at room temperature for 15 min. To inactivate the lysozyme, 2 µl of proteinase K working solution was added. The samples were incubated first at 37°C for 15 min, then 10 min at 50°C and finally at 98°C for 30 min to inhibit all enzyme activity. The samples were centrifuged for 10 min at 6000 x g. From the supernatant 2 µl were used for the second round real-time PCR according to the protocol described in chapter 3.

## **Results**

In an initial experimental setup, lysozyme treatment was simply introduced to the PCR protocol after the DNase digestion and the denaturation of the DNase by heating to 98°C.

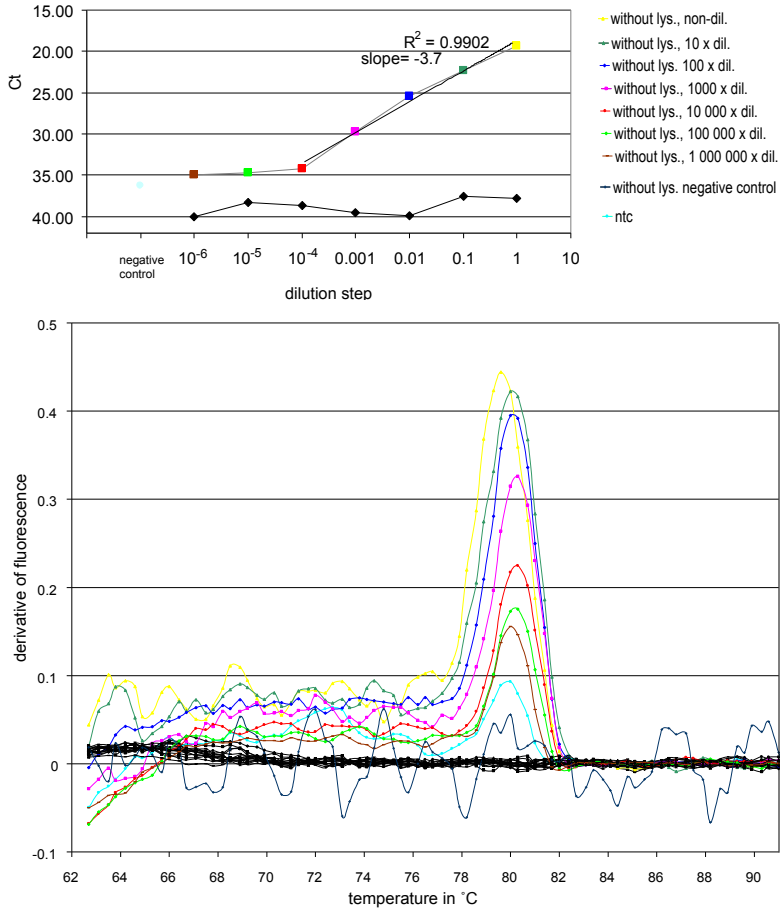


Figure 5.4.1:

Panel A: Ct values obtained by real-time PCR from a dilution series of *E. coli* cells stored in PBS. Before the PCR, one dilution series of samples was treated with lysozyme (diamonds). The control samples were processed in parallel the same way but without lysozyme treatment (squares).

Panel B: Dissociation curves for each amplification product after the completed PCR, plotted as derivatives. The colour of each line corresponds to the Ct value of panel A.

In figure 5.4.1 panel A, the control samples processed in parallel without lysozyme treatment were amplified quantitatively. This is manifested in the well fitted standard curve with a slope of  $-3.7$ , which indicates a theoretically good, almost fully efficient PCR for a 10-times-dilution series plotted on a semi-logarithmic graph. No significant decrease in Ct values with increasing target concentration was observed with the lysozyme treated samples. The dissociation curves (derivative fluorescence Rn) of all these analysed samples (figure 5.4.1, panel B) show the characteristic peaks at around  $80^{\circ}\text{C}$  of the amplified PCR product. However only the samples without lysozyme addition formed the peak. The dissociation curves of the samples with lysozyme treatment remained flat, which indicates that no amplification occurred during PCR.

