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DISSERTATION

Development and evaluation of molecular assays for microbial water quality assessment

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Abstract

Water is essential for life. Still in the 21th century waterborne outbreaks remain a challenge in developing countries but even in industrial countries.

In Austria the water quality is related to sensoric, chemical and microbial parameters and their assessment is defined by the Austrian Drinking Water Directive (Austrian DWD; BGBl. II Nr. 304/2001). Currently used techniques for determination of microbial parameters are cultivation-based. These are well established, but drawbacks are identified in the long duration of analysis and the complexity of execution (in general several cultivation steps and biochemical confirmation tests are required). Alternative DNA-based techniques, such as real-time PCR (RT-PCR), enable more rapid and highly specific analysis of target organisms, where high-throughput of samples can be achieved. However, the application is still hampered by the innate inability to discriminate between target DNA from living and dead organism, which is of uttermost importance in quality assessment, as only viable cells are determined by standardized techniques and may pose a health risks.

Therefore, in order to facilitate viable cell detection, RT-PCR combined with propidium monoazide (PMA) treatment was developed for rapid microbial water quality assessment. Propidium monoazide, a DNA-intercalating substance, is used to modify DNA from cells with compromised membrane, thereby allowing selective PCR amplification of unmodified DNA from viable cells. For all microbial parameters defined in the Austrian DWD (2001) (*E. coli*, coliforms, *Enterococcus* spp., *P. aeruginosa* and heterotrophic plate count) PMA-RT-PCR assays were established.

In the proof of principle study (Gensberger et al., 2013) live/dead discrimination potential was successfully shown by utilization of 10µM PMA, which resulted in significant (3 $\log_{10}$) or complete signal reduction of DNA from heat-killed *E. coli* and *P. aeruginosa* cells in samples with an abundant water microflora. The application potential of PMA-RT-PCR in comparison to conventional reference methods and RT-PCR without PMA was approved in an extended evaluation with a set of drinking and process water samples. This was highlighted by the complete compliance of PMA-RT-PCR compared to conventional microbiological assessment for *E. coli* and further 100% specificity for detection of *E. coli*, *Enterococcus* spp., *P. aeruginosa* in process water evaluation. A major challenge remained in sensitivity of the both molecular assays (PMA-RT-PCR and RT-PCR), which was presumably due to insufficient sample preparation (i.e. concentration of bacteria and DNA extraction), rather than the limit of detection of PMA-RT-PCR (1-10 cells/reaction). For the detection of indicators (coliforms and total bacterial count), both encompassing a diverse group of bacteria, no adequate assessment on molecular basis could be facilitated, because of differences in the detection spectrum.

The heterotrophic plate count method (HPC) used as quality parameter was assessed in detail for the composition of culturable HPC community under different cultivation conditions (R2A and yeast extract agar at 22°C and 37°C) by 16S rRNA gene sequence analysis. HPC communities revealed significant differences in composition and abundance of determined taxa accordingly to cultivation condition applied. Our data confirmed that temperature has a major effect ($p < 0.01$) on the composition and therefore utilization of the basic concept of two temperatures is essential.

Summarizing the findings, the HPC method for the assessment of water quality should be reconsidered. HPC method represents a valuable tool for monitoring of fluctuations of disinfection processes, but may be of less significant value for single isolated assessment of a water sample.

At present cultivation-based methods for water quality assessment cannot be replaced by molecular assays, but given the careful optimization for improving sensitivity and refinements in PMA-RT-PCR, molecular assays represent a promising and valuable detection method due to the high specificity and rapid analysis. First applications could be considered for detection of *E. coli*, *Enterococcus* spp. and *P. aeruginosa* from treated drinking water, as regular analysis is essential in this field, to allow immediate actions in case of a determined contamination.

Kurzfassung

Wasser ist ein essenzielles Lebensmittel. Im 21. Jahrhundert besteht nach wie vor die Herausforderung von mikrobiologisch kontaminiertem Wasser und damit verbundenen Wasser-assoziierten Krankheiten, einerseits in Entwicklungsländer und andererseits auch in Industrieländern.
Österreichs Trinkwasser obliegt gesetzlichen Anforderungen (Trinkwasserverordnung (TWV; BGBl. II NR. 304/2001) und eine regelmäßige Überwachung auf sensorische, chemische und mikrobiologische Parameter ist verpflichtend. Die mikrobiologische Analyse basiert auf der Verwendung von standardisierten Kultivierungsverfahren, jedoch sind diese sehr zeitintensiv und bedingen einen hohen Laboraufwand (Notwendigkeit mehrere Kultivierungsschritte und biochemische Bestätigungstests durchzuführen). Fortschritte in der Anwendung alternativer molekularer DNA-basierter Methoden, wie zum Beispiel der real time (RT)-PCR, ermöglichen eine schnellere und spezifischere Analyse mit hohem Probendurchsatz. Dennoch wurde die bisherige Anwendung erschwert, nicht zwischen DNA aus lebenden und toten Bakterienzellen unterscheiden zu können. Denn dies erlaubt weder den direkten Vergleich zu standardisierten Kultivierungsverfahren noch spiegelt es das Gefahrenpotential der lebenden Zellen wider.

Das Ziel der vorliegenden Arbeit befasste sich mit der Entwicklung und Etablierung von molekularen Methoden für die schnelle Wasserqualitätsbewertung durch Verwendung der real-time (RT-PCR) in Kombination mit der Behandlung von Propidium Monoazide (PMA). PMA ist ein DNA-interkalierender Farbstoff, der selektiv in tote bzw. Membran-geschädigte Zellen diffundiert und deren DNA durch irreversible Modifikation in der Analyse hemmt. Dadurch wird die selektive Detektion nicht modifizierter DNA aus lebenden Zellen ermöglicht. Für alle in der Trinkwasserverordnung (TVO, 2001) definierten mikrobiellen Parameter (*E.coli*, coliforme, *Enterococcus* spp., *P. aeruginosa* und Keimzahlbestimmung (KBE)) konnten PMA-RT-PCR Analysen etabliert werden.

In der Entwicklungsphase (Gensberger et al., 2013) wurde das Potential der Lebend/Tot Differenzierung durch Verwendung von 10 µM PMA anhand der signifikanten ($3\log_{10}$) oder kompletten Unterdrückung von DNA aus hitze-getöteten *E. coli* und *P. aeruginosa* Zellen in einer Probe mit abundanter Wassermikroflora demonstriert. Das Anwendungspotenzial der PMA-RT-PCR wurde in einer Evaluierung zu Referenzverfahren und RT-PCR ohne PMA mit einer Vielzahl an Trinkwasserproben und Prozesswasserproben geprüft. Die Analyse von Prozesswasser resultierte in der Korrelation der *E. coli* PMA-RT-PCR zu Referebzverfahren und zudem 100%ige Spezifitätsraten in der Analyse von *E. coli*, *Enterococcus* spp., *P. aeruginosa*. Hingegen zeigte sich die Limitation der Sensitivität, ermittelt für beide molekulare Methoden (PMA-RT-PCR und RT-PCR), die vermutlich

auf eine unzureichende Probenvorbereitung (Konzentration von Bakterien und DNA Extraktion) zurückzuführen ist und nicht aufgrund des Detektionslimits der PMA-RT-PCR (1-10 Zellen/Reaktion). Die Umsetzung der PMA-RT-PCR für Indikatororganismen (coliforme Bakterien und KBE) erlaubte keinen direkten Vergleich des Detektionsspektrums, da beide Parameter eine diverse Bakteriengruppe miteinschließen. In diesem Zusammenhang wurde die KBE Bestimmung als Wasserqualitätsparameter auf deren bakterielle Ökologie bei verschiedenen Kultivierungsbedingungen (R2A und Hefeextrakt Medium bei 22°C und 37°C) durch 16S rRNA Sequenzanalyse untersucht. Signifikante Auswirkung auf das präsente Bakterienspektrum konnte durch den Kultivierungsparameter der Temperatur ($p < 0.01$) statistisch bewertet werden und bestätigte das Konzept der Analyse zweier unterschiedlicher Temperaturen.

Zusammenfassend lässt sich sagen, dass die Keimzahlbestimmung bezugnehmend auf deren Aussage des Qualitätszustands einer Wasserprobe überdacht werden sollte. Die KBE Bestimmung eignet sich für das Monitoring von Fluktuationen bei der Wirksamkeitsprüfung von Desinfektionsprozessen, jedoch kann die Einzelbestimmung einer Wasserprobe in einer unzuverlässigen Qualitätsbewertung resultieren.

Kultivierungsmethoden als standardisierte Verfahren sind derzeit in der Wasserqualitätsbewertung noch nicht durch molekulare Methoden zu ersetzen. Gelingt es die Sensitivität zu verbessern, birgt sich eine gute Verwertungschance in der PMA-RT-PCR Methode durch die hohe Spezifität und die Gewährleistung der schnelleren Aussage um rasches Eingreifen im Kontaminationsfall zu ermöglichen. Eine Verwendung für mikrobiologische Parameter *E. coli*; *Enterococcus* spp. und *P. aeruginosa* könnte beispielsweise in der Wirksamkeitsprüfung bei der Trinkwasseraufbereitung angedacht werden, da in diesem Anwendungsgebiet eine regelmäßige Überprüfung essenziel ist.

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I am looking back to exciting times and I look forward to new challenges ahead in my scientific career

Table of content

Abbreviations

ALC	Active labile compounds
CCA	Canonical correspondence analysis
CFU	Colony forming unit
Cq	Quantification cycle
DGGE	Denaturing gradient gel electrophoresis
DNA	Desoxyribonucleic acid
dNTP	Desoxyribonucleotide triphosphates
DWD	Drinking Water Directive
EMA	Ethidium monoazide
EPA	Environmental Protection Agency
FISH	Fluorescence in situ hybridization
HPC	Heterotrophic plate count
ISO	International Standardization Organization
LAMP	Loop mediated isothermal amplification
LOD	Limit of detection
NASBA	Nucleic acid sequence based amplification
NGS	Next generation sequencing
NHMRC	National Health and Medical Research Council

NPV	Negative predictive value
OECD	Organisation for Economic Cooperation and Development
OTU	Operational taxonomic units
PCoA	Principal coordinate analysis
PCR	Polymerase chain reaction
PMA	Propidium monoazide
PPV	Positive predictive value
RNA	Ribonucleic acid
RT-PCR	Real-time polymerase chain reaction
TGGE	Temperature gradient gel electrophoresis
Tm	Melting temperature
T-RFLP	Terminal-restriction fragment length polymorphism
U.S.	United States
WHO	World Health Organization
YEA	Yeast extract agar

Introduction

1. Worldwide water demands

Water is essential for life and therefore should be protected as a human right (Vidar and Mekouar, 2002). This implies at the minimum sufficient quantities to meet basic human needs in terms of drinking and domestic use such as cooking, sanitation, bathing and cleaning (WHO, 2002).

Today, access to safe drinking water is currently available only for 83% of the human population (WHO statistics, 2004). Almost 1 billion of people lack the access to a public water supply and even 884 millions of people do not have safe drinking water (http://water.org/water-crisis/water-facts/water/; http://www.who.int). In Europe and America high quality drinking water is available, but in developing areas like Africa or some parts of Asia the availability of drinking water sources is limited (Figure 1) and efficient treatment processes often cannot be facilitated, thus leading to unsafe water.

Figure 1. Access to drinking water throughout the world
http://news.bbc.co.uk/nol/shared/spl/hi/world/10/water_maps/img/water_natural_624.jpg

People in Africa have to live with only 20 liter water per day. In contrast, in Europe the water demand is about 150 liter per capita per day and in North and Central America the double amount of 300 liter is needed in every day's life (http://www.wasserwerk.at/home/alles-ueber-wasser/wasserressource/17). In Austria the per capita consumption of water is nearly allocated to the European demand with 135

liter per day used for drinking, sanitary needs such as hand washing, bathing, showering and for other domestic purposes (Figure 2) (http://duz.lebensministerium.at/duz/duz/theme/view/1533951). In big industrialized countries the fresh water withdrawal is still continuously increasing and in the last 300 years a raise of 35 times was recorded (http://www.wasserwerk.at/home/alles-ueber-wasser/wasserressource/17). Therefore strong emphasize is set to protect our water resources and argue for Water Safety plans from World Health Organization (WHO, 2011), which involve improvements in drinking water supply in developing countries through better sanitation, inexpensive approaches for treatment purposes and water quality assessment.

Figure 2. Per capita water demand of Austrian households
http://images04.kurier.at/wasser.jpg/3.468.741;
http://www.holdinggraz.at/typo3temp/pics/9ad078724f.jpg

2. Health impacts and waterborne outbreaks

The real burden of waterborne diseases worldwide is unknown; however it was illustrated with a total number of 1.4 billion annual episodes through transmitted diarrhea infections in children less than five years of age, leading to 4.9 million of death cases (Medema et al., 2003; Huttly, 1989). Actually 88% of all diarrhea cases are attributed to ingestion of contaminated drinking water due to unsafe water supply, sanitation facilities and hygiene (WHO, 2011). The total disease burden worldwide is estimated for 5.7% through water-related severe infections (Medema et al., 2003). However, it is likely that many disease cases are underestimated by sporadic illnesses or people suffering from mild symptoms. The best surveillance system and documentation for waterborne outbreaks is allocated to the U.S., in the period of 2009 - 2010, 33 drinking water outbreaks in 17 states were reported and resulted in 1.040 illnesses and nine deaths (MMWR, 2013). There the outbreaks were identified from plumbing distribution net (57.6%), untreated groundwater (24.2%) and distribution system deficiency (12.1%), causing either gastrointestinal illnesses (92.6%) or respiratory

diseases (57.6%) (MMWR, 2013). Consequently, vast majority of disease outbreaks in industrialized countries like U.S. and also in Europe are attributed to mismanagement of freshwater resources, technological failure and/or inappropriate detection measures (Brettar and Höfle, 2008; OECD, WHO; 2003). According to WHO, in Europe in the period 2000 – 2007, 354 outbreaks of waterborne diseases were related to drinking-water and resulted in over 47 617 episodes of illness from microbial contaminated water.

In contrast to industrialized countries the disease burden in rural areas and developing countries is estimated to be much higher, as safe drinking water is not a rule and thus infection are rather common (Figure 3). However, surveillance systems are not commonly established, so that exact number of disease burden is unknown.

Figure 3. Deaths caused through water transmitted diseases from unsafe water, sanitation and hygiene
http://www.who.int/heli/risks/water/en/webwshmap.jpg

Most substantial numbers of infections and waterborne outbreaks are caused by transmission of microbial contaminants. A huge diversity of microorganisms can be found in water habitats comprising autochthonous but also allochthonous organisms from surrounding environment. As outlined by Zinger, Gobet and Pommiers (2012) that aquatic ecosystem origins a diverse pool of non-pathogenic and pathogenic organisms with an estimate of 10^6 eukaryotic cells (Brown et al., 2009), 10^8 prokaryotic cells (Whitman et al., 1998) and 10^9 – 10^{11} virus like particles per liter of water (Wilhelm and Matteson, 2008).

Health impacts of bacteria found in water

By far most bacteria derived from water are still unclassified (Revetta et al., 2010). A huge diversity can be found in water including Proteobacteria, Cyanobacteria, Flavobacteria, Planctomyces, Verruncomicrobiales and other representative phyla (Liu et al., 2013; Kwon, 2011; Revetta et al., 2010). Among non-pathogenic waterborne bacteria, some are described to be opportunistic pathogens and may cause disease in children, elderly and immune-compromised people. Potential opportunistic species include the genera of *Pseudomonas*, *Aeromonas* and *Klebsiella*, which are often associated with gastrointestinal infection caused by ingestion of contaminated drinking water (Edberg and Allen, 2004; Rusin et al., 1997). Prevalent bacterial pathogens found in polluted water and associated with waterborne diseases are *Yersina* spp., *Legionella* spp., *Vibrio cholera*, *Campylobacter* spp., *Helicobacter* spp., *Salmonella* spp., *Shigella* spp. and pathogenic *E. coli* (Botes, Kwaadsteniet and Cloete, 2013; Egli, Köster and Meile, 2002). They can cause a serious of severe infections such as legionellosis, cholera, pneumonia, acute gastritis, bacteremia and septicemia. Mostly reported illnesses are associated with gastrointestinal infections causing symptoms as nausea, diarrhea, vomiting, abdominal pain (Ashbolt, Grabow and Snozzi, 2001; Medema et al., 2003). The vast majority of gastrointestinal and enteric illnesses are water transmitted diseases. One example is *Camplyobacter* abundantly present in environment and often associated with diarrhea cases worldwide through transmission route to water (WHO, 2011). Other pathogens such as *E. coli* O157 and *Shigella* spp., with low infection dose as 10-100 organisms, cause over 2 million of infections each year with 60.000 deaths (WHO, 2011).

Health impacts of viruses and protozoa found in water

Viruses are assumed to be present in water in even higher numbers than reported in literature (Zinger, Gobet and Pommiers, 2012; Girones et al., 2010). They remain often undetected, because of the lack of sensitive detection methods (WHO, 2011). In recent years discussions raised that their monitoring should also be included in water quality assessment, because they are expected to be more stable against disinfection processes than bacteria and thus unlikely to be adequately removed during drinking water treatment. Therefore, viruses can be apparently present in drinking water and associated with persistent infections (Payment and Robertson, 2004). Viral outbreaks arise for the most part due to ingestion of drinking water polluted with enterovirus, adenovirus, norovirus, rotavirus and hepatitis A virus (Girones et al., 2010; Sinclair, Jones and Gerba, 2009; Betrand et al., 2004). The health impacts are not as clearly described to date and further focus should be set to.

Beside viruses also protozoa represent a challenge in drinking water. Especially, cysts of *Cryptosporidium* and *Giardia* are highly resistant against disinfectants in sewage treatment and

survive for weeks to months (Medema et al., 2003). Therefore these members are a major causative agent of parasitic infections worldwide (Girones et al., 2010). Interestingly, majority of cases of parasitic infections are attributed to USA and Europe, which could be explained by better surveillance systems than in developing countries (Botes, Kwaadsteniet and Cloete, 2013; Karanis, Kourenti and Smith, 2007).

Severe waterborne outbreaks and future emerging diseases

Waterborne outbreaks from bacterial, viral or parasitic pathogens are most frequently transmitted from outside pollution from fecal derived sources. These pathogens have low infectivity dose, are often associated with resistance to disinfection and persistence in distribution net (Table 1).
Diseases are mainly attributed to enteric infections caused by water polluted with animal (livestock) or human fecal excreta (insufficient sewage treatment) (Cabral, 2010; Pavlov et al., 2004; Medema et al., 2003). Zoonotic pathogens account for approximately 75% of water-transmitted diseases including bacteria (*Campylobacter*, *E. coli*, *Salmonella* spp., *Shigella* spp.), viruses (adenovirus, enterovirus, norovirus, rotavirus) and protozoa (Cryptosporidium, Entamoeba, Giardia) (WHO, 2011). Most severe outcomes and high mortality rates were reported from *Vibrio cholerae*, *E. coli* O157:H7 and hepatitis E virus (Medema et al., 2003).
In the 19th century, *Vibrio cholerae* accounted for millions of deaths from contaminated drinking water. The largest documented outbreak in U.S. history in Milwaukee (1993) was caused by drinking water contaminated with *Cryptosporidium parvum*, which were not adequately removed by chlorine treatment. The outbreak affected over 400.000 people and healthcare costs were estimated with $96 million (http://www.waterandhealth.org/milwaukee-1993-largest-documented-waterborne-disease-outbreak-history/).

Still in the 21th century waterborne diseases through microbial pathogens remain a major problem and pose health risks with estimates of 250 million of new cases each year (Zhou et al., 2011; Al-Qadiri et al., 2006). One of main challenges in future will be the upcoming emerging diseases through new or (multi)-resistant pathogens surviving water treatment and inhabiting distribution systems (biofilm formation). Intensive agriculture, migration, the increase in human population and climate change will be directing emergence of future waterborne diseases (Aw Gim and Rose, 2012; Medema et al., 2003). Therefore there is and will be a strong need in ensuring water quality assessment strategies and rapid testing methods of hygienic parameters and pathogens.

Table 1. List of relevant pathogens (bacteria, viruses and protozoa) associated with health impacts (WHO, 2011)

Pathogen	Health significance[b]	Persistence in water supplies[c]	Resistance to chlorine[d]	Relative infectivity[e]	Important animal source
Bacteria					
Burkholderia pseudomallei	High	May multiply	Low	Low	No
Campylobacter jejuni, C. coli	High	Moderate	Low	Moderate	Yes
Escherichia coli – Pathogenic[f]	High	Moderate	Low	Low	Yes
E. coli – Enterohaemorrhagic	High	Moderate	Low	High	Yes
Francisella tularensis	High	Long	Moderate	High	Yes
Legionella spp.	High	May multiply	Low	Moderate	No
Leptospira	High	Long	Low	High	Yes
Mycobacteria (non-tuberculous)	Low	May multiply	High	Low	No
Salmonella Typhi	High	Moderate	Low	Low	No
Other salmonellae	High	May multiply	Low	Low	Yes
Shigella spp.	High	Short	Low	High	No
Vibrio cholerae	High	Short to long[g]	Low	Low	No
Viruses					
Adenoviruses	Moderate	Long	Moderate	High	No
Astroviruses	Moderate	Long	Moderate	High	No
Enteroviruses	High	Long	Moderate	High	No
Hepatitis A virus	High	Long	Moderate	High	No
Hepatitis E virus	High	Long	Moderate	High	Potentially
Noroviruses	High	Long	Moderate	High	Potentially
Rotaviruses	High	Long	Moderate	High	No
Sapoviruses	High	Long	Moderate	High	Potentially
Protozoa					
Acanthamoeba spp.	High	May multiply	High	High	No
Cryptosporidium hominis/ parvum	High	Long	High	High	Yes
Cyclospora cayetanensis	High	Long	High	High	No
Entamoeba histolytica	High	Moderate	High	High	No
Giardia intestinalis	High	Moderate	High	High	Yes
Naegleria fowleri	High	May multiply[h]	Low	Moderate	No

3. Standard water quality assessment

As outlined by WHO in 1979: "*It is of utmost importance to control the hygienic quality of water supply and the bacteriological examination should be carried out frequently and regularly"* (Medema et al., 2003).

Routine assessment of microbial safety of drinking water is generally defined for monitoring of bacterial parameters such as *E. coli*, coliforms (total or thermotolerant) and heterotrophic bacteria, whereas some countries also include enterococci, *P. aeruginosa* and *C. perfringens*.
The microbial parameters defined in Europe and Austria (Table 2 and 3) is regulated by the Council Directive 98/83/EC on the quality of water intended for human consumption (EC, 1998) and the Austrian Drinking Water Directive (DWD, BGBl. II, Nr. 304, 2001). Other countries and their regulations, for example the U.S. Water Directive (Environmental Protection Agency (EPA), 2009), Australian Drinking Water Guidelines (NHMRC, 2011) or WHO recommendations (WHO, 2011) are more permissive and do not include the strict routine assessment of whole range of parameters as defined in Europe.

Table 2. Microbiological parameters and the parametric values defined for drinking water in European Communities (Council Directive 98/83/EC)

Parameter	**Parametric value**
Escherichia coli (*E. coli*)	0/100 ml
Enterococci	0/ 100 ml
coliforms	0/100 ml

The following applies to water offered for sale in bottles or containers:

Parameter	**Parametric value**
Escherichia coli (*E. coli*)	0/250 ml
Enterococci	0/250 ml
coliforms	0/250 ml
Pseudomonas aeruginosa	0/250 ml
Colony count 22°C	100/ml
Colony count 37°C	20/ml

[1] indicator parameter

Clostridium perfringens (including spores) in drinking water influenced by surface water (0/100 ml)

Table 3. Microbiological parameters and the parametric values according to Austrian Drinking Water Directive (BGBl. II Nr. 304/2001)

Parameter	Parametric value non-disinfected water	Parametric value immediate after disinfection	Parametric value water offered in bottles or containers
Escherichia coli (*E. coli*)	0/100ml	0/250ml	0/250ml
Enterococci	0/100ml	0/250ml	0/250ml
Pseudomonas aeruginosa	0/100ml	0/250ml	0/250ml
Colony count 22°C[1]	100/ml	10/ml	100/ml
Colony count 37°C[1]	20/ml	10/ml	20/ml
Coliforms[1]	0/100 ml	0/250 ml	0/250ml
Clostridium perfringens (including spores)[2]	0/100 ml	0/250 ml	0/100ml

[1] indicator parameter; [2] drinking water influenced by surface water

The basis of regular control of microbial quality of drinking water is outlined by the assessment of defined organisms as called indicator or index organism, themselves not harmful. In routine these organisms are monitored as they were described to correlate with the presence of potential pathogenic disease causing bacteria in water sources. The indicator concept was established because pathogens themselves cannot easily be detected and it is even impossible to monitor the entire range of relevant ones (Payment and Robertson, 2004; Hach, 2000). Furthermore the concept allowed for a routine monitoring of water quality with less costs. The indicator concept was introduced in 1892 and today still remains the basis of quality assessment (Medema et al., 2003; Payment, Waite and Dufour; 2003; Hach, 2000).

The indicator concept follows the criteria (Medema et al., 2003):

- Indicators should be absent in clean water and abundantly present and co-occur with pathogenic organism
- Indicators should not be able to multiply in environment
- Indicators should be present in greater numbers than the pathogen
- Indicators should respond and behave in a similar manner as the pathogen, i.e. treatment processes
- Indicators should be easy to isolate, identify and enumerate
- The test should be inexpensive thereby permitting that numerous samples to be taken
- The indicator should not be a pathogen

With the application of the indicator concept also shortcomings have been simultaneously introduced. In fact, it has been demonstrated that several proposed indicator organisms are poorly correlating to pathogens and thus their potential for predicting health based threats is questionable (Savichtcheva and Okabe, 2006; Edberg and Allen, 2004). Especially questionable is the utilization of coliforms, as their absence does not necessarily indicate the absence of pathogenic bacteria (Hach, 2000). This was shown by the extended evaluation of river water samples, in which fecal derived pathogens could be detected but coliforms and also enterococci were absent in the same sample (Atoyan, Herron and Amador, 2011). Similar findings were reported by Harwood and colleagues (2005), who revealed that indicators readily disappeared after disinfection treatment, but pathogens were still present due to longer survival rates and in some cases even showed resistance to treatment processes. In addition, it has been stated that some members of the coliform group (e.g. *Citrobacter* spp., *Enterobacter* spp.) can also be naturally found and are able to multiply in water habitats, contrarily to the indicator concept. However, to date they continue to be used in water quality assessment as primary indicator. EPA has determined that *E. coli* is most adequate and suitable predictive parameter correlating with the presence of pathogenic bacteria usually derived from fecal contamination, whereas fecal coliforms were identified as poorest indicator associated with presence of potential health risk (http://water.epa.gov/type/rsl/monitoring/ vms511.cfm).

To improve the constraints of quality assessment the refinement to fecal indicator concept was considered and alternative indicators were suggested. Savichtcheva and Okabe (2006) described that adequate indicators should be i) consistently present in feces, ii) unable to multiply outside the intestinal tract, iii) as resistant as pathogens in environmental and treatment conditions and iv) have strong association with the presence of pathogens. In consistency to this description alternative indicators were proposed: *Bacteroides* and *Bifidobacterium* (most prevalent in feces), spore forming *Clostridium*, viruses (adenovirus) or cryptosporidal cysts (Cabral, 2010; Girones et al., 2010; Savichtcheva and Okabe, 2006). The *Bacteroidetes* as marker for fecal contamination was intensively studied by Reischer et al. (2007 and 2006) to differentiate between human (*BacH- Bacteroidetes* human) and ruminant derived (*BacR- Bacteroidetes* ruminant) specific pollution in spring and carstic water and was evaluated as promising indicator for source tracking. In addition also monitoring of viral particles is considered, because the nature of viruses is very different from bacteria as they are more stable against treatment processes. Human adenovirus was suggested as indicator virus to be monitored in Europe, because it is assumed to be highly present in drinking water there (Botes, Kwaadsteniet and Cloete, 2013).

