PANDHUB

Grant Agreement No:	607433
Project Acronym:	PANDHUB
Project Title:	Prevention and Management of High Threat Pathogen Incidents in Transport Hubs

D3.2 – Rapid detection

Lead participant:	VTT
Authors:	Johanna Maukonen, Anniina Salmela, Satu Salo, Anna-Maria Veijalainen, Pertti Pasanen, Ilpo Kulmala
Reviewers:	Paul Riley/PHE and Audrey Berthier/Medes
Dissemination level:	PU = Public
Date	26.04.2016

This project has received funding from the European Union's Seventh Framework Programme for research, technological development and demonstration under grant agreement n° 607433

Summary

Transportation plays an important role in the spread of infectious diseases. In addition to naturally occurring infectious diseases, threats also exist from the intentional use of biological agents. Transport sector is critical to our society and any disturbances in the major hubs can have wide-spread consequences. Primary challenge in the transportation hubs is to control rather than eradicate the microbes. Furthermore, rapid detection methods are needed in order to be able to react quickly in possible pandemic cases. Knowledge about the microbiota present will help in the controlling, since specific characteristics of each microbiota can be considered as preventative / control measures are applied. It should be remembered that sampling is a crucial step in the characterization / identification procedure. If it is performed inadequately, the characterization of microbiota will inevitably be biased.

Ideal properties for a rapid detection method include representative sampling system, quick results, high throughput, reliable operation (repeatable, precise, low false alarm rate), sensitive, selective to target agents, low cost (easy to use, userfriendly). Additionally, it includes interpretation of results and automatic distribution of information to relevant stakeholders and suggestions for actions. In crowded public places it is also important to take into consideration the variability in microbial populations and continuous sampling possibility. Selection of target microbes or indicators suitable for different cases are important.

Environments (incl. transport hubs) in which microbial communities are of interest are extremely diverse. However, regardless of the environment in question, representative microbial sampling is often challenging, due to the spatial and temporal heterogeneity of microbial communities and technical problems. Microbes are often tightly attached to the surfaces and sampling sites may contain parts that are difficult to access thereby hampering the representative sampling. Conditions during sample transportation have a major impact on the sample quality and therefore the time between sampling and sample processing should be limited to the minimum. It should be kept in mind that regardless of how sophisticated method applied for the characterization of the microbial community is, the result can be only as good as the sample which arrived in the laboratory. Furthermore, the sample processing method has to be chosen based on the properties of the target microbial groups, and the method used in the subsequent detection step.

This literature review describes the advantages and limitations of traditional (culture-based detection and molecular identification) and molecular methods (hybridization-based, PCR-based, and other options) used for microbial detection and identification. Out of the reviewed microbial detection and identification methods, ethidium monoazide (EMA) / propidium monoazide (PMA) in combination with quantitative PCR (qPCR) seems to be the most interesting alternative for detection of specific high threat pathogens. qPCR would allow rapid, specific, and sensitive quantification and EMA/PMA treatment would enable the specific amplification of viable (membrane-intact) bacteria. Moreover, there are portable qPCR machines, which would allow the performance of the analysis outside the laboratory (e.g. the handheld advanced nucleic acid analyzer (HANAA), which weighs less than 1 kg and has been evaluated with *E. coli* enumeration; Higgins *et al.* 2003).

In conclusion, there is currently no suitable method allowing online sampling, detection and identification neither for air nor for surfaces. However, there are rapid methods, which take several (2 to 6) hours for detection and identification. Because there are no valid commercial

methods, the best way is to invest in prevention as well as to develop new rapid detection and identification techniques.

Abbreviations

ARDRA	amplified rDNA restriction analysis
DGGE	denaturing gradient gel electrophoresis
DHPLC	denaturing high-performance liquid chromatography
DNA	deoxyribonucleic acid
EMA	ethidium monoazide
FISH	Fluorescent in situ hybridization
FISH-MAR	fluorescent in situ hybridization combined to microautoradiography
HANAA	handheld advanced nucleic acid analyzer
mRNA	messenger RNA
NanoSIMS	nano-secondary ion mass spectrometry
NASBA	nucleic sequence-based amplification
PCR	polymerase chain reaction
PMA	propidium monoazide
qNASBA	quantitative NASBA
qPCR	quantitative real-time PCR
Raman-FISH	FISH coupled with Raman microscopy
RNA	ribonucleic acid
RNA-SIP	RNA-based stable isotope probing
SSCP	single-stranded conformation polymorphism
TGGE	temperature gradient gel electrophoresis
T-RFLP	terminal restriction fragment length polymorphism

Contents

1. Introduction

Large numbers of people pass transiently through places like airports, railway stations, and ports making these places extremely susceptible to the potential spread of disease. Helsinki-Vantaa airport, where 16 000 000 travelers passed through during the year 2014 (Finavia https://www.finavia.fi/fi/tietoa-finaviasta/tilastot/2014/), is an example of this kind of a hub. Besides direct contact between people, there is also indirect contact via surfaces or via breathing air. Infected substances, that include bacteria and viruses e.g. saliva droplets, may be dispersed onto surfaces and into the air when infected people sneeze, cough, talk, breathe, touch the surfaces, or if we are considering bioterrorism, spread the disease intentionally e.g. via ventilation ducts. Contact with any of the above may lead to spread of pathogenic bacteria or viruses among passengers and personnel. Sufficient contamination control of surfaces and minimizing the spread of pathogenic bacteria and viruses is thus necessary to prevent propagation of diseases. The contamination control is extremely important, especially in regard of highly pathogenic bacteria and viruses that cause pandemia. Security control needs also to be taken into account when intentionally spread bioaerosols are considered (e.g. bioterrorism). However, since the most important aerosol source spreading the pathogen risk for human health is humans themselves, routine actions are needed for prevention of infections (Verreault *et al.* 2008).

In an ideal case the detection and identification of microbes on surfaces and in the air would be specific and accurate, and the results of the analysis would be available rapidly, preferably on-line. This is not yet reality. As it stands, first the appropriate samples, in terms of number and distribution, need to be taken, processed and analyzed. The gold standard of analysis for many species of bacteria remains cultivation which takes hours, days or even weeks which is clearly far too long a time period in emergency cases. Therefore if we are going to be able to influence the spread of a pandemic or mitigate the impact of a bioterrorist attack, rapid detection and identification methods are needed so that we know what we are dealing with and can instigate improved control measures such as intensification of cleaning or diversion of people

Reliable detection of possible pathogenic microbes from surfaces and air is challenging due to the intense passenger flow in transport hubs. Pathogen carrier particles are quickly diluted by indoor air to very low concentrations (Yang *et al.* 2011). In addition, air is full of particles, air volumes are large, and there are no clear particle depositions. The size range of microbes is wide, starting from 10 nm (e.g. influenza and Ebola viruses >80 nm, *Yersinia pestis* >0.5 µm and *Bacillus anthracis* >1 µm) (Hinds 1999, Verreault *et al.* 2008). Microbes can also adhere to other particles (virus- and/or bacteria-containing droplets). While single virus particles exist in the air, they tend to aggregate rapidly. It is important to realize that the size of the viral particle itself is close to the airborne particle size. The similar size of the particles makes the detection challenging: bacteria, which are bigger in size than viruses, are easier to distinguish. Although the indoor environment is generally unfavorable to microbes, some can and will survive on the surfaces and in the air for a significant period of time therefore pandemic or bioterrorism threats need to be considered. Viability of viruses and bacteria is dependent on ambient temperature, amount of UV-radiation, and relative humidity (Verreault *et al.* 2008, Nikitin *et al.* 2014). Also the surface properties of the materials such as roughness can affect to viability, incidence, and prevalence (Rose *et al.* 2003).

In order to be able to reduce the spread of diseases, good contamination control tools are needed. These include reliable detection methods for sampling, analysis and identification of the contaminating microbes, as well as effective cleaning and disinfection methods.

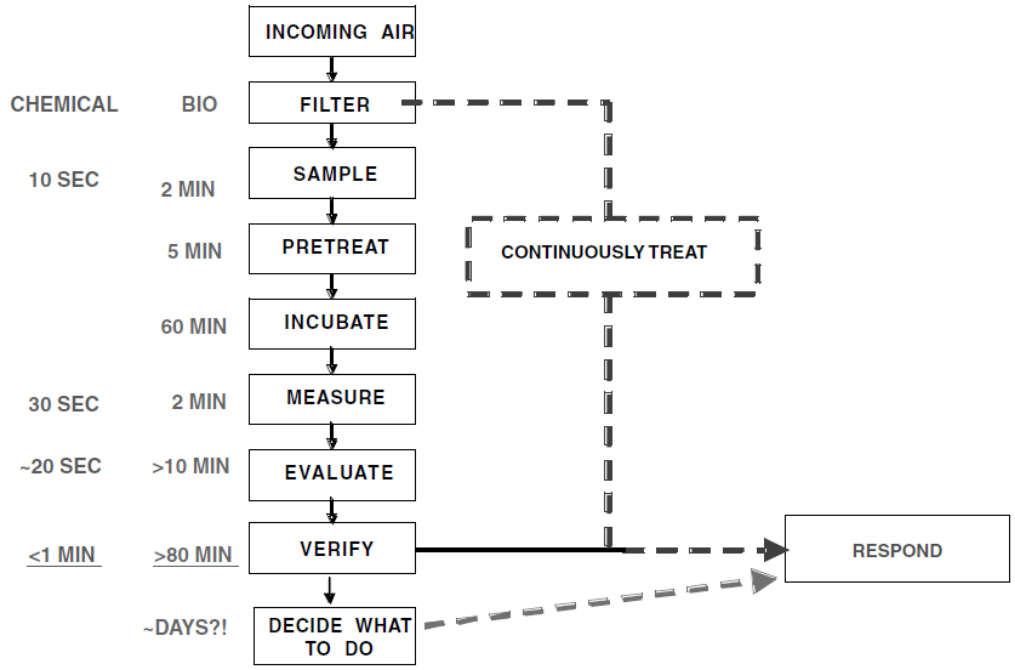

Figure 1. Typical detection times for chemical and biological agents (left-hand side) (NRC 2006).

2. Goal

The aim is to review microbial contamination detection methods applicable to crowded public spaces and areas typical to transport hubs. The review results will then be applied in future field studies. The available recommendations including standards for surface contamination monitoring are analysed and summarised.

3. Sampling methods

3.1 Surface

The importance of sampling cannot be over emphasized. Regardless of how sophisticated the method applied for the characterization of the microbial community is, the result can be only as good as the sample which arrives in the laboratory. Furthermore, the sample processing method has to be chosen based on the properties of the target microbial groups, and the method used in the subsequent detection step. Sampling consists of actual sample collection, sample handling, and sample preparation.

Sufficient samples need to be collected, which give a high degree of confidence that they are representative of the environment from which they are collected, before microbial analyses can be performed Sampling location selection can be different for various purposes; for routine monitoring the sampling location should represent critical control point and for tracing of contamination source all possible routes should be sampled. In some cases, analyzing capacity can be increased by pooling samples. Sampling methodology is also dependent on sample type (air, water, or surface) and detection method (for culture-based detection, sampling must not injure microbes, but for DNA-based PCR microbial viability is not critical).

Microbes are often tightly attached to the surfaces and places to be sampled may contain parts such as dead ends or bends in pipes that are difficult to access. Different methods such as swabbing, rinsing, agar flooding, and contact agar methods have been employed for sampling in industrial environments (Wirtanen *et al.* 1996 & 1997, Ganesh Kumar & Anand 1998, Salo *et al.* 2002, Raaska *et al.* 2002).

Sampling and transportation affect the quality of the samples, besides the analysis techniques applied. Conditions during sample transportation have a major impact on sample quality and therefore the time between sampling and further processing and storing should be minimized. It should be kept in mind that regardless of how sophisticated the applied method for characterization of the microbial community is, the result can be only as good as the sample which arrives in the laboratory. Furthermore, the sample processing method must take into account both the properties of the target microbial groups and the method used in the subsequent detection step (Maukonen 2012). Most of the steps in the processing of samples affect the results obtained. The sampling and storage conditions affect the results, although not as extensively as the steps following, especially the DNA/RNA extraction (Maukonen *et al.* 2012).

Sample preparation is a key aspect for all the technologies compared. Currently sample preparation is usually manual and optimized by each laboratory for each purpose. Some equipment already include automated DNA/RNA extraction, optimized for the specific targets for which they have been designed.

3.2 Air

3.2.1 Sample collection

There are no commercial devices for continuous detection of airborne microbes. However, some particle counters can detect biological particles on-line, but they do not identify the species. Monitoring of environmental biocontamination is based on conventional methods, in which sampling and detection are separated (Table 1). Sampling is usually performed manually and detection is based on either culture-based or microscopic methods, which are time-consuming and days are needed to get the results. Moreover, methods are mostly designed for bacteria and fungi, but not for viruses.

Techniques that are traditionally used for the collection of bioaerosols from the air include centrifugal settling, electrostatic precipitation, filtration, liquid impingement, impaction, and gravitational deposition onto plates (Table 1). In many of the traditional sampling methods the samples need to be pre-handled before the analysis and identification. However, samples that are collected directly into fluid can be directly used by various analysis and identification techniques. Routine sampling for monitoring biological contamination of air needs to be reliable and rapid. If sampling is not performed continuously, the sampling time needed

for a representative sample should be long enough to get reliable results. Currently the most common sampling methods to get information on bioaerosols and their size distributions are based on impaction directly on growth media. There are also more recent methods based on cyclone samplers, which can collect samples up to flow rate of 100 L/min. However, since most samplers cannot efficiently trap particles with an aerodynamic size of <500 nm, filters are frequently used to sample airborne microbes (Verreault *et al.* 2008). For filter sampling there are devices that can sample air trough filter e.g. 50 l/min (Azhar *et al.* 2014). When cyclone samplers (fluid sample) or filter sampling (extraction to fluid) is used, the sample then needs to be analyzed e.g.by molecular biological techniques such as quantitative PCR. This combination of modern techniques is still more rapid than traditional cultivation techniques.

Modern methods for bioaerosol detection are based on recognition of physico-chemical properties of marker molecules of microbial origin (e.g. autofluorescence), biochemical responses, adsorption of light with specific wavelengths and recognition of nucleic acids. These techniques enable automated, fast, and specific analysis of microbes or their fragments. However, there are only a few applications for rapid real-time detection

Table 1. Characteristics of traditional methods for environmental monitoring (Morris et al. 2000; Martinez et al. 2004)

	Principle of sampling	**Principle of analysis**	**Total time needed**	**Microbe**
Impactor	Impaction onto agar	Cultivation	>1 day	Bacteria
Cyclone	Impaction into liquid or onto agar	Cultivation / microscopy	>1 day	Bacteria, virus
Centrifugal	Centrifugal impaction onto agar	Cultivation	>1 day	Bacteria
Impinger	Impingement into liquid	Cultivation / microscopy	>1 day	Bacteria
Filter collection	Filtration; impaction, diffusion, electrical and gravitational forces	Cultivation / microscopy	>1 day	Bacteria, virus
Deposition plate	Gravitational settling	Cultivation	>1 day	Bacteria

3.2.2 Particle counters

There are no commercial devices for rapid detection, which can count the concentration and identify bioaerosols online. Viruses create their own challenge for detection. There are several applications for detection of bacteria and also viruses in the laboratory but suitable field applications are missing. In the laboratory, different kinds of techniques can be applied such as flow cytometry, laser induced breakdown microscopy, electron- and fluorescent microscopy and even chemical analytics e.g. gas chromatograph. In the field, particle counters and

particle counter with laser-induced fluorescence can be used for the detection of the amount biological particles, but not for identification of what species those particles represent.

Particle counters are used to detect the concentrations and size distribution of airborne particles (both viable and non-viable). In particle counters, the air sample is drawn directly into the measurement chamber to minimize particle losses during the sampling. A sheath air flow is used to surround the sample flow, which focuses the aerosol to enhance size resolution, and keeps the optics clean to improve longevity of the counter. The flow rates in the OPCs are carefully controlled using real-time feedback to ensure concentration accuracy. In the optical chamber, the aerosol crosses a laser beam, creating a light pulse. The intensity of the flash is used to count and measure the particles. The shape of the laser beam, the size of the viewing volume, the type of detector, and the signal processing algorithms are designed to provide optimal resolution over the optical size range e.g. 0.3 to 10 µm (TSI model 3330, Manual TSI 2011).