In a more complex setup a DNA isolation step with Quiagen Genomic Tips was used to separate the released DNA from the PCR inhibiting lysozyme. The Ct plots of the test series and its corresponding control samples without lysozyme treatment show that the lysozyme was indeed removed and the amplification was successful (figure 5.4.2).

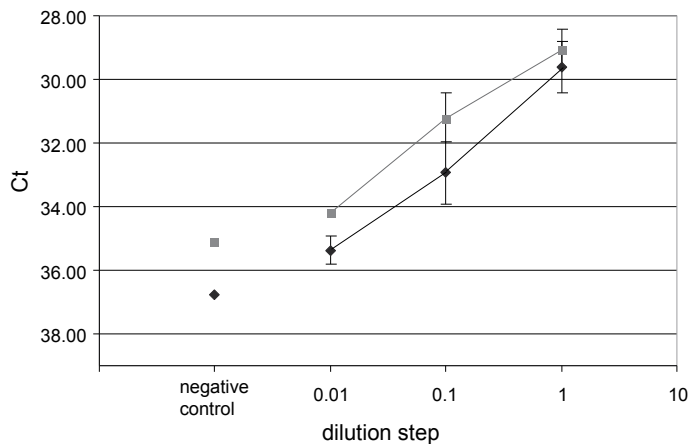


Figure 5.4.2: Ct values obtained by real-time PCR from a dilution series of *E. coli* stored in PBS. The data points with diamonds indicate the results obtained from the preparation protocol with lysozyme treatment and subsequent DNA isolation with Quiagen Genomic Tips. The squares indicate the control series without lysozyme treatment.

However, the samples with lysozyme treatment still showed a slightly lower yield than the control series without initial lysozyme addition.

In the following it was investigated whether the laborious and expensive DNA isolation step could be omitted by adding proteinase K to digest the lysozyme. A pair of larger test series showed that addition of lysozyme led to a distorted "standard curve" (figure 5.4.3). The correlation coefficient  $R^2$  of 0.47 was poor compared to the control series (0.96).

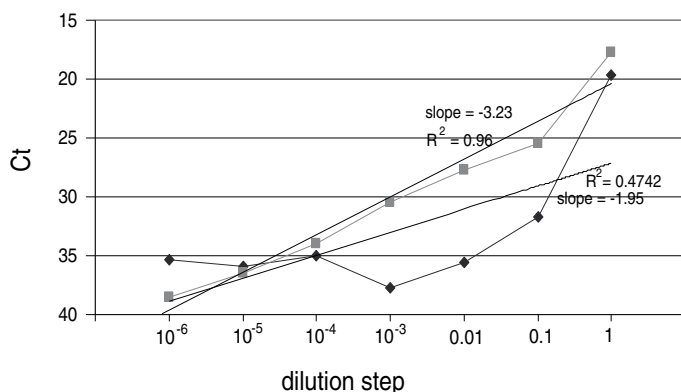


Figure 5.4.3: Ct values obtained by real-time PCR from a 10 times dilution series of *E. coli* stored in PBS. All the samples were treated additionally with proteinase K. Lysozyme was added to the samples indicated with diamonds previously to the proteinase K treatment. The  $R^2$  value indicates the correlation coefficient of the fitted standard curve over the data points of the dilution series.

## Discussion

Some qualitative inhibition of PCR by lysozyme has been seen before, when PCR products after lysozyme treatment were loaded on an ethidium bromide stained agarose gel. No bands were visible on the gel (data not shown). The effect was reproducible with different PCR conditions and DNA staining dyes.