Trends towards establishing a broader range of alternative indicators and also including pathogens would ensure the safety of supplied water. Currently, they are still under investigation and not applied in routine assessment (Savichtcheva and Okabe, 2006).

Thitherto coliforms, *E. coli*, enterococci, *P. aeruginosa*, *C. perfringens* and heterotrophic bacteria remain the standard parameters assessed in monitoring of water.

Coliforms and *E. coli*

Coliforms were introduced as first specific indicator (Payment and Robertson, 2004) for water quality monitoring and are stipulated in all regulations worldwide. According to the definition of WHO, coliforms are rod-shaped, non-spore forming, gram-negative bacteria, capable of growth in bile salts (Ashbolt, Grabow and Snozzi, 2001). Biochemical characterization is described as cytochrome oxidase negative and able to ferment lactose with ß-galactosidase under the production of acid, gas and aldehyde. Phylogenetically coliforms belong to the family of *Enterobacteriaceae*, which is a heterogeneous diverse group including a range of genera and species. However, not all members of the *Enterobacteriaceae* family are coliforms like *Salmonella* spp., *Yersinia* spp. or *Shigella* spp. (non-coliforms), which don't exhibit the biochemical characteristics of coliforms (Figure 4).

In the past coliform parameter was subjected to extensive changes to ensure conformity with the indicator concept. Initially the total coliforms were defined as the first indicator for fecal contamination of water, because members (*Klebsiella* spp. and *E. coli*) were isolated from stool (Medema et al., 2003). However, some other members of the coliform group, such as *Enterobacter*, were identified to be natural inhabitants of water and beyond that able to multiply there. Therefore the predictive value of coliforms concerning the indication of pathogens and fecal pollution was questioned (Payment and Robertson, 2004; Hach, 2000). As a consequence, thermotolerant (fecal) coliform description has been implemented to better meet requirements of indicator concept and act as warning signal for fecal contamination (illustrated in Figure 4). Thermotolerant species are restricted to *E. coli*, *Klebsiella pneumonia* and Citrobacter *freundii*, having the same fermentation properties as the total coliforms but being able to fermentate lactose at 44°C (Payment, Waite and Doufour, 2003; Rompré et al., 2002). However, studies also elucidated that the use of the thermotolerant indicators showed low reliability as *Klebsiella* spp. are frequently derived from non-fecal environments such as paper mill and potato industry (Payment and Robertson, 2004; Medema et al., 2003). Currently the definition of coliform bacteria differs slightly between regulations and countries (Rompré et al., 2002), but in practice in drinking water utility bodies the monitoring of total coliforms is still the standard test, regarding that total coliforms potentially indicate insufficient disinfection processes and that the total coliform method is simpler than the determination of thermotolerant indicators.

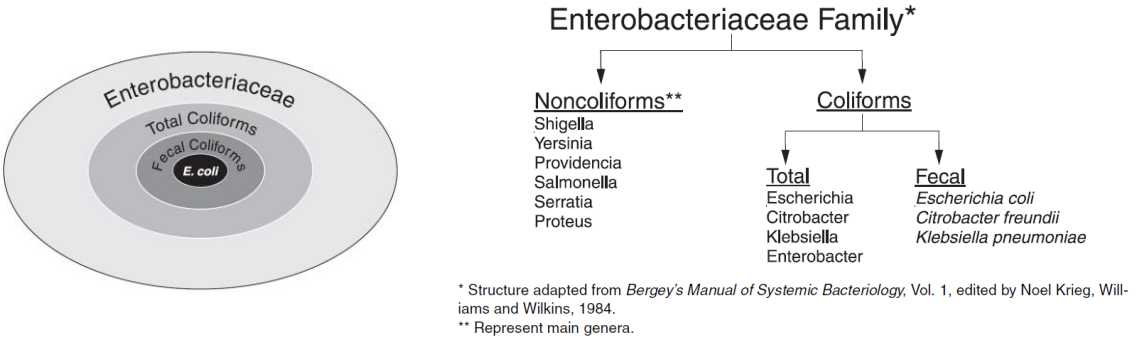

Figure 4. Relationship of coliforms in the *Enterobacteriaceae* family (Hach, 2000)

The most reliable coliform member, invariably associated with faeces, is *E. coli*. Authorities included this indicator in regulations as primary quality parameter for monitoring of drinking water (Payment and Robertson, 2004), because of its abundance in human feces (10^9 cells/g stool, accounting 90% of stool bacteria) and therefore is a good indication of pollution from warm blooded animals and humans (Medema et al., 2003; Hach, 2000). *E. coli* is a thermotolerant coliform and able to produce indole from tryptophan at 44°C (Rompré et al., 2002). Most members of *E. coli* are non-pathogenic, but the pathogenic strains are associated with severe waterborne diseases. The most prevalent strain *E. coli* O157:H7 accounts for several 100 million cases of diarrhea and tens of thousands of death each year (Cabral, 2010). Potential drawback of *E. coli* as indicator is reported in its sensitivity to disinfectants readily disappearing and not correlating to pathogens.

EN ISO 9308-1 regulates the enumeration of coliform bacteria and *E. coli*. The test method is based on membrane filtration of water sample and subsequent cultivation on non-selective and selective media, with biochemical confirmation of targeted organisms after 2-3 days. The norm also includes a rapid testing for *E. coli*.

Briefly, bacteria are concentrated on membrane filters and incubated on solid lactose agar at 36±2°C for 21±3 h. Then yellow colonies are sub-cultured on non-selective agar at 36±2°C for 21±3 h and furthermore in tryptophan bouillon. Colonies are tested for their oxidase activity on a filter paper with 1-2 drops of oxidase reagent. The appearance of dark blue color accounts for oxidase positive colonies. Furthermore indole formation is in parallel tested by using Kovacs' reagent resulting in red colorization. Colonies with negative oxidase reaction and positive indole test are confirmed as *E. coli*. Oxidase and indole negatives are considered to be coliforms.

Rapid test for *E. coli* uses membrane filtration and incubation on casein agar at 36±2°C for 4-5 h. The membrane is then transferred to trypton soy agar (TSA) and incubated at 36±2°C for 4-5 h and final incubation is performed on trypton-galle-agar at 44±0.5°C for 19-20h. After incubation the membrane

is transferred to a filter paper saturated with indole reagent and positive *E. coli* colonies show red colorization under UV-light.

Application of this ISO method is often concluded to be difficult because coliforms consist of a heterogenic group often impeding the confirmation by atypical colonies and the excessive crowding of colonies (Rompré et al., 2002). Furthermore the practice is labor and time consuming. Therefore faster detection tests based on chromogenic/fluorogenic principles have been developed for detection of coliforms and *E. coli* from water samples within 18 h. Colilert®-18 (IDEXX laboratories, Germany) has become included into the new International Organization for Standardization (ISO) standard 9308-2:2012. Furthermore it was approved by U.S. Environmental Protection Agency and stipulated in Standard Methods for Examination of Water and Wastewater (http://www.idexx.com). Colilert®-18 is a defined enzyme substrate technology based on the presence of coliform specific enzyme β-galactosidase and ß-D-glucuronidase specific for *E. coli.* Chromogenic detection is based on the metabolization of o-nitrophenyl-galactopyranoside (ONPG) through the β-galactosidase enzyme appearing as chromogenic yellow colorization on positive samples. *E. coli* is identified through the metabolization of 4-methyl-umbelliferly-ß-D-glucuronide by ß-D-glucuronidase and results in emission of fluorescence under UV-light (Figure 5). In addition to the defined enzyme substrate technology, coliform and *E. coli* detection can also be facilitated with chromogenic media. Chromocult Coliform agar (Merck, Germany) enables differentiation of pink (*E. coli*) and blue-violet (total coliform) colonies.

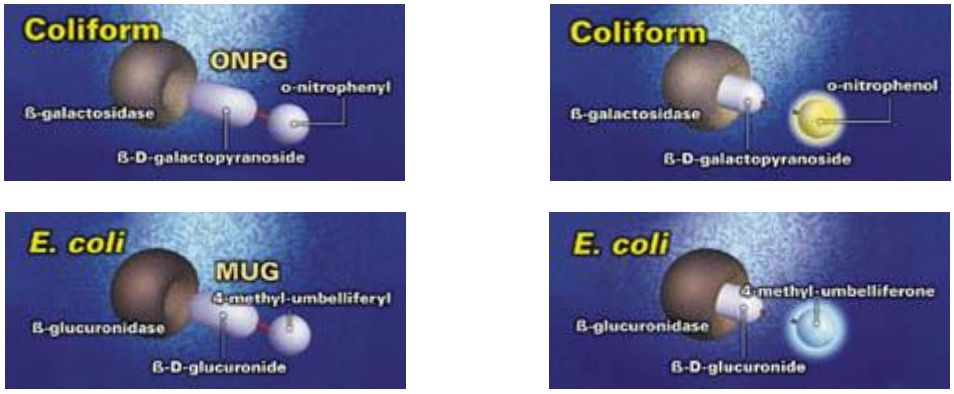

Figure 5. Principle of defined enzyme substrate technology of Colilert®-18 (IDEXX laboratories, Austria) for the fast (18 h) detection of coliforms and *E. coli* http://www.idexx.com/view/xhtml/en_us/water/products/colilert-18.jsf

***Enterococcus* species**

In addition to coliforms and *E. coli*, enterococci are second most important indicator for fecal pollution. Enterococci are defined to be a subgroup of the fecal streptococcus group and are gram positive, catalase negative cells able to grow at pH 9.6 at 10°C and 45°C and reduce 0.1% methylene blue (Cabral, 2010; Ashbolt, Grabow and Snozzi, 2001). *E. faecalis*, *E. faecium*, *E. durans* and *E. hirae* have been predominately isolated from fecal samples and are supposed as indicator for animal derived fecal contamination in water sources (Ashbolt, Grabow and Snozzi, 2001; Hach, 2000).

EN ISO 7899-2 prescribes the enumeration of intestinal fecal enterococci based on membrane filtration method. The first step after membrane filtration is the incubation (at 36±2°C for 44±4 h) on Slanetz and Bartley agar. After incubation the membrane is transferred to sodium azide selective media and incubated at 44±0.5°C for 2 h. Biochemical confirmation is performed using 2,3,5-triphenyltetrazolium chloride that is chemically reduced from colorless to red formazan, thereby positive colonies appear red, dark brown or pink. At least another confirmation step is needed by incubation of the positive assumed colonies on bile-aesculin-azide agar. After 2 h at 44°C with the presence of enterococci a yellow-brownish colorization in the media appears.

The ISO method is rather cheap but time-consuming and requires handling with toxic sodium azide substance (Köster et al., 2003). An alternative defined enzyme substrate technology is also commercially available for testing of enterococci in water. The Enterolert-E test (IDEXX laboratories, Germany) is based on the metabolization of the nutrient indicator 4-methy-umbelliferyl-ß-D-glucoside by the presence of enterococcal ß-glucosidase at the growth optimum of 44°C with results obtained in 24 h (Figure 6). The detection meets EU standardization and correlates to ISO 7899-1.

Figure 6. Principle of defined enzyme substrate technology of Enterolert-E (IDEXX laboratories, Austria) for the fast detection of enterococci from water http://www.idexx.com/view/xhtml/en_us/water/products/enterolert-e.jsf

Pseudomonas aeruginosa

P. aeruginosa is a specific parameter to be assessed in Europe and Austria for bottled water. It is not targeted as indicator, but it is related to the cleanliness of water (Payment, Waite and Dofour, 2003). It is a gram-negative, rod shaped, oxidase positive, non-spore forming bacteria. *P. aeruginosa* was defined to be an opportunistic pathogen commonly found in feces, water and in sewage sludge and with the ability to multiply in aquatic systems (Allen, Edberg and Reasoner, 2004; Payment, Waite and Dofour, 2003). *P. aeruginosa* is stable against treatment processes and therefore has to be monitored in bottled drinking water.

EN 12780:2002 defines the determination of *P. aeruginosa* in water samples. Water sample is filtered through a membrane, which is then incubated on selective cetramide agar (CN-agar) at 36±2°C for 44±4 h. Colonies with blue-green pigmentation are confirmed to be *P. aeruginosa* and (pyocyanine)-fluorescence colonies under UV-light are suspected to be *P. aeruginosa*. Other colonies with red-brownish pigmentation have to be further tested with oxidase reagent resulting in a deep blue to magenta colour. Oxidase positive colonies have to be sub-cultivated on King's B media for 5 days are then confirmed by fluorescence under UV-light. Further confirmation is required through sub-cultivation in acetamide-broth at 36±2°C for 22±2 h and testing for the production of ammoniac with Nessler reagent resulting in a yellow or brick-red precipitate.

Testing of *P. aeruginosa* has the shortcoming of long duration of analysis, which requires several days and needs the utilization of several cultivation media. Alternative defined enzyme substrate technology called Pseudalert (IDEXX laboratories, Austria) has recently become available, but it is still not included into standards.

Clostridium perfringens

C. perfringens is a specific parameter only assessed in case that drinking water abstraction is influenced by surface water. *C. perfringens* is an anaerobic, gram positive, spore forming rod commonly found in soil and surface water and can also reside within faeces of warm blooded animals and is present to 13-25% in human faeces (Ashbolt, Grabow and Snozzi, 2001). *C. perfringens* has the ability to ferment lactose, sucrose and inositol with the production of gas, reduce nitrate, hydrolyze gelatin and produce lecithinase and acid phosphatase (Cabral, 2010). The implementation of *C. perfringens* and its use as quality parameter is mainly limited to European regulations and was included because of strong resistance to treatment processes (UV-irradiation, chlorination) due to the formation of resistant spores (Ashbolt, Grabow and Snozzi, 2001).

According to the Austrian DWD (2001) *C. perfringens* including spores are tested on membrane filter incubated anaerobically on m-CP-agar at 44±1°C for 21±3 h. Colonies which turn under vaporization with sodium hydroxide to pink or red are confirmed as *C. perfringens*.

Heterotrophic plate count (HPC)

HPC has been introduced to monitor the treatment efficiency for drinking water (Allen, Edberg and Reasoner, 2004), but is nowadays a commonly analyzed parameter for quality measure of water samples. HPC method aims at the enumeration of heterotrophic bacteria, which are able to grow and produce visible colonies under prescribed cultivation conditions (Payment, Waite and Dofour, 2003). In scope of water quality assessment parametric values were defined, in Austria and Europe parametric values should not exceed 20 CFU/ml (HPC 37°C) and 100 CFU/ml (HPC 22°C). The U.S is more permissive and only recommends a parametric range of 1 - 500 CFU/ml, which are normally considered acceptable for non-disinfected drinking water (Pavlov et al. 2004; Sartory et al. 2004).

According to the EN ISO 6222:1999, culturable microorganisms are enumerated by heterotrophic plate count method. Pour plate method is recommended, using 1 ml of water sample and yeast extract agar. Two sets of incubation temperatures have to be assessed, 36±2°C for 44±4 h and 22±2°C for 68±4 h. The colonies are counted for each plate and results are represented as CFU/ml. However, the method is not globally standardized and different HPC protocols and cultivation conditions are available. Other HPC measurements include the use of different practices (membrane filtration or spread plate method) and defining a range of incubation temperatures (20°C to 40°C), incubation times (48 h - 7 days), and variable formulations of media (e.g. low and high nutrient media). These differences in cultivation parameters were shown to result in variable HPC outcomes and may lead to biased conclusions according to the applied protocol (Reasoner 2004; Bartram et al. 2004). Beyond that, HPC as hygienic quality measure was questioned as not all heterotrophic bacteria can be determined, because growth is restricted to culturable ones. Štursa and co-authors (2009) described that only 90-99% of all microorganisms can be cultivated on non-selective media. Nevertheless, it remains as one of the most commonly assessed parameter in regulations.

Limitations of cultivation-based techniques for quality assessment

In past cultivation was the only technique to identify microorganisms from various matrices such as water (Köster et al., 2003; Medema et al., 2003). Therefore, cultivation is basically the 'golden standard' technique for routine monitoring as outlined by ISO norms for assessment of microbial parameters. This practice is simple as no specialized equipment is required and thus is cost efficient. However, the use as analytical diagnostic tool has been repeatedly questioned based on identified practical and technical shortcomings. ISO methods are usually time consuming, lab intensive and lack detection of non-culturable organisms. The detection of selected organisms may require several days, because primary cultivation on non-selective media and sub-culturing on selective media is necessary. Most often sub-culturing has to be performed on several nutrient media containing different supplements. Several cultivation steps are prerequisite to detect target organisms and to minimize co-enrichment of non-target species. Finally, a confirmation by biochemical test(s) is required. The enumeration of colonies can be facilitated but often overcrowding or atypical colonies make the quantification difficult.

In addition to these practical drawbacks, it has to be taken into account that certain bacteria are unable to grow on cultivation media. It is known that cultivation methods are restricted for the growth and detection of culturable cells only, which misses out a considerable part of microorganisms. In fact, that with cultivation techniques, as mentioned before, only about 1-10% of prokaryotic diversity can be identified, known as great plate count anomaly (Hammes and Egli, 2010; Dorigo,Volatier and Humbert, 2005). Furthermore with traditional cultivation, stressed and starved bacterial cells often cannot propagate. Microbial indicator organisms such as *E. coli* and *Enterococcus faecalis* have been reported to be able to switch in a so called viable but non-culturable state (VBNC) (Oliver, 2005). VBNC is very common in oligotrophic habitats such as water, because fluctuations in temperature, oxygen level and nutrient availability can stimulate the dormant, physiological inactive state of many bacteria (van Frankenhuyzen et al., 2011; Oliver, 2005). The health based impacts are contradictory, as it is expected, that dormant cells with low metabolic activity are not able to cause symptoms. However, Jones and Roworth (1996) demonstrated that VBNC *Campylobacter jejuni* caused death in inoculated mice. In addition, VBNC cells are able to resuscitate in their active state. This has been shown for example for *V. vulificus* as cold temperature below 10°C cause the VBNC status, but a temperature increase resulted in resuscitation to culturable state (Oliver, 2005; Wolf and Oliver, 1992). So either health impacts could be caused in their VBNC state remaining their pathogenicity or capacity to resuscitate in their active state prior not been determined by cultivation.

Another limitation is the culturability of pathogens. They are not routinely assessed but in scope of outbreak cases and risk assessment, the detection of pathogens is limited by cultivation methods. Several pathogens are rarely growing on nutrient media (e.g. *Crytosporidium*, *Campylobacter* spp.). If pathogens can be detected by cultivation, this technique often limits the appropriate discrimination of

different closely related pathogenic strains (pathogenic *E. coli*) or impossibility to differentiate between serotypes (*Salmonella* serotypes).
Due to the range of shortcomings, WHO already reviews alternative test methods for risk based and water quality assessment (WHO, Water Quality and Health 2013-2020).

4. Alternative cultivation-independent molecular methods

The detection of microorganisms was in the past restricted to cultivation techniques, but with the era of molecular assays, the potential of cultivation independent detection and identification evolved. In the field of water quality assessment a demand for new technologies arose in the last decades because of above discussed shortcomings of cultivation-based techniques. Considerable interest in novel molecular assays mainly nucleic acid (DNA)-based detection methods is seen as these hold several advanatges. The potential is seen for selective detection of specific sequence region of interest (discriminative down to species and strain level), more rapid analysis, quantification and higher sample throughput.

First applications of nucleic acid-based techniques for quality assessment were microscopic techniques such as fluorescence in situ hybridization (FISH) or flow cytometry. Both techniques rely on the detection of fluorescently labeled DNA sequence. FISH most often uses the universal bacterial probe EUB338 able to cover 90% of the domain bacteria (Amann et al., 1990), visualized in epifluorescence microscope. Flow cytometry also utilizes fluorescence markers such as SyberGreen I, Syto9 or propidium iodide and allows for automated separation and quantification of DNA according to the fluorescent labeling. Both methods opened new insights into microbial water complexity and quantification thereof. Hammes and Egli (2008) could reveal that there was a constant underestimation of biodiversity and detected amount of bacteria through cultivation based methods, such as HPC. Amann and Ludwig (2000) revealed that HPC gives approximately 2-4 orders of magnitude lower concentrations than microscopic techniques. Furthermore these techniques showed that even 10^4-10^5 cells/ml can be found in treated drinking water, not detectable with cultivation (Hammes et al., 2008; Rinta-Kanto et al., 2004; Hoefel, 2003).
Other alternative detection methods such as microarrays for simultaneous detection of indicators and pathogens were also considered for drinking water and food industry (Fusco and Quero, 2012; Cao, 2001). For example the phylochip for the detection of 50,000 bacteria, archaea and microalgae was developed for testing wastewater samples (Hazen et al., 2010). A microbial diagnostic microarray for the detection of relevant bacterial food- and water-borne pathogens and indicator organisms was developed by Kostic et al. (2010) or another PCR-based DNA microarray was established by Zhou and authors (2011) for the simultaneous detection of 26 drinking water relevant pathogens. However,

most of these diagnostic microarrays yielded only 10^4 CFU ml^{-1} sensitivity, although biological pre-enrichment steps were performed. Most microarrays have never been validated for application and are hampered by the lack in appropriate sensitivity; moreover they are rather cost-intensive. However, microarrays bear the potential and should not be disregarded for serotyping or for functional genomics in highly parallel manner.

The evolving field of PCR technologies and advances by real-time PCR displaced most of the other nucleic acid (DNA)-based detection tools. For example as shown by Lee and coauthors (2006) that RT-PCR was able to detect 2 gene copies in comparison to 140 gene copies from PCR-based DNA microarray. Furthermore beside the more sensitive detection, they are more practicable methods intended for routine use for quality assessment purposes and nowadays most focus is set to development and establishment of RT-PCR analysis.

In comparison to above mentioned techniques used for detection of microorganisms, molecular assays contributed to the better understanding of microbial communities in water ecosystem.
For example fingerprinting techniques like denaturing/temperature gradient gel electrophoresis (DGGE/TGGE) or terminal-restriction length polymorphism (T-RFLP) allowed for profiling of microbial populations and encompassing spatial or temporal dynamics in community composition. DGGE/TGGE resolves fingerprinting pattern according to the nucleotide polymorphism (nucleotide variability) and the melting properties through denaturing agent or a temperature gradient in gel electrophoresis. In accordance T-RFLP allows for community profiling due to creating different fragment lengths by a restriction digest through variability of sequences (Dorigo, Volatier and Humbert, 2005). For example DGGE application was shown for fecal source tracking of different isolated *E. coli* strains in water, resolved in species fingerprint from isolates (Farnleitner et al., 2000) and a comparison of community patterns in marine water was illustrated by T-RFLP (Matz and Jurgens, 2003). Both methods contributed considerably to the insights and monitoring of microbial communities, but these fingerprinting technologies are almost disappearing as more information, concerning the identification is achieved through cloning and sequencing. In addition with the evolving field of NGS methods they allow for detection and identification of complex communities and to identify the *rare biosphere* (Zinger, Gobet and Pommiers, 2012).

A large array of different nucleic acid-based techniques is available to date, proposed for different applications in water for understanding the water ecosystem and microbial community by fingerprinting techniques (DGGE/TGGE or T-RFLP), identification thereof (sequencing strategies) or to act as diagnostic detection tool (PCR technologies, microarray, FISH) (Figure 7).

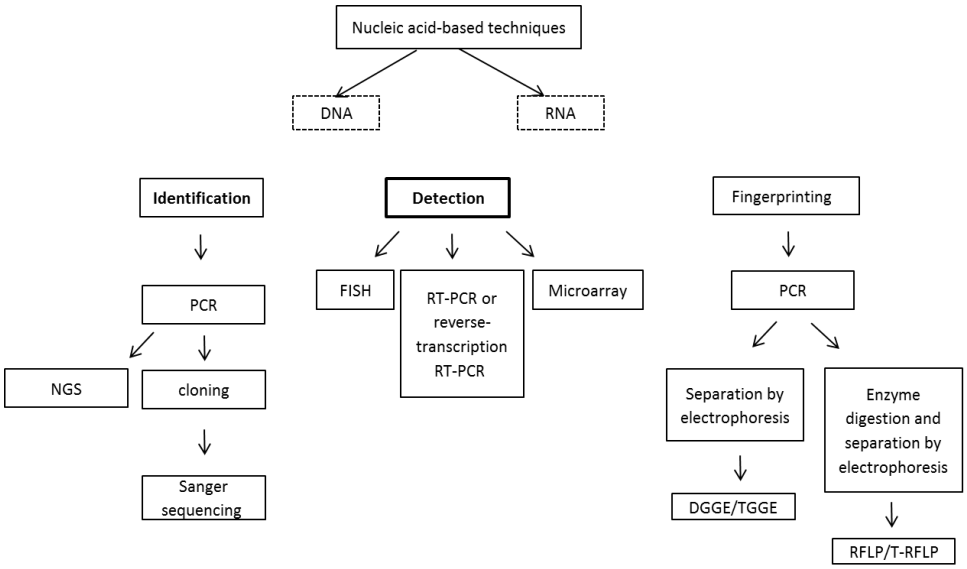

Figure 7. Overview of applications for nucleic-acid based techniques

Quality assessment with DNA-based techniques

By far DNA-based approaches, especially PCR technologies are most promising candidates for detection of microorganisms (bacteria, protozoa and virus) from various matrices such as environment, water and food. The application of PCR techniques revolutionized already the field of clinical diagnostics because they exhibit several advantages. In an expert meeting of the OECD the use of PCR has already been considered in the framework of quality management for drinking water to allow for more routine testing (Ashbolt, Grabow and Snozzi, 2001). Major advantages lie in the detection of non-cultivable microbes or viable but non-cultivable (VBNC) bacteria, reduced analysis time (hours instead of days), specificity of analysis through high selectivity of assays and high-throughput due to automated procedures. DNA-based approaches require methodical procedures such as i) selection of the sequence region of interest, ii) sample preparation for concentration of microorganisms and DNA extraction and iii) analysis/detection of the target sequence.

Selection of target DNA sequences

In DNA-based analysis the DNA sequence serves as basis for detection, thus enabling accurate and specific taxonomic resolution based on genotype rather than phenotypic characteristics as in cultivation (Beneduce, Fiocco and Spano, 2007; Medema et al., 2003). Most frequently gene-encoding sequence regions are targeted; either phylogenetic markers or species-specific genes can be utilized. Primarily for the detection of bacteria the universal phylogenetic marker the 16S rRNA gene sequence is used, because it is widespread in all bacteria, having essential function and be present in multiple

copies. The 16S rRNA gene has a length of approximately 1500 bp and contains 9 hypervariable regions (V1-V9), despite the phylogenetic conservation there is considerable variability in sequence among different bacteria allowing mainly resolution to family, genus and species level (Beneduce, Fiocco and Spano, 2007). It is the most frequently used universal marker as also huge 16S rRNA databases are available. Other phylogenetic markers such as the 23S rRNA gene, ribosomal polymerase B (*rpoB* gene), heat shock protein (*hsp60* gene), gyrase B (*gyrB* gene) were also proposed as suitable targets. The intergenic spacer (IGS) of the 16S rRNA - 23S rRNA gene also demonstrated good discrimination among strains, species and genera through their different length and variability (Zhou et al., 2011; Amann and Ludwig, 2000; Gürtler and Stanisich, 1996). However, for deeper resolution, especially for discrimination between different strains or even serotypes (Beneduce, Fiocco and Spano, 2007), functional markers genes are more suitable targets. For example detection of *EHEC* O157:H7 by shiga toxins (*stx1* or *stx2*), *Salmonella* spp. by the invasion encoding region (*invA*) or gene regions encoding a toxin (*regA*) for *P. aeruginosa* (Fusco and Quero, 2012) can be utilized. The selection of a suitable DNA region has a considerable effect on the subsequent detection specificity and sensitivity. Therefore every assay should be carefully designed *in silico* and tested *in vitro*. Furthermore, copy numbers of the respective marker has to be considered when quantification is required.