Particle counters with laser-induced fluorescence

All biological materials contain fluorescent molecules (Hill *et al.* 2009, Despres *et al.* 2011). Most viable organisms, including bacteria and viruses, have natural auto-fluorescence due to biochemical fluorophores, such as fluorescent coenzymes nicotinamide-adenine dinucleotide (NADH) and nicotinamide-adenine dinucleotide phosphate (NADPH), the coenzymes flavin mononucleotide (FMN) and flavin adenine dinucleotide (FAD), amino acids (e.g. tryptophan), and riboflavin (vitamin B_2, B_6, B_9) which when excited by UV/visual radiation of a suitable wavelength, fluoresce (Brosseau *et al.* 2000, Hill *et al.* 2009, Pöhlker *et al*. 2012, Taketani *et al.* 2013). Viable bacteria usually exhibit a coenzyme signal due to increased metabolic activity and an amino acid signal due to proteins and peptides (Pöhlker *et al.* 2012). In contrast, viruses exclusively show a protein signal. Tryptophan, NADH, amino acids, and proteins have their own peaks in emission spectra (Manninen *et al.* 2009, Putkiranta *et al.* 2010, Pöhlker *et al.* 2012). Vegetative bacterial cells can be found when excited by light in the 405 nm and bacterial spores when excited by light in the 355nm (Pan *et al.* 2007, Manninen *et al*. 2009).

Using a laser-induced fluorescence (LIF) technique, Laser particle counters (LPCs) have been shown to be useful for discriminating between biological and non-biological particles (Hill *et al.* 1999). It detects fluorescence emission from auto-fluorescence molecules when particles are radiated by laser light. LIF allows non-invasive, real-time, *in-situ* detection of particles from air with high temporal resolution (Pöhlker *et al.* 2012) without separate sampling and sampling pretreatments with a detection limit in the range of 0.1-1 particle/m^3. A major weakness of LIF is that it is not yet capable of identifying microbial groups or species; only size and number of biological particles can be detected.

Recently, many LIF based real-time rapid biologic detection devices, such ioLaz (Particle Measuring Systems, Inc. CO, US), BioVigilant IMD (BioVigilant, Inc. AZ, US) and BioScout X (Environics Ltd. FI) and WIBS (the University of Hertfordshire, UK), have been developed

3.2.2.1 4th level heading

4. Detection and identification

The application of molecular techniques for the identification and/or enumeration of different microbial groups has increased enormously during the past decade. Molecular techniques enable faster detection and enumeration of specific bacteria than conventional culture-base methods. However, culture-based methods are still the only validated methods able to quantitate viable bacteria. In addition to the analysis techniques used, sampling and transportation may also affect the quality of the samples. Conditions during sample transportation have a major impact on sample quality and therefore the time between sampling and further processing and storing should be limited to the minimum. It should be kept in mind that regardless of how sophisticated the applied method for characterization of the microbial community is, the result can be only as good as the sample which arrives in the laboratory. Furthermore, the sample processing method must be chosen taking into account both the properties of the target microbial groups and the method used in the subsequent detection and/or identification step. When molecular techniques are applied to study microbial communities from complex environments, it should be kept in mind that in most cases only viable bacteria are relevant / of interest. Therefore, it is of utmost importance to understand also the limitations related to detection and/or identification techniques applied.

4.1 Culture based methods

The sample processing method used is dependent on the properties of the target microbial groups, and the method used in the subsequent detection step. If detection is performed by culture-based methods, the target strains must retain viability and culturability during sample processing. The sample matrix plays an important role when a decision on the method used for microbial detection and identification is made.

Cultivation and the subsequent identification of the isolates using conventional techniques are time-consuming. It may take weeks to obtain the complete results (Curtis & Lee 1995, Restaino *et al.* 1999). Much effort has been put into the development of more rapid culture techniques, many of which are based on the use of fluorogenic and chromogenic culture media. An ideal method for studying microbial communities would detect and enumerate all microbial species present in the samples with equal efficiency. It has been speculated that many microbial communities are too diverse to be counted exhaustively, which has led to the application of statistical approaches for estimation of diversity (Hughes *et al.* 2001).

Microbial community analysis by cultivation is extremely laborious, especially when complex samples with high diversity are studied. If cultivation is applied to microbial community analysis, several non-selective and selective culture media and different growth conditions including different temperatures and gaseous atmospheres, followed by accurate identification of a large number of isolates from each medium, should be included to get an overview of the diversity of the microbial population in the sample. The dominant cultivable population is recovered from non-selective media, whereas selective media allows detection of groups or species that are present at lower numbers. Identification of isolates with traditional methods is based on assessment of several phenotypic features, which is often inaccurate, and may lead to underestimation of the species diversity. Currently, this is combined with molecular identification techniques, e.g. sequencing. It is generally known that conventional cultivation methods recover less than 1 % of the total species of microbes present in environmental

sample (Giovannoni *et al.*, 1990; Ward *et al.*, 1990, Amann *et al.* 1995), partly due to the poor ability of the routinely used culture media and growth conditions to recover a large fraction of the microbial population (Palleroni 1997). When information below species level is needed, different typing methods are used (Maukonen *et al.* 2003, Ludwig 2007). Due to the limitations of culture-based analysis (it can be both time-consuming and labor-intensive), the focus has more and more shifted to molecular techniques. Culture-based studies are, therefore, nowadays used to a lesser extent. However, culture-based studies also enable phenotypic as well as genotypic identification, and especially in those cases where phenotypic identification is crucial (e.g. antibiotic resistance (Saarela *et al.* 2007)), culture-based studies can still offer more information than purely genotypic studies.

Examples of standard methods and other recommendations for surface colony count are presented in Table 2.

Table 2. Examples of standard methods and other recommendations for surface colony count.

Reference	Application/field	Method	Remarks
ISO 18593:2004 Microbiology of food and animal feeding stuffs -- Horizontal methods for sampling techniques from surfaces using contact plates and swabs	surface sampling methods for the detection or enumeration of bacteria in food processing area and equipment / food & feed industry	sampling using contact plates or swabs	does not describe when sampling should be performed or what areas should be sampled does not give sufficient guidance or advice for detection of specific microbes (for instance *Listeria monocytogenes*)
NMKL 5, 5.ed. 2005 Aerobic microorganisms and presumptive Enterobacteriaceae. Enumeration on surfaces and utensils	sampling of food processing surfaces and surfaces in food production areas, as well as in catering / Food industry	using swabbing or contact plates to enumerate aerobic microorganisms and presumptive Enterobacteriaceae	does not give the absolute number of bacteria but an estimate of the hygienic status after cleaning procedures as well as the level of contamination at critical steps in food production processes. commercially available validated and quality controlled dipslides and culture plates can also be used.
Commission regulation (EC) No 2073/2005 on microbiological criteria for foodstuffs	defining microbiological criteria for foods	This Regulation lays down the microbiological criteria for certain microorganisms and the implementing rules to be complied with by food business operators when implementing the general and specific hygiene measures referred to in Article 4 of Regulation (EC) No 852/2004.	Sampling processing areas and equipment for *L. monocytogenes* on a routine basis according to a sampling scheme is thus necessary and mandatory
Guidelines on sampling the food processing area and equipment for the detection of *Listeria monocytogenes* Version 3 – 20/08/2012 Brigitte Carpentier and Léna Barre, EURL for *Listeria monocytogenes*, Maisons-Alfort Laboratory for Food Safety, ANSES, France	*Listeria monocytogenes* / Food industry	Sampling with wipe sampling devices stick swab and sponge, woven or unwoven cloth or gauze pads	Wipe sampling methods (swab and sponge/ cloth method) are the only appropriate methods to use for *L. monocytogenes* The total sampled area during a sampling campaign should be as large as possible to increase the probability to detect *L. monocytogenes*
SCAN-CM 61:02 SCAN-P 82:02 Accepted 2002	food contact material / paper industry	Microbiological examination – Surface colony number	no limits for the number of microbes in materials for use in contact with food are stipu-

Mechanical and chemical pulps, paper and paperboard		using the dry rehydratable film method	lated in any law In SCAN-test Method, the dry rehydratable film, Petrifilm Count Plates, is used for determination of bacteria as well as yeast and moulds.

4.1.1 Typing of bacterial isolates

Genetic fingerprinting techniques can be used to characterize bacterial communities or single bacterial isolates. Genetic fingerprinting techniques of microbial communities provide a pattern or a profile of the community diversity based upon the physical separation of unique nucleic acid sequences (Stahl & Capman, 1994). Fingerprinting of bacterial isolates can be performed by a variety of techniques including e.g. ribotyping, amplified ribosomal DNA restriction analysis (ARDRA), pulsed-field gel electrophoresis (PFGE), randomly amplified polymorphic DNA (RAPD), repetitive element sequence-based-PCR (rep-PCR), and amplified fragment length polymorphism (AFLP). All these techniques aim at differentiating bacterial isolates at the subspecies level, preferably even at the strain-level.

Prior to molecular techniques phenotypic methods such as biotyping and serotyping were used for the bacterial strain differentiation. These techniques are still used today, but with molecular techniques more reliable and often less laborious fingerprinting can be achieved. Regardless of whether phenotypic or genotypic techniques are applied, the fingerprinting is preceded by culture and single-colony subculture steps. Thus, even though PCR and hybridization can be used both in bacterial detection and fingerprinting, the techniques applied differ in a profound way: While detection methods are able to find the target organisms in a sample containing hundreds of other bacteria, fingerprinting methods are not genus or species specific and can therefore be applied to pure bacterial cultures only. When molecular techniques were first applied for bacterial fingerprinting, restriction endonuclease analysis (conventional REA) of the genomic DNA and plasmid profiling were used. Both techniques have their limitations. With conventional REA complicated patterns with hundreds of restriction fragments are obtained, which makes the profile comparison difficult. With plasmid profiling far simpler profiles are obtained, but this technique is suitable only for bacteria carrying (several) plasmids (for a review see Vaneechoutte 1996).

4.1.1.1 Ribotyping

When conventional REA is combined with a hybridization step far simpler and thus more easily comparable fingerprints are obtained. This technique, where genomic restriction fragments are separated by gel electrophoresis, transferred to a nylon membrane and hybridized to a probe, is called restriction fragment length polymorphism (RFLP). By far the most widely applied RFLP technique is (classical) ribotyping, in which rRNA genes (usually both 16S and 23S rRNA genes or a whole rRNA operon containing 16S, 23S and 5S rRNA genes and their spacer regions) are used as a probe. Since rRNA operon contains both conserved and hypervariable regions the same probe (originating e.g. from *Escherichia coli*) can be used in ribotyping of different bacterial species (Grimont & Grimont, 1986). The strain differentiation in ribotyping is thus based on the unique hybridization pattern (fingerprint) obtained and not on the specificity of the probe. Differences in the hybridization patterns originate from restriction endonuclease recognition site variation within variable regions of rRNA genes and

their spacer regions. The discriminatory ability of ribotyping is greatly influenced by both the probe (whole rRNA operon vs. a single gene) and the restriction endonuclease applied. Obviously the best discrimination is obtained when the whole operon is used as a probe and an optimal restriction endonuclease for each bacterial genus is selected from a panel of restriction endonucleases tested. However, when ribotyping is used as a taxonomical tool, riboprints of isolates representing different genera and species are compared, and thus the same restriction endonuclease has to be applied for all bacteria. The invention of the automated ribotyping system (Riboprinter®) has greatly facilitated the bacterial fingerprinting, thus allowing larger numbers of bacterial isolates to be characterized and compared than previously, when ribotyping had to be performed manually.

4.1.1.2 Amplified rDNA restriction analysis (ARDRA)

In addition to classical ribotyping, rDNA-based fingerprints can be obtained by a technique called ARDRA (amplified rDNA restriction analysis). In ARDRA bacterial rRNA gene(s) are first amplified by PCR using conserved sequences of rDNA as primers. The PCR amplification product is then digested with restriction endonuclease and restriction fragments are resolved electrophoretically to obtain a fingerprint (Vaneechoutte *et al.* 1992). Although ARDRA fingerprinting is faster to perform than classical ribotyping, its discriminatory power is often inferior to that of ribotyping. This is due to the fact that in ARDRA smaller areas of the rRNA operon (and none of the sequences surrounding the rRNA genes) are targeted than in ribotyping.

4.1.1.3 Pulsed-field gel electrophoresis (PFGE)

Due to the problems encountered with conventional REA of bacterial genomes, a technique, where profiles consisting of fewer numbers of larger sized genomic restriction fragments were obtained, was developed for bacterial fingerprinting (Ely & Gerardot, 1988). In this technique bacterial genomic DNA is restricted *in situ* (in a gel block) with a restriction endonuclease that cuts DNA rarely such as *Sma*I, *Sfi*I, *Not*I, and *Bss*HII and the restriction fragments are separated by pulsed-field gel electrophoresis (PFGE; a special technique capable of resolution of large DNA fragments). With PFGE highly discriminative fingerprinting of bacterial isolates can be performed. Of the different molecular fingerprinting methods PFGE has in most cases proved to be the most discriminative one. However, PFGE is a laborious technique and it is not usually applied in studies where large numbers of isolates are characterized.

4.1.1.4 Random amplified polymorphic DNA (RAPD)

In RAPD fingerprinting one or two primers (usually 10-12 bp long) are arbitrarily selected and allowed to anneal to the bacterial genomic DNA template at a low stringency. In RAPD several amplification products of varying sizes are obtained. These products are resolved electrophoretically to yield a RAPD-fingerprint (Welsh & McClelland 1990, Power 1996). RAPD typing is fast to perform, especially in the cases where fingerprinting can be performed directly on single-colonies growing on an agar plate. Due to the low stringency of the PCR amplification RAPD-fingerprints can show some variation (especially in band strengths) and therefore the fingerprint comparisons have to be done visually by an experienced person. However, when strictly identical conditions (same thermocycler, reagents etc.) are used, the method usually works well (Meunier & Grimont, 1993). RAPD banding pattern reproducibility can be improved by using a procedure where the same strains are exposed to three different annealing temperatures (with increasing stringency) and by identifying the stable amplicons

(Cusick & O'Sullivan 2000). This triplicate procedure naturally makes the RAPD fingerprinting technique more laborious.

RAPD is best suited for studies where a specific bacterial strain (e.g. a certain food-borne pathogen) is looked for among large number of isolates. With RAPD, bacterial isolates with clearly different fingerprints can be quickly identified and rejected and remaining (fewer) strains then further characterized with another, more laborious technique (e.g. ribotyping, PFGE, AFLP). RAPD is not well suited for inter-laboratory or for taxonomical studies or for studies where the aim is to develop a fingerprint database.

4.1.1.5 Repetitive element sequence-based-PCR (rep-PCR)

Repetitive chromosomal elements, which are found randomly distributed in bacterial genomes, are the targets of repetitive element sequence-based-PCR (rep-PCR) amplification. In the rep-PCR primers anneal to repetitive parts of the chromosome and amplification occurs when the distance between primer binding sites is short enough to enable it (Versalovic *et al.* 1991). The repetitive DNAs can be classified either as "short sequence repeats" (SSRs) or "variable number of tandem repeats" (VNTRs). Variations of rep-PCR include ERIC-PCR (enterobacterial repetitive integenic consensus-PCR), BOX-PCR, REP-PCR (repetitive extragenic palindromic unit sequence-PCR, and VNTR-PCR (van Belkum *et al.* 1998).

4.1.1.6 Amplified fragment length polymorphism (AFLP)

AFLP involves restriction of total bacterial DNA with two restriction enzymes of differing cutting frequencies (e.g. *Hind*III and *Taq*I) followed by ligation of fragments to oligonucleotide adapters complementary to the sequences of the restriction sites (restriction-half-site specific adapters). Selective PCR amplification of subset of fragments is achieved using primers corresponding to the contiguous sequences in the adapter, restriction site plus few nucleotides in the original target DNA. When only one of the primers is labeled only a subset of amplified fragments are detected during visualization (Vos *et al.* 1995, Janssen *et al.* 1996). A variation of this technique where only a single restriction enzyme is used has also been developed (Gibson *et al*. 1998).

4.2 Independent molecular techniques

Molecular techniques can be used together with other traditional methods or independently starting directly from the sample without need for cultivation. A molecular technique used for the detection of pathogens must be capable of detecting low numbers of target bacteria in samples which may contain considerable background of interfering microorganisms and several matrix-derived compounds that may hamper the detection. In microbial community analysis the method should allow detection of different groups or species present in the ecosystem with similar efficacy to avoid biases in evaluation of species distribution and complexity of the microbiota. Biases may already be introduced by sample handling (Muyzer & Smalla 1998), as well as during the extraction of nucleic acids from microbes in the sample.