The working conditions for the enzymes were chosen as far as possible according to the supplier's instruction. The recommended working conditions for lysozyme are indicated as 25°C or room temperature, pH7. Lysozyme can be stable up to temperatures of 100 °C in acid solution, however in alkalic environments the activity decreases rapidly at higher temperatures. The isoelectric point is very high at 10.6-10.9. The proteinase K is active in a wide pH range

from 6.5-9.5, 65°C. Rapid denaturation of the proteinase K occurs at temperatures above 65°C. Proteinase K is not inhibited by metal chelating agents such as EDTA. The isoelectric point is 8.9. There is not much literature available on PCR inhibition mechanisms by enzymes and proteins (Lantz *et al.*, 2000). Publications from the food industry described PCR inhibitory effects of proteins with high DNA binding properties (Sitohy *et al.*, 2001). Proteins with a positive charge can form DNA-protein complexes due to the negative charge of DNA. It can be assumed that the high isoelectric point of the lysozyme could have been a cause of inhibition. At the high pH of 9 in the PCR buffer, this protein is still positively charged. Removing the protein by column DNA separation or degradation by proteinase K only decreased the inhibitory effects.

Proteinase K seems not to influence PCR efficiency. Maybe this treatment could be useful to inactivate the DNase after the digestion step of free DNA.

The introduction of lysozyme did not cause the expected higher yield of available DNA for the PCR but seriously increased the risk of inhibition of the PCR. With a relatively simple measure like addition of proteinase K the problem could only slightly be reduced. Only the expensive and work intensive DNA isolation with columns reduced the lysozyme effect to an acceptable degree. The problems being introduced with a lysozyme step seem unreasonably bigger than the ones that should have been solved by this measure.

## 5.5 Bioprecipitation of CaCO<sub>3</sub>

### Introduction

While investigating the performance of the PCR protocol for the detection of *E. coli* in chapter 3, we looked for explanations for the phenomenon of the wide standard deviations of the Ct values at certain time points of the long-time-incubation experiments.

The wide standard deviations were of concern that turned up in the experiments with the exponentially grown *E. coli* released to drinking water after 24h and after 6 to 9 days of incubation. This was most apparent in the microcosms with middle cell number, where the initial *E. coli* number was 100 per 50 ml. At time-points after 24h and 9 days, 1 repetition of the 3 failed to produce a detectable PCR product. The calcium carbonate precipitation onto the cell

surface, thus inhibiting PCR was indicated as a possibility by colleagues working on bioprecipitation (D. Mavrocordatos and M. Dittrich). Precipitation of biogenic calcite is quite common (Braissant *et al.*, 2002) and was also found in association with *E. coli* (Katkova *et al.*, 1994). We speculated that in the hard water used for the incubation experiments, precipitation of calcium carbonate could occur on the surface of *E. coli*. In case of a complete encrustation, the cell may become completely protected from lysis of the cell wall and penetration of the enzyme Taq polymerase. We repeated the experiments with exponentially grown *E. coli* cells to reproduce the result of the loss in detection efficiency of the PCR protocol after 24 h of incubation in water and/or after 2 weeks of incubation. Samples from these time points were also prepared for microscopic inspection.

## Material and Methods

### *Cultivation of bacteria and media*

For the 3 independent experiments in this chapter strain *E. coli* ML30 (DSM1329) was used. It was cultivated in LB medium as described in chapter 3 to an OD of 0.33, 0.4 and 1.1, respectively.

For the first experiment pristine drinking water was used as described in chapter 3. For the second experiment, the source of drinking water was a tap of the drinking water distribution of Schlieren (Switzerland). The water was twice sterile filtrated through 0.2 µm pore size into sterile Erlenmeyer flasks. The water of Schlieren has a hardness of 22-32 fH° (Stadt Schlieren Wasserversorgung, 2007) and a tendency to precipitate calcium carbonate. For the third experiment, drinking water from the distribution net in Dübendorf (Switzerland) (25-26 fH°, Genossenschaft Wasserversorgung Dübendorf, 2001) was sterile filtrated in the same way as in the second experiment.

### *Spiking and incubation of the drinking water*

In the first experiment, cells were grown to the exponential phase in LB medium and then diluted in PBS (described in chapter 3). They were inoculated into the prepared drinking water microcosms in the 2 L Erlenmeyer flasks to an initial concentration of 100 cfu/50 ml.