Sample preparation

Sample preparation is the most important and at same the most challenging step in pipeline of DNA analysis. Sample preparation procedures are generally separated into concentration of microorganisms from sample matrix and extraction of DNA.

The proper concentration method is prerequisite to achieve an efficient isolation of target organisms from sample. This is especially important in case of rare targets present in low numbers. Heterogeneity of microorganisms in the samples (i.e. presence of target and non-target organisms) has to be considered, as well as the presence of possibly interfering compounds, which may strongly influence the efficiency of downstream application. Inhibitory substances such as humic and fulvic acids or heavy metals are abundantly present in environment and have to be appropriately removed as they interfere with subsequent DNA-based analysis, especially PCR amplification, which serves as basis for most other molecular assays. Therefore inhibitors in the extracts have to be tested, which was initially outlined by autoclaving the water sample and then inoculation of pure culture to proof the detection possibility. However, as this is not practicable for huge numbers of samples and routine use, commonly DNA extracts are diluted to remove the inhibitors. Another possibility is the use of internal amplification controls, acting as positive control in the same assay, to evaluate its detection efficiency. Most commonly dilution procedures are performed because diluting the sample can be easily

performed and that design of internal amplification controls is often difficult and needs to be constructed for each assay.

Basically for efficient determination of target organisms, the concentration of large volumes of water is required, ranging from 100 ml up to 1L. Commonly applied for bacterial recovery from water is membrane filtration with porosities 0.22-0.45 µm. Other organisms such as protozoa are conventionally enriched through cartridge filtration (porosities < 2 µm) (Girones et al., 2010; Köster et al., 2003) and for the recovery of viral particles, several methods are proposed such as electro-negative/positive filters, tangential or hollow fiber ultrafiltration, and immuno-magnetic separation (Liu et al., 2012; Yang et al., 2011). For processing larger volumes of water above 1L, ultra-centrifugation can be the method of choice, but needs the facility and is impracticable for routine use (Köster et al., 2003). In single cases where no efficient concentration method can be applied to achieve enough concentration of target organism, the pre-enrichment on cultivation media may be essential. However, this limits the ability of quantification with molecular approach.
The best case scenario would be a single efficient target concentration method for bacteria, viruses and protozoa, but to date most concentration methods have to be optimized for each target to allow for maximal yield.

After concentration of microorganisms, the extraction of DNA is required. Many different DNA extraction protocols are established but most commonly used are commercially available kits. They are either based on enzymatic lysis of bacterial cells by lysozyme and proteinase K or mechanical bacterial disruption by bead milling procedures. In the extraction of DNA commonly a treatment step with RNAse is conducted to remove interfering RNA species. Afterwards DNA is cleaned from interfering cell material and proteins through precipitation and washing steps. At least in commercial available kits, the DNA is bound to silica membrane columns to enhance the purity. Finally, pure DNA is eluted and can be used as template for analysis.
Many different DNA extraction kits are available for specialized purposes, such as DNA extraction from water or environmental samples. These kits include inhibitor removal steps, however, sometimes these kits are suboptimal in their extraction efficiency and ‘older’ recommended techniques are more appropriate as freeze thawing procedures, simple boiling or organic solvent such as phenol/chloroform extraction (Köster et al., 2003). In the sample preparation procedure, the extraction of DNA is described to be the most critical step and extraction efficiency is strongly dependent on the used protocol (Mothershed and Whitney, 2005). Therefore the initial comparison of different extraction protocols is strongly recommended, to achieve maximal yields of target DNA (van Frankenhuyzen et al., 2011). Nevertheless, all sample preparation steps should be evaluated and optimized for the special purposes to result in maximal efficiency for downstream DNA analysis such as PCR.

5. Polymerase chain reaction (PCR) technologies

PCR technologies are currently the most used molecular technique due to its versatility (Beneduce, Fiocco and Spano, 2007). PCR facilitates the amplification (copying) of particular DNA sequence region (phylogenetic marker or functional marker sequences) to result in million fold copies. Amplification is facilitated by forward and reverse oligonucleotide primers (most commonly 15-24 bp long) that complementary bind to DNA. PCR reaction composes of denaturation of double stranded DNA, primer annealing and synthesis step through polymerase. The whole procedure takes approximately 2-3 hours (Köster et al.; 2003). Therefore, it is a rapid and specific method for determining the presence of a target sequence of interest. Furthermore conventional PCR serves as basis for most other molecular downstream analysis.

Conventional end-point PCR

Several studies reported on PCR application in food and water mainly for detection of bacteria and some for viruses. For example Chung et al. (1996) could demonstrate that the detection efficiency of enterovirus and hepatitis A virus was 50% increased by PCR compared to cell culture alone (Toze, 1999). The application of PCR was also shown for microbial water quality indicators such as *E. coli* and coliforms, which were validated against enzymatic based Colilert®-18 (Bej 1990; 1991). Specific primers for detection of *E. coli* based on the *uidA* (ß-glucuronidase) and coliforms on the *lacZ* (ß-galactosidase) resulted in detection of corresponding targets. Primers developed for the *uidA* gene included the positive detection of *E. coli* and four strains of *Shigella* species (*S. sonnei*; *S. flexneri*; *S. boydii* and *S. dysenteriae*) and facilitated also detection of *E. coli* not determined with enzymatic test (Bej et al., 1991). The coliform detection was demonstrated, but showed dependence on PCR conditions to exclude some potentially related non coliform members such as *Salmonella* species (Bej et al., 1990). Another PCR was developed for the determination of pathogens *C. jejuni*, *C. coli* and *Y. enterocolitica* from highly contaminated wastewater samples. Results showed successful detection with pure cultures, but application of the PCR on wastewater showed approximately one order of magnitude decreased sensitivity (Alexandrino et al., 2004).

Conventional end-point PCR technologies were in the past approved for detection, but often remained hampered in the detection sensitivity. Furthermore post amplification procedures, such as visualization by gel electrophoresis, are necessary for detection and estimation of the concentration.

Modified PCR strategies (nested PCR, NASBA and LAMP)

Some other modified PCR strategies, such as nested PCR, with two rounds of PCR reaction with outer and inner primers were developed for more sensitive detection also of rare target organism and pathogens in water (Tantawiwat et al., 2005; Juck et al., 1996). The study by Cellini et al. (2004) applied nested PCR for revealing the transmission route of pathogen *Helicobacter pylori* in seawater and could show that minimal amount of 62 CFU per 100 ml water could be detected and that even in an background of coliforms, fecal coliforms and enterococci.

An isothermal amplification method, nucleic acid sequence based amplification (NASBA) facilitates the amplification of rare RNA targets with viral reverse transcriptase, RNAseH and T7 RNA polymerase. NASBA application allowed for sensitive detection and furthermore for discrimination of subspecies of *Mycobacterium avium* subspecies paratuberculosis from water (Motershed and Whitney, 2005; Rodriguez-Lazaro et al. 2004).

The loop mediated isothermal amplification (LAMP) was shown for detection of pathogenic bacteria from water based on specific amplification and strand displacement using six primer pairs and allowing the PCR be performed at one temperature of 60°C. The developed LAMP achieved high sensitivity (81.3%) and specificity (96.6%) when compared to isolation by direct plating detection for detection of *C. jejuni* and *C. coli* (Yamazaki et al., 2008).

Concluding, these PCR strategies may allow for more sensitive detection than conventional PCR and isothermal amplification could be considered as alternative promising PCR technology, because of its advantage that no special equipment is necessary and that PCR reaction is conducted at one temperature. However, they are still not well established and as all conventional PCR technologies were readily outcompeted by the progress and evolvement of real-time PCR (RT-PCR), as they provide technical advances such as real-time measurement enabling quantification through automated procedure and enhanced sensitivity in comparison to conventional PCR technologies.

Real-time PCR (RT-PCR)

Real-time PCR represent most promising candidate for detection and quantification of microorganisms. The quantification in RT-PCR also named quantitative PCR (qPCR) is facilitated through automated real-time fluorescence measurement during amplification process. Fluorescence emission is automatically plotted against cycle number and when cycle threshold fluorescence (Cq) of sample is higher than background, the starting concentration can be calculated (Postollec et al., 2011). For quantification a standard series has to be included in the run and the copy number of the target gene has to be known that cell numbers can be calculated (van Frankenhuyzen et al., 2011). Either

absolute quantification can be facilitated or relative quantification in case gene expression analysis is aimed at.

Various fluorescence chemistries are available for RT-PCR, which have the same principle of fluorescence measurement, but differ in their binding strategy such as DNA intercalating dyes or probe based systems. The selection of technology depends on the detection needs, specificity and the costs of assays.

SyberGreen® chemistry

DNA intercalating dyes such as SyberGreen® (illustrated in Figure 8) are commonly applied. As the RT-PCR progresses the more dye intercalates into the PCR amplicons and fluorescence intensity increases proportionally and at the fluorescence threshold the initial concentration can be evaluated (Postelloc et al., 2011; Wong and Medrano, 2005).

SyberGreen® RT-PCR was successfully applied for the detection of pathogens such as *C. jejuni* and *Salmonella* spp. ($3.0x10^1$ and $7.0x10^1$ gene copies) from 100 ml water sample (Ahmed et al., 2009).

Advantages of the SyberGreen® technology are the lower costs compared to other fluorescence probe-based chemistries and that longer amplicon length >100 bp can be facilitated as it is not the case for probe-based RT-PCR. However, the use of SyberGreen® technology is often prone to false positive detections as unspecific products may also be detected, because dye is intercalating in any amplified double stranded PCR product (Wong and Medrano, 2005). Therefore careful design of primers and optimization in running condition is essential to prevent unspecific signals (Botes, Kwaadsteniet and Cloete, 2013). Further melting curve analysis has to be performed after the amplification reactions to proof the specificity of amplified product. This is done by increasing the temperature to melt double stranded DNA and measure fluorescence decline to pin point melting curve peak from targeted sequence. Disadvantages are that optimization of each assay is necessary to achieve efficient and specific results and that melting curve analysis has to be performed. In addition, SyberGreen® allows for no multiplex detection of several targets in one assay as it can be facilitated with TaqMan® chemistry (outlined below).

TaqMan® chemistry

TaqMan® chemistry (illustrated in Figure 8) improved the specificity and reliability in RT-PCR. High specificity of TaqMan® chemistry also known as 5' nuclease chemistry or hydrolysis probes is facilitated by a dual labeled fluorogenic probe. Probe has a quencher fluorochrome at the 3' end, which reduces fluorescence of reporter fluorochrome at the 5' end. TaqMan® probes are complementary to DNA sequence and during synthesis the 5' nuclease of DNA polymerase activity hydrolyzes the probe. Thereby, the quencher is removed and reporter fluorescence emission is ensured.

For example Maheux and coworkers (2011) demonstrated rapid detection of the quality indicator *Enterococcus* spp. and *Enterococcus faecalis/faecium* from potable water by using TaqMan® RT-PCR. The facilitation of hydrolysis probes with a previous whole genome amplification step allowed a detection limit of 4.5 CFU/100 ml similar to reference tes of membrane filtration (EN ISO 7899-2) with 2.3 CFU/100 ml (Maheux et al., 2011). The application of TaqMan® RT-PCR was also shown for virus detection from water. Albinana-Gimenez et al. (2009) demonstrated the detection of human adenovirus (10^1-10^4 genome copies/L) and polyomavirus (10^0-10^3 genome copies/L) from drinking water treatment plants also able to detect rare virus particles in the samples.

Generally TaqMan® RT-PCR studies show high detection specificity due to target sequence complementary probes. Therefore hydrolysis probes often outcompete SyberGreen® technology in the discrimination of specific sequence, but careful design of primers and probe is essential for achieving high assay specificity and selectivity. Another big advantage is that TaqMan® RT-PCR reduces analysis time to approximate 1h as no melting curve analysis is necessary and further has the ability for multiplex detection of several targets in one assay. In multiplexing a maximum of six probes can be utilized in a single assay either targeting different organisms or gene clusters.
Multiplex PCR for determination of three toxin genes from EHEC O157:H7 in wastewater was represented by Ibekwe et al. (2002). Murinda et al. (2004) showed the simultaneous detection of four different species (*Escherichia coli*, *Listeria monocytogenes*, *Campylobacter jejuni*, and *Salmonella* spp.) and Kong and coworkers (2002) even demonstrated the successful multiplexing of six pathogens from marine water. In all assays parallel detection was accomplished but lower sensitivity and cross-reactivity was often the case. So that multiplexing is a nice task to lower overall analysis time and costs, but the careful design and evaluation is necessary and often single assays for each target are preferred due to higher selectivity of assays.

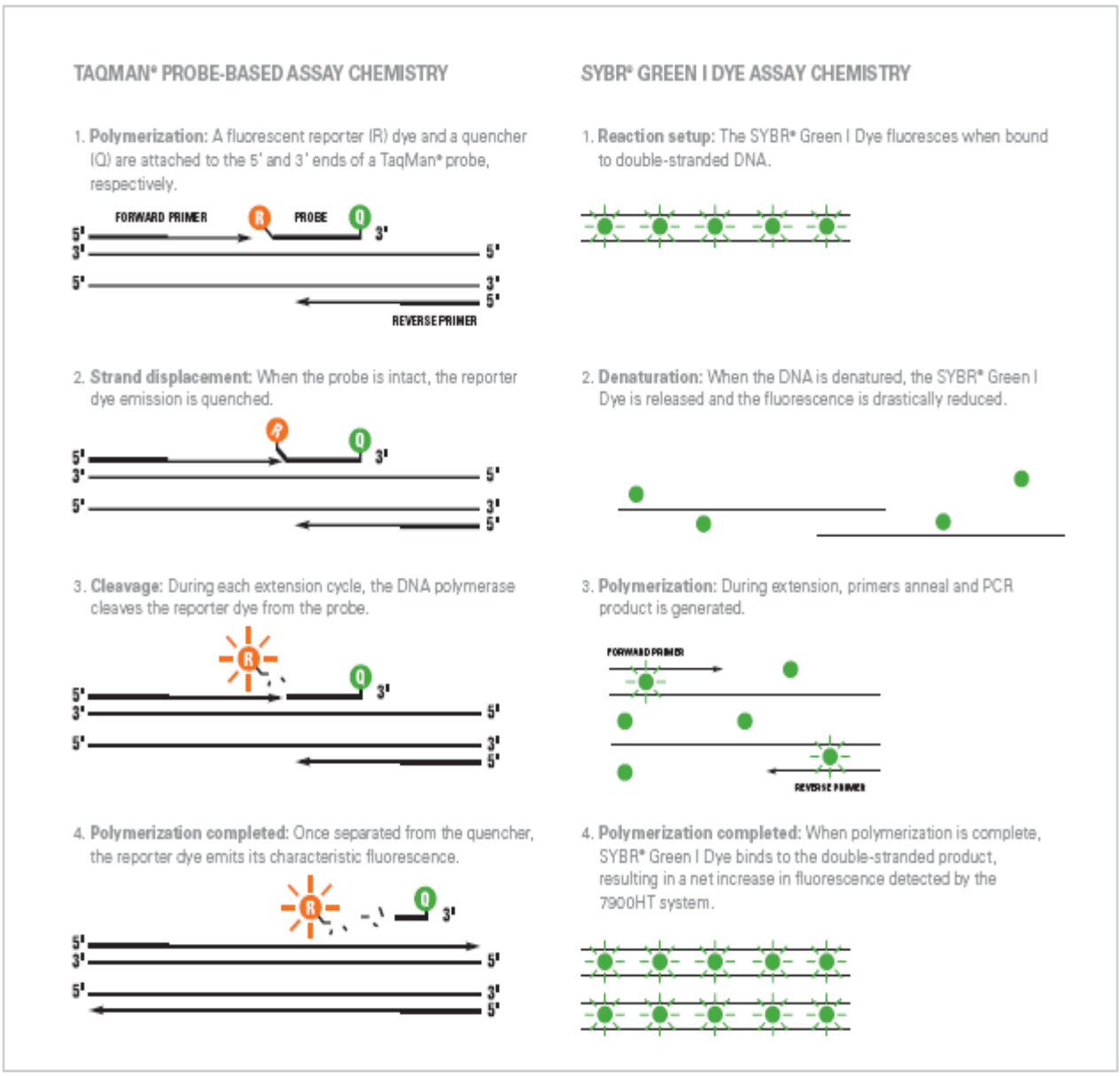

Figure 8. RT-PCR based on TaqMan® fluorescent hydrolysis probes or the intercalating dye SyberGreen® technology

http://www.lifetechnologies.com/at/en/home/life-science/pcr/real-time-pcr/qpcr-education/taqman-assays-vs-sybr-green-dye-for-qpcr.html

Molecular beacons and scorpion primers

Other probe-based strategies have been developed recently like molecular beacons or scorpion primers (illustrated in Figure 9). Molecular beacons are hairpin probes forming a secondary structure in inactive unbound state. As all hydrolysis probes have on their opposite ends a quencher and a reporter fluorochrome and upon binding to target sequence the reporter and quencher are separated in proximity and therefore allow the emission of reporter fluorescence and detection in RT-PCR. Scorpion primers are structured similar to molecular beacons, although they additionally have one primer directly attached to stem loop structure separated by a PCR blocker, preventing the synthesis of probe side (Wong and Medrano, 2005). The binding of scorpion primers results in the opening of the stem loop structure and separation of quencher region from reporter, which allows the increase in fluorescence emission in amplification. These secondary structured probes are reported for higher specificity and ability to recognize as one mismatch in the target sequence (Wong and Medrano, 2005). Furthermore, scorpion primers are able to decrease the reaction time as described that binding is a one collision step with target sequence due to bi-probe (primer bound probe), whereas for others a two collision step is necessary (Wong and Medrano, 2005).

A molecular beacon RT-PCR targeting *invA* gene of *Salmonella* spp. was developed for the detection *Salmonella* spp. from surface and potable water. The assay enabled sensitive detection of 1 to 10 genomic equivalents from an enriched background of non-pathogenic *E. coli* (10^8 CFU ml^{-1}). The study demonstrated that molecular beacon RT-PCR was 100 times more sensitive than conventional PCR with detection limit of 10^4 CFU ml^{-1} (Jyoti et al., 2010). RT-PCRs using molecular beacons result in high specificity and sensitivity therefore represent promising future perspectives that should be extensively validated.

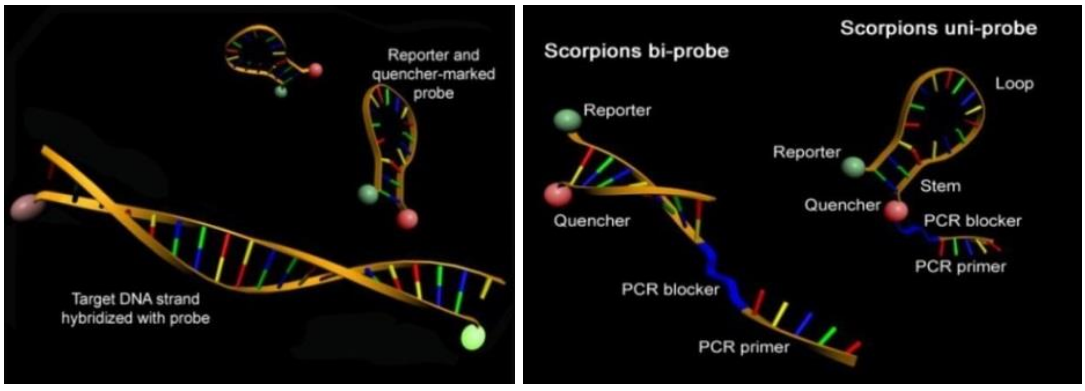

Figure 9. Molecular beacons and scorpion primers for application in RT-PCR
http://upload.wikimedia.org/wikipedia/commons/0/05/Molecular_Beacons.jpg

Limitation of RT-PCR

Numerous RT-PCR methods are currently established for microbial risk assessment in water (Lamendella et al., 2012; Revetta et al., 2010; Layton et al., 2006) and detection of single indicator organisms or pathogens (Ahmed et al., 2012; Varma et al., 2012). However, acceptance requires more extensive validation and establishment by regulatory authorities. Some RT-PCR techniques for the detection of pathogens have already been included into governmental guidelines (Varma et al., 2009, Beneduce, Fiocco and Spano, 2007) and U.S. regulatory authorities consider the application of RT-PCR for fecal bacteria (enterococci and *Bacteroidales*) (Botes, Kwaadsteniet and Cloete, 2013).

Actual implementation is often hampered on the one hand that extensive validation is missing, but on the other hand by inadequate correlation of RT-PCR to standard methods. Only few studies compared RT-PCR to conventional cultivation based detection. Lavender and Kinzelman (2009) demonstrated overestimations of *E. coli* and enterococci with RT-PCR from municipal water samples, which was further confirmed by Colford et al (2007) that 12 fold higher amounts of enterococci were quantified by RT-PCR than by membrane filtration or enzymatic reference test. Differences of 1-5 $\log_{10}$ units were determined for detection of *E. coli*, *Salmonella* spp. and *Clostridium perfringens* with RT-PCR compared to cultivation techniques (Wery et al., 2008). Also the detection of *E. coli* and enterococci from wastewater resulted in significant differences and authors stated that RT-PCR quantified VBNC bacteria and thereby results varied (Sivaganesan, Varma and Haugland, 2008). However, higher quantification is accounted to a more considerable part to the inability to discriminate DNA targets from living and dead organisms (van Frankenhuyzen et al., 2011; Nocker, Cheung and Camper, 2006; 2007; Rudi et al., 2005).

Since viability is a major issue in drinking water quality assessment, viable cell detection has to be facilitated with RT-PCR, as only viable cells are likely to pose a health risk and are infectious (Nocker and Camper, 2008). Furthermore given the conventional methods for water quality assessment are cultivation-based and detect only viable (i.e. culturable) bacteria and therefore it is also of uttermost importance to selectively detect of viable cells in molecular assays. In fact, that they have to be validated against these reference methods. Despite the RT-PCR is a novel tool for monitoring of quality assessment, it is not widely implemented in policy frameworks in Europe or other countries, because of the problem for live/dead discrimination and viability assessment.

6. DNA-intercalating dyes-based viability assessment combined with RT-PCR

RT-PCR holds a range of advantages, but the main disadvantage remains in the detection of DNA targets originated from dead or membrane comprised cells, whereas cultivation based techniques are selective for the growth of viable organisms only (Medema et al., 2003). Furthermore, as dead cells are not uncommon in water habitats, the viability assessment has to be facilitated. Studies from Kalish and co-workers (2011) revealed by live/dead staining that approximately 21% of cells in drinking water may have damaged membranes, which would cause erroneous false positive detections with PCR analysis. Furthermore extracellular DNA in environment can persist for prolonged periods of days up to 3 weeks, due to DNA's negative charges binding to cations and therefore being protected from nucleases (van Frankenhuyzen et al., 2011; Nocker, Cheung and Camper, 2006; England et al., 2004; Keer and Birch, 2003; Köster et al., 2003; Schmittgen et al., 2000).

One possibility to approximate viability detection is to target RNA rather than DNA, because messenger RNA (mRNA) is reported to have shorter half-life (0.5-2 min) and have been described to be rapidly degraded by RNAse after cell death (Malorny et al., 2003). In fact, mRNA persistence after death is strongly dependent on RNA molecule targeted as exemplified that rRNAs may be present even after loss of viability (Yáñez et al., 2011; Girones et al., 2010). The application of reverse transcription RT-PCR for rapid and viable testing of *E. coli* and *Enterococcus* spp. were reported by Bergeron et al. (2011). Hence, difficulties in sample preparation make reverse transcription RT-PCR not suitable for quality assessment purposes (Yáñez et al., 2011; Köster et al., 2003).

Therefore, more promising approach for viability assessment with RT-PCR detection is related to the use of DNA-intercalating dyes, such as ethidium monoazide (EMA) and propidium monoazide (PMA). The application of these dyes in combination with RT-PCR methods was demonstrated for several bacteria, protozoa and viruses (Alonso, Amoros and Guy et al., 2014; Parshioniker et al., 2008; Nocker and Camper, 2007; Nocker, Cheung and Camper, 2006).

The DNA-intercalating dyes are able to inhibit DNA from dead cells in the amplification, thereby enabling viable cell detection. The membrane integrity provides the discrimination potential for these dyes. Viable cells have intact membranes, which act as barriers and render the entry of the dye, whereas dead cell lose membrane integrity and dye can penetrate through comprised damaged membranes (Nocker and Camper, 2008). Treatment of samples prior to DNA extraction facilitates the discrimination (Figure 10). Theoretically, the dye penetrates through damaged or comprised cell membranes of dead cells and preferentially binds to double stranded DNA with high affinity of one dye molecule per 4-5 bases (van Frankenhuyzen et al., 2011; Waring, 1965; www.biotium.com). Upon a photo-activation, i.e. exposure to visible light, a photo-reactive azide group is converted to highly reactive nitrene radical, which readily reacts with carbon moiety of double stranded DNA to form

stable covalent nitrogen-carbon bonds (www.biotium.at). This causes irreversible modification of DNA from dead cells. This modification inhibits amplification in PCR, whereas viable cell DNA renders unmodified and can be amplified (Nocker, Cheung and Camper, 2006). Unbound excess compound gets readily inactivated by water molecules forming unreactive hydroxylamine (Fittipaldi, Nocker and Codony, 2012).

For the application of intercalating dyes with RT-PCR different dye concentrations and light incubation times have to be tested in order to result in most efficient exclusion of dead cells from analysis. Generally light exposure times of 2-5 minutes are commonly established and concentration range of 6-200 µM for EMA and PMA are reported (Nocker and Camper, 2008; Nocker, Cheung and Camper, 2006; Rudi et al., 2005). Different studies revealed different efficiencies dependent on the dye concentration and concentration ratio of dead cells to viable cells. Therefore it is necessary to approve the best conditions for selected assay and purposes.

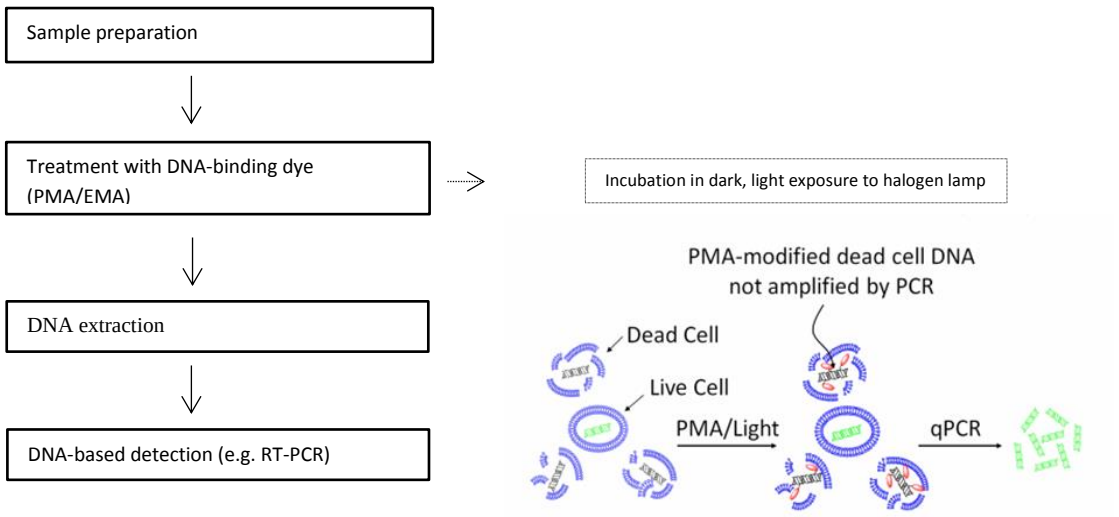

Figure 10. Viability detection with nucleic-acid based RT-PCR in combination with pre-treatment of samples with monoazide dye
Flow chart adapted from van Frankenhuyzen et al., 2011; www.biotium.com

Ethidium monoazide (EMA)

First concepts of the use of DNA intercalating dyes in combination with RT-PCR were shown for EMA (Amino-8-azido-5-ethyl-6-phenylphenanthridinium bromide, Biotium U.S.) (Figure 11).