Molecular techniques can be utilized in the detection and identification of microbes in two ways: a) identification is performed directly from sample material, or b) identification is based on combined culture and molecular detection. The sample matrix studied plays an important role when the decision between the two choices is made. If the matrix is known to contain factors that can inhibit e.g. PCR reaction and which are difficult to remove, it is often best to use the combination of a culture technique and a suitable molecular technique. There are two major techniques applied in the molecular detection and identification of bacteria, PCR

and hybridization. When molecular tools were first introduced for detection and identification of microbes, hybridization methods were widely applied. The rapid evolution of PCR techniques has led to the present situation where hybridization is mainly used in combination with PCR. Development of DNA microarrays allows, due to the automatization of the procedure, simultaneous identification of huge number of specific sequences by hybridization (Hacia *et al.* 1998).

Various molecular techniques enable faster detection and enumeration of specific bacteria than conventional cultivation, but special emphasis should be put into the detection method in question to ensure that a correct molecule (for the specific need in question) is targeted. In addition, each molecular technique has its own advantages and limitations (e.g. Lauri & Mariani 2008). Furthermore, biases may already be introduced by sample handling (Muyzer & Smalla 1998), as well as during the extraction of nucleic acids from microbes in the sample. Different types of microbes require a different sample preparation method for optimal cell lysis. In regard of bacterial cell lysis, *Escherichia* spp. and *Salmonella* spp. as Gram-negative and *Clostridium difficile* as Gram-positive bacteria have different cell wall structures and compositions, and therefore the optimal DNA-extraction method for these two groups is different. Gram-negative bacteria are more easily lyzed and if too rigorous DNA-extraction method is used, it may result in detecting lower numbers and diversity of Gram-negative species. With Gram-positive bacteria, instead, more rigorous DNA-extraction methods are needed, especially with complex matrices (Maukonen *et al.* 2012).

Since DNA mainly remains intact in dead cells, the methods targeting DNA (of e.g. 16S rRNA gene) enumerate both viable and dead cells (Josephson *et al.* 1993). Another commonly used target for bacterial detection is structural RNA such as 16S rRNA (Ludwig 2007, Tringe & Hugenholtz 2008). RNA is more labile than DNA and more susceptible to degradation caused by treatments such as heating (Sheridan *et al.* 1998). Due to the fact that environmental conditions influence the cellular rRNA content, the amount of rRNA is considered to correlate with the growth rate (Poulsen *et al.* 1993, Amann *et al.* 1995). However, the rRNA molecule remains stable for some time after cell death - e.g. Sheridan *et al.* (1998) found *E. coli* 16S rRNA 16 hours after thermal inactivation at 60°C, 80°C, and 100°C. Therefore, the rRNA content of a bacterium may not correctly reflect its physiological status, if the rRNA-based enumeration technique is performed directly after the disinfection step (which should be the case in detection of final disinfection efficiency). A more attractive indicator molecule for bacterial viability is therefore messenger RNA (mRNA), due to its fast degradation after cell death (Bej *et al*. 1996, Sheridan *et al.* 1998). However, the extent of degradation varies according to the type and severity of the treatment used for killing of the bacteria. Furthermore, since detection of mRNA in viable cells may depend on the physiological status, cells which are stressed, or viable but non-culturable, may contain too low mRNA quantities for the detection (Barer & Harewood 1999). However, in a recent study (Gao *et al.* 2007) a whole-community RNA amplification was developed to provide sufficient amounts of mRNAs for the microarray analysis of environmental samples. Moreover, since bacteria have to be killed before the nucleic acids may be extracted, there can be no absolute correlation between the presence of mRNA and culture-based viability (Sheridan *et al*. 1998). Since the methods involving mRNA are usually relatively complicated to perform, especially with environmental samples, these methods have not yet been widely used (Min & Baeumner 2002, Chen *et al.* 2004, Zhao *et al.* 2006, Thieme *et al.* 2008). Besides 16S rRNA, other housekeeping or functional genes – such as genes associated with virulence factors e.g. listeriolysin O (*hly*) toxin producing gene in *Listeria monocytogenes* (Soejima *et al.* 2008) or specific metabolic pathways (Luton *et al*. 2002) – can be used as targets for PCR and/or hybridization. However, the sequence databases for other genes besides 16S rRNA genes contain

only limited numbers of sequences (Ludwig 2007) limiting their use in microbial ecological studies.

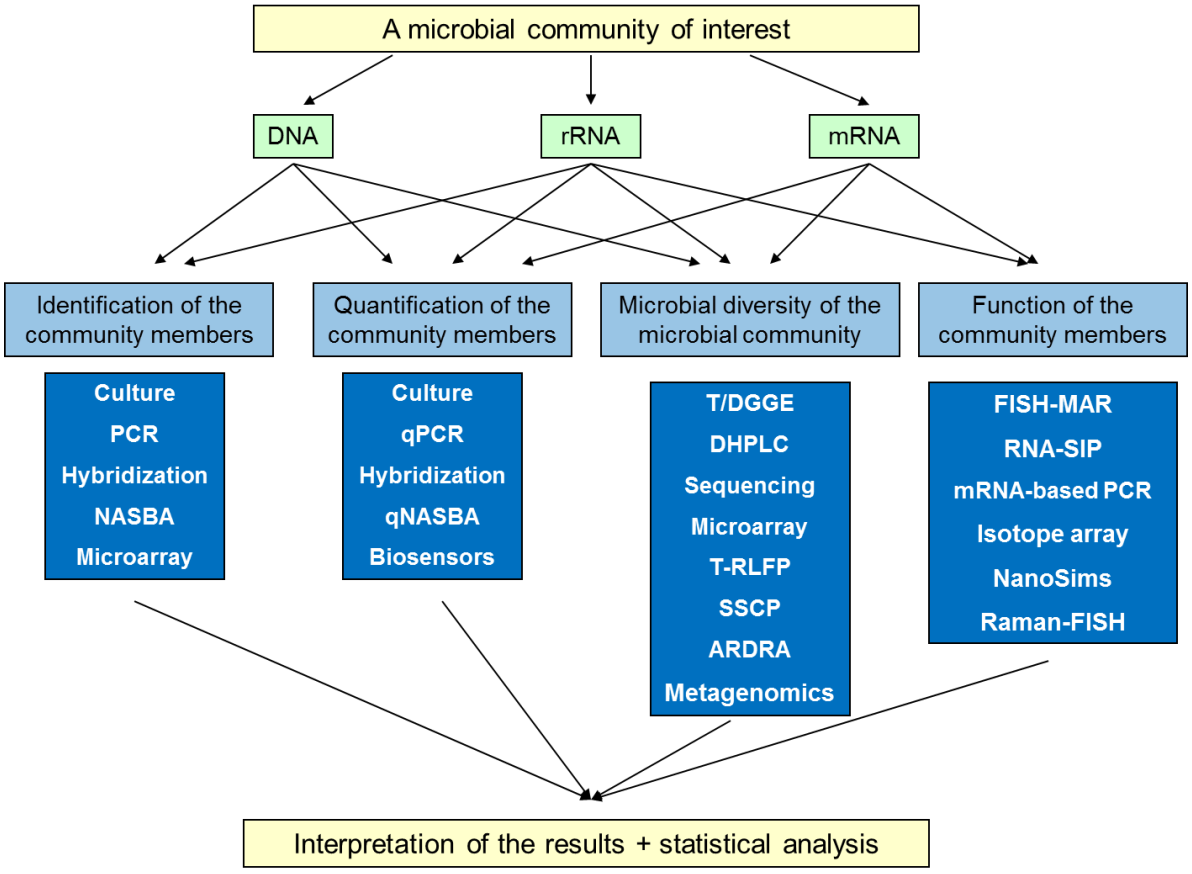

Figure 1. Techniques most commonly used for the characterization of microbial communities. (rRNA: ribosomal RNA, mRNA messenger RNA, PCR: polymerase chain reaction, NASBA: nucleic sequence-based amplification, qPCR: quantitative real-time PCR, qNASBA: quantitative NASBA, TGGE: temperature gradient gel electrophoresis, DGGE: denaturing gradient gel electrophoresis, DHPLC: denaturing high-performance liquid chromatography, T-RFLP: terminal restriction fragment length polymorphism, SSCP: single-stranded conformation polymorphism, ARDRA: amplified rDNA restriction analysis, FISH-MAR: fluorescent in situ hybridization combined to microautoradiography, RNA-SIP: RNA-based stable isotope probing, NanoSIMS: nano-secondary ion mass spectrometry, Raman-FISH: FISH coupled with Raman microscopy). Adapted from Maukonen & Saarela, 2009.

Viral detection

Viruses are identified by their shape (rods, spheres), size, nucleic acid type (RNA, DNA), size, single or double stranded, number of nucleic acid pieces, type of vector for spread and serological characteristics of the coat or capsid protein. There are several different laboratory applications that may be used for identification of viruses (Table 3). There are also several valid methods for bacterial identification besides viruses. Identification methods can be separated as follows e.g. non-immunological methods (including histologic staining, electron microscopy, haemadsorption (HAD), haemagglutination (HA), challenge interference (CI), and DNA probes) and immunological methods (including immunofluorescence (IF), immunoperoxidase (IP), quantitative polymerase chain reaction (qPCR), enzyme-linked immunosorbent assay (ELISA), neutralization (NEUT), haemagglutination inhibition (HAI), passive

agglutination (PA), radioimmunoassay, and immune adherence haemagglutination.) or e.g. to conventional methods: *(*amplification methods (PCR, SDA, 3SR, NASBA, LAMP), NA hybridization (FISH, Southern & Northern blot) and novel techniques*: (*Pan-viral microarrays (available in different formats), metagenomic analysis (deep sequencing, MPS))*.* However, in this section we focus only on identification methods, which are suitable for both viruses and bacteria, and take only hours like DNA- and RNA-based methods such as PCR-methods. Traditional culture-based identification is slow and complicated and it is not considered here.

Table 3. Applications for viral identification (Pankaj 2013)

Technique	Detection Principle	Reproducibility	Time	Labour	Cost
viral plaque assay	infectivity assay	poor	days	high	inexpensive
$TCID_{50}$, LD_{50}, EID_{50}	infectivity assay	poor	days	high	inexpensive
immunofluorescence foci assays	infectivity assay	poor	days	high	expensive
qPCR	viral nucleic acid	excellent	hours	moderate	expensive
immunoblotting	viral protein	good	days	moderate	inexpensive
immunoprecipitation	viral protein	good	days	moderate	inexpensive
ELISA	viral protein	good	hours	moderate	inexpensive
haemagglutination assay	viral protein	good	hours	moderate	inexpensive
viral flow cytometry	viral particle	excellent	hours	high	expensive
transmission electron microscopy	viral particle	excellent	weeks	high	expensive

4.2.1 Hybridization

Hybridization techniques can be used in bacterial identification either alone or combined with a preceding PCR step. In hybridization, a labeled probe anneals to a denatured target DNA / RNA with sequence homology (Southern 1975, Alwine *et al.* 1977). Target DNA / RNA can be directly blotted onto a membrane, or if size information of the hybridization target is warranted, the target DNA / RNA is first run through an agarose gel with DNA markers of a specific size and then transferred to a membrane. Detection of hybrids is based on radioactive signal, fluorescence, or color reaction, depending on the type of label. By determining the intensity of the hybridization signal, the number of target organisms can be estimated (Stahl *et al.* 1988). However, it should be noted that the relative amount of an rRNA sequence does not reflect the true abundance of the microbe, since the amount of rRNA per cell varies according to the species and the metabolic activity of the bacterial cell (Klappenbach *et al.* 2000). Nevertheless, the relative quantity of rRNA provides a reasonable measure of the relative physiological activity of a specific population (Vaughan *et al.* 2000).

Microarrays, which are miniaturized and automated forms of a dot-blot hybridization, facilitate the study of large numbers of genes simultaneously by hybridization of DNA or mRNA to a high-density array of immobilized probes (Fodor *et al.* 1993, Schena *et al.* 1995, Lockhart *et al.* 1996). There are two major types of DNA microarrays; an oligonucleotide-based array and a PCR product-based array. The detection of whole bacterial cells via labeling of specific nucleic acids with fluorescently labeled oligonucleotide probes is called fluorescent *in situ* hybridization (FISH). FISH requires no cultivation and cells can be fixed before analysis, thus enabling the storage of samples prior to analysis (DeLong *et al.* 1989, Amann *et al.* 1990). The whole-cell or *in situ* hybridization technique has become a much used molecular tool in

microbial ecology, since organisms or groups of organisms can be identified with minimal disturbance of their environment and spatial distribution.

4.2.1.1 Fluorescent in situ hybridization (FISH)

The detection of whole-bacterial cells via labeling of specific nucleic acids with fluorescently labeled oligonucleotide probes is called fluorescent *in situ* hybridization (FISH). FISH requires no cultivation and cells can be fixed before analysis which enables the storing of samples prior analysis (DeLong *et al.*1989, Amann, *et al.* 1990a, Amann *et al.* 1990b, Amann, *et al.* 1992, Amann 1995a&b, Stahl 1995, Manz *et al.* 1998). The whole-cell or *in situ* hybridization technique has become a very used molecular tool in environmental microbiology, since organisms or groups of organisms can be identified with minimal disturbance of their environment and spatial distribution. Due to the fact that environmental conditions influence the cellular rRNA content, the amount of rRNA is considered to correlate with the growth rate (Poulsen *et al.* 1993), and *in situ* hybridization using rRNA targeted oligonucleotides can therefore be a powerful tool for the assessment of bacterial activities.

FISH in combination with epifluorescence microscopy has become a widely applied method to analyze microbial communities (Amann *et al.* 1995). The sensitivity and objectivity can be greatly enhanced by digital image analysis (Ramsing *et al.* 1996). The application of FISH combined with conventional fluorescence microscopy for the analysis of complex microbial biofilms can be impaired by biofilm thickness, background fluorescence caused by humic substances or detritus, and the inherent autofluorescence of phototrophs. These problems can be circumvented by using FISH with confocal scanning laser microscopy (CSLM; Wagner *et al.* 1994; for a review see Wagner *et al.* 1998b). The advantage of CSLM for the study of complex environments is that undisturbed samples can be analyzed without removal or homogenization of biofilm or other material (Lawrence *et al.* 1991). Sample thickness is not limiting since light from out-of-focus planes is excluded (Manz *et al.* 1999).

Since non-automated microscopic examination is rather time-consuming, flow cytometry (FCM) has been used in numerous studies for the enumeration of FISH hybridized cells (e.g. Lay *et al.* 2005). A flow cytometer is able to analyze 10 000 cells in a few seconds, and once it is adjusted and ready to use, it takes only 1-2 min to complete whole procedure for a single cell suspension sample. A flow cytometer, however, may recognize and analyze aggregated cells as one particle unlike microscopic counting. Furthermore FCM requires only small sample sizes and differentiates between individual cells according to the number of particles (if fluorescently labeled) each of them has accumulated (Avery *et al.* 1995). The ability to rapidly and precisely detect, characterize and identify cells in mixed population demonstrates its potential as a tool for the analysis of microbial populations (Diaper *et al*. 1992). The flow cytometry devices using nanochannels have higher suitability and could therefore be considered. Another possibility to detect FISH hybridized cells rapidly with an automated system would also be a fluoroscan (see e.g. Alakomi *et al.* 2005).

Hybridization with peptide nucleic acid (PNA) oligonucleotides to target DNA/RNA offers many advantages as compared to “normal” oligonucleotides; they can hybridize rapidly, hybridization times can be reduced to 30 min allowing hybridization and detection to be completed within a few hours, hybridization is independent of the salt concentration, they are resistant to nuclease and protease attack, they bind more specifically, and shorter probes can be used for greater sensitivity (Prescott & Fricker 1999). FISH has also been used in combination with direct viable count (DVC) for the quantification of coliforms. The DVC procedure involves exposing bacterial cells to a revival medium containing antibiotics preventing cellular division and thereafter elongated cells are enumerated as viable cells (Garcia-Armisen *et al.* 2005). In FISH-DVC, 16S rRNA based probes have also been the most com-

monly used probes (Garcia-Armisen & Servais 2004, Armisen & Servais 2004, Garcia-Armisen *et al.* 2005). Besides the standard FISH protocols, an approach utilizing oligonucleotide probes labeled with a single near-infrared dye has been developed in combination with FISH to target both rRNA and mRNA (*rpsH* gene; 30S ribosomal subunit Protein S8) of *E. coli* in the same FISH experiment (Coleman *et al.* 2007).