In the second and the third experiment, the cells were taken from the exponentially grown batch culture, pelleted by centrifugation and washed twice in PBS to remove nutrients from

the batch medium. They were finally re-suspended in sterile filtrated drinking water, diluted and spiked to the Erlenmeyer flasks with the prepared water. In the second experiment, 2 different initial concentrations were prepared, each in triplicate. In the third experiment, 3 different initial concentrations were spiked (but only 1 microcosm was prepared per concentration level).

All microcosms were incubated at 4°C and continuously shaken at 100 rpm.

#### *Detection and enumeration*

At different time-points during incubation, samples were taken to analyse in parallel with the culture method and the PCR protocol described in chapter 3.

#### *Preparation of samples for microscopy*

Fifty ml of sample was concentrated by filtration through 0.4 µm Isopore membrane filters (HTBP01300, Millipore). Before the last ml of the sample was filtered through the membrane, the vacuum was relieved. 10 µl of SYBR Green I (Molecular Probes, Leiden, Netherlands) stock solution was diluted in the water that covered the filter. After 15 min. of incubation at room temperature in the dark, vacuum was applied again and the remaining water was removed. The filter was transferred onto a slide, mounted in Citifluor™ (CITIFLUOR LTD, London UK) and examined with a fluorescence microscope (Olympus, Japan) connected to a CCD-camera.

### **Results**

In 3 independent experiments which were performed at different time periods, a decrease in PCR efficiency occurred after 24 h of incubation of the *E. coli* cells in the water microcosms in all 3 experiments. Table 5.5.1 summarises the 3 experiments. The error bars in the graphs indicate the standard deviation of 3 replicates. (In the last experiment shown in Table 5.5.1, only 1 microcosm per cell concentration was run, therefore no standard deviation could be calculated.) The replicates from three individual microcosms were analysed at the same time. The tap water used for all microcosms contained high amounts of carbonates.

Table 5.5.1: Ct curves of the incubation of exponentially grown *E. coli* cells in drinking water.

Experimental conditions		Ct values of PCR protocol over the time of incubation in water
Water used for the incubation	tap water (Dubendorf)	
Initial concentration	100 cfu/50 ml	
Water used for the incubation	sterile filtered tap water (Schlieren)	
Initial concentration	$10^5$ and $2.5 \cdot 10^4$ cfu/50 ml, respectively	
Water used for the incubation	sterile filtered tap water (Dubendorf)	
Initial concentration	8800, 880 and 88 /50 ml, respectively	

Aliquots of samples at some of the investigated time points were stained with SYBR Green. Figure 5.5.1 shows a picture after 24 h of incubation in the drinking water of Schlieren. Cells containing stained DNA are visible. No evidence of bioprecipitation is seen.

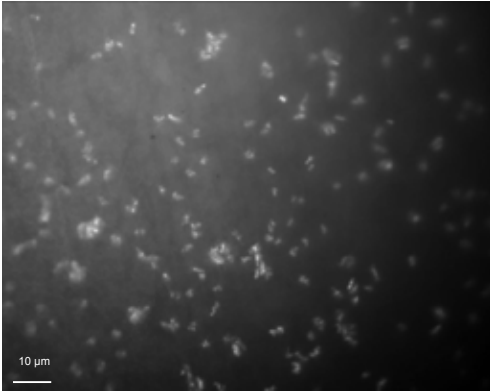


Figure 5.5.1: *E. coli* cells after 24 h incubated in very hard drinking water (second experiment) stained with SYBR Green and examined with the fluorescence microscope.

After 24 days, another sample was prepared with a nucleic dye and examined with the fluorescence microscope. The picture in Figure 5.5.2 was taken while the object was excited with UV light. The cells are still discernible containing the stained DNA. The morphology of the cells did not manifestly change after this relatively long incubation time.