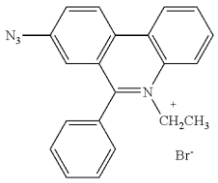

Figure 11. Chemical structure of EMA

www.biotium.com

The covalent crosslinkage of EMA to DNA from dead cells resulted in strong inhibition of PCR amplification (Nocker, Cheung and Camper, 2006; Wang and Levin, 2006). The first application of EMA was approved for viable detection combined with RT-PCR for *C. jejuni* with 4 logs reduction of heat-killed cells (Rudi et al., 2015). Wang and Levin (2006) demonstrated almost complete RT-PCR signal inhibition in RT-PCR of 1x 10^7 heat killed *V. vulnificus* with incubation of 2.5 µg/ml EMA followed by 5 min photo-activation. More recent studies reported that 50µM EMA are optimal to allow exclusion of DNA from dead cells, demonstrated by inhibition of 5 $\log_{10}$ heat inactivated *Legionella* cells (Chang et al., 2010). Beyond the exclusion of dead cell DNA with EMA, it has been repeatedly reported that EMA with higher dye concentration shows cytotoxicity on viable cells and penetration to intact cells (Yáñez et al., 2011; Chang et al., 2010; Nocker, Cheung and Camper, 2006). For example Nocker and coworkers (2006) revealed that EMA resulted in loss of 60% of viable cells.

Therefore, as an alternative, similar dye propidium monoazide (PMA) was extensively studied.

Propidium monoazide (PMA)

PMA (phenanthridium, 3-amino-8-azido-5-[3-(diethylmethylamino)propyl]-6-phenyl dichloride, Biotium U.S.) (Figure 12) has been chemically modified from propidium iodide (PI) with addition of an azide group to the phenanthridine ring to allow for same characteristics as EMA molecule to be crosslinked to DNA through a photo-activation step (Nocker, Cheung and Camper, 2006).

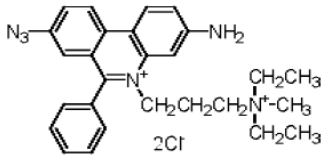

Figure 12. Chemical structure of PMA

www.biotium.com

In analogy to fluorescent stain of PI the PMA molecule diffuses only to permeabilized cells (Nocker, Cheung and Camper, 2006). The higher selectivity is assumed in chemical structure of PMA containing two positive charges and higher molecular weight (MW = 512.5), whereas EMA contains only one and a molecular weight of 420.3 (Nocker, Cheung and Camper, 2006). Detailed chemical mechanism of PMA is presented in Figure 13. The amino group gets activated through visible light to form a reactive nitrene intermediate, which readily reacts with carbon moiety from DNA, thereby covalently crosslinking to DNA.

Comparison of EMA and PMA capacities demonstrated that higher PMA concentrations are necessary to result in same reduction of dead cells. A four-fold higher concentration of PMA (200 µM) has to be applied for inhibition of 5 $\log_{10}$ of heat killed *Legionella* cells in comparison to only 50 µM EMA (Chang et al., 2010). However, the EMA concentration of 50µM showed 1 log reduction also on viable cells, whereas PMA resulted in no negative effect. Nocker and colleagues (2006) studied different light exposure times of 1 min to 15 min and different PMA dye concentrations (3 µM, 30µM and 240µM PMA) on the inhibition of PCR amplification on heat killed EHEC O157:H7 and *Streptrococcus sobrinus* cells. They revealed best conditions to induce PCR inhibition of dead cells with a concentration of 50 µM PMA and 5 min photo-activation. Similar findings were presented by Yáñez et al. (2011) that 50 µM PMA could almost reduce 4 $\log_{10}$ heat killed cells from an initial concentration of 5.3 x 10^4 CFU/ml *Legionella* cells. Slimani et al. (2011) could demonstrate the successful exclusion of dead *Legionella* cells (3 log reduction) with lower dye concentration of 6.25 µM.

Therefore, it is concluded that PMA is a better viability marker than EMA. PMA in combination with RT-PCR provides a valuable viability detection tool but several factors have to be considered such as dye concentration, sample matrix and even species difference to enhance the affectivity and capacity of the assay.

Other applications of PMA in combination with molecular assays were shown for microbial community studies by DGGE and 454 sequencing. But with these methods PMA did not seem to be suitable for revealing viable populations as only slight differences in PMA and non-PMA treated community were observed. This was explained by dense community in DGGE analysis reaching PMA's capacity or short read lengths of NGS not catching PMA modification in sequence (Nocker et al., 2010; Nocker and Camper, 2007). Furthermore PMA-PCR based diagnostics microarray was developed by Nocker and coworkers (2009) for detection of viable *P. aeruginosa*, *Listeria monocytogenes*, *Salmonella typhimurium*, *Serratia marcescens* and *E. coli* O157:H7. This microarray was only evaluated to date for pure cultures (viable and heat killed ones) but not for real samples.

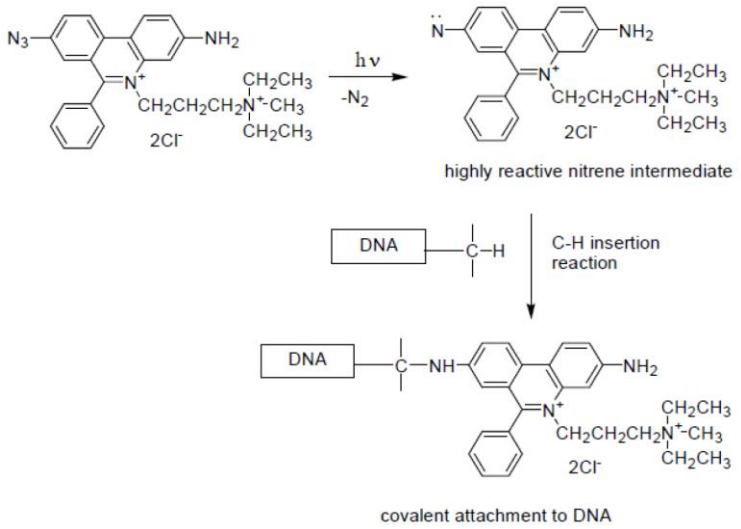

Figure 13. Chemical mechanism of PMA to covalent crosslinkage to DNA upon photo-activation

www.biotium.com

7. Standardization of new detection methodologies

For the acceptance of newly developed technologies in the diagnostic or quality assessment sector, a validation to defined ISO norms or established reference tests is essential, to provide evidence that method is capable for intended purposes (Köster et al., 2003).

However, it has to be noted that ISO defined techniques for water quality assessment were established long times ago and have never been validated themselves, although shortcoming are commonly known.

Several criteria were determined by Malorny and authors (2003) for standardization of diagnostic PCR. Following factors have to be provided: i) analytical accuracy and selectivity for detection of

targets from non-target organisms by performing inclusivity and exclusivity testing including closely related targets, ii) detection specificity for excluding non-target species linked to false positives, iii) sensitivity which utilizes detection of target organisms present in a sample not resulting in false negatives, iv) detection limit of the method should be in the range of 10-100 copies for bacterial DNA, v) robustness of the method, to achieve reproducible data concerning interfering substances, pipetting error, batches of reagents and equipment (Figure 14).

All these factors have to be considered in an evaluation procedure of new analytical diagnostic technologies such as illustrated for PCR technologies, because these is the main challenge for their potential application and implementation as alternative test systems in accordance to conventional defined ISO-norms.

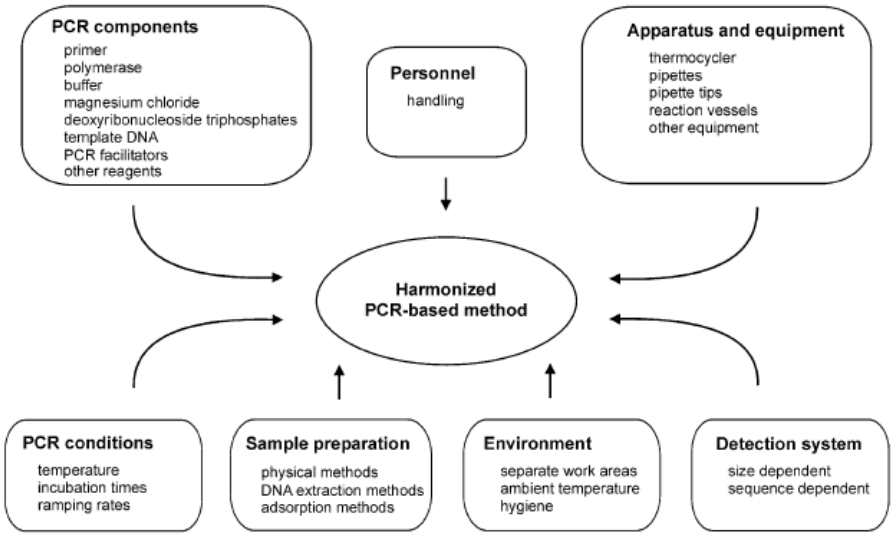

Figure 14. Standardization of diagnostic PCR

Malorny et al., 2003

8. Next era of detection and identification by sequencing strategies

Sequencing is the only tool for detection and identification of uncharacterized organisms (Aw Gim and Rose, 2011). In future with the raise of emerging diseases and upcoming resistance of organisms and evolving of new pathogens, next generation sequencing (NGS) technologies will gain in their importance.

Sanger sequencing

The first advent of sequencing was in the mid-1970s with the development of the dideoxy method for sequencing of DNA, based on *in vitro* synthesis coupled with chain termination by radioactive labeled dideoxyribonucleoside triphosphates (Alberts et al., 2002). Sanger sequencing (1990) progressed the sequencing by using four different fluorescent labeled dNTPs and automated detection through capillary electrophoresis, which industrialized sequencing as cheap and high-throughput method (Shendure and Ji, 2008). To date Sanger sequencing is most commonly applied technology as it is beneficial for higher read length of 1000bp and basically highest achievable accuracy 99.9% (Pareek, Smoczynski and Tretyn, 2011; Nowrousian, 2010; Shendure and Ji, 2008). However, Sanger sequencing gets readily outcompeted by the fast evolving field of NGS strategies that allow sequencing of multiple samples in parallel and generation of billions of base reads in a single run, thus improving the probability of detection of rare organisms due to increased depth coverage (Zinger, Gobet and Pommiers, 2012; Raffan and Semple, 2011). Sanger sequencing compared to NGS is limited to analysis of 96 to 386 samples per run and in the fact that preparation necessities the cloning procedure which is lab intensive and may be strongly biased (Zinger, Gobet and Pommiers, 2012; Nowrousioan, 2010).

Next generation sequencing (NGS)

Next generation sequencing, as defined by Zinger, Gobet and Pommiers (2012), is the era of the access to the *rare biosphere* and new genomes to be characterized (Pareek, Smoczynski and Tretyn, 2011). Several different NGS platforms were developed and are still under refinement. Most common NGS technologies are 454 sequencing (Roche); Illumina approach and Ion Torrent™. Benefits of NGS rely in millions of reads in parallel in as single reaction, no preparation of clone library and miniaturized platforms allow for high-throughput (Aw Gim and Rose, 2013).

At the moment most studies represent data from pyrosequencing (454) technology as it was the former NGS method. The technology is based on a bioluminescence reaction that develops upon the DNA synthesis and dNTP incooperation when a pyrophosphate is released. Briefly, this released

pyrophosphate coverts ATP, used for activating the enzyme luciferase to convert oxiluciferin, which transfers the light signal. The methodical practice is based on an emulsion PCR with beads in a synthetic oil water mixture, which facilitates that at best one DNA molecule is bound to one bead, where sequencing reaction can take place. Benefits of 454 technology is the short run time of 23 h and the fact that large number of parallel sequencing reaction can be conducted.
Some studies already demonstrated by pyrosequencing the identification of complex microbial communities from drinking water or water treatment plants. Kwon (2011) demonstrated by 16S rRNA pyrosequencing in drinking water treatment plant the increased number of detected microorganisms with about 1700 observed OTUs, compared to less than 100 OTUs derived with common cloning and Sanger sequencing (Eichler, 2006). Similar findings were obtained from pyrosequencing of freshwater habitat, including detection of rare pathogens (Liu et al., 2013).

In 2011 released sequencing platforms include the Ion Torrent and Illumina Miseq, which were intended as sequencing equipment for routine testing and diagnostic purposes in clinical area (Quail et al., 2012).
The Ion Torrent technology is a technology based on the direct translation of chemical information through the release of a H^+ upon dNTPs incooperation, detected by a semiconductor chip (www.life-technology.com). The charge causes a shift in the pH directly measured by an ion sensor. Advantages were described by the less expensive equipment but the limitation is lower throughput (~1Gb per run), which might be the cause that it is not readily used (Quail et al., 2012).
Illumina approach is most promising NGS candidate, able to come up with millions of new sequence data the next years. Both amplicon sequencing and whole genome sequencing are proposed by this method. It is based on sequencing by synthesis using reversible terminators on a solid surface of a flow cell. The basis is a solid phase bridge amplification, where single stranded DNA is attached to the surface via an adapter. The solid phase bride amplification is facilitated on the flow cells with a dense layer of primers, which forms bridges during synthesis with attached single stranded DNA. This form of amplification creates up to 1000 identical copies of each single template in close proximity on the flow cell which results in gigabases data from a single run from both ends. Illumina sequencing allows therefore for greater coverage and has the advance of multiplexing by barcoding and pooling up to 96 samples with read length of 200bp from paired ends (Zinger, Gobet and Pommiers, 2012; Nowrousion, 2010; Shendure and Ji, 2008). Disadvantage relies in higher costs and long sequencing run time of 3-5 days.

NGS represents a perspective tool, but improvements for widespread application are still needed as they are all limited in their short reads (150-450 bp) and lower quality of sequences linked to overall higher cost, although the run per base is lower than in Sanger sequencing (Quail et al., 2012, Zinger,

Gobet and Pommiers, 2012). Whole genome approaches with NGS are already established for smaller genomes like microbes (3-5 Mb), but are still complicated for *de novo* assembly of large genomes (Hall, 2007). In addition, improvements of software and bioinformatics pipelines are needed for evaluation of NGS data (Nowrousian, 2010; Hall, 2007). Therefore it is expected that third generation would be optimized in read length, quality, rapidness and lower costs and user friendly bioinformatics tools (Zinger, Gobet and Pommiers, 2012; Pareek, Smoczynski and Tretyn, 2011, Nowrousian, 2010).

Next generation sequencing technologies will offer novel tools for identification of microorganisms in risk assessment and epidemiology studies in outbreak cases. Moreover, deeper insights in terms of comparative genomics, transcriptomics (RNAseq) and metagenomics will be a future upcoming field and for better understanding microbial complexity in water.

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Outline of the thesis

Conventional drinking water analysis, as defined in the Austrian Drinking Water Directive (Austrian DWD; BGBl. II Nr. 304/2001), relies on the monitoring of microbiological parameters based on ISO defined cultivation techniques. Analyzed microbial parameters include the determination of *E. coli*, *Enterococcus* spp. and *P. aeruginosa* and indicators such as coliforms and heterotrophic plate count (HPC). Generally, mandatory methods are based on the cultivation of microorganisms and confirmation by biochemical test, accordingly they are very time consuming. Some analysis may even require up to 7 days, e.g. the confirmation of *P. aeruginosa*. Another, recently recognized, disadvantage of cultivation-based methods is their inability to detect viable but non-culturable cells (VBNC). In the past few years extensive discussions regarding the limitations of conventional and potential of novel nucleic acid-based methods evolved and tremendous developments of new technologies for the quality assessment were reported.
Molecular assays represent promising alternative method for the assessment of microbial parameters in scope of water quality assessment, allowing for the high-throughput analysis. DNA-based techniques such as real time PCR (RT-PCR or qPCR) enable a rapid and specific detection of target organism. However, their application is hampered by the inability to discriminate between living and dead bacterial cells.

Therefore, the objective of the thesis was focused on the establishment of molecular assays for more rapid, specific and parallel analysis and furthermore viability assessment of microbial water quality parameters. Real time PCRs (RT-PCR) combined with the propidium monoazide (PMA) treatment were established for all defined parameters according to Austrian DWD (2001). The PMA treatment was included in the RT-PCR protocol in order to facilitate the live/dead differentiation and to allow comparison to conventional tests.

The proof of principle study addressed the integration of PMA treatment into established RT-PCR detection assays (Chapter 1). Therefore artificially prepared samples including viable and heat killed bacterial cells were analysed and furthermore, for approximating natural samples, experiments were performed in natural background water microflora.

After the successful proof of principle, the application potential of established PMA-RT-PCR assays was evaluated on an extended set of drinking water and process water (drinking water treatment, cooling towers) samples. Performance characteristic of the PMA-RT-PCR and RT-PCR were evaluated to conventional microbiological reference methods for all in Austrian DWD (2001) defined microbial parameters (Chapter 2).

Furthermore heterotrophic plate count (HPC) quality parameter was assessed in detail. HPC for enumeration of total bacteria in water samples is routinely applied. However, different protocols are available to date and accordingly no information of recovered composition of heterotrophic bacteria is available. Therefore in Chapter 3, the HPC method was approved for their microbial cultivable community composition to investigate the effect of different proposed cultivation conditions (low (R2A) and high (yeast extract agar) nutrient media and incubation temperatures of 22°C and 37°C) by 16S rRNA gene cloning and sequencing.

Establishment and integration of PMA treatment in RT-PCR

Proof of principle of PMA-RT-PCR

(Chapter 1)

Propidium monoazide qPCR for viable E. coli and P. aeruginosa detection from abundant background microflora

↓

PMA-RT-PCR evaluation on drinking and process water samples

(Chapter 2)

Evaluation of molecular assessment of microbial water quality parameters by real time PCR with PMA treatment

↓

Approvement of HPC method and different cultivation condition (YEA/R2A media at 22°C and 37°C) by community analysis of 16 rRNA cloning and gene sequencing

(Chapter 3)

Effect of different heterotrophic plate count methods on the composition of culturable microbial

Chapter 1

Propidium monoazide qPCR for viable *E. coli* and *P. aeruginosa* detection from abundant background microflora

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Introduction

Conventional water quality assessment parameters and corresponding detection methods are defined in the EU in the Council Directive 98/83/EC on the quality of water intended for human consumption [1]. Similar practices are also used in other countries, e.g. in the U.S. [2] and Australia [3]. Generally, mandatory methods are based on the cultivation of microorganisms and are accordingly very time consuming. Some analyses may even require up to 7 days, e.g. the confirmation of *P. aeruginosa* [4]. Another, recently recognized, disadvantage of cultivation-based methods is their inability to detect viable but non-culturable cells (VBNC) [5, 6].

In the past few years extensive discussions regarding the limitations of conventional and potential of novel nucleic acid-based methods evolved [7-10]. Even though nucleic acid-based methods such as PCR exhibit a whole range of advantages like speed, specificity, multiplexing and automation potential, one major disadvantage remains, which is the inability to differentiate between living and dead cells [11]. The inclusion of the live/dead differentiation into nucleic acid-based assays is an absolute prerequisite for application of these methods for microbial water quality assessment.

DNA intercalating dyes, e.g. propidium monoazide (PMA), may be used for live/dead differentiation. Briefly, PMA is cell membrane-impermeable and can therefore be used to selectively modify (i.e. bind to) free DNA and DNA from cells with compromised membrane integrity. Upon photo-activation, the PMA molecule forms stable covalent nitrogen-carbons bond with a DNA molecule, resulting in irreversible DNA modification. This modification inhibits PCR amplification of DNA from dead cells, allowing selective PCR amplification of unmodified DNA from viable cells [12, 13]. Performance of quantitative PCR including PMA treatment (PMA-qPCR) has been studied using pure cultures of single waterborne organisms [12, 14-19]. However, a major challenge is a complex microbial community usually occurring in natural samples. For real-life applications it is essential to demonstrate that reliable detection of few relevant organisms in a complex microbial background can be achieved. Therefore, our aim was to establish and test a PMA-qPCR assay, which allows the detection of viable water quality indicators (*E. coli* and *P. aeruginosa*) in a complex microbial background.

Material & Methods

Overnight cultures of *E. coli* (DSM 30083) and *P. aeruginosa* (DSM 50071) were grown at 37°C in liquid Luria Bertani medium (Sigma-Aldrich, Germany) and Brain Heart Infusion broth (Sigma-Aldrich, Germany), respectively. A ten-fold dilution series was prepared for each organism and cultivable cell numbers were estimated by plating on corresponding agar plates. Heat killed cells were generated by heat-inactivation at 75°C for 10 min and loss of viability was also confirmed by plate counting. For the detection and quantification of *E. coli* and *P. aeruginosa* qPCR assays based on the *uidA* [20] and the *regA* gene [21] were used. Quantitative PCR was performed using SsoFast™ Probes Supermix (Bio-Rad, Austria), 0.25 µM probe and 0.5 µM primers, and 2 µl total gDNA as a template. Cycling conditions were as follows: initial denaturation for 2 min at 95°C, followed by 40 cycles with 5 sec at 95°C and 30 sec at 62°C.

The background microflora was prepared from a well water sample that was filtered through a 0.45 µm filter (Millipore, Germany) and subsequently incubated on yeast extract agar (Sigma-Aldrich, Germany), according to the heterotrophic plate count method [22]. The diversity of obtained bacterial community was determined by 16S rRNA gene sequence analysis. Briefly, primers 8f (5'-AGAGTTTGATCCTGGCTGAG-3', and 1520r (5'-AAGGAGGTGATCCAGCCGCA-3') were used for the amplification of the 16S rRNA gene [23, 24]. Amplicons were cloned using StrataClone cloning kit (Agilent, Germany) and sent to Agowa (Germany) for sequencing with T3 (5'-AATTAACCCTCACTAAAGGG-3') and T7 (5'-TAATACGACTCACTATAGG-3') primers. Absence of *P. aeruginosa* and *E. coli* from the sample was tested by cultivation [4] or an enzymatic-based assay (Colilert®18, IDEXX Laboratories, Germany) and qPCR.

Result & Discussion

Efficiency of PMA (Biotium Inc., USA) treatment was tested using artificial bacterial mixtures (Table 1). Each mixture consisted of a viable background microflora in excess (2 - 3 $\log_{10}$ more than the target organism) and either viable, heat-killed or both cultures of targeted organism(s) respectively. Two different PMA concentrations, 10 µM and 50 µM, previously reported as optimal concentrations for live/dead differentiation [18, 25], were tested. Briefly, samples were incubated with PMA in the dark for 5 min on ice, followed by photo-activation with a 500 W halogen light for 5 min on ice. Subsequently, DNA was isolated with WaterMaster™ DNA Purification Kit (Epicentre®, U.S.A) according to the manufacturer's instructions and eluted in 100 µl sterile water. Finally, 2 µl were used as a template in qPCR assay.

Table 1. Sample composition of artificial mixtures containing listed CFU amounts (as determined by plate count method) and PMA induced signal/cell number reduction from conventional qPCR (w/o PMA) compared to PMA-qPCR (10 µM) in A) high multiple indicator spike and B) low multiple indicator spike. Standard deviations are calculated from three independent replicates

A) high indicator spike – *P. aeruginosa* and *E. coli* in background microflora							
	Sample composition [a]	**viable**		**mix**		**dead**	
Spike CFU	viable cells (*P. aeruginosa*)	7,0E+05		7,0E+05		x	
	heat killed (*P. aeruginosa*)	x		7,0E+05		7,0E+05	
	viable cells (*E. coli*)	8,0E+05		8,0E+05		x	
	heat killed cells (*E. coli*)	x		8,0E+05		8,0E+05	
	background microflora	1,0E+08		1,0E+08		1,0E+08	
	***P. aeruginosa* qPCR**	**Cq±SD**	**cells**	**Cq± SD**	**cells**	**Cq± SD**	**cells**
PMA [µM]	0	24,74±0,017	3,9E+03	25,49±0,030	2,2E+03	30,37±0,116	5,5E+01
	10	27,20±0,055	6,1E+02	26,99±0,093	7,2E+02	<LOD [b]	<LOD [b]
	***E. coli* qPCR**	**Cq±SD**	**cells**	**Cq±SD**	**cells**	**Cq±SD**	**cells**
PMA [µM]	0	24,66±0,224	1,5E+04	25,45±0,057	8,6E+03	29,33±0,164	5,3E+02
	10	26,34±0,131	4,5E+03	25,93±0,178	6,1E+03	31,19±0,170	1,4E+02
B) low indicator spike – *P. aeruginosa* and *E. coli* in background microflora							
	Sample composition [a]	**viable**		**mix**		**dead**	
Spike CFU	viable cells (*P. aeruginosa*)	5,4E+03		5,4E+03		x	
	heat killed (*P. aeruginosa*)	x		5,4E+03		5,4E+03	
	viable cells (*E. coli*)	1,6E+03		1,6E+03		x	
	heat killed cells (*E. coli*)	x		1,6E+03		1,6E+03	
	background microflora	5,0E+05		5,0E+05		5,0E+05	
	***P. aeruginosa* qPCR**	**Cq±SD**	**cells**	**Cq± SD**	**cells**	**Cq± SD**	**cells**
PMA [µM]	0	26,65±0,279	3,5E+02	26,03±0,131	5,4E+02	27,80±0,327	1,6E+02
	10	30,05±0,225	3,4E+01	30,40±0,104	2,7E+01	<LOD [b]	<LOD [b]
	***E. coli* qPCR**	**Cq±SD**	**cells**	**Cq±SD**	**cells**	**Cq±SD**	**cells**
PMA [µM]	0	28,90±0,265	1,2E+02	28,27±0,138	1,8E+02	30,04±0,263	6,6E+01
	10	30,80±0,131	3,4E+01	31,98±0,178	1,6E+01	<LOD [b]	<LOD [b]

[a] 50x lower template amounts were used for qPCR analysis (under assumption of 100% DNA extraction efficiency)

[b] Limit of detection (LOD) of qPCR analysis (10 cells) is considered.

In order to reflect, as close as possible, the naturally occurring conditions, a background microflora sample was prepared from well water. Community composition of the sample was analyzed by 16S rRNA sequence analysis and the data revealed dominance of two bacterial phyla, i.e. *Proteobacteria* (89%) and *Firmicutes* (11%). The highest diversity was seen in the family of *Enterobacteriaceae* with *Citrobacter* spp. (53%) as the most abundant bacterial genus. Additional genera including *Serratia* spp. (3%), *Klebsiella* spp. (3%), *Cedecea* spp. (2%), *Hafnia* spp. (2%) were detected. Also *Bacillus* spp. (10%), *Aeromonas* spp. (3%) and *Vogesella* spp. (2%) were found. For some sequences (3%) unambiguous resolution down to the genus level was not achieved, however, these were identified as members of the *Enterobacteriaceae*, *Moraxelleceae* and *Bacillaceae* family. These findings clearly demonstrate the complexity and relevance (presence of both Gram-positive and Gram-negative microorganisms) of the used background microflora.

The absence of *P. aeruginosa* and *E. coli* in the background microflora sample was confirmed by qPCR and through conventional assays.