4.2.1.2 Nucleic sequence-based amplification (NASBA)

NASBA is specifically designed to detect RNA. The genomic DNA from the target bacteria does not become a template for amplification, since it remains double-stranded at the temperature NASBA is performed. Therefore, a DNase treatment is not needed. In principle, the requirements for RNA purity are similar to those of quantitative PCR (Cook 2003), but in practice much simpler cell disruption methods have proved to be valid (Min & Baeumner 2002). The reaction is performed at a constant temperature, usually at 41ºC. This eliminates the need for specialized equipment, such as PCR thermocyclers, since only a heating block or a water bath is needed (Simpkins *et al.* 2000). However, the NASBA reaction itself is more complex than PCR and the effect of potential inhibitors have not been studied in detail. Substances which can combat the presence of matrix-derived inhibitors have however been identified (Cook 2003). The product obtained from NASBA reaction is mainly single-stranded RNA, which may be detected by e.g. gel electrophoresis, or if confirmation of specificity is wanted, with an additional hybridization step (for a review, see Cook 2003). One example where an additional hybridization step has been performed, is the use of the electrochemiluminescence (ECL) detection technique (Min & Baeumner 2002). The ECL technique has been shown to be a fast and sensitive hybridization technique.

4.2.1.3 Sandwich hybridization

Sandwich hybridization is a hybridization assay based on the hybridization of a target nucleotide (usually RNA) with differentially labeled capture and detector probes (Wicks *et al*. 1998). The capture probe labeled with e.g. biotin is used to immobilize the target sequence on a solid support and the detection probe is labeled with a detectable marker such as enzymes (Wicks *et al*. 1998), digoxigenin (DIG) (Rowan *et al.* 2005, Leskelä *et al*. 2005, Huhtamella *et al*. 2007, Thieme *et al.* 2008), quantum dot labeled probes (Satterfield *et al*. 2008), molecular beacons (MB: Zuo *et al*. 2007, Satterfield *et al.* 2008) or tentacle probes (TB: Satterfield *et al.* 2008). The use of MBs or TBs as detection probes enables the elimination of wash steps after the final hybridization step thus shortening the procedure time (Zuo *et al.* 2007, Satterfield *et al*. 2008). In addition, helper probes may be used to prevent “incorrect” secondary structures (Rowan *et al.* 2005, Thieme *et al.* 2008). Sandwich hybridization can be performed in a liquid format, where the capture probe is utilized for removal of hybridized cells with magnetic beads (Rowan *et al*. 2005, Leskelä *et al.* 2005, Huhtamella *et al*. 2007, Thieme *et al.* 2008), or in an immobilized format where the capture probe is used to immobilize the target cells into a detection platform (Zuo *et al.* 2007, Satterfield *et al.* 2008). The latter application has also been utilized in biosensors (e.g. Baeumner *et al*. 2004).

4.2.1.4 Molecular beacon assay

Molecular beacons (MB) are single-stranded nucleic acid sequences that possess a stem loop structure, which is double labeled with a fluorescent dye and a universal quencher at the 5´ and 3´ends, respectively. In the presence of the target sequence, they unfold, bind and fluoresce (Tyagi & Kramer 1996). In a study of Kuechenmeister *et al*. (2009) molecular beacons were utilized for the simultaneous enumeration of mRNA abundances and mRNA degradation properties of well-characterized *Staphylococcus aureus* virulence factors. In their

assay, MB hybridization was performed in a quantitative PCR machine (without a PCR step), which allowed a fast quantification of the hybridized targets.

4.2.1.5 Microarrays

DNA microarrays facilitate the study of large numbers of genes simultaneously by hybridization of DNA or mRNA to a high-density array of immobilized probes. DNA microarray is basically a miniaturized form of dot blot hybridization in a high-throughput format. There are two major types of DNA microarrays; an oligonucleotide based array and a PCR product based array. Microarrays allow the production of 'gene expression profile' or 'signature' for particular organisms under certain environmental conditions. These can be used to study the variability between the same or related species and between the ancestor and the descendants. As a result, microarrays provide information on the molecular basis of microbial diversity, evolution, and epidemiology (for reviews see, Delpech 2000, Lucchini *et al.* 2001, Ye *et al.* 2001, Ball & Trevors 2002, Gibson 2001).

The microarrays (or microchips) consist of thousands of diagnostic gene probes (oligonucleotides = library of 16S probes), which are spotted in high density in a predetermined pattern on a slide. In the PCR product based array, the 16S rRNA gene pool is amplified via PCR, after DNA-extraction. The amplicons are afterwards labeled and hybridized to the surface of the microarray. Positive reaction (=hybridization) will give a fluorescence signal, which will be recognized by a laser scanner. Since the spots can be attributed to certain microbial species, information can be gained with respect to the microbial community composition. Additionally, the brightness of a spot gives information about the abundance of certain microbial taxa.

Samples from soil, blood, tissues, water, air etc. (Brodie *et al.* 2007) can quickly be analyzed after the sample is brought into the liquid phase. Accuracy is ensured by using multiple probes. Another advantage is the sensitivity, allowing underrepresented microorganisms in a sample to be tracked by PhyloChip technology. PhyloChip microarray is a low-cost Affymetrix GeneChip microarray and it is ideal for monitoring and screening since highly reliable and reproducible. It is very fast technology, there are no sequencing errors and it can (to a certain degree) be customized (selection of probes, see “superchip”). Microarray (PhyloChip) technology is highly reproducible and very low abundant taxa can be detected. However there are some disadvantages also, for microarray quite extensive equipment is needed and it relies on DNA extraction and subsequent PCR reaction (may be biased).

4.2.2 PCR-based detection / identification

In PCR a thermostable DNA polymerase enzyme is used to exponentially amplify a target DNA sequence defined by two oligonucleotide primers (Saiki et al. 1985, Mullis et al. 1986, Mullis 1987a, Mullis, 1987b). The amplified DNA fragment can be visualized by agarose gel electrophoresis, which also allows the size determination of the PCR product, or by hybridizing the PCR product with a labeled probe. Combining PCR with a hybridization step improves the sensitivity and specificity of the assay. PCR technique is very sensitive, and small amounts of contaminating DNA from many sources can give false-positive results.

PCR techniques are very sensitive, and small amounts of contaminating DNA can give false positive results. In addition, fecal samples contain factors which can either totally inhibit the PCR reaction or cause partial inhibition leading to a non-exponential amplification of the target (Wintzingerode *et al.* 1997). Inhibition may be avoided or reduced by pre-PCR sample manipulations such as dilution of the sample material, optimization of the used DNA/RNA

extraction protocol, or by harvesting the bacterial cells from the sample e.g. by centrifugation, filtration, or using immunomagnetic beads coated with specific monoclonal antibodies to the target organism. However, even partial inhibition of the PCR reaction inevitably leads to reduced sensitivity and excludes the possibility of performing quantitative PCR. To minimize the risk of obtaining false-negative amplification results suitable external standards, which are co-amplified together with the target DNA in the PCR reaction, may be used (Reischl & Kochanowski 1995).

Quantification of the initial amount of target gene is not possible in traditional end-point PCR because the amount of PCR product is determined when the reaction has already reached the plateau phase. In real-time PCR (quantitative PCR – qPCR), the amount of PCR product is measured at each cycle, also during the exponential phase, which enables the quantification of the initial template amount. The real-time measurement is based on fluorescent dyes that either bind to double stranded DNA or hybridize to a specific sequence. Since real-time PCR is especially vulnerable to inhibitory compounds, internal standards should always be used when complex sample matrices are studied (Reischl and Kochanowski, 1995).

4.2.2.1 DNA/RNA extraction

When PCR is applied to environmental samples several problems arise including inhibition of PCR amplification by co-extracted contaminants, differential PCR amplification, formation of PCR artefacts, e.g. chimeric molecules (leading to description of non-existing species), and DNA contamination. It should also be noted that 16S rRNA gene sequence variations due to rrn operon heterogeneity can interfere with the analysis (for a review, see Wintzingerode *et al.* 1997). When PCR is used in direct bacterial detection from sample materials containing other microbes, the validation of the protocol applied is of utmost importance. The chosen method has to be tested on a large panel of strains representing the target species, closely related species and other microbes commonly present in the sample material. This, and the fact that different methods have to be applied to overcome the inhibitory effects of different sample matrixes, necessitates the use of tailor-made approaches for each microbe-sample matrix pair.

Reliable and reproducible lysis of microbial cells, as well as the extraction of intact nucleic acids from any habitat is a demanding task (Wheeler & Stahl 1996, von Winzingerode *et al.,* 1997). In addition, removal of substances, which may interfere with hybridization or PCR amplification, may be difficult (Wheeler & Stahl 1996). The cell lysis can be performed by enzymatic (e.g. lysozyme, lyticase or proteinase), chemical (e.g. detergents or guanidium isothiocyanate), or mechanical procedured (e.g. freeze-thaw/freeze-boil cycles, bead-beating, or microwave heating). In many cases, e.g. for the identification or fingerprinting of isolates obtained by culture, the crude cell lysate can be used directly in a subsequent molecular analysis. However, since environmental samples can contain inhibitory compounds, crude cell lysates are usually not sufficient when direct molecular detection methods are applied. Further processing steps include removal of proteins, which has commonly been performed by phenol-chloroform extraction, followed by precipitation of nucleic acids by ethanol, isopropanol or polyethylenglycol precipitation, and purification of nucleic acids (Wallace 1987, Roose-Amsaleg *et al.* 2001).

There are also several commercial kits available for the DNA and RNA extractions (Li *et al.* 2003, Li *et al.* 2007, Nechvatal *et al.* 2008, Ariefdjohan *et al.* 2010, Maukonen 2012). The common finding of all the before mentioned articles has been that the results/numbers of Gram-positive bacteria have been better/higher if a rigorous mechanical disruption step has been included in the DNA-extraction protocol. On the other hand, if only Gram-negative bacteria have been targeted, enzymatic DNA-extraction has been sufficient. When DNA-

extraction is performed from fecal material using solely chemical treatments combined with heat treatments, it has been shown that the results for some bacterial groups may be as much as 4 log-values lower as compared to a DNA extraction method in which rigorous mechanical disruption is applied (Maukonen *et al.* 2012). Therefore the used DNA/RNA extraction may have a great impact on the results obtained from any kind of samples.

A laser-induced cell lysis system has also been developed that enables instantaneous cell lysis of *E. coli*. The protocol did not denature proteins under conditions that caused 80% cell lysis, whereas the intracellular mRNA molecules were released and were detectable. Moreover, miniaturization of this system will make it available for the field use (Dhawan *et al.* 2002).

The utilization of ethidium monoazide (EMA) or propidium monoazide (PMA) for amplification of only viable cells

Viable bacteria (in this case bacteria with intact membranes) may also be targeted by using DNA-intercalating dyes ethidium monoazide (3-amino-8-azido-5-ethyl-6-phenyl-phenanthridinium chloride; EMA) (Nogva *et al.* 2003, Rudi *et al.* 2005a, Rudi *et a*l. 2005b, Nocker & Camper 2006, Nocker *et al.* 2006) or propidium monoazide (PMA) (Nocker *et al.* 2006, Nocker *et al.* 2007a, Nocker *et al.* 2007b, Nocker & Camper, 2009; Nocker *et al.*, 2009) prior to DNA extraction. Although the mechanism of action of EMA and PMA has not been fully elucidated yet, the overall result of treatment of membrane-compromised cells is a reduction in PCR amplification (Nocker *et al.* 2009). EMA can enter only bacterial cells with compromised cell walls and cell membranes (Rudi *et al.* 2005a). EMA and DNA become cross-linked by covalent bond after visible light irradiation (maximum absorbance at 460 nm), after which the normally reversible intercalation becomes irreversible. Monoazide substituent is converted with photolysis to nitrile, which can form a covalent linkage to DNA and other molecules. Simultaneously the free EMA in solution is inactivated through a reaction with water molecules. Thereafter the free EMA is transformed to hydroxylamino ethidium which has only little DNA binding ability (Nocker & Camper 2006, Soejima *et al.* 2007). It has been proposed that the selective loss of genomic DNA from dead cells takes place during the DNA-extraction procedure (Nocker & Camper 2006) or that cross-linking strongly inhibits PCR (Nocker *et al.* 2007). However, in the study of Soejima *et al.* (2007) no chromosomal DNA was found after treatment with 100 µg/ml of EMA (before DNA-extraction step). The loss of the DNA on the gel was neither due to the change of DNA from double- to single-strand as confirmed by SYBR Gold staining nor due to hydroxylamino ethidium remaining in DNA preparation. In fact, electron microscopy studies confirmed that the decreased band intensity in electrophoresis was mainly due to the direct random breaks of double-stranded DNA by the EMA treatment (Soejima *et al.* 2007). EMA did not have a bactericidal effect on *E. coli,* when treated with less than 10 µg/ml of EMA for 3 hours. However, high concentrations (100 µg/ml) showed strong bactericidal effect (Soejima *et al.* 2007). It has also been found that with some bacterial species EMA is able to penetrate also the viable cells thus inhibiting the DNA amplification from viable cells as well as from dead cells (Nocker & Camper 2006, Nocker *et al.* 200, Cawthorn & Witthurn 2008).

It has been found that the concentration of EMA (Wang & Levin 2006, Chang *et al.* 2009), the photoactivation time (Wang & Levin, 2006), and the matrix in which the bacteria are embedded (Pisz *et al*. 2007) are critical for the maximal discrimination of viable cells from dead cells. In addition, in a study of Chang *et al* (2009), they found that the amplification of partial 16S rRNA gene (406 bp) was the most discriminative towards viable *Legionella* cells as compared to the amplification of 5S rRNA (108 bp) and *mip* genes (649 bp). Therefore they concluded that if EMA randomly binds and cleaves DNA sequences, a smaller DNA region would not be affected by EMA treatment and would be amplified with PCR (Chang *et al.* 2009). Moreover, Soejima *et al.* (2008) demonstrated with *L. monocytogenes* that with longer

PCR-products (894 bp) EMA was specific, whereas with short PCR-products (113 bp) the used EMA-protocol could not reliably differentiate between viable and dead cells. Therefore Soejima *et al.* (2008) developed an alternative protocol, in which DNA gyrase/topoisomerase IV and mammalian topoisomerase poisons (T-poisons) were added to improve the specificity of the EMA-protocol. T-poisons could penetrate heat-treated *L. monocytogenes* cells to cleave chromosomal DNA by poisoning activity within 30 min. The EMA-protocol with added T-poisons was able to detect 10 live *L. monocytogenes* cells from spiked milk samples (Soejima *et al.* 2008).

PMA has been shown to be more specific to membrane-compromised cells than EMA in a few studies (Nocker *et al.* 2006, Cawthorn & Witthurn 2008), whereas in a study of Lee & Levin 2009) EMA was found to be more specific than PMA. PMA is identical to propidium iodide (PI), which has been widely used for the detection of dead cells, except that PMA has an additional azide group that allows cross-linking with DNA (Nocker *et al.* 2006) similarly to EMA. Since the PMA molecule has a higher charge than the EMA molecule (two positive charges compared to one, respectively) and PI has been successfully used in a wide variety of studies, PMA was suggested to be a better alternative than EMA for selective amplification of viable cells (Nocker *et al.* 2006).

4.2.2.2 Polymerase chain reaction (PCR) – practical considerations

Many types of sample matrixes contain factors which can either totally inhibit the PCR reaction or cause partial inhibition leading to a non-exponential amplification of the target DNA (Rossen *et al.* 1992, Lantz *et al.* 1994, Scheu *et al.* 1998). Inhibition may be avoided or reduced by pre-PCR sample manipulations such as dilution of the sample material, short enrichment culture, extraction of the DNA from the sample, or by harvesting the bacterial cells from the sample e.g. by centrifugation, filtration, or by using immunomagnetic beads coated with specific monoclonal antibodies to the target organism. However, even partial inhibition of the PCR reaction inevitably leads to reduced sensitivity and excludes the possibility of performing quantitative PCR. To minimize the risk of obtaining false-negative amplification results suitable external standards, which are co-amplified together with the target DNA in the PCR reaction, should be used (Reischl & Kochanowski, 1995). Sensitivity of the PCR assay can be improved by enrichment culture prior to PCR (Agersborg *et al*. 1997, Wang *et al.* 1997), but this also precludes attempts to quantify the number of target organisms in the sample. Thus amplification of target DNA sequences from sample materials containing inhibitory factors for PCR can provide information on the presence, but not on the numbers and usually not on the viability of target organisms (except in the case where enrichment step is included). It should also be remembered that PCR will also detect nonviable cells, as long as intact target nucleic acid sequences are available as templates (Josephson *et al.* 1993).

When PCR is applied to environmental samples several problems arise including inhibition of PCR amplification by co-extracted contaminants, differential PCR amplification, formation of PCR artefacts e.g. chimeric molecules (leading to description of non-existing species), and DNA contamination. It should also be noted that 16S rDNA sequence variations due to rrn operon heterogeneity can interfere with the analysis (for a review, see von Wintzingerode *et al.* 1997). If only *in silico* testing is performed, the specificity of the applied PCR may not necessarily be as expected. This, and the fact that different methods must be applied to overcome the inhibitory effects of different sample matrices, necessitates the use of tailor-made approaches for each microbe-sample matrix pair. A positive control for each analysis is important in the confirmation that inhibitory substances do not interfere with the detection and cause false-negative results (Hoffman & Wiedman 2001).