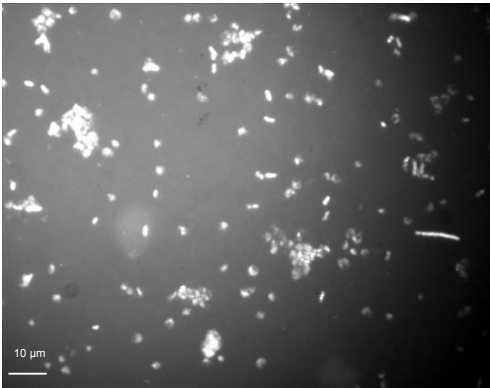


Figure 5.5.2: *E. coli* cells after 24 days of incubation in drinking water and examined with the fluorescence microscope.

After adding white light, crystals of calcium carbonate became visible in the sample after 24 days of incubation (Figure 5.5.3). In the negative control without addition of bacteria, the calcium carbonate precipitated about as well as in the samples with bacteria (not shown). This indicates no active involvement of added *E.coli* cells to calcium precipitation.

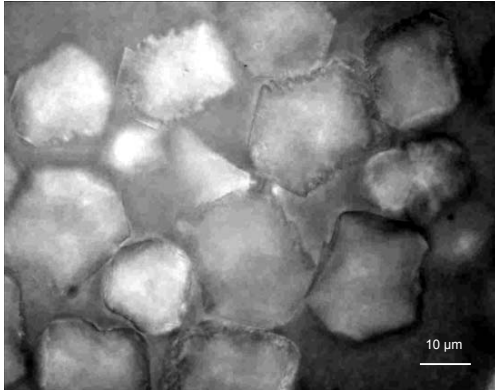


Figure 5.5.3: Calcium crystals in hard drinking water with added *E. coli* cells after 24 days of incubation, microscopically examined with white light and UV light.

### Discussion

The drinking water used as medium for the long term incubation was over-saturated with carbonates, which precipitated in course of the experiment. If the bacteria provided surfaces to initiate precipitation, it was not possible to show it with these experiments. The microscopic inspection indicated rather, that the precipitation occurs later than 24 h after the inoculation. As the negative control showed similar precipitation behaviour, the *E. coli* cells probably play a minor role.

The increase of the Ct value (decrease of available target DNA or amplification efficiency) after 24 h remains an enigma. However, to exclude artefacts or uncontrolled coincidences more repetitive experiments should be performed to confirm the observed phenomenon.

## 5.6 Inhibition of the PCR by substances in water from the effluent of a waste water treatment plant

### Introduction

In most of the reports about the use of PCR to amplify specific DNA in a complex sample of whatever matrix, the problem of inhibitory substances is mentioned. However, there is little knowledge on the exact mechanisms and detailed classification of such substances that inhibit the PCR (Lantz *et al.*, 2000).

The problem of inhibitory substances was also considered here, in the development of our direct PCR protocol for drinking water samples. In the PCR protocol designed for the detection of *E. coli*, the sample is concentrated by filtration through a filter with 0.45  $\mu\text{m}$  pore size. We expected that the dissolved compounds and the ones that are smaller than 0.2  $\mu\text{m}$  would have passed the mesh of the filter which would reduce the risk of inhibition of the following PCR.

Here an experiment is described, to explore the risk of inhibitory substances to the PCR protocol to detect *E. coli* in water samples. Therefore we selected strongly polluted water that contained (natural) *E. coli* cells as well as various kind of environmental substances with the potential to inhibit the PCR. An extended discussion including experiences described in literature and from other chapters of this work is given to develop a possibly realistic model of the inhibition problems occurring in our PCR protocol.

### Methods

Water was taken from the effluent of the local waste water treatment plant. An aliquot of the sampled water was sterile filtrated twice through a sterile membrane filter, 0.45  $\mu\text{m}$  in pore size and 47mm in diameter (HAWG047S1, Millipore, Bedford MA). The non-filtrated waste-water was diluted in tenfold steps with the sterile filtrated waste water. From these samples, 50-ml-aliquots were analysed with the classical culture method, as described in chapter 3 and with the PCR protocol in parallel, as described in chapter 2.

### Results

The PCR results are shown in figure 5.6.1 as bands in the ethidium bromide stained agarose gel. Triplicate analysis was performed for each dilution step of the raw waste water. The according result obtained with the culture method is indicated below each lane.