In the preliminary study (data not shown), optimal concentration of PMA for intended application was determined. For this purpose single indicator spike (*P. aeruginosa* (1.5×10^5 viable, heat killed or both cells) in the background microflora (10^8 viable cells)) was used. Complete reduction of false positive signal (approx. 3 $\log_{10}$ units) obtained from the heat killed cells was achieved. Better performance was observed with 10 µM than 50 µM PMA concentration, because higher concentration showed stronger cytotoxic effects on viable cells as reported previously [18]. Therefore, following experiments were performed with 10 µM PMA.

In case of the high multiple indicator spike (Table 1A and Figure 1A) PMA treatment resulted in complete suppression of false positive signals arising from heat killed *P. aeruginosa* cells. For *E. coli* only partial inhibition of qPCR signal from heat killed cells could be achieved. There are two possible explanations for this. Firstly, as it was shown before, higher levels of dead cells in the mixture seem to have a negative effect on PMA performance [18, 25, 26]. In fact, the reported limit of PMA differentiation capacity lies about $10^4 - 10^5$ dead cells and is close to the amounts used in this experiment. However, this spike was intentionally set to this elevated level in order to compensate for other potential biases such as insufficient sample preparation [27, 28]. Another explanation could be previously observed species- and sequence-dependent differences in the efficiency of PMA binding and treatment efficiency [29].

In order to further demonstrate the usefulness of the proposed approach, low multiple indicator spike was prepared and analyzed. In this case the concentration of background microflora was somewhat lower (5×10^5) and representative for naturally occurring raw waters [30]. Targeted bacteria were also spiked at significantly lower levels (1.6×10^3 and 5.4×10^3), and consequently, also in this case, high abundance of background microflora was ensured (Table 1B). As expected, at this lower cell concentrations even better PMA performance was observed. Accordingly, complete signal reduction of false positive signals from heat killed cells was achieved for both *P. aeruginosa* and *E.coli* (Table 1B and Figure 1B). The observed negative effects were also somewhat greater, however, no false negative results were observed. At this point it has to be emphasized that the water quality assessment does not require quantitative determination of the indicator organisms. Actually, regulatory norms define zero tolerance [1-3]. Therefore, it is only important to ensure the correct detection of presence or absence. Presented data clearly demonstrate that conventional qPCR (w/o PMA) would always result in false positive detection of heat killed bacteria. With implementation of PMA (10 µM), this could be alleviated in 4 out of 5 cases. Only high spike of heat killed *E. coli* would be diagnosed as falsely positive with proposed method and this sample is, as discussed above, not really

representative for intended application. On the other hand, even in case of low spike, no false negatives were observed.

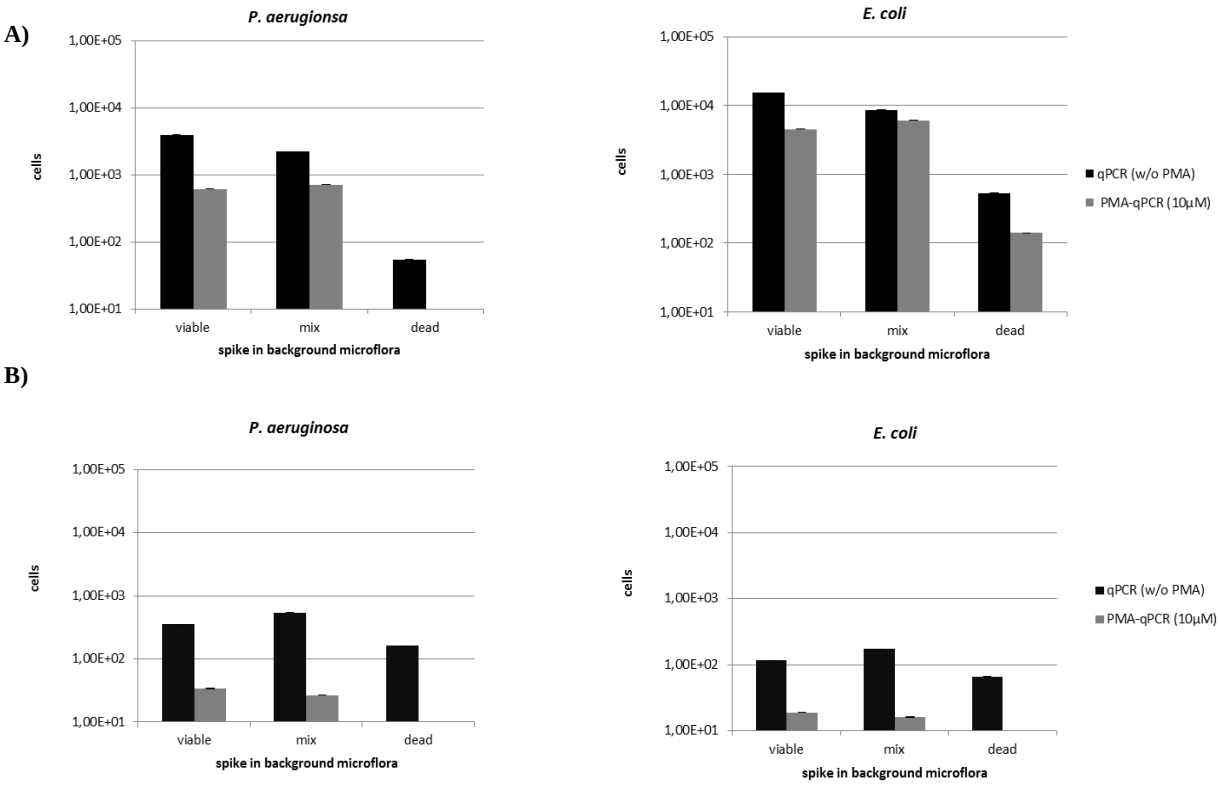

Figure 1. Impact of PMA-qPCR (10 µM) compared to conventional qPCR (w/o PMA) in a (A) high multiple indicator spike and (B) low multiple indicator spike of heat killed and viable *P. aeruginosa* and *E. coli* in a viable background microflora. Background microflora sample was *P. aeruginosa* and *E. coli* negative (data not shown). Error bars in diagrams represent standard deviations from three independent replicates. Limit of detection (LOD) of qPCR analysis (10 cells) is considered

Conclusion

In conclusion, the described PMA-qPCR approach resulted in the significant suppression of false positive signals arising from the amplification of DNA from the dead cells in conventional qPCR. It has to be emphasized, that these results were achieved in the presence of the abundant (2 - 3 $\log_{10}$ higher concentration than targeted species) and a complex background microflora. Therefore, we conclude, that given the careful optimization and validation, PMA-qPCR can be a valuable tool for monitoring microbial water quality parameters.

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Chapter 2

Evaluation of molecular assessment of microbial water quality parameters by real-time PCR with PMA treatment

This chapter has been submitted to:

Water Research

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Introduction

In scope of routine cultivation-based analysis of water, total bacterial enumeration is determined by heterotrophic plate count (HPC), which requires culturing at 37°C and 22°C for 48 h and 72 h, respectively. Further microbiological parameters (*E. coli*, coliforms, *Enterococcus* spp., *Pseudomonas aeruginosa*) are monitored on selective agar plates followed by biochemical confirmation tests. In general, those culture-based techniques are time consuming and laborious (Agudelo et al., 2006).
In recent years the potential of molecular assays was recognized, enabling more rapid, specific and high-throughput detection of target organisms from a variety of matrices (Aw and Rose, 2012). Developed PCR techniques for the detection of pathogens in water have been already included in some governmental guidelines in the U.S. (Varma et al., 2009). Furthermore, numerous RT-PCR-based methods were proposed for microbial risk assessment in water (Layton et al., 2006; Revetta et al., 2010; Lamendella et al., 2012; Sivaganensan), but to our best knowledge RT-PCR analysis has not been considered yet for the detection of the whole set of microbial parameters defined for water quality assessment.

As standard microbiological methods are based on viable cell detection, some adaptions of RT-PCR are of concern, because nucleic acid-based methods have the innate inability to discriminate between DNA from living and dead bacterial cells. A combination of RT-PCR with propidium monoazide (PMA) treatment was previously investigated in several studies for specifically monitoring of viable target bacteria (Nocker, Sossa and Camper, 2007; Yokomachi and Yaguchi, 2012; van Frankenhuyzen et al., 2013). PMA is a DNA intercalating molecule with the capacity to diffuse into dead or membrane compromised cells thereby irreversibly modifying DNA by forming stable covalent nitrogen-carbon bonds upon photo-activation. Consequently, this modification inhibits PCR amplification of DNA from dead cells, allowing selective PCR amplification of unmodified DNA from viable cells (https://ca.vwr.com/store/catalog/product.jsp?product_id=8286393, Nocker and Camper, 2009). Successful application of PMA-RT-PCR for detection of *E. coli* and *P. aeruginosa* in complex matrices was shown previously in our studies, achieving substantial reduction (3logs) or complete suppression of amplification arising from DNA of dead cells (Gensberger et al., 2013).
Therefore this study focuses on the investigation of the application of molecular assays (RT-PCR and PMA-RT-PCR) to rapidly assess water quality. RT-PCR-based assays were established and optimized for all microbial parameters defined according to the Austrian drinking water directive (DWD, 2001), i.e. *E. coli*, coliforms, *Enterococcus* spp., *P. aeruginosa* and the estimation of total bacteria. Performances parameters (specificity and sensitivity) were comparatively determined to the respective conventional microbiological method using a variety of drinking water and process water samples.

Material & Methods

Primer and probe selection

For all microbiological parameters defined in the Austrian drinking water directive (DWD, 2001) PCR primers were selected from the published literature and extensively tested (with special focus on primer/probe specificity and limit of detection of the assays).

Initially, RT-PCR assays were established based on the intercalating dye technology (EvaGreen® mix, Solis Biodyne, Estonia). Accordingly, PCR primers were selected that were either already used in intercalating dye RT-PCR systems or that fulfilled certain basic criteria, i.e. PCR amplicon length between 100 and 300 bp. As within this study the need for the implementation of more specific detection became evident, the TaqMan® technology (based on the use of hybridization probes) was selected and accordingly a new set of primers tested. The list of selected and used primers for both intercalating dye (EvaGreen®) and TaqMan® chemistry is given in Table 1. The complete list of tested primers can be found in Table S1.

Primer specificity check

The specificity of the selected primers was tested using a set of 26 bacterial target and non-target microbial species with known association to water (S2). The identity of bacteria was confirmed by 16S rRNA gene sequencing. The genomic DNA from overnight cultures grown in 10% TSB (Merck, Austria) at 37°C was isolated with the GenElute™ Bacterial Genomic DNA kit (Sigma Aldrich, Germany). DNA concentration was measured with Nanodrop1000 (Fisher Scientific, Austria) and adjusted to 25 ng μl^{-1}. Two µl (50 ng) were used as a template in the PCR reaction.

Initially primers were tested in conventional PCR (using recombinant *Taq* DNA polymerase, Invitrogen, U.S. and following the conditions given in the original publication) and specificity was assessed via gel electrophoresis. Promising primers were further investigated in the RT-PCR. All RT-PCR experiments were performed in triplicates and included a non-template control (NTC).

Table 1. Oligonucleotide primers and probes established for RT-PCR analysis.

Target name	Gen	Primer and probe name *	sequence [5'-3']	Amplicon size (bp)	Reference
(A) intercalating dye - EvaGreen®					
Total bacteria[1)]	16S rRNA	516-F	CCACCAGCAGCCGCGGTA	391	Yu and Morrison, 2004
		907-R	CCGTCAATTCCTTTGAGTTT		
E. coli & *Shigella* spp.	*uidA*	ECN125-F	GCAAGGTGCACGGGAATATT	75	Takahashi et al., 2009
		ECN1328-R	CAGGTGATCGGACGCGT		
Enterococcus spp.	16S rRNA	g-Encoc-F	ATCAGAGGGGGATAACACTT	337	Matsuda et al., 2009
		g-Encoc-R	ACTCTCATCCTTGTTCTTCTC		
Enterobacteriaceae[2)]	23S rRNA	En-lsu-3-F	TGCCGTAACTTCGGGAGAAGGCA	428	Matsuda et al., 2009
		En-lsu-3-R	TCAAGGACCAGTGTTCAGTGTC		
Pseudomonas aeruginosa	*oprL*	oprL-F	ATG GAA ATG CTG AAA TTC GGC	504	Xu et al., 2004
		oprL-R	CTT CTT CAG CTC GAC GCG ACG		
(B) hybridization probe TaqMan® system					
Total bacteria[1)]	16S rRNA	331-F	TCCTACGGGAGGCAGCAGT	466	Nadkarni et al., 2002
		797-R	GGACTACCAGGGTATCTAATCCTGTT		
		probe	CGTATTACCGCGGCTGCTGGCAC		
E. coli & *Shigella* spp.	*uidA*	ECN1254-F	GCAAGGTGCACGGGAATATT	75	Takahashi et al., 2009
		ECN1328-R	CAGGTGATCGGACGCGT		
		probe	CGCCACTGGCGGAAGCAACG		
Enterococcus spp.	23S rRNA	ECST748-F	AGAAATTCCAAACGAACTTG	92	Haugland et al., 2005
		ENC854-R	CAGTGCTCTACCTCCATCATT		
		probe	GGTTCTCTCCGAAATAGCTTTAGGGCTA		
Pseudomonas aeruginosa	*regA*	Paer-F	CTGCTGGTGGCACAGGA	64	Lee et al., 2008 modified
		Paer-R	GTTGGTGCAGTTCCTCATTG		
		probe	CCAGATGCTTTGCCTCAACGTCGA		

*Same primer and probe selection for PMA-RT-PCR

[1)] Quantification of total bacteria as substitution for heterotrophic plate count

[2)] *Enterobacteriaceae* as alternative assay for coliform test

Standard preparation for RT-PCR

Overnight cultures of *E. coli* DSM 30083, *P. aeruginosa* DSM 50071 and *Enterococcus faecalis* DSM 20478 were grown in liquid Luria Bertani medium (Sigma-Aldrich, Germany) and in Brain Heart Infusion broth (Sigma-Aldrich, Germany), respectively, all at 37°C.

Enterobacteriaceae standard was prepared from the mixture of several species belonging to this family, i.e. *E. coli* DSM 30083, *Enterococcus faecalis* DSM 20478, *Salmonella* spp. AIT-AM13, *Citrobacter* sp. DSM 30041, *Raoultella terrigena* DSM 2687, *Yersinia enterocolitica* DSM 11502, *Enterobacter asburiae* AIT-AM 9, *Shigella fexneri* DSM 4782 and *Citrobacter freundii* CCM 4475. All cultures were grown over night in liquid plate count media (Sigma-Aldrich, Germany) at 37°C and turbidity was measured with a spectrophotometer (Eppendorf, Austria). The standard for total bacteria (analysed by 16S rRNA gene analysis) was prepared from a well water sample that was filtered through a 0.45 µm filter (Millipore, Germany) and subsequently incubated on yeast extract agar at 37°C (Sigma-Aldrich, Germany), according to the heterotrophic plate count method (ISO 6222:1999). For all cultures a 10-fold dilution series was prepared to estimate cultivable cell numbers (CFU/ml) in parallel by plating. The genomic DNA was extracted from cultures with the GenElute™ Bacterial Genomic DNA kit (Sigma Aldrich, Germany) and eluted in 100 µl sterile water. The cells in standard stock solutions were calculated from the corresponding elution volume and from determined cultivable cell numbers (CFU/ml).

Limit of detection

The limit of detection (LOD) and accordingly the sensitivity of RT-PCR assays was verified from the 10-fold serial dilution of each prepared standard for all microbiological parameters. The measured standard curves ranged from $10^6 – 10^0$ target cells and were examined in triplicates. NTC served as negative controls in RT-PCR amplification runs. Amplification efficiencies were automatically calculated with the CFX software 3.0 (Biorad, Austria) calculating the standard curve from the cycle threshold (Cq) of each measured standard and the efficiency according to the formula $E= 10^{-1/s}-1$, where s is the slope of the standard curve (Garrido et al., 2013). For LOD determination cut-off principle from NTC was applied (Caraguel et al., 2011), i.e. Cq (LOD) = Cq (NTC) – 3. In cases where negative control was not detectable within RT-PCR, LOD was set at Cq of the last detectable standard dilution. The samples with Cq values higher than Cq (LOD) were classified as non-determined and Cq values lower were classified as positive.

Assay repeatability was determined by analyzing the standard dilution series ($10^6 – 10^0$ target cells) with five replicates per dilution. Experiments were repeated on three different days by the same operator and on the same CFX96™ cycler (Biorad, Austria).

RT-PCR conditions

For specificity testing 50 ng genomic DNA was used as a template in a 20 µl reaction mix. Control of RT-PCR performance was facilitated by running a corresponding bacterial standard in a range of 10^6 to 10^0 bacterial cells.

Hot FirePol EvaGreen® mix (Solis BioDyne, Estonia) and SsoFast™ Probes Supermix (Bio-Rad, Austria) were used for RT-PCR assays. Reaction volumes of 20 µl contained either 1x Hot FirePol EvaGreen® mix or 1x SsoFast™ Probes Supermix, 0.5 µM primers and 0.25 µM probe. EvaGreen® system cycling conditions started with initial denaturation for 15 min at 95°C, followed by 40 cycles with 30 sec at 95°C, 40 sec 52°C, 60 sec at 72°C and final 60 sec at 86°C. Melting curve analysis was performed after each PCR. For this, samples were heated at 95°C for 60 sec, cooled at 55°C for 60 sec and subsequently the temperature ramped from 55°C for 60 sec in 0.5°C increments per cycle. Fluorescence was measured at the end of each cycle.
The cycling parameters for TaqMan® assay were as follows: initial denaturation for 2 min at 95°C, followed by 40 cycles with 5 sec at 95°C and 30 sec at 62°C (*E. coli*, *P. aeruginosa*, total bacteria) or 20 sec at 60°C (*Enterococcus* spp.).
The threshold baseline, slopes and efficiencies were automatically calculated by the Bio-Rad CFX Manager version 3.0 software (Biorad, Austria).

Water sample collection

Water samples were collected from different locations in Lower Austria, Vienna and Burgenland, Austria. A total volume of 3 L was sampled at each site according to DIN EN ISO 19458 in sterile polypropylene plastic bottles (VWR, Austria). Samples were transported (refrigerated) to laboratory for analysis and stored at 4°C until further processing (max. 18 h).

In total 100 drinking water samples were collected, comprising of 65 well water samples, 16 spring water samples and 19 samples from public water supply. Further 30 process water samples were collected from 16 cooling towers, 6 samples from a drinking water treatment plant and 8 samples from purification plant.

Standard water quality assessment

Standard cultivation-based techniques defined in EN 12780:2002 and ISO 6222:1999 were used for the detection of *P. aeruginosa* and determination of heterotrophic plate counts (at 22°C and 37°C), respectively. For the detection of *Enterococcus* spp. and coliforms/*E. coli* alternatively approved chromogenic/fluorogenic tests (Enterolert®-DW and Colilert®-18; IDEXX, Austria) were used.

Sample preparation and PMA treatment

For each molecular assay, 1L water aliquots were filtered through a 0.45 µm nitrocellulose filter membrane (Millipore, Germany) and the bacteria were washed off with a 0.01% Tween20 solution. Bacterial cell suspension of the first 1L aliquot, intended for analysis with conventional RT-PCR, was pelleted by centrifugation at 10 000 x g for 5 min. Bacterial cell suspension of the second aliquot, intended for pre-treatment with PMA, was directly mixed with 10 µM PMA dye (Biotium Inc., USA). Firstly, samples were incubated for 5 min in dark, and then subsequently placed on ice and horizontally exposed to 500 W halogen light (distance 20 cm) for 5 min. After photo-activation, cells were pelleted at 10 000 x g for 5 min. DNA isolation was performed with WaterMaster™ DNA Purification Kit (Epicentre, U.S.).

DNA extraction from water samples

Several genomic DNA extraction protocols were tested including both an enzymatic (Lysozyme) based extraction using GenElute™ Bacterial Genomic DNA kit (Sigma Aldrich, Germany) and a mechanical test kit (bead-beating) such as the UltraClean®Water DNA isolation kit (MO BIO, U.S.) according to manufacturer's instructions. Furthermore, conventional DNA extraction using phenol/chloroform-based purification and ethanol extraction was tested (Maniatis, Fritsch and Sambrook, 1982) (data not shown). The genomic DNA from water samples in evaluation study was extracted with a WaterMaster™ DNA Purification Kit (Epicentre, U.S.), containing a specific inhibitor removal technology. Briefly, bacterial pellets from sample preparations were lysed by enzymatic treatment with Lysozyme (45 mg/ml) and Proteinase K (50 mg/ml). RNA was degraded by adding RNAse (5 mg/ml). DNA was precipitated with isopropanol followed by purification through a spin column (incl. inhibitor-removal step). DNA was eluted in 60 µl sterile water and 5 µl was used as template in (PMA)-RT-PCR amplification.

Inhibition tests

PCR inhibition arising from the sample matrix can lead to false negative results (Staley et al., 2012), therefore potential inhibition of DNA from water samples was assessed. For this, an inhibition test based on dilution was selected. Briefly, undiluted and 1:10 diluted DNA samples were quantitatively analysed for total bacteria (PMA)-RT-PCR targeting 16S rRNA genes. Higher quantitative values for diluted samples indicated an inhibition, similar or lower quantities revealed no interference in amplification reaction. Samples showing an inhibition were tested with further dilutions (1:20 and 1:50) and subsequently analysed with the diluted DNA template for the other targets.

Diagnostic values

Method validation included the calculation of analytical performance characteristics through evaluation of the specificity and sensitivity of the test. Therefore firstly the rates of true positives (TP) and true negatives (TN) and moreover the rates of false positives (FP) and negatives (FN) were determined. The results of standard water quality assessment were taken as a reference and considered as "true". Diagnostic sensitivity is the proportion of true positives (TP) and correct positives scored with the validated method, according to the formula: $Sensitivity = TP / (TP + FN)$. Specificity is the proportion of true negatives and false positives samples assigned by the test, according to the formula: $Specificity = TN / (FP + TN)$.

Further the accuracy of a method was described by the evaluation of calculated positive and negative predictive values (PPV and NPV). PPV indicates the probability that a positive test results correctly identifies the presence of a contamination ($PPV = TP / (TP+FP)$) while NPV indicates the probability that a negative result correctly identifies the absence of a contamination ($NPV = TN / (FN+TN)$). (Caraguel et al., 2011; Alberg, 2004; Nutz, Döll and Katrlovsky, 2011)

Results

Establishment and optimization of real time PCR

The establishment of RT-PCR assays was more straightforward for single organisms (*E. coli*, *P. aeruginosa* and *Enterococcus* spp.) than for groups of indicator organisms (coliforms) or heterotrophic plate counts. Nevertheless, for each target several PCR assays were tested before the best assay was selected.

Initially RT-PCR assays were established using intercalating dye technology (EvaGreen® mix, Solis BioDyne, Estonia). However, we observed that this approach did not perform reliably when tested with environmental samples. Obtained melting curves often had several peaks that were shifted around the expected melting temperature (Tm) peak. Accordingly the assessment was extremely challenging or even impossible if the obtained signal can be called positive or was unspecific and called non-determined, which is illustrated in the example of *E. coli* detection with EvaGreen® (Fig. 1). This is explained by the fact that DNA binding dyes intercalate randomly in double stranded DNA and thus also accumulate in un-specific non-target PCR products and/or primer dimers. Therefore, a more discriminative TaqMan® technology was selected for the further development of RT-PCR assays, allowing a more specific detection of target sequences through the application of a complementary fluorogenic hybridizing probe.

In general it was noted that the performance of assays developed many years ago was sub-optimal (especially with regard to specificity). This can be explained by the fact that the primer/probe design is only as good as the database it is based on and the extent of its *in vitro* validation. Availability of sequences increases rapidly over time (Lagesen et al., 2010), and accordingly *in silico* specificity of primer might prove as unreliable as new sequences become available. Similarly, as the knowledge of the microbial diversity increases, the list of targets relevant for *in vitro* validation can expand beyond the initially tested species.

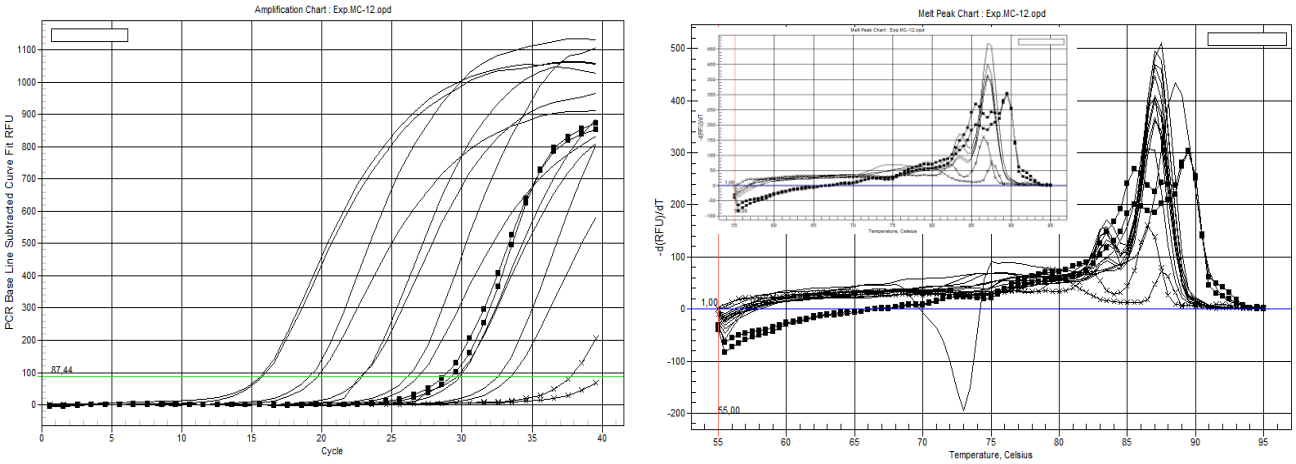


Fig. 1. Application of intercalating dye (EvaGreen®) on environmental water sample. Melting curve for RT-PCR exemplified for detection of *E. coli* showed several not clearly assignable peaks of sample. The identification of sample (squares) was positive between 10^3 - 10^2 cells from standard curve (accented zoom of melting curve analysis), but melting curve at the melting temperature showed several peaks, which challenged if sample can be considered as true positive or peaks raised from unspecific signals. Negative control is represented by line indicated with cross.

Specificity test

The focus of primer specificity testing was on exclusivity, i.e. ensuring that selected primers will not yield false positive signals with species known to be native in water samples. Accordingly a set of 26 bacteria was selected for specificity testing (Table S2).

Primers selected for the detection of *E. coli*/*Shigella* spp., *P. aeruginosa* and *Enterococcus* spp. (Table 1) were indeed highly specific and yielded positive amplification results only with targeted species.
More challenging was selection of a PCR assay that could be used for the detection of coliforms. Coliforms are defined on the basis on their common biochemical characteristics and do not represent a unique phylogenetic group, accordingly functional genes represent most suitable targets (Rompré et al., 2002).
Firstly, a range of PCR primers targeting the *lacZ* gene (encoding for *β-galactosidase*) was tested (Bej et al., 1990, 1991). Primers LZL-389 and LZL-653 yielded good results with *E. coli* DSM 30083 and *S. flexneri* DSM 4782, however, amplification efficiency of other tested coliform bacteria (*Citrobacter* sp. DSM 30041, *C. freundii* CCM4475 and *K. oxytoca* DSM 5175) was poor, even when DNA from pure cultures was used as a template. In silico analysis of the primers revealed that in the case of *Citrobacter* spp. there was no significant homology between the tested primers and the target gene. In case of *Klebsiella* spp. tested primers were homologous with *K. pneumoniae* but not with other *Klebsiella* spp. (incl. test strain *K. oxytoca*).