Quantification of the initial amount of target is not possible in traditional end-point PCR because the amount of PCR product is determined when the reaction has already reached the plateau phase. In real-time PCR, the amount of PCR product is measured at each cycle, also during the exponential phase, which enables the quantification of the initial template amount. The real-time measurement is based on fluorescent dyes that either bind to double strand DNA or hybridize to a specific sequence. Since real-time PCR is especially vulnerable to inhibitory compounds, internal standards should always be used when complex sample matrixes are studied (Reischl & Kochanowski, 1995).

Quantitative reverse transcriptase–polymerase chain reaction (RT-qPCR) -based approaches represent fast, effective methods enabling the quantification of gene and/or transcript numbers within environmental samples (including unculturable and culturable fraction), providing unparalleled specificity and sensitivity to target sequences present within a mixed community background (Smith and Osborn 2009). RT-qPCR based techniques are widely used for RNA detection. While RT-PCR is used to qualitatively detect gene expression through creation of complementary DNA transcripts from RNA, qPCR is used to quantitatively measure the amplification of DNA using fluorescent probes. RT-PCR is currently the most sensitive technique available for the quantification of steady-state mRNA levels (Doak and Zaïr 2012). It requires only a small amount of RNA for analysis. RT–qPCR affords a sensitive and rapid approach for quantifying low levels of bacteria and viruses (Yang *et al.* 2011) when sample is collected to e.g. filter.

PCR also requires less operator skill and training to carry out and can be automated to process large numbers of samples. It is very sensitive, although it can be vulnerable to contamination and cannot distinguish infective viruses. Most PCR methods also require some costly equipment and are not suitable for use outside the laboratory. Some recent advances have been made to reduce the complexity, the volume and the weight of qPCR instruments. However, it leads most of the time to a decrease in throughput and multiplexing capabilities. The most interesting improvement in the family of qPCR instruments has been proposed by BIOFIRE Diagnostics. The FilmArray of BIOFIRE is a multiplex PCR that integrates sample preparation, amplification, detection and analysis all into one system. It requires just a few minutes of hands-on-time and its turnaround time is approximately one hour. The FilmArray pouch stores all the necessary reagents for sample preparation, reverse transcription-PCR, PCR, and detection in a freeze-dried format, and the user has just to add a hydration solution and an unprocessed sample.

4.2.2.3 Next generation sequencing technologies

Next generation sequencing technologies (NGS) allow deeper analysis of the microbial communities (Zwolinski 2007). The capability of large-scale sequencing techniques to generate billions of reads at low cost with high speed is useful in many applications such as whole-genome sequencing, metagenomics, metatranscriptomics, and proteomics. Metatranscriptomics allows monitoring of microbial gene expression profiles in natural environments by studying global transcription of genes by random sequencing of mRNA transcripts pooled from microbial communities at a particular time and place (Moran 2009).
Recent developments in new sequencing chemistries, bioinformatics, and instruments have revolutionized the field of microbial ecology and genomics.

Pyrosequencing

One of the next generations DNA sequencing technologies (NGS) is pyrosequencing, which has increased the resolution and detectable spectrum of microbial communities and of the

dynamics of microbial ecosystems in environmental samples. The principle of 454 pyrosequencing technique is that each incorporation of a nucleotide by DNA polymerase results in the release of pyrophosphate, which initiates a series of downstream reactions that ultimately produce light by the firefly enzyme luciferace (Mardis 2008). As a result of enzymatic reactions, light can be detected by a charge coupled device (CCD) camera and as a peak in a pyrogram. The light intensity is proportional to the amount of ATP used, and the height of each peak is proportional to the number of nucleotides incorporated (Rothberg & Leamon 2008, Mardis 2008, Siqueira *et al.* 2012). The advantage of pyrosequencing is that it provides a high amount (even hundreds of thousands) of sequence reads in single run, and thus the analysis has broad sampling depth including both the dominant and rare members of investigated microbial community (Siqueira *et al.* 2012). The sensitivity of NGS techniques are in the range of PCR. La Duc *et al.* (2012) have shown by using 454 pyrosequencing method that the detected microbial diversity is 100-1000-fold greater than that analyzed by using cultivation methods in a spacecraft. Also single genomes and genes can be explored efficiently.

However, according to Mardis (2008) commercial available instruments allow highly streamlined sample preparation steps prior to DNA sequencing, which provides significant time savings and minimal need for other equipment. After pre-treatment genomic DNA is isolated, fragmented, ligated to special adapters and separated into single strands in liquid sample (Siqueira *et al.* 2012, Rothberg & Leamon 2008). It is possible to sequence only viable cells if propidium monoazide (PMA) treatment is used to differentiate living and dead cells.

Bridge sequencing

Illumina's sequencing by synthesis (SBS) technology is the most successful and widely adopted next-generation sequencing platform worldwide. SBS technology uses four fluorescently labelled nucleotides to sequence the tens of millions clusters on the flow cell surface in parallel. During each sequencing cycle, a single labelled deoxynucleotide triphosphate (dNTP) is added to the nucleic acid chain. The nucleotide label serves as a terminator for polymerization, and therefore after each dNTP incorporation, the fluorescent dye is imaged to identify the base and then enzymatically cleaved to allow incorporation of the next nucleotide. Base calls are made directly from signal intensity measurement during each cycle. Unlike pyrosequencing (Roche/454 pyrosequencing), the DNA chains are extended one nucleotide at a time and image acquisition can be performed at a delayed moment, allowing for very large arrays of DNA colonies to be captured by sequential images taken from a single camera (Ansorge 2009, Metzker 2010).

Bridge sequencing is a semi-quantitative method. *Illumina* launched in 2011 the MiSeq, first commercial application based on bridge sequencing. MiSeq promises to go from DNA to data in just over 8 hours (1.5 hours for sample preparation, 4 hours for sequencing, 3 hours for analysis). Although it is presented as a compact, all-in-one platform that incorporates cluster generation, paired-end fluidics, sequencing by synthesis chemistry, and complete data analysis, some steps have to be performed by a trained operator (15 minutes of manipulation for the sample preparation, 20 min for sequencing). The main advantage of currently available bridge sequencing applications is the user-friendly data-analysis of Illumina's MiSeq which allows the user to follow the performance of the reaction in real-time. MiSeq has therefore been used as an example in the trade-off-table. Figure 2 shows an example of phylogenetic pie chart from Illumina MiSeq.

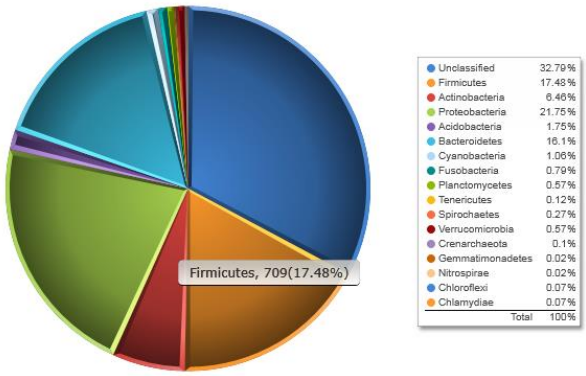

Figure 2. Example of presentation of phylum-level data from MiSeq, Illumina (MiSeq Reporter Metagenomics Workflow Reference Guide)

In addition, *Illumina* will complete its product range with the launch of two new sequencers, HiSeqX and NextSeq 500. HiSeq X Ten is a factory scale sequencer for population sequencing and NextSeq 500 is a new desktop high-throughput sequencer. NextSeq will have the power of HiSeq, but with the size of MiSeq.

Nanopore sequencing

Nanopore sequencing is a semi-quantitative method. *Oxford Nanopore Technologies* is the only manufacturer that provides a commercial instrument based on Nanopore sequencing, the Oxford nanopore GridION, with its miniaturized version the MiniION. Oxford Nanopore passes an ionic current through nanopores and measures the changes in current as biological molecules pass through the nanopore or near it. The information about the change in current can be used to identify that molecule. Nanopore sequencing has been a promising alternative for NGS-platforms for over five years, ever since Oxford Nanopore Technologies licensed the technology in 2008. Advantages of the Oxford MinION nanopore sequencing include small size (more a sensor than a platform), and single-use concept, which eliminates the need for equipment repairing. The MinION™ system is a disposable device that contains the sensor chip, ASIC and nanopores that are needed to perform a complete single molecule sensing experiment. Plugging directly into a laptop or desktop computer through a USB port, it is a self-contained device to deliver real-time experimental data. Sequencing is mostly automated, but the data-analysis is not yet automatic. However, development of data-analysis options is in-progress.

One interesting trend on nanopore sequencing is that Illumina has made a patent-licensing deal between themselves and the University of Washington (UW). The licensing deal gives Illumina, developer of integrated systems for genetic variation analysis, exclusive worldwide rights to develop and market the nanopore DNA sequencing technology that is based on the engineered pore of UW. Since Illumina is currently the market leader in NGS sequencing, rapid development in nanopore sequencing may be expected in the next few years.

It is important to stress that sufficient computing power is vital to the performance of these analyses and that these techniques also require specific training for interpretation of results. However, in a longer term perspective and with the rapid advances in this field, the suitability of this method should also be evaluated.

Other sequencing methods are SOLiD™ System (Applied Biosystems, USA), the HeliScope Single Molecule Sequencer (Helicos BioSciences, USA), and the Single Molecule Real Time technology (SMRT, Pacific Biosciences, USA). The technology of these platforms shares

characteristic of performing massively parallel sequencing (clonally PCR-amplified products (the first three platforms) or single DNA molecule (last two)), when amplification of DNA fragments is not needed prior to sequencing (Mardis 2008, Siquera *et al.* 2012).

Metagenomics

Metagenomics may be defined as the application of NGS to a DNA-template obtained directly from a sample (Kunin *et al.* 2008). In most studies, the metagenomic approach is applied to study the genes present in the microbiota. As with any NGS-technique, the amount of data obtained is enormous. However, metagenomic studies are usually performed with the Illumina HiSeq-technique, and thus the obtained sequence length is usually <200 bp. This makes the bioinformatics part even more demanding than when NGS is applied to a PCR-product.

4.2.3 Other options

4.2.3.1 Biosensors

In general, a biosensor consists of a biorecognition element that provides the assay with specificity to an analyte of interest, and a transducer as well as other components, such as amplification elements and solid supports. Ideally all these elements would be assembled within one housing (Baeumner *et al.* 2004). Within the environment of interest in this project the most relevant biosensors would detect bacteria and/or viruses of interest. There are three different types of biosensors; a direct biosensor, a competitive indirect biosensor and a non-competitive indirect biosensor. In the direct biosensor, the analyte is bound by its biorecognition element, which is detected directly. In indirect biosensor an additional reaction (either competitive or non-competitive) has to occur in order to detect the binding of analyte and biorecognition element. Direct and non-competitive indirect biosensors provide signals that are proportional to the analyte concentration, whereas the competitive indirect biosensor provides an indirect proportionality (Baeumner 2003).

In a study of Baeumner *et al.* (2003), a highly sensitive and specific RNA biosensor utilizing the NASBA based quantification of *clpB* gene coding for a heat shock protein of *E. coli* was developed. The newly developed biosensor, which was a membrane based DNA/RNA hybridization system using liposome amplification, allowed a rapid detection and quantification of *E. coli* mRNA in only 15-20 min. In addition, the developed biosensor was portable, inexpensive and very easy to use. The detection limit using a synthetic target sequence was determined as 5 fmol per sample, which was similar to that of their previous study (Min and Baeumner 2002).

In a later study of the same research group (Baeumner *et al*. 2004a), a dipstick-type biosensor with liposome amplification, based on a sandwich assay format with optical detection, was combined with a simple coupling reaction that allows the transformation of the generic biosensor components to target ones with just a simple 20-min incubation step. Thereafter an 8-10 min membrane assay is performed. No special personnel training and no specific equipment other than heating block or water bath were needed to perform the analysis. In addition, the signals could be quantified using a computer scanner or a portable reflectometer. The lower and upper detection limits of *E. coli* were defined as 5 fmol and 1000 fmol, respectively (Baeumner *et al.* 2004a). In an improved version (Baeumner *et al.* 2004b) of the previous biosensor (Baeumner *et al.* 2004a), the detection limit was lowered to 1 fmol and the dynamic range between 1-750 fmol (Baeumner *et al.* 2004b).

Chen *et al.* (2008) have developed a circulating-flow piezoelectric biosensor, which is based on an Au nanoparticle (Merkoçi 2007) amplification and verification method. The target gene was first amplified and thereafter detected with the piezoelectric biosensor with a detection limit of 10^2 cfu/ml of *E. coli* O157:H7 (Chen *et al.* 2008). In a study of Sun *et al.* (2006) a sensitive (23 *E. coli* cells) flow through piezoelectric quartz crystal (PQC)/DNA biosensor assay was developed, in which sequencial flow PCR products were denaturated before the hybridization of single stranded DNA. The hybridized products were thereafter detected with PQC (Sun *et al.* 2006). Furthermore, Sun *et al.* (2008) developed a nano-silver modified PQC/DNA biosensor, which was able to detect a single *E. coli* cell from 100 ml of water.

There are also studies in which array format biosensors (Gabig-Ciminska 2006) have been developed. In a study of Farabullini *et al*. (2007) a disposable electrochemical genosensor was developed, whereas Johnson-White *et al.* (2007) developed an immunoassay format array biosensor, in which *E. coli* was one of the target bacteria. After the immunoassay, which confirmed the identity, a viability staining was performed to confirm the viability of the targeted bacteria. Furthermore, cells captured by the immobilized antibodies can be cultured for further confirmation of the viability or a PCR analysis may be performed from the targeted cells. The detection limit of the immunoassay as such was 10^5 cfu/ml and after PCR 10^2 cfu/ml of *E. coli* (Johnson-White *et al.* 2007). Furthermore, Straub *et al*. (2005) have developed a BEADS (biodetection enabling analyte delivery system) platform to automatically concentrate and purify target analytes utilizing immunomagnetic separation (IMS). Captured cells are thereafter amplified in a flow-through PCR and PCR products are detected by hybridization to a DNA suspension array (Straub *et al.* 2005).

4.2.3.2 Lab-on-a-chip

Lab-on-a-chip (LOC) is a term for devices that integrate (multiple) laboratory functions on a single chip of only millimeters to a few square centimeters in size. They are capable of handling extremely small fluid volumes down to less than pico liters. In general, they consist of a microchip containing wells and channels where samples and reagents are stored and transported to reaction chambers to create a microfluidic network. The chip is connected to an instrument for control and detection (biosensor) and to a computer performing data analysis and relay information to required locations (Gilbride *et al*. 2006).

In a study of Zhao *et al.* (2006), a microsystem compatible approach was developed for the detection of viable *E. coli* utilizing *GroEL* heat shock protein. On a microchip with controlled heating modules, the bacterial cells are first subjected to high temperature treatment to stimulate the mRNA transcription of the heat shock proteins. On the next step, the target mRNA is specifically hybridized to the complementary biotinylated capture probe. Thereafter, the *GroEL* mRNA is separated from cell lysates with streptavidin-coated magnetic particles. Then the target mRNA is converted and amplified into large amount of biotinylated cDNA. Next, streptavidin-coated gold nanoparticles will bind to biotin end of the amplicons – after the amplicons have hybridized to the immobilized probe on the working electrode surface. Finally, the electrochemical (EC) signal of gold catalyzed silver reduction is detected with the EC method to represent the existence of amplicons (Zhao *et al*. 2006).

There are also other studies in which different bacteria have been detected with lab-on-a-chip assay or an assay that may be integrated to a lab-on-a-chip (Zaytseva *et al.* 2005, Yeung *et al.* 2006), e.g. continuous flow PCR (Hashimoto *et al.* 2004, deMello 2006), DNA extraction with laser irradiation and magnetic beads (Lee *et al.* 2006) and nanodroplet real-time PCR system with laser assisted heating (Kim *et al.* 2009). Furthermore, in a study of Dimov *et al.* (2008) an integrated microfluidic tmRNA purification and real-time NASBA device were developed. Moreover, in a study of Lee *et al.* (2008b) a polymer LOC for reverse

transcriptase PCR (RT-PCR) of human immunodefiency virus was designed, fabricated and validated. In addition, a portable analyzer consisting of a non-contact infra-red based temperature control system for RT-PCR process and an optical detection system for on-chip detection, enabling the analysis to be performed in less than 1 hour, were also developed (Lee *et al*. 2008b).