In the raw, non-filtrated waste water sample very weak bands were distinguishable on the gel, at least in 1 sample of the triplicate. With the PCR protocol, lucid evidence of present *E. coli* was only produced in the 100-times-diluted raw waste water (lanes 7 to 9 in figure 5.6.1).

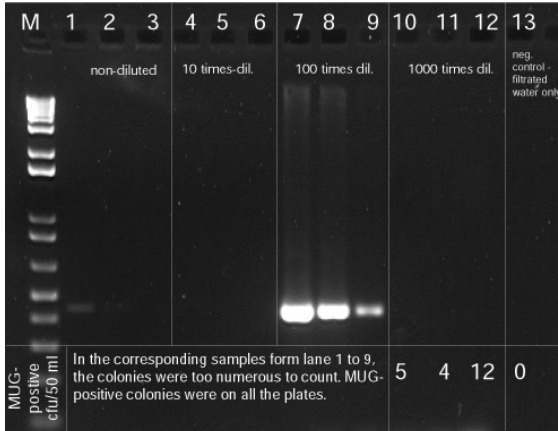


Figure 5.6.1: Ethidium bromide stained agarose gel with the PCR products of the samples that had been prepared with waste water effluent. Non-treated waste water was diluted in the same water that had been previously sterile filtrated. The corresponding results to the sample in each lane performed with the culture method is indicated in the lower part of the figure. See text for the detailed experimental setup.

In samples with high bacteria concentrations from the raw waste water effluent, no single colonies could be differentiated on the plates because they were densely overgrown. However, among the cultures on the plates, MUG positive fluorescence was discernible, indicating the presence of *E. coli* in the sample.

The negative control was performed with the twice sterile filtrated waste water only. The negative result indicates that in the prepared water for the experiment the filtration had successfully removed the initially present *E. coli* cells.

## Discussion

This experiment indicates inhibition effects on the PCR protocol due to inhibitory substances in the water sample. In the non-diluted, pristine waste water a high concentration of *E. coli* was expected which was confirmed by the culture method. With the analysis of the culture method, we estimated that at least 7000 *E. coli* cells were present per 50 ml. However, the

bands which showed the according PCR results (lanes 1 to 3 in figure 5.6.1), were very faint. Probably also the concentration of inhibitory substances were high in these samples. In the samples of 10 time diluted raw water, the inhibitory substances seemed to suppress the PCR activity in its detection of the present *E. coli* DNA. Interestingly, in the next dilution step, where the number of *E. coli* cells per 50 ml samples was only about 70, inhibitory effects were not anymore that evident. Probably the inhibitory substances were diluted to an extent, were they became more unlikely to interfere. Probably in line 9, interference was responsible for the fainter band. According to the experiments that estimated the detection limit in chapter 2, a mean cell number of 7 per sample should be detectable by the PCR protocol. In this example, however, none of the three replicates delivered a positive band. Eventually, weak inhibition is effective enough in case of low target concentration to return a false negative result. With the dilution of the particles in filtrated waste water, also the inhibition effect to PCR seems to become reduced. This let us conclude that the main inhibition potential is associated to particles larger than 0.45  $\mu\text{m}$  and much less associated to diluted compounds in waste water.

When we started practising our PCR protocol on environmental samples, the prominent problems of inhibition of the PCR arouse. Also in our case we were only able to describe the observations. The very reason of an inhibition event remained speculative. Probably for different situations also different mechanisms of inhibition played a role. In the following, the assumed relevant mechanisms for our PCR protocol and drinking water as matrix are summarised, using the classification of Wilson, (1997).