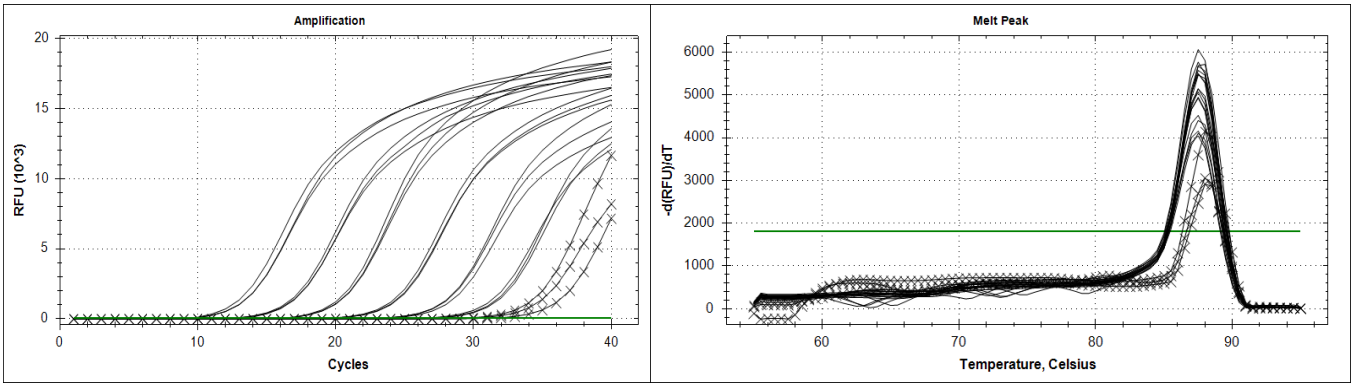
In general, the observed diversity of *lacZ* sequences from different coliform species clearly indicated that the design of group specific primers based on this gene is not feasible. Consequently, an alternative solution had to be implemented. We decided to develop a RT-PCR assay detecting the taxon *Enterobacteriaceae*. *Enterobacteriaceae* encompass members of coliform groups but also other genera including pathogens like *Salmonella* spp. and *Yersinia* spp. Selected PCR primers targeted 23S rRNA genes, and positive signals were obtained for all tested *Enterobacteriaceae*. For *Enterobacteriaceae* we were not able to find an established TaqMan® RT-PCR system or to design TaqMan® probe that could be integrated in the selected PCR system, so accordingly EvaGreen® was used. In this system melting curve analysis yielded unambiguous peaks even in case of water samples.
For the quantification of total bacteria in the sample the utilization of different universal markers as target was investigated (Santos and Ochman, 2004). Although, the 16S rRNA gene is the most frequently marker used in bacterial taxonomy and diversity analyses, it had to be considered that in this case quantitative analysis was required, which might be biased by the variability 16S rRNA copy numbers in different organisms (Klappenbach et al., 2001). Accordingly, our initial focus was on other universal, single copy genes such as *rpoB* (ribosomal polymerase B subunit; Powell et al., 2006) and *RNase P* (ribonuclease-P, Dolan et al., 2009) gene. However, tested primers failed to yield positive amplification with all tested strains. For other genes (e.g. *gyrB* - DNA gyrase subunit B) no suitable primers could be found or designed. Therefore, at the end the 16S rRNA gene had to be utilized. For this purpose a range of different primer combinations were tested (Yu and Morrison, 2004) and the best performing primers were selected for implementation. In order to accommodate for the copy number variabilities a mixed standard was used.

Limit of detection

For selected RT-PCRs, the LOD and accordingly sensitivity was determined by analyzing a 10-fold serial dilution of the corresponding standard. The standard curves of *Enterobacteriaceae*, *Enterococcus* spp. and total bacteria were linear and robust over 6 log units from 10^6 – 10^1 cells (Fig. 2A and B). The standard dilution 10^0 had to be excluded from the analysis based on the cut-off established from negative control. Signals from negative controls from runs targeting universal bacterial phylogenetic markers (16S rRNA or 23S rRNA genes) could be explained by the fact that polymerase preparations inevitably contain contaminating microbial DNA (Corless et al., 2000, Spangler; Goddard and Thaler, 2009). The RT-PCRs for *E. coli* and *P. aeruginosa* allowed the detection of 10^6 - 10^0 targets, because negative controls were not detected and accordingly no cut-offs had to be set. In accordance to the assay development also repeatability was determined. For example RT-PCR targeting *Enterococcus* spp. achieved efficiencies of 98-103% with high correlation coefficient of 0.997. The standard curves from three independent days confirmed the robust

repeatability of the assay with ΔCq = 3.27-3.35 and standard deviations of 0.065-0.190. Other RT-PCRs showed similar results.

A)



B)

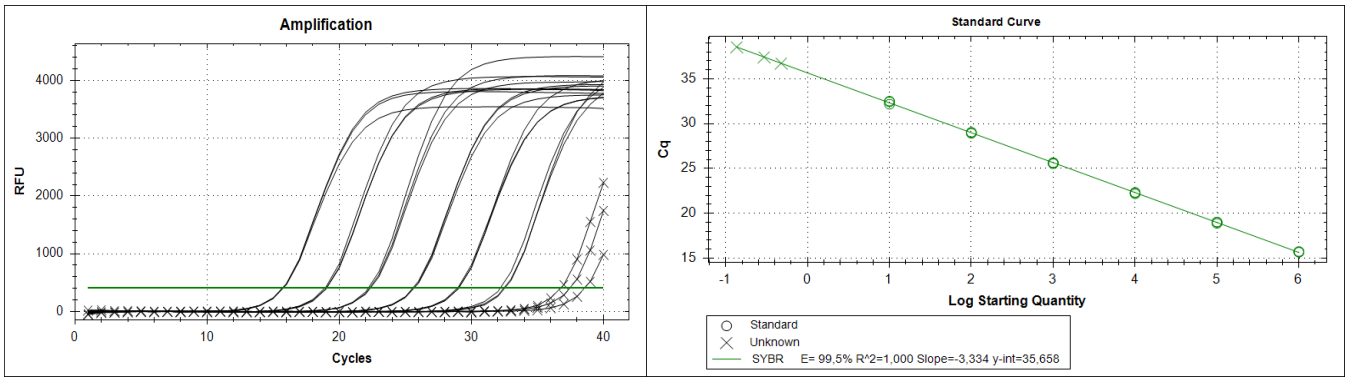


Fig. 2. Amplification chart of RT-PCR. The RT-PCR amplification of the standard curve is illustrated for A) *Enterobacteriaceae* (EvaGreen®) including melting curve analysis. B) *Enterococcus* spp. based on the use of TaqMan® technology. Standard curve for both RT-PCRs was robust over 6 log units but cut-off principle from negative control (cross) had to be set.

Evaluation of PMA-RT-PCR and RT-PCR for water quality assessment

Standard water quality assessment

Water quality assessment with standard microbiological methods (Fig. S1) revealed that 20 out of 100 drinking water samples and 6 out of 30 process waters were acceptable as a drinking water according to the Austrian DWD (2001). The most frequent contaminations found in drinking water samples were coliforms (69/100) followed by the exceeding of the threshold values from HPC 37°C. Less abundant were positive samples for *E.coli* (22/100) and *P. aeruginosa* (20/100). For process water numerous HPC 37°C exceeding (12/30) and further contaminations with coliforms and *Enterococcus* spp. with 9 positive samples out of 30 were determined. Microbiological reference values for confirmation of *P. aeruginosa* were not obtained for 8 samples collected from cooling towers, because selective media plates were overgrown by a fungal contamination.

Inhibition tests

Performance assessment was based on 96 samples only, because in 4 samples molecular analysis was not possible due to problems during DNA extraction (inefficient elution). First inhibition was tested; 24 (25%) drinking water samples and 17 (57%) process water samples exhibited inhibition. The highest inhibition rate was seen for well waters and waters from cooling towers. For these samples all (PMA)-RT-PCR analysis were performed with 1:10 dilution of original DNA. However, the detected inhibition could not be directly assigned to a sensitivity problem. Another problem that might have affected the overall sensitivity of the molecular analysis was due to solid organic material blocking filtration. This occurred in three samples, from which only 450-700 ml were filtrated. However, these samples did not exhibit any sensitivity problems.

Performance assessment of PMA-RT-PCR and RT-PCR

Results from both molecular assays were compared to each other and further to reference values obtained from standard water quality assessment (considered to be a "true" result). Conventional RT-PCR (without PMA treatment) was included to reveal PMA-induced reduction of false positives.

Per legal definition in the Austrian DWD (2001), the absence (0/100 ml water) of *E. coli*, coliforms, *Enterococcus* spp., and *P. aeruginosa* has to be assured. Accordingly, the results from RT-PCR were only qualitatively specified, presented as positive detections (Cq > LOD) and non-determined (Cq < LOD). For total bacterial estimation, quantification of cells through standard curve was performed.
The performances of both RT-PCR and PMA-RT-PCR for assessing the quality of drinking and process water samples are summarized in Table 2 and were depicted as correlation rates, false negatives and false positives for target bacteria. The correlation rates were on average 70-81% for RT-

PCR and 66-84% for PMA-RT-PCR in drinking water evaluation. Best performance in validation was identified for *E. coli* and *P. aeruginosa* PMA-RT-PCRs both having correlation rates of 84%, i.e. 81 from 96 samples were correctly assigned. The treatment with PMA induced reduction of false positives, e.g. for *E. coli* a decrease of 6% was determined and for *P. aeruginosa* 4%. Further also for *Enterococcus* spp. the number of false positives in RT-PCR could be reduced to 6% with PMA-RT-PCR. However, for this assay the correlation rate (75%) was somewhat lower when compared to reference results.

The lowest correlation rates were assigned to *Enterobacteriaceae* in both assays (RT-PCR and PMA-RT-PCR) because high rates of both false positives (12-17%) and false negatives (13-22%) were determined. These assays showed insufficient performances when compared to reference method, thus strongly indicating that the detection of the phylogenetic assigned *Enterobacteriaceae* group is not well correlating and can hardly be compared to biochemically characterized coliform group. However, it was reported that the used enzymatic based Colilert®-18 may be biased by environmental factors strongly regulating the activity of the enzyme and that high numbers of heterotrophic bacteria in the sample may also interfere with the chromogenic reaction (Maheux et al., 2008; Maheux et al., 2014).

In process water evaluation, in general higher correlation rates of PMA-RT-PCR compared to RT-PCR were observed for all indicator bacteria (with exception of *Enterobacteriaceae*). Actually PMA-RT-PCR for the detection of *E. coli* correlated 100% to results from reference test. Furthermore, no false positive detection could be assigned in PMA-RT-PCR for *Enterococcus* spp. and *P. aeruginosa*. Conformation to reference test was achieved from 24/30 samples (*Enterococcus* spp.) and 20/22 (*P. aeruginosa*).

Although, both molecular assays (RT-PCR and PMA-RT-PCR) yielded a certain amount of false negative detections in drinking (up to 21/96) and as well as process water (up to 7/30). This bias is assumed to be due to insufficient sample preparation (i.e. filtration, DNA extraction), leading to the loss of cells, especially for initially low numbers of target bacteria, which might result in negative outcomes.

Table 2. Correlations between RT-PCR and PMA-RT-PCR in comparison to reference values. Rates of correlation, false negatives and false positives are represented for all defined bacteria (Austrian DWD, 2001) for drinking water and process water.

	Enterobacteriaceae	***E. coli***	***Enterococcus* spp.**	***P. aeruginosa***
Drinking water (96 samples)				
		RT-PCR		
Correlation	68 (70%)	76 (79%)	76 (79%)	78 (81%)
False negative	12 (13%)	11 (12%)	11 (12%)	13 (14%)
False positive	16 (17%)	9 (9%)	9 (9%)	5 (5%)
		PMA-RT-PCR		
Correlation	63 (66%)	81 (84%)	72 (75%)	81 (84%)
False negative	21 (22%)	12 (13%)	21 (22%)	14 (15%)
False positive	12 (12%)	3 (3%)	3 (3%)	1 (1%)
Process water (30 samples)				
		RT-PCR		
Correlation	23 (76%)	27 (90%)	22 (73%)	16 (73%)
False negative	2 (7%)	0 (0%)	5 (17%)	2 (9%)
False positive	5 (17%)	3 (10%)	3 (10%)	4 (18%)
		PMA-RT-PCR		
Correlation	20 (67%)	30 (100%)	24 (80%)	20 (91%)
False negative	7 (23%)	0 (0%)	6 (20%)	2 (9%)
False positive	3 (10%)	0 (0%)	0 (0%)	0 (0%)

Diagnostic parameters for PMA-RT-PCR and RT-PCR

Diagnostic values and the performance characteristics of RT-PCR and PMA-RT-PCR are illustrated in Table 3. Both assays resulted in relatively high specificity values, with improvement over 15% by PMA-RT-PCR. Accordingly, also the PPV were higher for PMA-RT-PCR, at best in process water with an optimization from 50% for RT-PCR to 100% with PMA-RT-PCR. This improvement was achieved for *E. coli*, *Enterococcus* spp. and *P. aeruginosa* (PPV of 100%), which is explained by the complete suppression of false positive detection and better correlation to reference methods. Best performance through diagnostic evaluation of the assays was attributed to *E. coli* PMA-RT-PCR with a 100% sensitivity and specificity for process water.

Nevertheless, problems were identified in the sensitivity for all other assays; with lowest of 26.3 % in drinking water. This was further reflected in NPV values, for both assays with almost similar values, e.g. 84.7% in RT-PCR and 84.4% PMA-RT-PCR for *P. aeruginosa* in drinking water. Both diagnostic parameters are an issue of false negative assignments in the evaluated test and as considered before, probably caused by inefficient sample preparation rather than the insufficient LOD of RT-PCR. Comparable values of RT-PCR and PMA-RT-PCR indicated that PMA treatment does not have a negative effect on overall sensitivity of the method.

Total bacterial quantification (PMA)-RT-PCR versus heterotrophic plate count

As analogue to the bacterial enumeration by HPC on the molecular basis, the total bacterial quantification was established in form of 16S rRNA (PMA)-RT-PCR assay. The direct comparability of these methods is not feasible, because two plate counts at 22°C and 37°C and threshold values of 100 and 20 colonies ml^{-1}, respectively, have to be compared to one quantitative cell number from molecular assay. Therefore, the heterotrophic counts were set in relation to cell quantifications from RT-PCR and PMA-RT-PCR in order to investigate if new threshold values for the molecular assays could be established. However, as illustrated in Fig. 3 no reliable threshold value could be determined, because no clear correlation or cut-offs could be ascertained considering the legal thresholds of the HPC method. This is exemplified through samples that have zero enumeration in HPC, but $10^0 – 10^5$ quantified cells in PMA-RT-PCR assay. Conversely, in some samples with extremely high HPC counts (≥ 500 colonies), PMA-RT-PCR yielded in cell quantifications values below the defined HPC threshold value. However, also HPC method itself showed in some cases that one temperature resulted in no exceeding (< threshold), whereas the other temperature enumerated substantial counts (>threshold) (e.g. HPC 37°C counted for 300 CFU/ml and HPC 22°C only for 37 colonies) leading to in-sufficient water quality as defined by the Austrian DWD.

Table 3. Diagnostic parameters for RT-PCR and PMA-RT-PCR. Rates of sensitivity, specificity, negative and positive predictive values are illustrated for target bacteria for drinking and process water.

Diagnostic value [%]	***Enterobacteriaceae***	***E. coli***	***Enterococcus* spp.**	***P. aeruginosa***
Drinking water samples (total 96)				
		RT-PCR		
Specificity	44.8	87.8	82.0	93.5
Sensitivity	82.1	50.0	76.1	31.6
NPV	52.0	85.5	78.9	84.7
PPV	77.5	55.0	79.6	54.6
		PMA-RT-PCR		
Specificity	58.6	96.0	94.0	98.7
Sensitivity	68.7	45.5	54.4	26.3
NPV	44.7	85.5	69.1	84.4
PPV	79.3	76.9	89.3	83.3
Process water samples (total 30)				
		RT-PCR		
Specificity	71.4	88.9	85.7	87.5
Sensitivity	88.9	100	44.4	33.3
NPV	88.2	100	78.3	77.8
PPV	61.5	50.0	57.1	50.0
		PMA-RT-PCR		
Specificity	81.0	100.0	100.0	100.0
Sensitivity	33.3	100.0	33.3	66.7
NPV	70.8	100.0	77.8	88.9
PPV	50.0	100.0	100.0	100.0

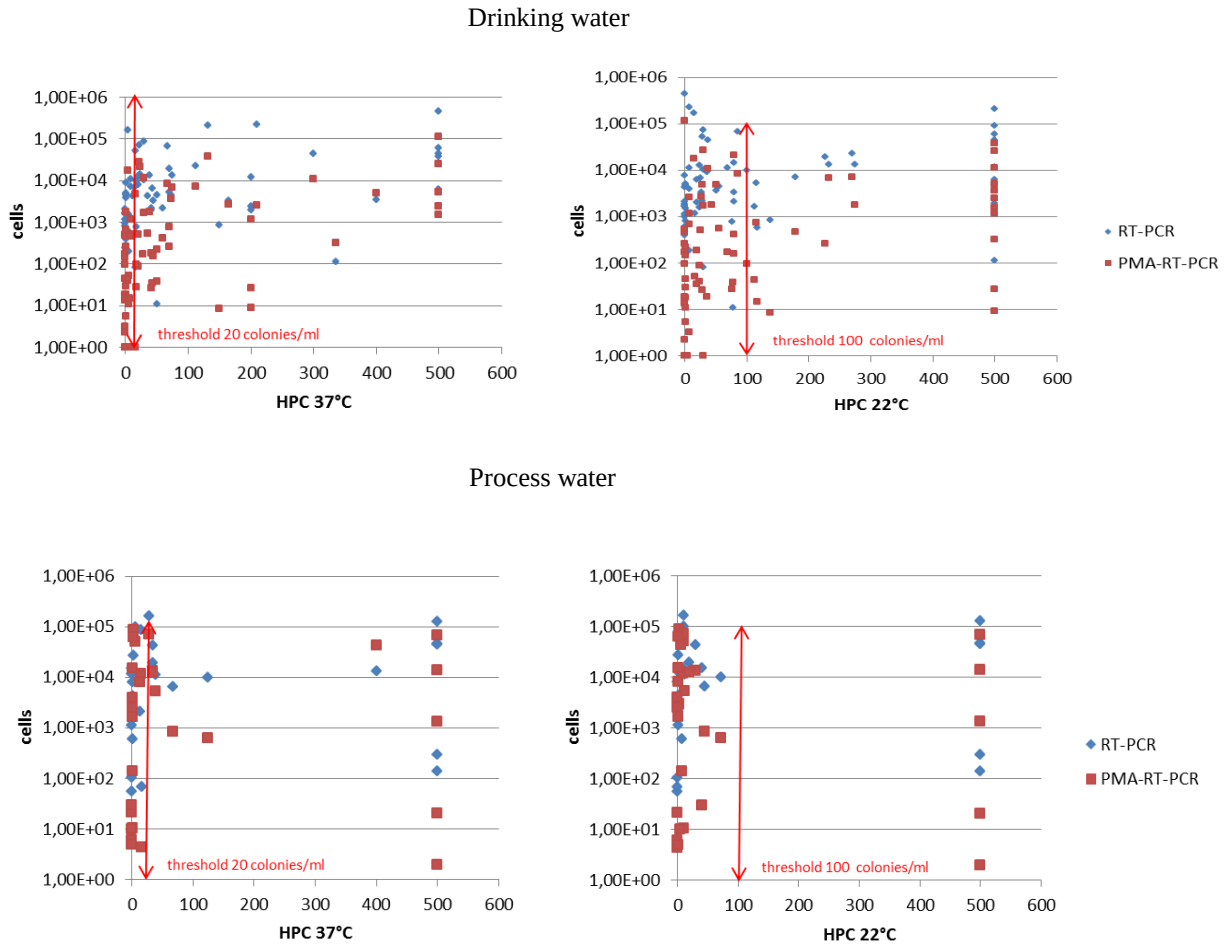


Fig. 3. Heterotrophic plate count versus total bacterial quantification with RT-PCR and PMA-RT-PCR. Threshold values (arrow) for HPC 22°C (100/ml water) and HPC 37°C (20/ml water) are illustrated according to Austrian DWD (2001). PMA-RT-PCR (diamonds) and RT-PCR (squares) are presented in cell quantification (cells) and are plotted against counted colonies from HPC method at both temperatures for drinking and process water. The maximal value of heterotrophic plate counts was set to 500 to allow for clearer plotting.

Discussion

Cultivation-based techniques for determination of microbial quality parameters have been applied for a long time and are well established standardized procedures (Brettar and Höfle, 2008; Aw and Rose 2012). However, demands to develop and implement more rapid and specific technologies, such as e.g. RT-PCR technology, were made in last decades. Nevertheless, it has to be considered that RT-PCR does not allow the differentiation between living and dead bacterial cells and thus has poor comparability to cultivation techniques. Some studies already combined RT-PCR with DNA intercalating dyes such as PMA for viable cell detection (Delgado-Viscogliosi et al., 2009; Slimani et al., 2011), but to our knowledge, not applied yet for entire microbial parameter set required in water quality assessment. Therefore this study focused on the establishment and evaluation of RT-PCR with PMA treatment for all microbiological parameters defined in Austrian DWD (2001) with diverse and representative water samples. The need of an implementation of a live/dead differentiation step (PMA treatment) into RT-PCR analysis was clearly observed in an initial method evaluation (data not shown), which resulted in high percentages of false positive detections in comparison to reference tests. Therefore implementation of a PMA-treatment was included into established RT-PCR assays and proof of principle was shown for indicator parameters in an abundant water background microflora successfully excluding dead cells from analysis (Gensberger et al., 2013).

In evaluation of PMA-RT-PCR with drinking water and process water samples, best performances were identified for *P. aeruginosa* and *E. coli* detection, achieved through the substantial reduction of false positives and therefore leading to high specificity (100% process water). The specificity of these assays is also suggested in the utilization of specific marker genes for these single indicators than for target groups.

E. coli was detected targeting the sequence encoding the β-glucuronidase, *uidA* (Takahashi et al., 2009) and the pathogenic island of the *regA* (Lee et al., 2008) provided the determination of *P. aeruginosa*. Especially, in the case of *E. coli* direct comparability to Colilert®-18 test is facilitated, because both target ß-glucuronidase. *E. coli* RT-PCR also includes the detection of *Shigella* spp., because of high homology in the *uidA* sequence between the two species (97 - 98%). This is not surprising given that the genus status of *Shigella* spp. is actually phenotypic (the ability to cause a specific type of diarrhea) and that from evolutionary perspective *Shigella* strains should be classified as *E. coli* (Pupo et al., 2002; Zhang and Lin, 2012). Contrarily to the high specificity, a low sensitivity, caused by continuous detection of similar values of false negative (12-22%) was obtained. However, this is not only attributed to PMA-RT-PCR, also RT-PCR resulted in almost same low calculated sensitivity in diagnostic parametric evaluation. Exceptions with slightly higher false negative scores with PMA-RT-PCR were identified for *Enterococcus* spp. and for *Enterobacteriaceae*, which could be due to a moderate cytotoxic effect of PMA to this target organism or species from target spectrum (Yáñez et al., 2012). The sensitivity problem is presumed in the sample preparation procedure rather

than the LOD of RT-PCRs. Molecular detection of bacteria from water requires concentration of bacteria and then extraction of DNA. Commonly applied method for concentration of bacteria from water is the filtration, because of the easy handling and inexpensive equipment. However, after filtration the re-suspension of bacteria from filters is necessary, which might result in a moderate recovery bias. All these methodical steps are not 100% efficient and consequently cause loss of target bacteria that are already present in low amounts in water samples (Brettar and Höfle, 2008; Agudelo et al., 2010). In fact, the RT-PCR LOD itself is rather low; taking into account that observed LOD is between 1 – 10 targets per RT-PCR reaction, and that 5 µl (out of 60 µl) DNA were used as a target, overall sensitivity of the detection would be 12 cells/100 ml water under assumption of 100% efficient sample preparation. Insufficient sample preparation is in general of major concern in the application of molecular assays and numerous studies revealed the inherently variable and inefficient recovery of DNA from kits utilizing spin filter columns (Lemarchad et al., 2005; Lloyd et al., 2010; Haugland et al., 2012; Staley et al., 2012). Optimization to higher sensitivity is required, which could be assumed in improvement of DNA extraction protocol or assembling the filtration, PMA treatment and DNA extraction to one procedure in order to prevent loss of bacteria in separate steps (Slimani et al., 2012).

In general poor correlation (specificity and sensitivity) was observed for molecular detection of *Enterobacteriaceae* (for both RT-PCR and PMA-RT-PCR) compared to results of enzymatic (β-galactosidase) coliform reference test (Colilert®-18). This is most probably due to the differences in target spectrum of the taxonomically assigned *Enterobacteriaceae* and biochemical characterized group of coliforms. Coliforms are described as rod-shaped, non-spore forming, gram-negative, oxidase positive, bacteria that are able to grow on bile salts and further ferment lactose with gas and acid production. Actually the definitions of coliforms differ slightly from total coliforms to thermo-tolerant species (Rompré et al., 2002). In contrast, the *Enterobacteriaceae* family encompasses the detection of coliforms but also a range of non-coliform genera like *Salmonella* spp. or *Yersinia* species.

For the coliforms the primer development is more difficult, because it is a diverse group containing many genera and primers must be specific to exclude some closely related non-coliforms (Rompré et al., 2002). However, as mentioned before the specificity of the tested *lacZ* was rather poor, not allowing the detection of some species belonging to the coliform group. In this scope no adequate assessment for coliforms on molecular basis could be determined.

Also total bacterial estimation with RT-PCRs (16S rRNA gene analysis) could not be related to the HPC enumerations. Both RT-PCR and PMA-RT-PCR commonly resulted in higher quantifications than HPC method. However, it is reported that molecular assays are able to detect much more bacteria from water samples than cultivation-based technique such as the heterotrophic plate count. As reported by Hammes and Egli (2010), non-chlorinated drinking water typically contains about 10^4-10^5 cells/ml of diverse populations of bacteria and only a small proportion can be detected with the heterotrophic plate count method. Only approximately 1 % of bacteria are culturable on standard cultivation media

(Amann, Ludwig and Schleifer, 1995; Walsh and Duffy, 2013) and further the presence of viable but non-culturable cells (VBNC), especially in oligotrophic habitats as water (Oliver, 2005) may lead to underestimations with HPC method. Furthermore, targeting the 16S rRNA gene, which may be present in multiple copies (~1-15 per genome), may lead to higher quantifications (Větrovský and Baldrian, 2013) in molecular detection. For this reason, single copy genes (*rpoB* and *RNaseP*) were initially assessed but obtained results showed inefficient specificity, and therefore these assays could not be used.

Actually, many studies reviewed and questioned the significance of HPC methods for monitoring of water quality, especially the utilization of different HPC protocols and the range of defined threshold making a comparison of values unreliable (Allen, Edberg and Reasoner, 2004; Bartram et al., 2004). However, it is globally defined as standard method and serves as reference for evaluation of alternative test methods. Accordingly, evaluation of alternative tests remains therefore a major challenge even when these allow more precise estimation of bacteria.

Conclusions

The inclusion of PMA treatment in RT-PCR analysis resulted in substantial or complete reduction of false positives signals in both drinking and process water evaluation. Best performances due to high specificity were achieved for the microbial parameters *E. coli*, *Enterococcus* spp. and *P. aeruginosa*. This was highlighted by the compilance to reference results for *E. coli* PMA-RT-PCR in process water evaluation with 100% specificity and sensitivity. The major challenge was seen in the evaluation of molecular assays for indicators (coliforms and HPC), which proved to be poorly correlated to results from reference tests.