4.2.4 The effect of the used method on the results

Most of the steps in the processing of samples from any given environment may affect the microbiological results obtained. The sampling and storage conditions have been shown to affect the results (Ott *et al.* 2004, Molbak *et al.* 2006, Roesch *et al.* 2009), although not as extensively as the following steps, especially the DNA extraction (Salonen *et al.* 2010, Dridi *et al.* 2009, Maukonen *et al.* 2012). The initial two week storage at -20°C has been shown to decrease the detected numbers of *Bacteroides* spp. by over 1 log unit as compared to the fresh samples, whereas the storage temperature has not been shown to have such a significant effect on the gram-positive bacteria (e.g. Firmicutes and Actinobacteria) (Maukonen et al. 2012). Since the analysis of fresh samples, especially in the case of large samplings, is impractical, the effect of the storage conditions on the results obtained should be taken into account when the results are discussed. When DNA-extraction is performed using solely chemical treatments combined with heat treatments, it has been shown that the results for some bacterial groups may be as much as 4 log-values lower as compared to a DNA extraction method in which rigorous mechanical disruption is applied (Maukonen *et al.* 2012).

Actinobacteria, in particular, have been shown to be underestimated with many currently used techniques, most probably due to their high G+C content (Krogius-Kurikka *et al.* 2009). In a study of Nakamura *et al*. (2009), it was shown that when the same samples were examined with qPCR (enzymatic DNA-extraction) and FISH, the difference in results was enormous. The proportions of bifidobacteria with qPCR was detected to be 0.1 -1.7%, whereas the proportion with FISH was 28 – 84% (Nakamura *et al.* 2009). Moreover, in studies in which clone libraries have been constructed, the primer Bact-8F (synonyms: e.g. 008F) targets bifidobacteria only poorly (Magne *et al.* 2006), and therefore in studies in which this primer has been used the abundance of bifidobacteria has been underestimated (e.g. Palmer *et al.* 2007, Eckburg *et al.* 2005, Suau *et al.* 1999). In addition, the normally used 95°C for denaturation in PCR-amplification may not be sufficient for high G + C content bacteria, such as Actinobacteria and SRB (Maukonen *et al.* 2012).

Another methodological aspect that may cause confusion is that different probes/primers target different bacterial populations, such as in the detection of Bacteroidetes and Firmicutes. In pyrosequencing/metagenomic studies the authors usually refer to Bacteroidetes, whereas in studies conducted with FISH/PCR, the studied bacterial population is usually narrower (e.g. *Bacteroides* spp. or *Bacteroides-Prevotella* group) (Hoyles & McCartney 2009). The same controversy applies to Firmicutes. In many pyrosequencing studies Firmicutes are discussed as a single group, whereas in fact it contains several different bacterial classes – e.g. Bacilli, Clostridia, Erysipelotrichia – which all have different metabolic properties. Furthermore, when PCR/FISH is used, each of these groups is usually studied separately.

When microbial communities are examined, especially using several different techniques and/or next-generation sequencing, the quantity of data generated is enormous. Therefore, the use of suitable algorithms for e.g. measurement of microbial diversity and correct interpretation of the results are of utmost importance (Corry *et al.* 2007, Ramette 2007, Rudi *et al.* 2007, Bent & Forney 2008, Lozupone *et al.* 2007, Werner *et al.* 2012, Kuczynski *et al.* 2011, Achtman *et al.* 2008). Moreover, we should also bear in mind that even if statistical significance is achieved, the observed changes may not be of biological significance.

5. Conclusions

Transportation plays an important role in the spread of infectious diseases. In addition to naturally occurring infectious diseases, threats also exist from the intentional use of biological agents. Transport sector is critical to our society and any disturbances in the major hubs can have wide-spread consequences. Primary challenge in the transportation hubs is to control rather than eradicate the microbes (Camper & McFeters, 2000). Furthermore, rapid detection methods are needed in order to be able to react quickly in possible pandemic cases. Knowledge about the microbiota present will help in the controlling, since specific characteristics of each microbiota can be considered as preventative / control measures are applied. It should be remembered that sampling is a crucial step in the characterization / identification procedure. If it is performed inadequately, the characterization of microbiota will inevitably be biased.

One of the challenges for microbial ecology is to gain more information below the bacterial community, genera, and even species level. Subspecies level identification is especially important when a source of contamination is traced in epidemic cases. DNA fingerprinting techniques provide effective molecular tools to identify and type microorganisms to subspecies level (Giraffa & Neviani 2001). While typing of the microbial isolates is performed, e.g. to trace a contamination source, the importance of including sufficient numbers of isolates from each sample site should be remembered. Once efficiently integrated, the typing techniques provide precise information on the heterogeneity of target bacterial population at a given time/space combination. However, fingerprinting methods are laborious and time consuming since isolation and cultivation of a large number of bacterial isolates cannot be avoided. Another limitation is that the unculturable strains present in natural ecosystems cannot be reached with typing methods.

The following list summarizes the methods, which have been excluded from the potential list of usable methods in PANDHUB. However, some of these methods could be useful as an additional or backup method. For instance autofluorescence does not provide the required information about microbiota but if combined with another technique, it could contribute to an optimal concept. The main advantages of autofluorescence method are on-line monitoring and functioning without reagents. It could thus be of interest for continuous prescreening and for selection of sampling points. The disadvantage is that separate instrumentation is needed for air and water samples, and surface samples need sample preparation.

The eliminated methods are:

- **Autofluorescence**: the method is based on counting fluorescent particles and thus cannot provide any information about microbiota. Separate instrumentation is needed for air and water quantification.

- **FISH:** FISH method is not suitable if analysis is conducted with microscopy. However, this method could be suitable if FISH is combined with flow cytometry.

- **Biosensors & Lab-on-a-chip**: Biosensors and Lab-on-a-chip do not providing all information required. They need to be further developed.

- **Pyrosequencing:** Pyrosequencing cannot be considered anymore, since the proprietary manufacturing company Roche has announced that research on the method has been stopped. Therefore it is removed from the list of potential methods. When the method existence in the future is questionable, the method has been eliminated from the table comparing other trade-off properties.

- **Metagenomics:** Metagenomics is not suitable, since computer capacity needed for metagenomics analysis is huge.

- **Metatranscriptomics:** This is even more complex than the previous method and therefore not suitable.

- **Proteogenomics:** not suitable.

In addition to these techniques, most hybridization based methods were eliminated, since PCR- and/or NGS-based methods seem to hold the most promising features for rapid detection of wanted pathogens. In addition, in case of new high treat microbial incidences, those methods may quickly be adapted for detection of new microbes.

After the rough elimination, the methods potentially suitable for rapid microbial contamination detection for the PANDHUB purposes are:

- **qPCR:** method fully quantitative, suitable for quantitative and qualitative approach.

- **NGS after a specific PCR (i.e. not the metagenomics approach):**

    - **Bridge sequencing (Illumina HiSeq/MiSeq) / Nanopore sequencing / Semiconductor-based sequencing (IonTorrent):** method not fully quantitative but suitable for semi-quantitative and qualitative approach.

It is important to stress that the successful use of each of these methods requires good sampling strategy, in addition to optimized DNA/RNA extraction. The detection of viability would also be important, besides the detection of microbial incidence / prevalence. Due to the fact that environmental conditions influence the cellular rRNA content, the amount of the growth rate is considered to correlate with the amount of rRNA; therefore it also can be used for preliminary screening of bacterial activity. In addition, PMA/EMA treated samples could also be used for screening of viability.

In conclusion, bacterial detection, identification and typing from environmental samples remains a laborious task, mainly due to the fact that frequently large numbers of samples need to be analyzed. Development of automated techniques that allow high throughput analysis of large numbers of samples will greatly facilitate the studies on microbial ecology.

EMA/PMA in combination with qPCR would be an interesting alternative for detection of specific high threat pathogens. qPCR would allow rapid, specific, and sensitive quantification and EMA/PMA treatment would enable the specific amplification of viable (membrane-intact) bacteria. Moreover, there are portable qPCR machines, which would allow the performance of the analysis outside the laboratory (e.g. the handheld advanced nucleic acid analyzer (HANAA), which weighs less than 1 kg and has been evaluated with *E. coli* enumeration; Higgins *et al.* 2003). In an article of Gedalanga & Olson (2009) EMA-qPCR, using *uidA* and *fliC* genes, was used for the enumeration of viable *E. coli* cells from wastewaters. Gedalanga and Olson (2009) got similar results with culture and EMA-qPCR from certain wastewaters, indicating that EMA-qPCR could be used for the monitoring of viable bacteria. However, they also found that in certain samples EMA-qPCR and culture results did not correlate well. As mentioned above, the concentration of EMA (Wang & Levin 2006, Chang *et al.* 2009), the photoactivation time (Wang & Levin 2006), and the matrix in which the bacteria are embedded (Pisz *et al.* 2007) are critical for the maximum discrimination of viable cells from dead cells, which necessitates the optimization of the EMA/PMA-qPCR protocol separately for each different environment.

There is no suitable method for online air and/or surface sampling which can be used for detection and identification at the same time. However, there are rapid methods, which take several hours for detection and identification. Because there are no valid commercial methods, the best way is to invest in prevention as well as to develop new rapid detection and identification techniques.

References

Achtman M & Wagner M (2008). Microbial diversity and the genetic nature of microbial species. Nat Rev Microbiol, 6:431–440.

Agersborg A, Dahl R & Martinez I (1997). Sample preparation and DNA extraction procedures for polymerase chain reaction identification of *Listeria monocytogenes* in seafoods. Int J Food Microbiol, 35:275-280.

Alakomi HL, Mättö J, Virkajärvi I & Saarela M (2005). Application of a microplate scale fluorochrome staining assay for the assessment of viability of probiotic preparations. J Microbiol Methods, 62:25-35.

Alwine JC, Kemp DJ & Stark GR (1977). Method for detection of specific RNAs in agarose gels by transfer to diazobenzyloxymethyl-paper and hybridization with DNA probes. Proc Natl Acad Sci USA, 74:5350-5354.

Amann RI, Binder BJ, Olson RJ, Chisholm SW, Devereux R & Stahl DA (1990a). Combination of 16S rRNA-targeted oligonucleotide probes with flow cytometry for analyzing mixed microbial populations. Appl Environ Microbiol, 56:1919-1925.

Amann RI, Krumholz L & Stahl DA (1990b). Fluorescent-oligonucleotide probing of whole cells for determinative phylogenetic and environmental studies in microbiology. J Bacteriol, 172:762-770.

Amann RI, Ludwig W & Schleifer K-H (1992). Identification and *in situ* detection of individual bacterial cells. FEMS Microbiol Lett, 100:45-50.

Amann RI (1995a). *In situ* identification of microorganisms by whole cell hybridization with rRNA-targeted nucleic acid probes. In: Molecular Microbial ecology Manual (Akkermans ADL, DJ van Elsas, and FJ de Bruijn, eds), 3.3.6, pp 1-15, Kluwer Academic Publishers, Dordrecht, The Netherlands.

Amann RI (1995b). Fluorescently labeled, rRNA-targeted oligonucleotide probes in the study of microbial ecology. Mol Ecol, 4:543-554.

Amann RI, Ludwig W & Schleifer KH (1995). Phylogenetic identification and *in situ* detection of individual microbial cells without cultivation. Microbiol Rev, 59:143-169.

Ansorge WJ (2009). Next-generation DNA sequencing techniques. New Biotechnol, 25:195-203.

Ariefdjohan MW, Savaiano DA & Nakatsu CH (2010). Comparison of DNA extraction kits for PCR-DGGE analysis of human intestinal microbial communities from fecal specimens. Nutr J, 9:23.

Armisen TG & Servais P (2004). Combining direct viable count (DVC) and fluorescent *in situ* hybridization (FISH) to enumerate viable *E. coli* in rivers and wastewaters. Water Sci Technol, 50:271-275.

Avery SV, Harwood JL & Lloyd D (1995). Quantification and characterization of phagocytosis in the soil amoeba *Acanthamoeba castellanii* by flow cytometry. Appl Environ Microbiol, 61: 1124-1132.

Azhar EI, Mashem AM, El-Kafrawy SA, Sohrab SS, Aburizaiza AS, Farraj SA, Hassan AM, Al-Saeed MS, Jamjoom GA & Madani TA (2014). Detection of the Middle East Respiratory Syndrome Coronavirus Genome in an Air Sample Originating from a Camel Barn Owned by an Infected Patient. Am Soc Microbiol (5), 4:e1450-14.

Ball KD & Trevors JT (2002). Bacterial genomics: the use of DNA microarrays and bacterial artificial chromosomes. J Microbiol Met, 49:275-284.

Barer MR & Harewood CR (1999). Bacterial viability and culturability. Adv Microb Physiol, 41:93-137.

Baeumner AJ (2003). Biosensors for environmental pollutants and food contaminants. Anal Bioanal Chem, 377:434-445.

Baeumner AJ, Jones C, Wong CY & Price A (2004a). A generic sandwich-type biosensor with nanomolar detection limits. Anal Bioanal Chem, 378:1587-1593.

Baeumner AJ, Pretz J & Fang S (2004b). A universal nucleic acid sequence biosensor with nanomolar detection limits. Anal Chem, 76:888-894.

Bej AK, Ng W-Y, Morgan S, Jones D & Mahbubani MH (1996). Detection of viable *Vibrio cholerae* by reverse-transcriptase polymerase chain reaction (RT-PCR). Mol Biotechnol, 5:1-10.

Bent SJ & Forney LJ (2008). The tragedy of the uncommon: understanding limitations in the analysis of microbial diversity. ISME J, 2:689–695.

Brodie EL, DeSantis TZ, Parker JPM, Zubietta IX, Piceno YM & Andersen GL (2007). Urban aerosols harbor diverse and dynamic bacterial populations. Proc Natl Acad Sci U S A, 104:299–304.

Brosseau LM, Vesley D, Rice N, Goodel K, Nellis M & Hairston P (2000). Differences in detected fluorescence among several bacterial species measured with a direct-reading particle sizer and fluorescence detector. Aerosol Sci Technol 32:545-558.

Camper AK & McFeters GA (2000). Problems in biofouling in drinking waters systems. In: Industrial biofouling; detection, prevention and control (Walker J, S Surman, and J Jass, eds), vol 1, pp 13-38, John Wiley & Sons, Chichester, UK.

Cawthorn DM & Witthuhn RC (2008). Selective PCR detection of viable *Enterobacter sakazakii* cells utilizing propidium monoazide or ethidium bromide monoazide. J Appl Microbiol, 105:1178-1185

Chang B, Sugiyama K, Taguri T, Amemura-Maekawa J, Kura F & Watanabe H (2009). Specific detection of viable *Legionella* cells by combined use of photoactivated ethidium monoazide and PCR/Real-time PCR. Appl Environ Microbiol, 75:147-153

Chen H, Ponniah, G, Salonen N & Blum P (2004). Culture-independent analysis of fecal enterobacteria in environmental samples by single-cell mRNA profiling. Appl Environ Microbiol, 71:4432-4439.

Chen S-H, Wu VCH, Chuang Y-C & Lin C-S (2008). Using oligonucleotide-functionalized Au nanoparticles to rapidly detect foodborne pathogens on a piezoelectric biosensor. J Microbiol methods, 73:7-17.

Coleman JR, Culley DE, Chrisler WB & Brickman FJ (2007). mRNA-targeted fluorescent *in situ* hybridization (FISH) of gram-negative bacteria without template amplification or tyramide signal amplification. J Microbiol Methods, 71:246-255.

Cook N (2003). The use of NASBA for the detection of microbial pathogens in food and environmental samples. J Microbiol Methods, 52:165-174.

Corry JE, Jarvis B, Passmore S & Hedges A (2007). A critical review of measurement uncertainty in the enumeration of food micro-organisms. Food Microbiol, 24:230–253.

Curtis GDW & Lee WH (1995). Culture media and methods for the isolation of *Listeria monocytogenes*. Int J Food Microbiol, 26:1-13.

Cusick SM & O'Sullivan DJ (2000). Use of a single, triplicate arbitrarily primed PCR-procedure for molecular fingerprinting on lactic acid bacteria. Appl Environ Microbiol, 66:2227-2231.

DeLong EF, Wickham GS & Pace NR (1989). Phylogenetic stains: ribosomal RNA-based probes for the identification of single cells. Science, 243:1360-1363.

Delpech M (2000). DNA chips. Ann Biol Clin, 58:29-38.

deMello AJ (2006). Control and detection of chemical reactions in microfluidic systems. Nature, 442:394-402.

Després VR, Huffman JA, Burrows SM Hoose C, Safatov AS, Buryak G, Fröhlich-Nowoisky J, Elbert W, Andreae MO, Pöschl U & Jaenicke R (2011). Primary biological aerosol particles in the athmosphere: a review. Tellus B, 2012, 64.