#### *Failure of lysis*

The cell lysis procedure by adding TET buffer and heating the sample was chosen because, it was evaluated and published (Goldenberger *et al.*, 1995) and it was possible to continue with the PCR in the same solution. However, as observed with the microscope (chapter 3), probably some components of the cell wall, especially the murein layer, stayed more or less intact. It is imaginable, that in certain situations the lysis could remain incomplete in a way that the Taq polymerase could not access the DNA. Presumably, such situations occur during starvation. However in the experiments in chapter 5.4 where we tried to improve the passing of DNase through the murein layer with lysozyme, we could not confirm that hypothesis. The hypothesis that precipitation of calcium could protect and hide the target bacterium from lysis

and access of the Taq polymerase, appeared to be worth exploring. However, the experiments to substantiate this hypothesis failed (chapter 5.5). Among the different environmental samples from geologically different regions also water quality and components in the water differed. Particles were present in some drinking water samples, especially from sources which probably deliver not long filtrated and deep ground water. It could be possible that cells were attached to such particles, eventually in a way that they were protected from the lytic conditions in the lysis step.

#### *Nucleic acid degradation and capture*

Experiments summarised in chapter 2 revealed, that fast grown *E. coli* cells used for spiking drinking water rendered more false negative results than the slowly growing ones. This strongly indicates that the DNase digestion step harbours a certain risk to digest target DNA, inside an otherwise culturable *E. coli* cell. There is no indication that other types of nucleic acid degradation plays a role in the systems we investigated.

Capture of DNA we consider a realistic scenario especially for environmental samples containing particles such as clay. In experiments described in chapter 5.5 strong binding of proteins to DNA was experienced. Wilson reviews that humic compounds are one of the most often cited reasons for PCR inhibition (Wilson, 1997). "The phenolic groups of humic compounds denature biological molecules by binding to amides or oxidise to form a quinone which covalently binds to DNA or proteins".

#### *Inhibition of the Taq polymerase*

Phenolic compounds such as reactive sites on humic acids or proteins in an environmental sample probably act also as direct inhibitor of the Taq polymerase (Tebbe *et al.*, 1993). The matrix of some samples seemed not to inhibit the PCR completely but to increase the detection limit. That could be interpreted in the way that the activity of the Taq polymerase is affected. This was observed in the experiment described in this chapter and in some environmental samples from natural sources (summarised in chapter 2). The risk of inhibition of PCR due to varying sample matrices signifies a bottleneck: in practice of drinking water monitoring it is considerably difficult if not impossible to estimate the risk of inhibition. With a good system of internal controls for the PCR, the data should become more reliable (Burkardt, 2000).

## 6. Concluding remarks

This PhD thesis was meant to focus on the applicability of molecular methods in drinking water monitoring. It was the goal to evaluate the advantages and disadvantages of the new methods as compared to the traditional cultivation methods. Protocols available from the literature had to be adapted to routine use and evaluated for their future potential in drinking water monitoring.

### *Potential of molecular methods as compared to proved traditional culture methods*

The implementation of PCR to different fields of application has been subject of fascinating research and project developments. The legislation regulates the possibility for development and application of new methods. It is possible to use an alternative method provided that it is properly validated and the result leads to the same interpretation as the result of the corresponding standard method.

PCR appears as fast and sensitive method. In a number of publications its implementation in the analysis of microbes in drinking water is described and discussed. However, a common ground seems not yet to be developed and every research group tends to have its favourite method and protocol.

One of the main reasons why the use of molecular methods in drinking water monitoring remains restricted to research laboratories is its high price per analysis. Molecular methods need expensive and highly purified reagents. In contrast, the standardised conventional methods are mostly based on cultivation of bacteria on inexpensive culture media. Furthermore there is a considerable risk of contamination by PCR products or bacterial DNA in the laboratory. This risk has to be minimised by meticulously cleaning and isolation of workplaces and equipment, and protecting them from possible artefacts. It might be a rational approach to decrease the costs by implementing automated systems. To become cost effective this would need a throughput of an enormous amount of samples. Potentials for automation also exist with the standard culture methods, however, they are not yet broadly utilised if they exist or their potential fully exploited as the investment for specialised equipment is high (e.g. automatic colony counting systems, or scanning systems, ChemScan, (Reynolds *et al.*, 1997). Fast (24 h) tests to detect *E. coli* and coliforms in water samples are available and in use. They success-

fully passed validation procedures (Niemela *et al.*, 2003).