The PMA-RT-PCR demonstrated to be a novel technique for application in water quality assessment and could also encompass the detection of relevant pathogens from water, but application PMA-RT-PCR still needs further optimization in sensitivity, validation of the assay (intra and inter-laboratory) and preparation of standardized protocols for legal harmonization.

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Supplements

Table S1. List of tested published primer sets for defined target organism for selection of RT-PCR.

total bacteria

- ***rpoB* gene (ribosomal polymerase B subunit) - Powell et al., 2006**

RT-PCR (343 bp)
1698F 5' CAA CAT CGG TTT GAT CAA C 3'
2041R 5' CGT TGC ATG TTG GTA CCC AT 3'

- ***rnp* gene (ribonuclease-P) - Dolan et al., 2008**

reverse transcription RT-PCR (299 bp)
G neg-rnp forward 5' gaa agt ccg ggc tcc ata 3'
G neg-rnp reverse 5' ata agc cgg gtt ctg t 3'

reverse transcription RT-PCR (197 bp)
G pos-rnp forward 5' gag gaa agtc c(a/g)(g/t) gct cgc ac 3'
G pos-rnp reverse 5' agg ggt tta ccg cgt tcc 3'

- ***rrs* gene (16S rRNA) - Yu and Morrison, 2004**

PCR (80 bp)
63fa 5' GCC TAA CAC ATG CAA GTC 3'
109r 5' ACG TGT TAC TCA CCC GT 3'

PCR (489bp)
63fa 5' GCC TAA CAC ATG CAA GTC 3'
518r 5' ATT ACC GCG GCT GCT GG 3'

PCR (424bp)
954fa 5' GCA CAA GCG GTG GAG CAT GTG G 3'
1369r 5' GCC CGG GAA CGT ATT CAC CG 3'

- **rRNA gene (16S rRNA) - Nadkarni et al., 2002**

RT-PCR (466 bp)
16S forward 5' TCCTACGGGAGGCAGCAGT 3'
16S reverse 5' GGACTACCAGGGTATCTAATCCTGTT 3'

Coliforms

- ***lacZ* gene (β-galactosidase) - Bej et al., 1991**

PCR (264 bp)
LZL-389 5' ATGAAAGCTGGCTACAGGAAGGCC 3'
LZR-653 5' GGTTTATGCAGCAACGAGACGTCA 3'

- ***lacZ* gene (β-galactosidase) - Bej et al., 1990**

PCR (326 bp)
ZL-1675 5' ATGAAAGCTGGCTACAGGAAGGCC 3'
ZR-2025 5' GGTTTATGCAGCAACGAGACGTCA 3'

Enterobacteriaceae

- **rRNA gene (23S rRNA) - Matsuda et al., 2009**

PCR (428 bp)
En-lsu-3F 5' TGCCGTAACTTCGGGAGAAGGCA 3'
En-lsu-3R 5' TCAAGGACCAGTGTTCAGTGTC 3'

E.coli* & *Shigella

- ***uidA* gene (β-glucuronidase) - Bej et al., 1990**

PCR (147 bp)
UAL-754 5' AAAACGGCAAGAAAAAGCAG 3'
UAR-900 5' ACGCGTGGTTACAGTCTTGCG 3'

- ***uidA* gene (β-glucuronidase) - Bej et al., 1990**

PCR (166 bp)
UAL-1939 5' TATGGAATTTCGCCGATTTT 3'
UAR-2105 5' TGTTTGCCTCCCTGCTGCGG 3'

- ***uidR* gene (*uidA* regulator) - Bej et al., 1990**

PCR (154 bp)
URL-301 5' TGTTACGTCCTGTAGAAAGCCC 3'
URR-432 5' AAAACTGCCTGGCACAGCAATT 3'

Pseudomonas aeruginosa

- ***regA* gene (toxin A synthesis regulating gene) - Lee et al., 2008**

PCR (64bp)
Paer-F 5' TGCTGGTGGCACAGGACAT 3'
Paer-R 5' TTGTTGGTGCAGTTCCTCATTG 3'

- ***oprL* gene (outer membrane lipoprotein) - Xu et al., 2004**

PCR (504 bp)
forward 5' ATG GAA ATG CTG AAA TTC GGC 3'
reverse 5' CTT CTT CAG CTC GAC GCG ACG 3'

- ***exo*A gene (exotoxin A) - Xu et al., 2004**

PCR (396 bp)
forward 5' GAC AAC GCC CTC AGC ATC ACC AGC 3'
reverse 5' CGC TGG CCC ATT CGC TCC AGC GCT 3'

Pseudomonas spp.

- **rRNA gene (16S rRNA) - Matsuda et al., 2009**

RT-PCR (215 bp)
PSD7F 5' CAAAACTACTGAGCTAGAGTACG 3'
PSD7R 5' TAAGATCTCAAGGATCCCAACGGCT 3'

Enterococcus faecalis

- ***groES* gene (heat-shock protein) - Lee et al., 2008**

RT-PCR (64bp)
Efaecal-F 5' TGTGGCAACAGGGATCAAGA 3'
Efaecal-R 5' TTCAGCGATTTGACGGATTG 3'

Enterococcus spp.

- **rRNA gene (16S rRNA) - Rinttila et al., 2004**

RT-PCR (144 bp)
forward 5' CCCTTATTGTTAGTTGCCATCATT 3'
reverse 5' ACTCGTTGTACTTCCCATTGT 3'

targets: *Enterococcus* spp. *Enterococcus faecalis*, *E. faecium*, *E. asini*, *E. saccharolyticus*, *E. casseliflavus*, *E. gallinarum*, *E. dispar*, *E. flavescens*, *E. hirae*, *E. durans*, *E. pseudoavium*, *E. raffinosus*, *E. avium*, *E. malodoratus*, *E. mundtii*, *E. azikeevi*, *E. canis*, *E. gilvus*, *E. rotate*, *E. haemoperoxidus*, *E. hermanniensis*, *E. moraviensis*, *E. pallens*, *E. phoeniculicola*, *E. villorum*

- **rRNA gene (16S rRNA) - Matsuda et al., 2009**

reverse transcription RT-PCR (337 bp)
g-Encoc-F 5' ATCAGAGGGGGATAACACTT 3'
g-Encoc-R 5' ACTCTCATCCTTGTTCTTCTC 3'

Table S2. List of 26 water relevant and non-waterborne bacteria that were used as reference strains for the primer specificity test.

Staphylococcus aureus SSM CI-1	*Comamonas acidovorans* AIT-AM 7
Escherichia coli DSM 30083	*Arthrobacter* spp. AIT-AM 3
Enterococcus faecalis DSM 20478	*Enterobacter asburiae* AIT-AM 9
Pseudomonas aeruginosa DSM 50071	*Bifidobacterium longum* AIT-AM 5
Clostridium perfringens DSM 756	*Bacteroides fragilis* AIT-AM 4
Salmonella spp. AIT-AM13	*Shigella flexneri* DSM 4782
Legionella pneumohila DSM 7513	*Mycobacterium tuberculosis* H37Rv
Citrobacter spp. DSM 30041	*Campylobacter jejuni* DSM 4688
Raoultella terrigena DSM 2687	*Streptococcus agalactiae* AIT-AM 6
Yersinia enterocolitica DSM 11502	*Helicobacter pylori* SSM 4138
Alcaligenes spp. AIT-AM 2	*Citrobacter freundii* CCM 4475
Acinetobacter calcoaceticus AIT-AM 1	*Klebsiella oxytoca* DSM 5175
Spingomonas paucimobilis AIT-AM 15	*Yersinia enterocolitica* DSM 4780

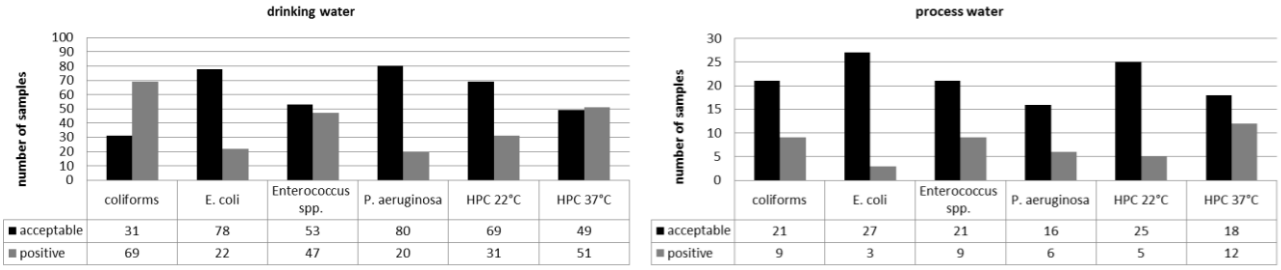

Fig. S1. Standard water quality assessment. The number of acceptable (negatives) and positives are illustrated for the analyzed subset of 100 drinking water and 30 process water samples. Legal definition prescribes the absence *E. coli*, coliforms, *Enterococcus* spp. and *P. aeruginosa* (0/100ml) in the assessed water. The thresholds values for HPC determination are 20 colonies/ml for 37°C and 100 colonies/ml for 22°C.

Chapter 3

Effect of different heterotrophic plate count methods on the composition of culturable microbial community

This chapter has been submitted to:

PeerJ

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Introduction

A huge diversity of bacteria can be found in water habitats comprising naturally present autochthonous waterborne bacteria but also allochthonous bacteria including opportunistic pathogens derived from fecal contamination of human or animal origin (Pavlov et al., 2004; Cabral, 2010). Therefore in order to ensure a high quality of water, safe for human consumption, a regular water quality assessment is a prerequisite.

The basis for water quality assessment is outlined in several national and international standards, e.g. in Europe in the Council Directive 98/83/EC on the quality of water intended for human consumption (EC, 1998), in the U.S. in the Water Directive (EPA, 2009), in Australia the Drinking Water Guidelines (NHMRC, 2011) or WHO recommendations (WHO, 2011). Even though regulations differ slightly, requirements generally include monitoring of microbial parameters such as fecal indicators (coliforms, *Escherichia coli*, *Enterococc*us spp.), the opportunistic pathogen *Pseudomonas aeruginosa* and the determination of the heterotrophic plate count (HPC).

The HPC is the enumeration of the growth of heterotrophic culturable microorganisms on a non-selective solid medium under defined cultivation conditions. The concept of HPC as water quality parameter was firstly proposed by Robert Koch in 1883 (Bartram et al., 2003) and to this day is included in most water quality regulations (Allen, Edberg and Reasoner, 2004). The heterotrophic plate count procedure has been subjected to extensive changes to ensure best possible recovery of heterotrophic organisms (Reasoner, 2004), thus contributing to the variations in methods between countries (Bartram et al., 2003). The commonly used practice for HPC determination is based on the pour-plate method, but also membrane filtration and spread plate method are proposed for analysis (Sartory, Gu and Chen, 2008). Beside the practices, variability further lies in the use of different cultivation conditions. HPC is stipulated by most regulations but still no globally standardized method is available and obligatory requirements, such as cultivation conditions, differ (Bartram et al., 2004). For example, DIN EN ISO 6222 (valid Europe-wide e.g. in Austria, Germany and Sweden) prescribes utilization of yeast extract agar and incubation at 37°C and 22°C for 48 h and 72 h respectively. Other regulations are more permissive and recommend a range of incubation temperatures (20°C to 40°C), incubation times (48 h - 7 days), and variable formulations of media (e.g. low and high nutrient media) (Reasoner, 2004; Bartram et al., 2004). Many questions that arose at the time when HPC methods were established (including media suitability, relationship of bacteria in water samples and corresponding HPC counts, interpretation of HPC counts) and remain the focus of discussion today (Bartram et al., 2003). Several studies have reviewed the significance of HPC measurement regarding their various different cultivation conditions (Carter et al., 2000; Allen, Edberg and Reasoner, 2004; Bartram et al., 2004; Reasoner, 2004). Some studies experimentally evaluated the effect of the cultivation condition on enumerated amount of colonies by HPC and results accordingly differed

(Lillis and Bissonnette, 2001; Allen, Edberg and Reasoner, 2004; Bartram et al., 2004; Reasoner, 2004; Inomata, Chiba and Hosaka, 2009). However, there is limited information on the composition of culturable bacteria in HPC assessment. To our best knowledge, only Wernicke and co-workers (1990) investigated composition of HPC populations obtained from different cultivation media and incubation times. Even though HPC populations were characterized only phenotypically, significant differences between tested media were observed (Reasoner, 2004). Farnleitner et al. (2004) examined profiles of HPC communities by denaturing gradient gel electrophoresis (DGGE) and clearly showed the cultivation-dependent variability of culturable HPC communities. As already stated by Burtscher and co-workers (2009), deeper insights into the structure of HPC population is essential, because it will lead to better understanding of the method itself and provide basis for selection of most suitable cultivation condition. Therefore our study addressed the effect of cultivation conditions, as proposed by EN ISO 6222:1999 and EPA, on the composition of culturable heterotrophic bacteria. Two different media: high nutrient yeast extract agar (YEA), commonly used in the EU, and R2A agar (designated as low nutrient media by Reasoner and Geldreich 1985), recommended in the U.S. (Allen, Edberg and Reasoner, 2004; Reasoner, 2004) were tested. In addition, the temperature effect (high and low temperature) was considered. The composition of bacterial communities cultivated under different conditions was determined by 16S rRNA gene sequence analysis. Furthermore, statistical analysis (permutation test on CCA and PERMANOVA) was applied in order to ascertain the significance of the tested cultivation conditions.

Materials and Methods

Water sampling

Water was collected from private wells from three different sites (IFA, N170 and N167) in Lower Austria. A total volume of 5 L was sampled at each site according to DIN EN ISO 19458 in polypropylene plastic bottles (VWR, Austria). Samples were transported and stored at 4°C until further processing (max. 18 h).

Sample preparation

Membrane filtration method was used for concentration of microorganisms, filtering a total volume of 1 L through a 0.45 µm membrane filter (Millipore, Germany) for each water sample and each tested cultivation condition (Reasoner and Geldreich, 1985; Köster et al., 2003). Membrane filters were incubated on yeast extract agar (Sigma Aldrich, Germany) and on R2A (Sigma Aldrich, Germany) and were tested at both incubation temperatures of 22°C (72 h incubation) and 37°C (48 h incubation). The four different cultivation conditions were abbreviated as YEA37, R2A37, YEA22 and R2A22. After the incubation, bacteria grown on the membrane filter were resuspended form filter with 1 ml of 0.01% Tween solution, pelleted by centrifugation and DNA was isolated using the GenElute genomic DNA Kit (Sigma Aldrich, Germany) according to the manufacturer's instructions and eluted in 100 µl sterile water. DNA concentration was measured with a Nanodrop spectrophotometer (Thermo Scientific, Austria) and confirmed with agarose (1%) gel electrophoresis.

16S rRNA amplification and cloning

The 16S rRNA gene was amplified with universal bacterial 16S rRNA oligonucleotide primers 8for (5'-AGAGTTTGATCCTGGCTGAG-3') and 1520rev (5'-AAGGAGGTGATCCAGCCGCA-3') (Edwards et al., 1989; Massol-Deya et al., 1995). A 25 µl reaction was prepared containing 1x Taq® buffer, 2.5 mM $MgCl_2$, 0.2 mM dNTP mix, 0.15 µM of each primer, 1 U of Taq® polymerase (Invitrogen, Austria) and 2 µl of template DNA. Cycling conditions were as follows: initial denaturation at 95°C for 5 min, 25 cycles of 95°C for 30 s, 54°C for 1 min, 72°C for 1 min, and a final elongation at 72°C for 10 min. PCR products were purified using Sephadex (Sigma Aldrich, Germany). Amplicons from three separate PCR amplifications were pooled for subsequent cloning and sequencing analysis. 16S rRNA gene clone libraries were constructed using the StrataClone PCR cloning kit following the manual (Agilent Technologies, Austria). White colonies containing the insert were picked from Luria Bertani (LB; Sigma Aldrich, Germany) plates containing 100 µg/ml ampicillin (Sigma Aldrich, Germany) and 80 µg/ml 5-Bromo-4-chloro-3-indolyl β-D-galactopyranoside (X-Gal; Biochem, Germany) and then grown overnight at 37°C in liquid freezing

media (Wittenberg et al., 2005) containing ampicillin (12.5 µg/ml). Then an aliquot of 1 µl was used for amplification of the insert using oligonucleotide primers M13for (5'-GTAAAACGACGGCCAG-3') and M13rev (5'-CAGGAAACAGCTATGAC-3'). Reaction mix (50 µl) contained 3 mM $MgCl_2$ and 2 U of Taq® polymerase and the same concentration of reaction buffer, dNTP mix and primers as for the 16S rRNA gene PCR. Cycling conditions included an initial denaturation step at 95°C for 5 min, 30 cycles of 95°C for 45 s, 58°C for 1 min, 72°C for 2 min, and a final elongation at 72°C for 10 min.

Partial 16S rRNA gene sequencing and sequence analysis

M13 PCR products were sent for sequencing to LGC Genomics (Germany) using the standard sequencing primer T3 (5'-AATTAACCCTCACTAAAGGG-3'). Electropherograms were then imported in the Geneious software for peak quality check. Fine sequences were generated by manual trimming (Kearse et al., 2012). Sequence data are available from http://dx.doi.org/10.6084/m9.figshare.1019902.

Preprocessed sequences and ancillary metadata were analysed using Quantitative Insights Into Microbial Ecology (QIIME) pipeline (Caporaso et al., 2010). Quality filtering consisted of excluding homopolymer runs (> 6 nt) and ambiguous bases (> 6 nt). Chimera removal and OTU selection were accomplished with USEARCH considering a pairwise identity percentage of 0.97 (Edgar et al., 2010; Edgar et al., 2011). Taxonomy assignment was performed employing the naïve Bayesian RDP classifier with a minimum confidence of 0.8 (Wang et al., 2007) against the last version (May 15, 2013) of the Greengenes database (http://greengenes.secondgenome.com/). The Greengenes tree was then used for phylogeny-based beta diversity calculations (McDonald et al., 2012).

Statistical analysis

A set of different alpha-diversity metrics based on richness (Chao's richness estimator) (Chao, 1984) and diversity (Simpson's diversity index) (Simpson, 1949) were calculated after a rarefaction step based on a randomly selected subset, depending on the number of sequences in the poorest sample.

The Good's coverage estimator was used for estimating the sampling completeness and calculating the probability that a random selected amplicon sequence from a sample has already been sequenced (Good, 1953). A two sided pairwise t-test with false discovery rate (FDR) correction was used for comparing the alpha diversity of samples.

An exploratory unsupervised classification approach was computed by means of hierarchic clustering with Ward´s criterion using the vegan R package (Ward, 1963; Ramette, 2007).

The community structure and diversity assessment was performed using multivariate analysis consisting of: i) an unconstrained ordination offered by principal coordinate analysis (PCoA), ii) a constrained analysis with reference to a specific hypothesis with canonical correspondence analysis (CCA), iii) a statistical test of the hypothesis and iv) a multivariate analysis of variances, v) a characterization of the taxa responsible for the multivariate patterns.

An unweighted Unifrac dissimilarity matrix (Lozupone & Knight, 2005) was calculated jackknifing read abundance data at the deepest level possible (35 sequences) after 100 reiterations. Overall dissimilarities in microbial community structures among samples were displayed using PCoA (Gower and Blasius, 2005) and plotted using KiNG (Chen, Davis and Richardson, 2009). Differences in community structure related to the specific categories were displayed by means of constrained ordination technique using the vegan R package. Significance of constrained ordination variables was tested via permutation test (Oksanen et al., 2013). The multivariate null hypotheses of no differences among *a priori* defined groups was investigated using the PERMANOVA approach originally developed by Anderson (2001) and re-implemented in the vegan R package as ADONIS function. The permutational multivariate analysis of variance was applied using the Unifrac distance matrix previously calculated for the multidimensional scaling ordination.

Taxon-group association analysis was calculated using the indicspecies R package (Cáceres and Legendre, 2009). Furthermore, a G-test of independence corrected with the FDR method was computed to determine whether the presence of one OTU was associated either to the R2A or to the YEA medium and to which water sample and temperature incubation. Similarly but for determining if the OTU relative abundance was different between the samples grouped by medium, water and temperature, an ANOVA with FDR method was applied. The latest and the previous tests were calculated in QIIME.

Results and Discussion

Bacterial diversity based on 16S rRNA gene sequence analysis

In total 650 clones were sequenced. Taxonomic assignments were based on 563 sequences, because potential chimeras were excluded from further analysis. This corresponds to an average of 46.9 ± 10.8 (n=12) sequences per sample, with an average read length of 484.8 bp and a min and max of 268 and 570 bp, respectively. Sequence clustering yielded a total number of 16 (6 ± 2.5) OTUs. When considered grouped by the medium, the number of sequences in R2A was 252 and 311 in YEA (281.5 ± 29.5), corresponding to 14 and 15 OTUs, respectively. The number of sequences in IFA, N167 and N170 water samples was 228, 174 and 161 (187.7 ± 29) respectively, corresponding to 14, 11 and 9 (11.3 ± 2) OTUs. Grouped by the temperature, sequences assigned to 22°C and 37°C incubation were 269 and 294 (281.5 ± 12.5), accounting for 9 and 16 OTUs (12.5 ± 3.5), respectively.

For all clone libraries saturation curves were plotted (data not shown), nearly reaching the plateau, which indicates good saturation level. This was further confirmed by comparing the observed OTU abundance (i.e. number of sequences assigned to distinct OTUs) and Chao1 richness estimator, as well as by Good's coverage estimator (Table 1).

Examination showed in four out of twelve cases 100% coverage. The coverage for the others was measured to be in the range of 91 - 97% and therefore close to saturation. One outlier (82.9% coverage) was identified in the case of YEA22-IFA, indicating the insufficient number of analyzed sequences, which could be a result of greater diversity in the sample as further indicated by highest Chao1 richness estimator. The observed diversity data further depicted a higher OTU abundance from cultivations at the temperature of 22°C (3 - 11 OTUs) than 37°C (3 - 5 OTUs) and overall the highest from R2A22. This is in agreement with previous phenotypic examinations from Wernicke and co-workers (1990) were highest diversity indexes were obtained from R2A agar at 20°C. R2A was developed by Reasoner and Geldreich (1985) and was designated as low nutrient media as it is considered to represent a lower carbon concentration and ionic strength (Reasoner and Geldreich, 1985; Allen, Edberg and Reasoner, 2004). Still, R2A contains nutrient concentrations significantly higher (800 x) than normally found in water habitats (Hammes et al., 2008). Nevertheless, lower temperatures and R2A media are closer approximates of environmental conditions (Allen, Edberg and Reasoner, 2004) and as observed in this study allow the recovery of a greater variety of culturable bacteria.

Potential effect of cultivation condition on diversity measures was investigated with pairwise comparison t-test. Significant differences between the observed OTUs ($p < 0.05$) and between the Simpson's diversity values ($p < 0.01$) were obtained for the temperature category.

Table 1. Bacterial diversity measure. Observed prokaryotic richness and diversity estimates based on OTU clusters.

Cultivation condition	Observed OTUs[a]	Chao1	Simpson %[a]	Coverage %[b]
		Water sample IFA		
YEA37	5.0	5.0	62	100
R2A37	4.0	5.0	52	94
YEA22	9.0	24.0	67	83
R2A22	11.0	11.0	88	94
		Water sample N167		
YEA37	3.0	3.0	57	100
R2A37	4.0	5.0	52	94
YEA22	6.0	6.0	74	97
R2A22	9.0	10.0	72	91
		Water sample N170		
YEA37	5.0	6.0	62	94
R2A37	3.0	3.0	58	100
YEA22	3.0	3.0	59	100
R2A22	6.0	6.0	73	97

[a] statistically significant differences ($p < 0.05$) between the observed OTUs according to the temperature and for the Simpon's diversity values always under the temperature condition ($p < 0.01$).

[b] estimated using Good's coverage estimator (Good, 1953)

Culturable microbial community composition based on 16S rRNA gene sequence analysis

The composition of the culturable microbial communities from three water samples (IFA, N167, N170) and four corresponding cultivation conditions is shown in Figure 1. In total 16 OTUs (i.e. sequence types) could be identified; related OTUs from same family were subsequently combined upon a threshold of 97% to one taxa resulting in 12 different taxa. The relative low number of OTUs is presumed in low resolution of partial 16S rRNA gene sequence that could be assigned at class to genus level only. Beyond that the number of OTUs is restricted trough the cultivation step. However, in the study from Farnleitner and co-workers (2004) similar numbers were detected with a maximum of 12 OTUs from culturable HPC population. An expanded spectrum would be expected from cultivation independent analysis, which would reveal OTUs missed out by selected cultivation conditions. However, in this study the focus was on the effects of different cultivation condition, and thus the cultivation-independent analysis was not performed.

Most taxa (eleven out of twelve) were affiliated and resolved at least to family or even genus level. One exception is Tax4 that could only be resolved to class level (*Bacilli*). Detailed examination of sequences assigned to Tax4 (*Bacilli*) and related Tax5 (*Bacillaceae*) revealed that these correspond to different regions of 16S rRNA gene. Sequences assigned to Tax5 were located in the 5' end and those assigned to Tax4 more towards the 3' end of 16S rRNA gene (consensus sequences were found to begin at *E. coli* position 28 and 1032 respectively). Although, both fragments span several hypervariable regions, previous publications suggest that 5' end hypervariable regions allow for more exact classification within this group (Goto et al., 2000; Wang et al., 2007). This explains the somewhat poor taxonomical resolution obtained for Tax4 and emphasizes the importance of proper fragment selection in case when only short reads are used.

The composition of the culturable community from three analyzed samples and further from different cultivation conditions appeared quite different (Figure 1). Sample IFA is characterized by most uniform composition of culturable microbial community throughout all tested cultivation conditions. Results from the sample IFA proposed that using different described HPC media, either R2A or YEA at both temperatures, would come up with almost overlapping spectrum. On the other hand, sample N167 exhibited highest diversity among tested cultivation conditions, where within each cultivation condition different abundances of recovered taxa were detected. Sample N170 was the only one with clear cultivation condition effect that could be contributed to temperature, with similar composition for high and low temperature. However, in all three water samples, a "core community" (Tax1 - 7) could be detected, albeit not using one single cultivation condition. Also the abundance of detected taxa in different samples and in different cultivation conditions varied.

Proteobacteria and Firmicutes were the two phyla that dominated the HPC cultivations. Firmicutes were observed to be unspecified *Bacillaceae* (Tax5) or *Bacilli* (Tax4). They were the dominant bacterial group identified in three out of twelve cultivations (R2A37-IFA, R2A37-N170 and YEA37-N170), and grew preferentially at higher temperature. Payment et al. (1994) already reported about frequent isolation of *Bacilli* with a temperature of 35°C from most drinking-water supplies. Beyond Firmicutes, Proteobacteria (Beta- and Gammaproteobacteria) dominated in nine out of twelve cultivations. The dominance of Proteobacteria was also shown by cultivation independent pyrosequencing techniques in well water samples or from treatment plant (Kwon et al., 2011; Liu et al., 2012).