Dhawan MD, Wise F & Baeumner AJ (2002). Development of a laser-induced cell lysis system. Anal Bioanal Cham, 374:421-426.

Diaper JP, Tither K & Edwards C (1992). Rapid assessment of bacterial viability by flow cytometry. Appl Microbiol Biotechnol, 38:268-272.

Dimov IK, Garcia-Cordero JL, O'Grady J, Poulsen CR, Viguier C, Kent L, Daly P, Lincoln B, Maher M, O'Kennedy R, Smith TJ, Ricco AJ & Lee LP (2008). Integrated microfluidic tmRNA purification and real-time NASBA device for molecular diagnostics. Lab Chip, 8:2071-2078.

Doak SH & Zaïr ZM (2012). Real-Time Reverse-Transcription Polymerase Chain Reaction: Technical Considerations for Gene Expression Analysis. Methods Mol Biol, 817:251-270.

Dridi B, Henry M, El Khechine A, Raoult D & Drancourt M (2009). High prevalence of *Methanobrevibacter smithii* and *Methanosphaera stadtmanae* detected in the human gut using an improved DNA detection protocol. PLoS ONE, 4:e7063.

Eckburg PB, Bik EM, Bernstein CN, Purdom E, Dethlefsen L, Sargent M, Gill SR, Nelson KE & Relman DA (2005). Diversity of the human intestinal microbial flora. Science, 308:1635-1638.

Ely B & Gerardot CJ (1988). Use of pulsed-field-gradient gel electrophoresis to construct a physical map of the *Caulobacter crescentus* genome. Gene, 68:323–333.

Farabullini F, Lucarelli F, Palchetti I, Marrazza G & Mascini M (2007). Disposable electrochemical genosensor for the simultaneous analysis of different bacterial food contaminants. Biosens Bioelectron, 22:1544-1549.

Fodor SP, Rava RP, Huang XC, Pease AC, Holmes CP & Adams CL (1993). Multiplexed biochemical assays with biological chips. Nature, 364:555–556.

Gabig-Ciminska M (2006). Developing nucleic acid-based electrical detection systems. Microbial Cell Factories, 5:9.

Ganesh Kumar C & Anand SK (1998). Significance of microbial biofilms in food industry: a review. Int J Food Microbiol 42:9-27.

Gao H, Yang ZK, Gebtry TJ, Wu L, Schadt CW & Zhou J (2007). Microarray-based analysis of microbial community RNAs by whole-community RNA amplification. Appl Environ Microbiol, 73:563-571.

Garcia-Armisen T & Servais P (2004). Enumeration of viable *E. coli* in rivers and wastewaters by fluorescent *in situ* hybridization. J Microbiol Methods, 58:269-279.

Garcia-Armisen T, Lebaron P & Servais P (2005). Beta-D-glucuronidase activity assay to assess viable *Escherichia coli* abundance in freshwaters. Lett Appl Microbiol, 40:278-282.

Gedalanga PB & Olson BH (2009). Development of a quantitative PCR method to differentiate between viable and nonviable bacteria in environmental water samples. Appl Microbiol Biotechnol, 82:587-596.

Gibson JR, Slater E, Xerry J, Tompkins DS & Owen RJ (1998). Use of an amplified-fragment length polymorphism technique to fingerprint and differentiate isolates of *Helicobacter pylori*. J Clin Microbiol, 36:2580-2585.

Gibson G (2001). Microarrays in ecology and evolution: a preview. Mol Ecol, 11:17-24.

Gilbride KA, Lee D-Y & Beaudette LA (2006). Molecular techniques in wastewater: Understanding microbial communities, detecting pathogens, and real-time process control. J Microbiol Methods, 66:1-20.

Giovannoni SJ, Britschgi TB, Moyer CL & Field KG (1990). Genetic diversity in Sargasso Sea bacterioplancton. Nature, 345:60-63.

Giraffa G & Neviani E (2001). DNA-based, culture-independent strategies for evaluating microbial communities in food-associated ecosystems. Int J Food Microbiol, 67:19-34.

Grimont F & Grimont PAD (1986). Ribosomal ribonucleic acid gene restriction patterns as potential taxonomic tools. Annal Institut Pasteur/Microbiol, 137B:165-175.

Hacia JG, Makalowski W, Edgemon K, Erdos MR, Robbins CM, Fodor SP, Brody LC & Collins FS (1998). Evolutionary sequence comparisons using high-density oligonucleotide arrays. Nat Genet, 18:155-158.

Hashimoto M, Chen P-C, Mitchell MW, Nikitopoulos DE, Soper SA & Murphy MC (2004). Rapid PCR in a continuous flow device. Lab Chip, 4:638-645.

Higgins JA, Nasarabadi S, Karns JS, Shelton DR, Cooper M, Gbakima A & Koopman RP (2003). A handheld real-time thermal cycler for bacterial pathogen detection. Biosens Bioelectron, 18:1115-1123.

Hill SC, Mayo MW & Chang RK (2009). Fluorescence of Bacteria, Pollens, and Naturally Occurring Airborne Particles: Excitation/Emission Spectra. ARL-TR-4722. Army Research Laboratory.

Hinds WC (1999). Aerosol Technology: Properties, Behaviour and measurement of airborne particles. John Wiley & Sons, Inc. 2nd edition.

Hoffman AD & Wiedmann M (2001). Comparative evaluation of culture- and BAX polymerase chain reaction–based detection for *Listeria* spp. and *Listeria monocytogenes* in environmental and raw fish samples. J Food Prot, 64: 521-1526.

Hoyles L & McCartney AL (2009). What do we mean when we refer to Bacteroidetes populations in the human gastrointestinal microbiota? FEMS Microbiol Lett, 299:175–183.

Hughes JB, Hellmann JJ, Ricketts TH & Bohannan BJM (2001). Counting the uncountable: statistical approaches to estimating microbial diversity. Appl Environ Microbiol, 67:4399-4406.

Huhtamella S, Leinonen M, Nieminen T, Fahnert B, Myllykoski L, Bretenstein A & Neubauer P (2008). RNA-based sandwich hybridization method for detection of lactic acid bacteria in brewery samples. J Microbiol Methods, 68:543-553.

Janssen P, Coopman R, Huys G, Swings J, Bleeker M, Vos P, Zabeau M & Kersters K (1996). Evaluation of the DNA fingerprinting method AFLP as a new tool in bacterial taxonomy. Microbiology, 142:1881-1893.

Johnson-White B, Lin B & Ligler FS (2007). Combination of immunosensor detection with viability testing and confirmation using the polymerase chain reaction and culture. Anal Chem, 79:140-146.

Josephson KL, Gerba CP & Pepper IL (1993).Polymerase chain reaction detection of nonviable bacterial pathogens. Appl Environ Microbiol, 59:3513-3515.

Kim H, Dixit S, Green CJ & Faris GW (2009). Nanodroplet real-time PCR system with laser assisted heating. Optics express, 17:218-227.

Klappenbach JA, Dunbar JM & Schmidt TM (2000). rRNA operon copy number reflects ecological strategies of bacteria. Appl Environ Microbiol, 66:1328–1333.

Krogius-Kurikka L, Kassinen A, Paulin L, Corander J, Mäkivuokko H, Tuimala J & Palva A (2009). Sequence analysis of percent G+C fraction libraries of human faecal bacterial DNA reveals a high number of Actinobacteria. BMC Microbiol, 9:68.

Kuczynski J, Lauber CL, Walters WA, Parfrey LW, Clemente JC, Gevers D & Knight R. (2011). Experimental and analytical tools for studying the human microbiome. Nat Rev Genet, 13:47–58.

Kuechenmeister LJ, Anderson KL, Morrison JM & Dunman PM (2009). The use of molecular beacons to directly measure bacterial mRNA abundances and transcript degradation. J Microbiol Methods, 76:146-151.

Kunin V, Copeland A, Lapidus A, Mavromatis K & Hugenholtz P (2008). A bioinformatician's guide to metagenomics. Microbiol Mol Biol Rev, 72:557–578.

La Duc MT, Vaishampayan P, Nilsson TT & Venkateswaran K (2012). Pyrosequencing-Derived bacterial, archaeal and fungal diversity of spacegraft hardware destined for Mars. Appl Environ Microbiol 78:5912-5922.

Lantz P-G, Hahn-Hägerdal B & Rådström P (1994). Sample preparation methods in PCR-based detection of food pathogens. Trends Food Science Technol, 5:384-398.

Lauri A & Mariani PO (2009). Potentials and limitations of molecular diagnostic methods in food safety. Genes Nutr, 4:1-12.

Lawrence JR, Korber DR, Hoyle BD, Costerton JW & Caldwell DE (1991). Optical sectioning of microbial biofilms. J Bacteriol, 173:6558-6567.

Lay C, Rigottier-Gois L, Holmstrøm K, Rajilic M, Vaughan EE, de Vos WM, Collins MD, Thiel R, Namsolleck P, Blaut M & Doré J (2005). Colonic microbiota signatures across five northern European countries. Appl Environ Microbiol, 71:4153-4155.

Lee J-G, Cheong KH, Huh N, Kim S, Choi J-W & Ko C (2006). Microchip-based one step DNA extraction and real-time PCR in one chamber for rapid pathogen identification. Lab Chip, 6:886-895.

Lee SH, Kim S-W, Kang JY & Ahn CH (2008b). A polymer lab-on-a-chip for reverse transcription (RT)-PCR based point-of-care clinical diagnostics. Lab Chip, 8:2121-2127.

Lee J-L & Levin RE (2009). A comparative study of the ability of EMA and PMA to distinguish viable from heat killed mixed bacterial flora from fish fillets. J Microbiol Methods, 76:93-96.

Leskelä T, Tilsala-Timisjärvi A, Kusnetsov J, Neubauer P & Breitenstein A (2005). Sensitive genus-specific detection of *Legionella* by a 16S rRNA based sandwich hybridization assay. J Microbiol Methods, 62:167-179.

Li M, Gong J, Cottrill M, Yu H, De Lange C, Burton J & Topp E (2003). Evaluation of QI-Aamp® DNA Stool Mini Kit for ecological studies of gut microbiota. J Microbiol Methods, 54:13–20.

Li F, Hullar MA & Lampe JW (2007). Optimization of terminal restriction fragment polymorphism (TRFLP) analysis of human gut microbiota. J Microbiol Methods, 68:303–311

Lockhart DJ, Dong H, Byrne MC, Follettie MT, Gallo MV, Chee MS, Mittmann M, Wang C, Kobayashi M, Horton H & Brown EL (1996). Expression monitoring by hybridization to high-density oligonucleotide arrays. Nat Biotechnol, 14:1675–1680

Lozupone CA, Hamady M, Kelley ST & Knight R (2007). Quantitative and qualitative beta diversity measures lead to different insights into factors that structure microbial communities. Appl Environ Microbiol, 73:1576–1585.

Lucchini S, Thompson A & Hinton JCD (2001). Microarrays for microbiologists. Microbiology, 147:1403-1414.

Ludwig W (2007). Nucleic acid techniques in bacterial systematics and identification. Int J Food Microbiol, 120:225-236.

Luton PE, Wayne JM, Sharp RJ, Riley PW (2002) The mcrA gene as an alternative to 16S rRNA in the phylogenetic analysis of methanogen populations in landfill. Microbiology, 148: 3521-3530

Magne F, Hachelaf W, Suau A, Boudraa G, Mangin I, Touhami M, Bouziane-Nedjadi K & Pochart P (2006). A longitudinal study of infant faecal microbiota during weaning. FEMS Microbiol Ecol, 58:563–571.

Manninen A, Putkiranta M, Saarela J, Rostedt A, Sorvajärvi T, Toivonen J, Marjamäki M, Keskinen J & Hernberg R (2009). Fluorescence cross sections of bioaerosols and suspended biological agents. Appl Optics, 48:4320-4328.

Manz W, Eisenbrecher M, Neu TR & Szewzyk U (1998). Abundance and spatial organization of Gram-negative sulfate-reducing bacteria in activated sludge investigate by *in situ* probing with specific 16S rRNA targeted oligonucleotides. FEMS Microbiol Ecol, 25:43-61.

Manz W, Wendt-Potthoff K, Neu TR, Szewzyk U & Lawrence JR (1999). Phylogenetic composition, spatial structure, and dynamics of lotic bacterial biofilms investigated by fluorescent *in situ* hybridization and confocal laser scanning microscopy. Microbial Ecol, 37:225-237.

Mardis ER (2008). Next-generation DNA sequencing methods. Ann Rev Genom Human Genet, 9:387-402.

Martinez K, Rao C & Burton N (2004). Exposure assessment and analysis for biological agents. Grana, 43:193-208.

Maukonen J, Mättö J, Wirtanen G, Raaska L, Mattila-Sandholm T & Saarela M (2003). Methodologies for the characterization of microbes in industrial environments. J Ind Microbiol Biotechnol 30:327-356.

Maukonen J & Saarela M (2009). Microbial communities in industrial environment. Curr Opinion Microbiol 12:238-243.

Maukonen J (2012). Characterization of the human predominant fecal microbiota with special focus on the Clostridial clusters IV and XIVa. VTT Science: 26, VTT, Espoo, Finland. 161 p. + app. 62 p.

Maukonen J, Simões C & Saarela M (2012). The currently used commercial DNA extraction methods give different results of clostridial and Actinobacterial populations derived from human fecal samples. FEMS Microbiol Ecol, 79:697-708.

Merkoçi A (2007). Electrochemical biosensing with nanoparticles. FEBS J, 274:310-316.

Metzker ML (2010). Sequencing technologies – the next generation. Nat Rev Genetics, 11:31–46.

Meunier J-R & Grimont PAD (1993). Factors affecting reproducibility of random amplified polymorphic DNA fingerprinting. Res Microbiol, 144:373-379.

Min J & Baeumner AJ (2002). Highly sensitive and specific detection of viable *Escherichia coli* in drinking water. Anal Biochem, 303:186-193.

Molbak L, Sommer HM, Johnsen K, Boye M, Johansen M, Møller K & Leser TD (2006). Freezing at 80°C distorts the DNA composition of bacterial communities in intestinal samples. Curr Issues Intest Microbiol, 7:29–34.

Moran MA (2009). Metatranscriptomics: eavesdropping on complex microbial communities. Microbe, 4:329–335.

Morris G, Kokki MH, Anderson K & Richardson MD (2000). Sampling of Aspergillus Spores in Air. J Spital Infect, 44:81-92.

Mullis K, Faloona F, Scharft S, Saiki R, Horn G & Erlich H (1986). Specific enzymatic amplification of DNA *in vitro*: the polymerase chain reaction. Cold Spring Harbor Symp Quant Biol, 51: 263-273.

Mullis K (1987a). US patent 4,683,195, July 1987.

Mullis K (1987b). US patent 4,683,202, July 1987.

Muyzer G & Smalla K (1998). Application of denaturing gradient gel electrophoresis (DGGE) and temperature gradient gel electrophoresis (TGGE) in microbial ecology. Ant van Leeuwenh, 73:127-141.

Nakamura N, Gaskins HR, Collier CT, Nava GM, Rai D, Petschow B, Russell WM, Harris C, Mackie RI, Wampler JL & Walker DC (2009). Molecular ecological analysis of fecal bacterial populations from term infants fed formula supplemented with selected blends of prebiotics. Appl Environ Microbiol, 75:1121–1128.

Nechvatal JM, Ram JL, Basson MD, Namprachan P, Niec SR, Badsha KZ, Matherly LH, Majumdar AP & Kato I. (2008). Fecal collection, ambient preservation, and DNA extraction for

PCR amplification of bacterial and human markers from human feces. J Microbiol Methods, 72:124–132.

Nikitin, N., Petrova, E., Trifonova, E. and Karpova, O. (2014). Influenza Virus Aerosols in the Air and Their Infectiousness. Advances in Virology, http://dx.doi.org/10.1155/2014/859090.

Nocker A & Camper AK (2006). Selective removal of DNA from dead cells of mixed bacterial communities by use of ethidium monoazide. Appl Environ Microbiol, 72:1997-2004.

Nocker A, Cheung C-Y & Camper AK (2006). Comparison of propidium monoazide with ethidium monoazide for differentiation of live vs. dead bacteria by selective removal of DNA from dead cells. J Microbiol Methods, 67:310-320.

Nocker A, Sossa KE & Camper AK (2007a). Molecular monitoring of disinfection efficacy using propidium monoazide in combination with quantitative PCR. J Microbiol Methods, 70:252-260.

Nocker A, Sossa-Fernandez P, Burr MD & Camper AK (2007b). Use of propidium monoazide for live/dead distinction in microbial ecology. Appl Environ Microbiol, 73:5111-5117.