The concept of quality assurance of drinking water production represents another important reason hampering the implementation of molecular methods. In a quality control system the microbial analysis of water samples at different production steps is important but not the only measure in a continuous survey.

To provide optimal drinking water safety, online monitoring is applied. Today's practicable online monitoring parameters comprise: turbidity, pH-value, flow rate (of sources), conductivity, free chlorine and furthermore, bio-indicators like living daphnia (Wasserversorgung Zürich, 2006). The online measurement of e.g. turbidity of raw source water and the connection to warning systems and to automatic discard, proved to be a powerful security measure. Up to now there are no online methods for the analysis of microbial parameters. As a certain time span passes from sampling to result (at least several hours, also with fast molecular methods), microbiological parameters remain parameters of random sampling and indicate the final quality of the drinking water. Experience shows that microbiological results often correlate well with online parameters such as turbidity. With numerous microbiological analyses the quality of such correlations can be improved. With such profound knowledge of a drinking water system, the operation can be optimised which increases the reliability of safe drinking water. In these cases, solely increasing the speed of microbiological analyses thus would not be of much benefit.

#### *Special situations for special features of analysis methods*

There are situations when fast methods for the analysis of microbial quality would be desirable, e.g., after construction, replacement, repair or overhaul of reservoirs or pipes and after cleaning and flushing of the water-bearing parts. Microbiological tests are strong indicators if the work is done properly or not. A speedier microbial testing would shorten the waiting time, and the water could be released sooner for consumption.

However there are two aspects that hamper the application of fast methods in the described situations: namely the legislative framework and the limited size of a potential market. In most legislation the parameter of the colony count (heterotrophic plate count) is an integral part of the microbiological quality testing for safe drinking water. This parameter is defined, amongst others, by the time of incubation, which is 3 days. Even if there are fast methods for

faecal indicators, water is released for consumption only after knowing the outcome of the colony counts.

Microbiological “high-speed” methods have their potential in emergency cases and situations such as release of the drinking water after construction. These are rather rare events, however. As a consequence, the market for fast (and expensive) methods in drinking water microbiology is limited. In the case of emergencies, methods, skilled personal, equipment and intact (not expired) reagents have to be ready at hand in a laboratory. The economies of maintaining such an emergency infrastructure goes beyond the possibilities of a routine laboratory and is restricted to a few specialists. The distance and as a consequence the time needed for transportation between the source to be analyzed and the specialized laboratory are additional challenges for the reliability of the data obtained.

Together with the still unsatisfactory sensitivity and specificity, all these additional obstacles limit the application range of high-speed microbiological analyses in practical use.

#### *Other applications for molecular methods in drinking water microbiology*

Molecular methods remain important and indispensable for special investigations such as direct analysis of pathogens. Cryptosporidia and human viruses are difficult to detect with cultivation methods or cultivation is not possible at all. They can better or only be detected with the help of molecular methods. In Switzerland these pathogens are not (yet) part of legislation. Their significance to public health in Switzerland is still object of research (Beuret *et al.*, 2002; Gassilloud *et al.*, 2003; Füchslin *et al.*, 2005).

In cases of waterborne outbreaks, the identification of the contamination source is of crucial importance. Molecular methods are in these cases indispensable tools. With help of molecular fingerprint techniques such as amplified fragment length polymorphism (AFLP), pulsedfield gel electrophoresis (PFGE)-*Sfi*I, and the arbitrarily primed–polymerase chain reaction (AP-PCR) it was possible to identify the contamination source in the largest outbreak of Legionellosis in Murcia, Spain in Summer 2001 (García-Fulgueiras *et al.*, 2003).

With a PCR method the DNA of non viable *E. coli* can be detected, as shown in chapter 3. This PCR method could be applied in cases where drinking water was contaminated with faecal bacteria several days before the sampling date.

Molecular methods are powerful tools in drinking water analysis. Their development will surely continue. Whether these methods become part of the routine analysis is not clear yet, at present it seems unlikely for the near future.

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- 1992-1998      Studies of Environmental Sciences at the Swiss Federal Institute of Tech-  
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