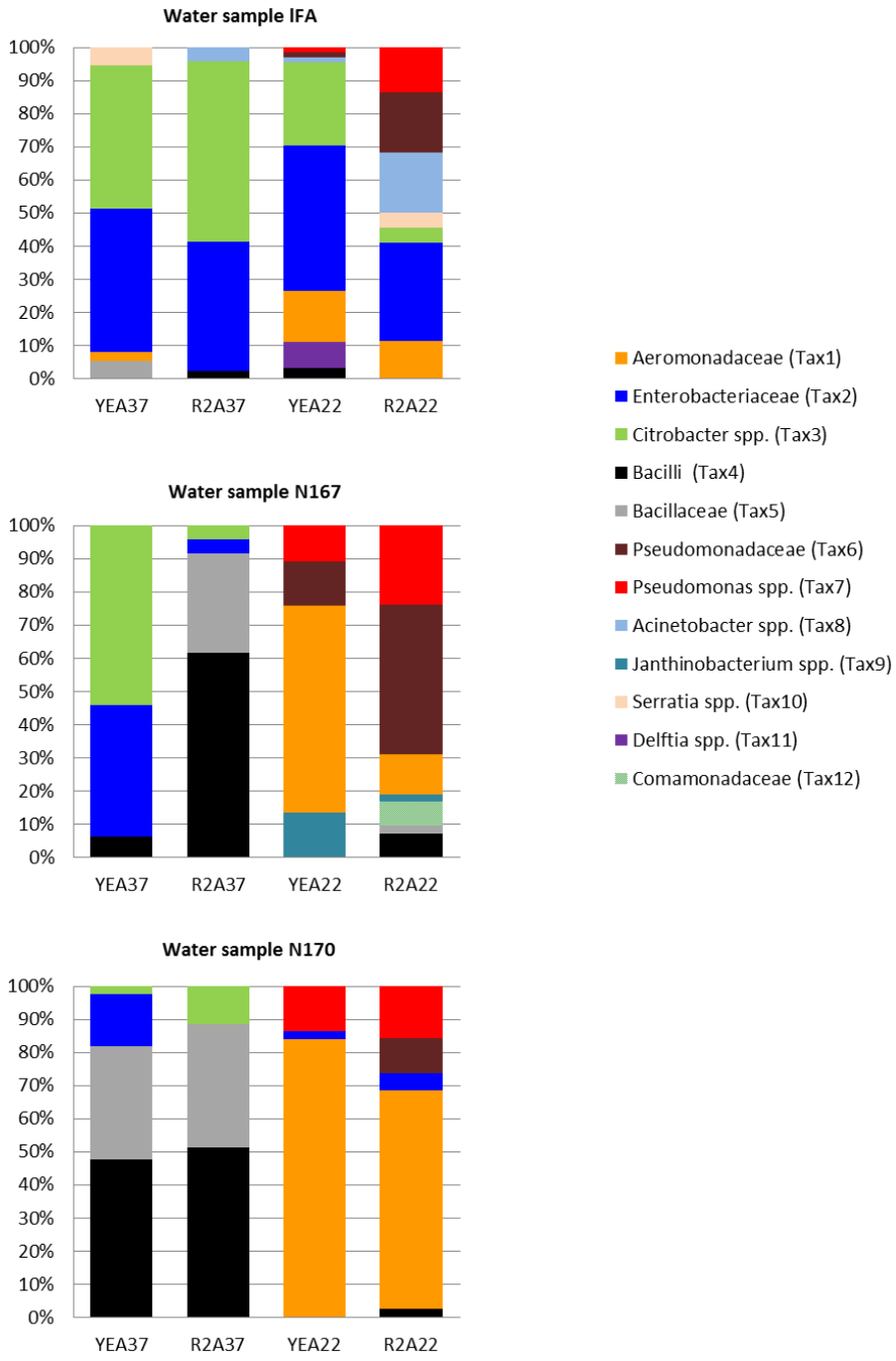


Figure 1. 16S rRNA gene sequence analysis. Composition of culturable microbial heterotrophic community under different tested cultivations (YEA37, R2A37, YEA22, R2A22) detected in three analyzed water samples (IFA, N167 and N170).

In our study Gammaproteobacteria were most dominant class among Proteobacteria, represented by four families (*Enterobacteriaceae, Aeromonadaceae, Pseudomonadaceae* and *Moraxellaceae*). *Enterobacteriaceae* (Tax2, 3 and 10) were predominantly found in the IFA sample in all tested cultivation conditions, indicating that this sample was probably enriched for this particular family. In general highest abundance was observed at YEA37. YEA and high temperature was introduced in Europe as 'body temperature count' in order to allow the growth of fecal bacteria from animal or human origins (Allen, Edberg and Reasoner, 2004). This is in congruency that *Enterobacteriaceae*, known to include a range of fecal derived bacteria, favored the growth at this cultivation conditions.

Aeromonadaceae (Tax1) and *Pseudomonadaceae* (Tax6 and 7) were detected almost exclusively at low temperature; the single exception was detection of *Aeromonadaceae* in YEA37-IFA cultivation. *Aeromonadaceae* prevailed on YEA and *Pseudomonadaceae* were more abundantly determined from R2A. Both are commonly known to inhabit water and more likely to grow on lower temperatures. This result is of particular interest, as these groups include members considered to be opportunistic pathogens (e.g. *P. aeruginosa*) associated with various infections (Allen, Edberg and Reasoner, 2004; Pavlov et al., 2004). Notably, it has been reported, that *Aeromonas* spp. are generally not easily detected from water samples by HPC method (APHA, 1998; Allen, Edberg and Reasoner, 2004), but our findings showed that high amounts of *Aeromonadaceae* could be recovered from cultivation at lower temperature, suggesting that YEA22 could be a good candidate for the detection. However, to ascertain this, a more comprehensive sample set has to be examined.

Moraxellaceae (Tax8 – *Acinetobacter* spp.) were detected only in sample IFA with highest abundance on R2A22, which correlates with previous findings by Reasoner (2004) that R2A is more suitable for their growth. *Betaproteobacteria* (families *Comamonadaceae* (Tax12) and *Oxalobacteraceae* (Tax9)) were rarely detected and if, then only at low temperature. Unique taxa were often assigned to a certain cultivation condition, as for example *Delftia* spp. (Tax11) to YEA22 or *Janthinobacterium* spp. (Tax9) to low temperature.

By statistical analysis no OTU was found to be associated uniquely to a particular cultivation, according to the G-test for independence. OTU category significance test (multipatt function in indicspecies R package) showed significant p-values for OTUs assigned to the *Aeromonadaceae* family (Tax1) ($p < 0.01$) and to *Pseudomonadaceae* (Tax6 and Tax7) ($p < 0.05$) for the 22°C incubation group. The only taxon turned out to be significantly correlated to the 37°C temperature group was Tax3 ($p < 0.05$), related to the *Enterobacteriaceae* family.

Different abundance significance test based on ANOVA showed that for the N170 sample the mean count of *Bacilli* was higher at 37°C (Tax 4 and Tax5), whereas the presence of *Aeromonadaceae* (Tax1)- and *Pseudomonadaceae* (Tax7)-related OTUs was more consistent at 22°C ($p < 0.05$).

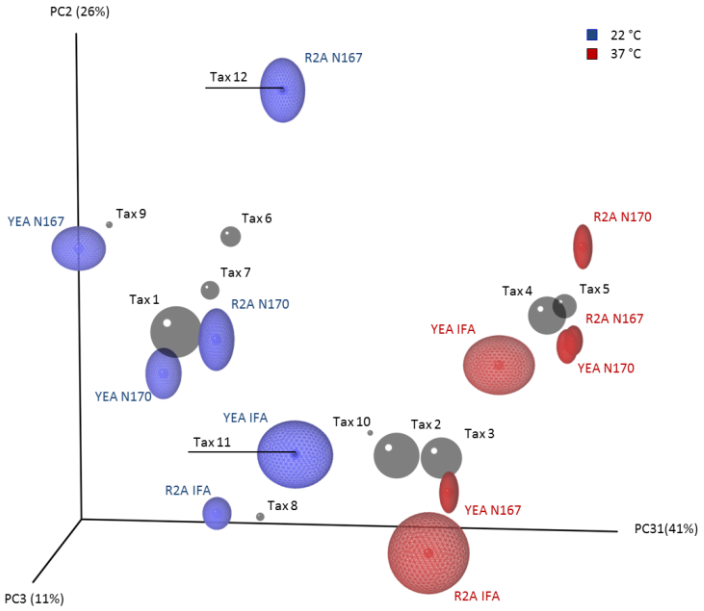
Multivariate statistical analysis of cultivation conditions and samples

To evaluate statistical significances of the cultivation parameters, i.e. temperature and media, an exploratory multivariate analysis based on hierarchic clustering was performed before making any hypothesis-driven assumption.

Unsupervised analysis offered by PCoA ordination method (Figure 2) clearly demonstrated the clustering of samples according to the temperature, illustrated in blue (22°C) and red (37°C) spheres. Grey spheres represent the taxa detected in the cultivations. Diameters of data points are proportional to the number of sequences assigned.

The close proximity of the cultivations is due to temperature and similarity of HPC composition (i.e. sharing of the same taxa). This is exemplified in the allocation of R2A22-N170 and YEA22-N170 to Tax1 (*Aeromonadaceae*) and of R2A37-N170 and YEA37-N170 to Tax4 and 5 (*Bacilli* and *Bacillaceae*). Also cultivation R2A37-N167 was located in the same cluster because of the abundant detection of Tax4 and 5. All cultivations from the IFA sample grouped together, described by the constant numerous detection of Tax2 (*Enterobacteriaceae*) and Tax3 (*Citrobacter* spp.). The small spheres represent less abundant taxa such as *Acinetobacter* spp. (Tax8) and *Serratia* spp. (Tax10). Outlying spheres represent unique taxa present in particular cultivations, as seen for R2A22-N167 by the detection of *Comamonadaceae* (Tax12). Therefore it becomes evident that, even though the temperature had the strongest effect, media also slightly influenced the growth of particular genera.

Previous findings of the PCoA plot (unconstrained model) were confirmed by the permutation test on CCA analysis (data not shown) as constrained model ($p < 0.001$). Clusters are also primarily formed according to the temperature 22°C and 37°C and their affiliated taxa. Multivariate analysis of variances (PERMANOVA) applied to the UniFrac dissimilarity matrix previously used for the PCoA provided a significant p-value ($p < 0.01$) for the cultivation temperature.



(Tax1 - *Aeromonadaceae*; Tax2 - *Enterobacteriaceae*; Tax3 - *Citrobacter* spp.; Tax4 - *Bacilli*; Tax5 - *Bacillaceae*; Tax6 - *Pseudomonadaceae*; Tax7 - *Pseudomonas* spp.; Tax8 - *Acinetobacter* spp.; Tax9 - *Janthinobacterium* spp.; Tax10 - *Serratia* spp.; Tax11 - *Delftia* spp.; Tax12 – *Comamonadaceae*)

Figure 2. Multivariate analysis by ordination method. The unconstrained model (PCoA plot) illustrated the clustering of sample according to temperature; blue (22°C) and red (37°C) spheres. The bioplots for each taxon were plotted with diameters proportional to the numbers of assigned sequences. Each sample data point was drawn as central point, surrounded by a semi-transparent cloud representing the variation in jackknifed Unifrac results.

Conclusions

Currently applied HPC methods vary in defined cultivation conditions, either high or low nutrient media are utilized, as well as high and low incubation temperature. We agree with the previously published reviews (Bartram et al., 2003; Allen, Edberg and Reasoner, 2004; Reasoner, 2004) that there will never be a single cultivation condition able to recover the entire heterotrophic bacteria. However, our study demonstrated that the choice of cultivation parameter can significantly affect the composition and abundance of detected heterotrophic bacteria, and this will influence the overall outcome of the water quality assessment. The most significant effect was observed for temperature with corresponding statistical value of $p < 0.01$. According to temperature a core community was identified, such as *Enterobacteriaceae* and *Bacilli* prevailed at high temperature (37°C) and opportunistic pathogens *Aeromonadaceae* and *Pseudomonadaceae* at lower temperature (22°C). In

general, incubation at 22°C allowed for the detection of higher OTU abundances; in combination with R2A medium, most comprehensive insight into diversity of recovered heterotrophic bacteria is obtained. Media type showed no significant effect in statistical analysis, but results indicated that media may direct the abundances of recovered taxa. Taking into consideration the quantitative nature of HPC analysis, this effect should not be disregarded. Furthermore the suitability of nutrient media is expected to be dependent on the composition of the native microflora present in the examined water sample and wherever practicable should be tested beforehand (Sartory, 2004). Ongoing developments in the field of culturomics raised the proportion of culturable microbial community beyond conventionally reported 1% (Amann, Ludwig and Schleifer, 1995). However, as demonstrated in study by Lagier et al. (2012), comprehensive determination of microbial communities with cultivation-based methods remains extremely complex (70 cultivation conditions were needed for the 100% recovery of species from gut microbiome) and beyond possibilities of routine analysis.

Generally our results confirm the importance of the basic concept (i.e. application of two incubation temperatures), but also indicate some potential limitations. Concluding, HPC method represents a valuable tool for trend monitoring of efficiency testing of treatment processes because of the detection of fluctuations, rather than absolute values. However, single assessment of a sample with one defined HPC method may be of less significant value and may result in biased conclusions of the quality of a water sample according to cultivation condition used.

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Final discussion

Molecular assays

Cultivation-based methods have been applied for a long time and are well established standardized procedures for water quality assessment (Brettar and Höfle, 2008; Aw Gim and Rose 2012). However, new technologies are constantly evolving and in the field of microbial detection, molecular approaches present a valuable alternative. To date a large array of molecular techniques is available and studied for their application in diverse fields such as clinical, environmental, food and water application.

Nucleic acid based technologies may overcome some shortcomings of cultivation-based methods to allow more reliable, specific and moreover rapid analysis. Therefore they are considered as promising alternative for water quality assessment. Highest potential was recognized for RT-PCR approaches. Recent applications of RT-PCR in the field of water quality were reported for microbial source tracking (Reischer et al., 2006; 2007) and detection of single fecal indicators and pathogens (Maheux et al., 2013; Yáñez et al., 2011). Developed RT-PCR techniques for the detection of pathogens in water have already been included in some governmental guidelines in the U.S. (Varma et al., 2009). However, the potential of RT-PCR was to date not shown for the entire set of quality parameters defined in the drinking water assessment regulations. Furthermore, the viability assessment in RT-PCR analysis for water quality assessment was not demonstrated.

Proof of principle of PMA-RT-PCR

Previous studies using RT-PCR without PMA-treatment resulted in substantial amount of false positives in comparison to cultivation-based ISO norms. This was due to the false positive detections of DNA from dead or membrane comprised cells in the analysis. Therefore the implementation of live/dead differentiation in RT-PCR is of uttermost importance for application in water safety.

Viability assessment was achieved through application of DNA intercalating dye, PMA, combined with RT-PCR methodology. In the proof of principle study the PMA treatment was included into the established protocol for RT-PCR analysis for all defined microbial parameters. The optimal dye concentration (10 µM), photo-activation time (5 min) and the ability to excluded dead cells from RT-PCR analysis was approved with artificially prepared samples comprising of mixtures of viable, heat killed and viable and heat killed *E. coli* and *P. aeruginosa* cells. The heat killing process (75°C for 10 min) was used, as it was reported as a practicable test method, which also impedes membrane damage (Nocker, Cheung and Camper, 2006; Yáñez et al., 2011). The heat killing process of bacteria may be

influenced by species type, but by approving cells viability on cultivation plate it was ensured that it was sufficient for tested species.

The successful proof of principle of PMA-RT-PCR was shown for a multiple spike of *E. coli* and *P. aeruginosa* in presence of a viable abundant background microflora (2 - 3 $\log_{10}$ higher concentration than targeted species). Application of PMA-RT-PCR resulted in significant (3 $\log_{10}$) or complete suppression of mixtures of heat killed target organism. Better PMA performance was observed for *P. aeruginosa* than for *E. coli* detection. In case of the high indicator spike, only partial inhibition of signal from heat killed *E. coli* cells was achieved. However, in that samples a high cell density (>10^8 cells/m) was present potentially interfering with the capacity of PMA, as the saturation limit is reported to be 4-4.5 $\log_{10}$ (Yáñez et al., 2011). Further species- and sequence-dependent differences in the efficiency of PMA binding was previously reported by Fittipaldi, Nocker and Codony (2012). Therefore the PMA-RT-PCR should be in best case optimized (dye concentration and photo-activation step) for each target organism to obtain complete suppression of DNA targets from dead cells in analyzed matrices. However, for the application in routine use, it is not practicable to perform different protocols for each target organism. The main challenge for the proposed application is the optimization of one protocol for multiple targets from complex matrices, which was partially achieved in the proof of principle study for low spike samples. Additional improvements in assay setup could be obtained by targeting longer amplicon lengths (>200bp), probably enhancing the discrimination potential of intercalating dye technology in RT-PCR (Contreras et al., 2011), however this is often not appropriate in use of hydrolysis probes, such as TaqMan® chemistry. Improved PMA performance could also be achieved by the addition of non-ionic detergents (e.g. IGEPAL or Triton-X-100), which is reported to increase the capacity of PMA dye (Coundray-Meunier et al., 2013). In here established protocol a 0.01% Tween20 solution was used, which could be the result of enhanced effect of PMA also in high spike samples.

In the analysis of artificially prepared viable mixtures slight reduction of viable cells due to PMA treatment was recognized. Moderate cytotoxicity of PMA on viable cells was also previously investigated for 50µM concentration with an average reduction of 1.3 log units in RT-PCR analysis (Yáñez et al., 2011). However, even higher cytotoxicity was reported from EMA on viable cells (2-3 log units). Even though, in this study a slight loss of DNA from viable cells was observed, but did not result in false negative outcomes in the RT-PCR analysis. It has to be notified that in case of tested parameters (*E. coli* and *P. aeruginosa*) qualitative and not quantitative analysis is required accordingly to zero tolerance in Austrian DWD (2001) and therefore no bias is ensued.

In addition, to PMA-based viability assessment other strategies have been proposed such as active labile compounds (ALC) designated by Nocker and Camper (2009). This approach should compensate

for the negative influence on the viable cells that was partially seen for PMA and even higher for EMA. ALC complement an intercalating agent such as PMA with an enzymatic cleavable linker and a crosslinking moiety. This chemical construct is inactive in viable cells through the cleavage of the linker by an active enzyme, whereas in dead cells with decreased metabolic activity the linker is not cleaved and consequently results in the intercalation of the ALC in the DNA. However, also ALC compounds have a limitation that metabolic activity can persist after cell death and thereby also might give false detections. Another idea would be to design new similar compounds to PMA, containing more positive charges and be structurally larger in order to optimize the discrimination potential and membrane permeability. Further studies are needed to investigate the chemical properties of these viability dyes, because to date it is not readily understood how much membrane damage is necessary for dye entry (van Frankenhuyzen, 2011).

Evaluation of PMA-RT-PCR for *E. coli*, *Enterococcus* spp. and *P. aeruginosa*

Successful application for PMA-RT-PCR was demonstrated in proof of principle study in artificially prepared samples, reflecting natural water habitats. However, to depict the performance of PMA-RT-PCR an extensive evaluation on real life samples is prerequisite.

Therefore diverse drinking water samples, including well water samples and expected to have some contamination, and further on process water samples (water treatment plant, cooling towers) were tested. Results demonstrated the high specificity of PMA-RT-PCR with best performances for *E. coli* and *P. aeruginosa* detection. This was highlighted in complete correlation of PMA-RT-PCR to reference test for *E. coli* detection. Results suggested that the utilization of specific marker, such as *uidA* gene (Takahashi et al., 2009) or *regA* (Lee et al., 2008) for detection of *E. coli* and *P. aeruginosa* respectively, improved the detection selectivity. Functional markers can achieve higher specificity than universal makers (16S rRNA and 23S rRNA) as these may be prone to detect also closely related non-target organisms. A low rate of false positive detections was observed in drinking water evaluation, which could be a result of additional detection of viable but non-culturable cells (VBNC) or starved cells not determined by reference test.

Beside the high specificity the main requirement for the application of diagnostic assays is the high sensitivity. Unfortunately, low sensitivity was observed for all PMA-RT-PCR assays. However, this was also seen in RT-PCR without PMA treatment, so it could be concluded that this is not a negative effect of PMA treatment. The bottleneck of sensitivity of nucleic acid-based detection techniques is attributed, in most cases, to insufficient sample preparation because first concentration of bacteria and then extraction of DNA is necessary. Sample preparation in established protocol is separated in i) concentration of bacteria through filtration, ii) resuspension of bacteria from filter membrane and iii)

extraction of DNA. In each sample preparation steps a loss the certain extent is expected (Agudelo et al., 2010; Brettar and Höfle, 2008). Membrane filtration was considered not to be the critical step, because even in cases were smaller volumes were filtrated (because of filter clogging due to particular material in the sample) no false detection was obtained.

The incubation of PMA and then resuspension of cells from filter membrane and transfer procedure to a microcentrifuge tube could lead to slight losses. Most challenging and frequently insufficient is the DNA extraction step (Haugland et al., 2012; Staley et al., 2012; Lloyd et al., 2010; Lemarchad et al., 2005). Therefore the testing of several protocols or commercially available kits for maximal DNA extraction efficiency is of uttermost importance. Different extraction protocols were tested in course of protocol establishment and the selected WaterMaster™ DNA Purification kit (Epicentre, USA) resulted in highest recovery compared to Bacterial GenElute Kit (Sigma Aldrich, Germany) or PowerWater® DNA isolation kit (Mo-Bio, USA) or phenol/chloroform extraction. In addition WaterMaster™ DNA Purification kit has an inhibitor removal technology, which was considered to be valuable for RT-PCR to be often sensitive against different contaminants.

Improvements in sample preparation could alleviate the sensitivity bias of the molecular detection assays. One idea could be to minimize number of steps, ideally assembling the single steps to one step, to increase the recovery. A more efficient recovery could be achieved as outlined by Slimani et al. (2011) where filtration was performed for concentration of bacteria and then PMA incubation was directly conducted on membrane filter and the filter was subjected to DNA extraction procedure.

Nevertheless, it has to be considered that probably some clogging material on filter may interfere with PMA treatment step.

Evaluation of PMA-RT-PCR for indicator organisms (coliforms and HPC)

For the detection of indicators PMA-RT-PCR evaluation showed that the utilization of alternative molecular assays such as *Enterobacteriaceae* for detection of coliforms and total bacteria as analogon to heterotrophic plate count was not adequate. This can be explained by the differences in the target spectrum. The applied reference test for coliforms, the chromogenic assay Colilert®-18 (IDEXX laboratories) utilizes the enzyme *β-galactosidase* for detection. On the other hand molecular detection assay was based on the 23S rRNA gene, targeting the complete *Enterobacteriaceae* family. The establishment on the *Enterobacteriaceae* family had to be applied because previously published and tested primers targeting *lacZ* gene (encoding for *β-galactosidase*) were not specific enough and excluded the detection of relevant coliform members such as *Citrobacter* spp. or *Klebsiella* species. Development of modified primer/probe system was attempted, but the observed diversity of *lacZ* sequences from different coliform species clearly indicated that the design of group specific primers based on this gene is not feasible. Phylogenetically coliforms belong to the family of

Enterobacteriaceae and therefore this family was selected as an alternative target group. However, not all members of the *Enterobacteriaceae* family are coliforms, and therefore it was expected that molecular detection of taxonomically assigned heterogeneous *Enterobacteriaceae* family would result in accumulation of "false positives" when compared to reference test. However, some shortcomings were also reported for chromogenic assays (Colilert®-18). In particular it was shown that assay was strongly influenced by environmental factors and that high abundance of microflora can cause interference in read outs of the assay (Maheux et al., 2014). Furthermore some non-coliforms were reported to give false positives in the Colilert®-18 such as *Aeromonas* spp. able to utilize substrate in this test (Rompré, 2002).

The second defined indicator regularly assessed in scope of water quality is the heterotrophic plate count (HPC). HPC relies on enumeration of colonies grown at 22°C and 37°C on a defined substrate in order to assess the total number of propagated heterotrophic bacteria. Both parametric values had to be compared to a single quantitative result obtained from total bacterial 16S rRNA (PMA)-RT-PCR. In general molecular assay regularly determined higher cell counts than reference methods. This overestimation can be attributed to several factors. Firstly, molecular analysis also enumerates the non-cultivable part in ecosystem, which was shown by Hammes and Egli, 2008 or Amann, 1990. However, utilization of 16S rRNA gene, a multi copy gene, results in higher quantitative values. Accordingly, our initial focus was to target single copy bacterial phylogenetic markers such as *rpoB* (ribosomal polymerase B; Powel et al., 2006) or *RNAseP* (ribonuclease P; Dolan et al., 2004), but tested primers failed to yield quantification of all selected bacteria in inclusivity tests. Furthermore for *gyrB* gene (encoding the subunit B of bacterial gyrase) gene no RT-PCR system could be established, therefore the 16S rRNA gene was selected. The intention was to establish quantitative threshold value for 16S rRNA PMA-RT-PCR that would allow comparable assessment of water quality as HPC method. However, the evaluation demonstrated that no reliable threshold value could be ascertained as PMA-RT-PCR resulted in wide distribution of ~5 $\log_{10}$ and furthermore yielded conflicting results in some cases in relation to HPC values. One option for the application of 16S rRNA PMA-RT-PCR could be used for monitoring of disinfection processes, thereby determining changes rather than absolute quantitative values. The advantage of molecular assay in this case could be postulated in more realistic and accurate ascertained values than determined with cultivation dependent analysis, which may be strongly biased by the non-cultivable cells.

HPC method itself showed in evaluation study in several cases controversial results that one parametric value at one temperature resulted in no exceeding (< threshold), whereas the other temperature enumerated substantial counts (>threshold). These discrepancies could lead to biased conclusions on quality of the analyzed water source. In past the HPC method has been often reviewed

and their significance and informative value in quality assessment was questioned. It was also subjected to extensive changes to ensure best possible recovery of heterotrophic organisms (Reasoner, 2004), thus resulting in variations of the method (Bartram et al., 2003). Currently approved and applied HPC protocols differ significantly and it is also well documented that depending on the method used the enumeration of HPC population varies (Allen, Edberg and Reasoner, 2004; Bartram et al., 2004; Reasoner 2004). Therefore the aim was to investigate the composition of heterotrophic plate count bacteria that grew under different proposed HPC cultivation conditions such as high nutrient media - yeast extract agar (DIN EN ISO6222) or low nutrient - media R2A (EPA) and temperatures of 22°C and 37°C. 16S rRNA gene sequence analysis revealed differences in the community composition and the abundance of detected taxa accordingly to cultivation condition applied. Statistical significance was determined for the temperature, which confirms the basic HPC concept of prerequisite analysis at two temperatures (22°C and 37°C) to allow for more complete view of heterotrophic culturable cells. Media type showed no significant effect in statistical analysis, but different abundances were obtained on different media type, which may be allocated to variable quantitative results of HPC. In conclusion, our results indicate that the HPC method should be reconsidered, especially in the case of the single assessment of a water sample as method could give controversial outcomes.

Future outlook

New technologies, such as RT-PCR with included viability assessment, hold major advantages over conventional methods. These are seen in rapid and specific analysis, ease of use, and ability to achieve high sample throughput. Accordingly, their application in quality assessment could enable a more exact and more regular analysis.

Given the careful optimization, validation and the preparation of a standardized protocol of PMA-RT-PCR, it represents a promising tool to supplement some culture methods. Indeed, some PCR assays are already approved by regulatory authorities. A need for improvement remains in the sample preparation procedures. Once these are optimized, first applications could be considered in monitoring of disinfection processes of treated drinking water as there a high sample throughput is essential.

Further direction of RT-PCR could encompass the detection of pathogens and viruses not only be approved for water but also for application in food diagnostics. Beside the RT-PCR analysis focus should be set to the upcoming field of NGS technologies to gain deeper insights in microbial communities and measures of microbial quality of water.

The implementation and acceptance of new molecular assays for water quality assessment will need persuasive data, standardized protocols and will be strongly dependent and driven by economic traits such as low costs, simplicity and automation potential.

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Curriculum Vitae

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02/09 – 03/2009	**Practical work** at **Vienna General Hospital (AKH)** Institute of Pathophysiology
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AppendixI

Virology Review

Novel tools for environmental virology

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