Nocker A & Camper AK (2009). Novel approaches toward preferential detection of viable cells using nucleic acid amplification techniques. FEMS Microbiol Lett, 291:137-142.

Nocker A, Mazza A, Masson L, Camper AK & Brousseau (2009). Selective detection of live bacteria combining propidium monoazide sample treatment with microarray technology. J Microbiol Methods,76:253-261.

Nogva HK, Drømtorp SM, Nissen H & Rudi K (2003). Ethidium monoazide for DNA-based differentiation of viable and dead bacteria by 5'-nuclease PCR. BioTechniques, 34:804-813.

National Research Council NRC (2006). Defending the U.S. Air Transportation System Against Chemical and Biological Threats. Committee on Assessment of Security Technologies for Transportation.

Ott SJ, Musfeldt M, Timmis KN, Hampe J, Wenderoth DF & Schreiber S (2004). *In vitro* alterations of intestinal bacterial microbiota in fecal samples during storage. Diagn Microbiol Infect Dis, 50:237–245.

Palleroni NJ (1997). Prokaryotic diversity and the importance of culturing. Ant van Leeuwenh, 72:3-19.

Palmer C, Bik EM, DiGiulio DB, Relman DA & Brown PO (2007). Development of the human infant intestinal microbiota. PLoS Biol, 5:1556–1573.

Pan YL, Eversole JD, Kaye PH, Foot MV, Pinnick RG, Hill SC, *et al.* (2007). Bio-Aerosol Fluorescence – Detecting and Characterising Bio-Aerosols via UV Light-Induced Fluorescence Spectroscopy. In Optics of Biological Particles, 1st ed., Edited by: Hoekstra, A., Maltsev, V. and Videen, G. 63–163. (NATO Science Series). Dordrecht: Human Press/Springer, The Netherlands. doi:10.1007/978-1-4020-5502-7.

Pankaj K (2013). Methods for Rapid Virus Identification and Quantification. Mater Methods, 3:207.

Pisz JM, Lawrence JR, Schafer AN & Siciliano SD (2007). Differentiation of genes extracted from non-viable versus viable micro-organisms in environmental samples using ethidium monoazide bromide. J Microbiol Methods, 71:312-318.

Poulsen LK, Ballard G & Stahl DA (1993). Use of rRNA fluorescence *in situ* hybridization for measuring the activity of single cells in young and established biofilms. Appl Environ Microbiol, 59:1354-1360.

Power EGM (1996). RAPD typing in microbiology - a technical review. J Hosp Infect, 34:247-265.

Prescott AM & Fricker CR (1999). Use of PMA oligonucleotides for the *in situ* detection of *Escherichia coli* in water. Mol Cell Probes, 13:261-268.

Putkiranta M, Manninen A, Rostedt A, Saarela J, Sorvajärvi T, Marjamäki M, Hernberg R & Keskinen J (2010). Fluorescence properties of biochemicals in dry NaCl composite aerosol particles and in solutions. Appl Physics B: Lasers and Optics, 1-11.

Pöhlker C, Huffman JA & Pöschl U (2012). Autofluorescence of atmospheric bioaerosols – fluorescent biomolecules and potential interferences. Atmos Measur Tech, 5:37-71.

Raaska L, Sillanpää J, Sjöberg A-M & Suihko M-L (2002). Potential microbiological hazards in the production of refined paper products for food applications. J Ind Microbiol Biotechnol 28:225-231.

Ramette A (2007). Multivariate analyses in microbial ecology. FEMS Microbiol Ecol, 62:142–160.

Ramsing NB, Fiossing H, Ferdelman TG, Andersen F & Thamdrup B (1996). Distribution of bacterial populations in a stratified fjord (Marianger fjord, Denmark) quantified by *in situ* hybridization and related to chemical gradients in the water column. Appl Environ Microbiol, 62:1391-1404.

Rastogi G & Sani RK (2011). Molecular Techniques to Assess Microbial Community Structure, Function, and Dynamics in the Environment. In: Ahmad, I., Ahmad, F. and Pichtel, J. (eds.). Microbes and Microbial Technology, Agricultural and Environmental Applications, pp. 29-57. Springer.

Reischl U & Kochanowski B (1995). Quantitative PCR - a survey of the present technology. Mol Biotechnol, 3:55-71.

Restaino L, Frampton EW, Irbe RM, Schabert G & Spitz H (1999). Isolation and detection of Listeria monocytogenes using fluorogenic and chromogenic substrates for phosphatidylinositol-specific phospholipase. Can J Food Prot, 62:244-251.

Roesch LF, Casella G, Simell O, Krischer J, Wasserfall CH, Schatz D, Atkinson MA, Neu J & Triplett EW (2009). Influence of fecal sample storage on bacterial community diversity. Open Microbiol J, 3:40–46.

Roose-Amsaleg CL, Garnier-Sillam E & Harry M (2001). Extraction and purification of microbial DNA from soil and sediment samples. App Soil Ecol, 18:47-60.

Rose LJ, Donlan R, Banerjee SN & Arduino MJ (2003). Survival of *Yersinia pestis* on Environmental Surfaces. Appl Environ Microbiol, 69:2166-2171.

Rothberg JM & Leamon JH (2008). The development and impact of 454 sequencing. Nature Biotechnol, 26:1117-1124.

Rossen L, Norskov P, Holmstrom K & Rasmussen OF (1992). Inhibition of PCR by components of food samples, microbial diagnostic assays and DNA-extraction solutions. Int J Food Microbiol, 17:37-45.

Rowan AK, Davenport RJ, Snape JR, Fearnside D, Barer MR, Curtis TP & Head IM (2005). Development of a rapid assay for determining the relative abundance of bacteria. Appl Environ Microbiol, 71:8481-8490.

Rudi K, Moen B, Drømtorp SM & Holck AL (2005a). Use of ethidium monoazide and PCR in combination for quantification of viable and dead cells in complex samples. Appl Environ Microbiol, 71:1018-1024.

Rudi K, Naterstad K, Drømtorp SM & Holo H (2005b). Detection of viable and dead *Listeria monocytogenes* on gouda-like cheeses by real-time PCR. Lett Appl Microbiol, 40:301-306.

Rudi K, Zimonja M, Trosvik P & Naes T (2007). Use of multivariate statistics for 16S rRNA gene analysis of microbial communities. Int J Food Microbiol, 120:95–99

Saarela M, Maukonen J, von Wright A, Vilpponen-Salmela T, Patterson AJ, Scott KP, Hämynen H & Mättö J (2007). Tetracycline susceptibility of the ingested *Lactobacillus acidophilus* LaCH-5 and *Bifidobacterium animalis* subsp. *lactis* Bb-12 strains during antibiotic-probiotic intervention International J Antimicrobial Agents, 29:271-280.

Saiki RK, Scharft S, Faloona F, Mullis KB, Horn GT, Erlich HA & Arnheim N (1985). Enzymatic amplification of β-globin genomic sequences and restriction site analysis for diagnosis of sickle cell anemia. Science, 230:1350-1354.

Salo S, Alanko T, Sjöberg A-M & Wirtanen G (2002). Validation of Hygicult® E dipslides in surface hygiene control: A Nordic collaborative study. J AOAC Int 85:388-394.

Salonen A, Nikkilä J, Jalanka-Tuovinen J, Immonen O, Rajilic-Stojanovic M, Kekkonen RA, Palva A & de Vos WM (2010). Comparative analysis of fecal DNA extraction methods with phylogenetic microarray: effective recovery of bacterial and archaeal DNA using mechanical cell lysis. J Microbiol Methods, 81:127–134.

Satterfield BC, Caplan MR & West JAA (2008). Tentacle probe sandwich assay in porous polymer monolith improves specificity, sensitivity and kinetics. Nucleic Acids Res, 36:e129.

Schena M, Shalon D, Davis RW & Brown PO (1995). Quantitative monitoring of gene expression patterns with a complementary DNA microarray. Science, 270:467-470.

Scheu PM, Berghof K & Stahl U (1998). Detection of pathogenic and spoilage microorganisms in food with the polymerase chain reaction. Food Microbiol, 15:13-31.

Sheridan GEC, Masters CI, Shallcross JA & Mackey BM (1998). Detection of mRNA by reverse transcription-PCR as an indicator of viability in *Escherichia coli* cells. Appl Environ Microbiol, 64:1313-1318.

Simpkins SA, Chan AB, Hays J, Popping B & Cook N (2000). An RNA transcription-based amplification technique (NASBA) for the detection of viable *Salmonella enterica*. Lett Appl Microbiol, 30: 75-79.

Siqueira J, Fouad A & Rôças I (2012). Pyrosequencing as a tool for better understanding of human microbiomes. J Oral Microbiol vol. 4.

Soejima T, Iida K-I, Qin T, Taniai H, Seki M, Takade A & Yoshida S-I (2007). Photoactivated ethidium monoazide directly cleaves bacterial DNA and is applied to PCR for discrimination of live and dead bacteria. Microbiol Immunol, 51:763-775.

Soejima T, Iida K-I, Qin T, Taniai H, Seki M & Yoshida S-I (2008). Method to detect only live bacteria during PCR amplification. J Clin Microbiol, 46:2305-2313.

Southern EM (1975). Detection of specific sequences among DNA fragments separated by gel electrophoresis. J Mol Biol, 98: 503-517.

Stahl DA, Flesher B, Mansfield HR & Montgomery L (1988). The use of phylogenetically based hybridization probes for studies of ruminal microbial ecology. Appl Environ Microbiol, 54:1079-1084.

Stahl DA & Capman WC (1994). Applications of molecular genetics to the study of microbial communities. NATO ASI Series, 35:193-206.

Stahl DA (1995). Application of phylogenetically based hybridization probes to microbial ecology. Mol Ecol, 4:535-542.

Straub TM, Dockendorff BP, Quiñonez-Díaz MD, Valdez CO, Shutthanandan JI, Tarasevich BJ, Grate JW & Bruckner-Lea CJ (2005). Automated methods for multiplexed pathogen detection. J Microbiol Methods, 62:303-316.

Suau A, Bonnet R, Sutren M, Godon JJ, Gibson G, Collins MD & Doré J (1999). Direct analysis of genes encoding 16S rRNA from complex communities reveals many novel molecular species within the human gut. Appl Environ Microbiol, 65:4799-4807.

Sun H, Zhang Y & Fung Y (2006). Flow analysis coupled with PQC/DNA biosensor for assay of *E. coli* based on detecting DNA products from PCR amplification. Biosens Bioelectron, 22:506-512.

Sun H, Choy TS, Zhu DR, Yam WC & Fung YS (2009). Nano-silver-modified PQC/DNA biosensor for detecting E. coli in environmental water. Biosens Bioelectron, 24:1405-1410.

Thieme D, Neubauer P, Nies DH & Grass G (2008). Sandwich hybridization assay for sensitive detection of dynamic changes in mRNA transcript levels in crude *Escherichia coli* cell extracts in response to copper ions. Appl Environ Microbiol, 74;7463-7470

Tringe SG & Hugenholtz P (2008). A renaissance for the pioneering 16S rRNA gene. Curr Opin Microbiol, 11:442-446.

Tyagi S & Kramer FR (1996). Molecular beacons: Probes that fluoresce upon hybridization. Nature Biotechnol, 14:303-308.

van Belkum A, Scherer S, van Alphen L & Verbrugh H (1998). Short-sequence DNA repeats in prokaryotic genomes. Microbiol Mol Biol Rev, 62:275-293.

Vaneechoutte M, Rossau R, De Vos P, Gilis M, Janssens D, Paepe N, De Rouck A, Fiers T, Claeys G & Kersters K (1992). Rapid identification of bacteria of the Comamonadaceae with amplified ribosomal DNA-restriction analysis (ARDRA). FEMS Microbiol Lett, 15:227-233.

Vaneechoutte M (1996). DNA fingerprinting techniques for microorganisms. Mol Biotechnol, 6:115-142.

Vaughan EE, Schut F, Heilig HGHJ, Zoetendal EG, de Vos WM & Akkermans ADL (2000). A molecular view of the intestinal ecosystem. Curr Issues Intest Microbiol, 1:1-12.

Verrault D, Moineau S & Dunchaine C (2008). Methods for Sampling of Airborne Viruses. Microbiol Mol Biol Rev, 72:413-444.

Versalovic J, Koeuth T & Lupski JT (1991). Distribution of repetitive DNA sequences in eubacteria and application to fingerprinting of bacterial genomes. Nucleic Acid Res, 19:6823-6831.

von Wintzingerode F, Göbel UB & Stackebrandt E (1997). Determination of microbial diversity in environmental samples: pitfalls of PCR-based rRNA analysis. FEMS Microbiol Rev, 21:213-229.

Vos P, Hogers R, Bleeker M, Reijans M, van de Lee T, Hornes M, Frijters A, Pot J, Peleman J, Kuiper M & Zabeau M (1995). AFLP: a new technique for DNA fingerprinting. Nucleic Acids Res, 23:4407-4414.

Wagner M, Assmus B, Hartmann A, Hutzler P & Amann R (1994). *In situ* analysis of microbial consortia in activated sludge using fluorescently labeled, rRNA-targeted oligonucleotide probes and confocal laser scanning microscopy. J Microsc, 176:181-187.

Wagner M, Hutzler P & Amann R (1998). Three-dimensional analysis of complex microbial communities by combining confocal laser scanning microscopy and fluorescence *in situ* hybridization. In: Digital Image Analysis of Microbes (Wilkinson MHF and F Schut, eds), pp 467-486, John Wiley & Sons Ltd, Chichester, U.K.

Wallace DM (1987). Large- and small-scale phenol extractions. Meth Enzymol, 152:33-48.

Wang R-F, Cao W-W & Cerniglia CE (1997). A universal protocol for PCR detection of 13 species of foodborne pathogens in foods. J Appl Microbiol, 83:727-736.

Wang S & Levin RE (2006). Discrimination of viable *Vibrio vulnificus* cells from dead cells in real-time PCR. J Microbiol Methods, 64:1-8.

Ward DM, Weller R & Bateson M (1990). 16S rRNA sequences reveal numerous uncultured microorganisms in a natural community. Nature, 35:63-65.

Welsh J & McClelland M (1990). Fingerprinting genomes using PCR with arbitrary primers. Nucleic Acid Res, 25:7212-7218.

Werner JJ, Koren O, Hugenholtz P, DeSantis TZ, Walters WA, Caporaso JG, Angenent LT, Knight R & Ley RE (2012). Impact of training sets on classification of high-throughput bacterial 16s rRNA gene surveys. ISME J, 6:94–103.

Wheeler AE & Stahl DA (1996). Extraction of microbial DNA from aquatic sediments. In: Molecular Microbial Ecology Manual (Akkermans ADL, van Elsas JD & de Bruijn FJ, eds). Kluwer Academic Publishers, Dordrecht, The Netherlands, 1.1.5, pp 1-29.

Wicks B, Cook DB, Barer MR, O'Donnell AG & Self CH (1998). A sandwich hybridization assay employing enzyme amplification for determination of specific ribosomal RNA from unpurified cell lysates. Anal Biochem, 259:258-264.

Wirtanen G, Husmark U & Mattila-Sandholm T (1996). Microbial evaluation of the biotransfer potential from surfaces with Bacillus biofilms after rinsing and cleaning procedures in closed food-processing systems. J Food Prot 59:727-733.

Wirtanen G, Salo S, Maukonen J, Bredholt S & Mattila-Sandholm T (1997). NordFood Sanitation in dairies. Espoo: The Technical Research Centre of Finland, VTT Publication 309, 47 p. + app. 22 p.

Yang W, Elankumaran S & Marr LC (2011). Concentrations and size distributions of airborne influenza A viruses measured indoors at a health centra, a day-care centre and on aeroplanes. J R SOC Interface, 8:1176-1184.

Ye RW, Wang T, Bedzyk L & Croker KM (2001). Applications of DNA microarrays in microbial systems. J Microbiol Methods, 47:257-272.

Yeung S-W, Lee TM-H, Cai H & Hsing I-M (2006). A DNA biochip for on-the-spot multiplexed pathogen identification. Nucleic Acids Res, 34:e118.

Zaytseva NV, Goral VN, Montagna RA & Baeumner AJ (2005). Development of a microfluidic biosensor module for pathogen detection. Lab Chip, 5:805-811.

Zhao W, Yao S & Hsing I-M (2006). A microsystem compatible strategy for viable *Escherichia coli* detection. Biosens Bioelectron, 21:1163-1170.

Zuo X, Yang X, Wang K, Tan W & Wen J (2007). A novel sandwich assay with molecular beacon as report probe for nucleic acids detection on one-dimensional microfluidic beads array. Anal Chim Acta, 587:9-13.

Zwolinski MD (2007). DNA sequencing: strategies for soil microbiology. Soil Sci Soc Am J, 71:592–600.