COMPONENT 3.5.1.

Development of FISH methods for detection of pathogens in biofilm

TECHNEAU

WP 3.5.1 Development of FISH methods for detection of pathogens in biofilm

Title
Development of FISH methods for detection of pathogens in biofilm

Author(s)
Simona Larsson, Talis Juhna, Linda Mezule (RTU), Liv Fiksdal (NTNU)

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PU = Public

Introduction

Untreated water contains many bacteria, protozoa, fungi and viruses. Some of them may pass into drinking water and become established in biofilm (a complex layer on the surface of the water distribution system). Attachment of organisms to surfaces has been shown to alter their physiology rendering them more active in absorbing nutrients as well as more resistant to environmental stress. It has been well documented that water, which reaches the consumer's tap, is often of inferior microbiological and sometimes chemical quality, when compared to that which left the waterworks.
In this report the development of fluorescence-in-situ-hybridization (FISH) method is described. The method can be used not only to identify the microorganisms in bulk water but also to identify the bacteria directly on the pipes and other surfaces in water distribution systems without removing the sample.

Importance

FISH method is (i) rapid to perform, (ii) able to detect the major groups of concern, (iii) reliable, (iv) inexpensive and (v) non destructive, *i.e.* such that it does not disturb the sample composition. An important problem in environmental samples has previously been the low signal-to-noise ratio and this report brings an improvement in this respect. FISH method does not require any expensive devices or reagents hence it is hoped that in the future also smaller and lesser-equipped laboratories all over the world could apply the method on a day-to-day basis.

Approach

We selected *Escherichia coli* as a model organism for pathogenic bacteria and *Aeromonas hydrophila* as model organism for the opportunistic pathogens, capable of regrowth, for the development of the method. The FISH protocol was the optimized for *E. coli* detection using PNA probe, both in lab and environmental samples. A novel PNA probe was designed for *A. hydrophyla* and the improved protocol was verified for this organism. Membrane biofouling was studied using DNA probes for detection of α-, β- and γ- proteobacteria.

Result

Improvements have been made for analyses of *E. coli* cells in (i) in lab-scale samples (water and biofilm) and (ii) environmental samples (water and biofilm) , namely,

- better signal intensity has been obtained
- hybridization time has been shortened
- washing step (consumes time and reagents as well as decreases signal) has been eliminated

It is possible to apply this protocol and detect *E. coli* directly on the surface using PNA probes and new protocol which ensures maximum signal intensity. The new protocol was also used for hybridization of *A. hydrophyla* with its corresponding probe.
A novel PNA probe for *A. hydrophila* rRNA has been designed and verified. Such probe to our best knowledge has not been designed and published previously.
The DNA probes for detection of α-, β- and γ- proteobacteria have been verified for pure cultures, spiked samples and real biofouling samples.

More information

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Quality control:	Joep van den Broeke (S::can)
Authors:	Simona Larsson, Talis Juhna, Linda Mezule (RTU), Liv Fiksdal (NTNU)
Contact person:	Talis Juhna, PhD
e-mail:	talisj@bf.rtu.lv
Phone:	+371 2 9226441
Address:	Department of Water Engineering and Technology, Riga Technical University, Latvia, Riga, Azenes 16/20.

TECHNEAU Knowledge Integrator (TKI) categorisation

Categorisation of Knowledge Packages

Categorisation (i.e. classification, contains and constraints) of knowledge packages (KPs) can be carried out by 'checking' the appropriate boxes in the attached tables. For example, for a KP investigating point-of-use treatment suitable for a developing world country, the following might be checked:

Classification: Process chain – Tap (Customer) – Point-of-use (POU).
Contains: Report; Literature review.
Constraints: Low cost; Simple technology; No/low skill requirement; No/low energy requirement; No/low chemical requirement; No/low sludge production; Developing world location.

Note that only the lowest level classification needs to be checked, e.g. Point-of-use (POU) in the above example.

Meta data can be included under the 'More Information' section of the Executive Summary Report, i.e. Author(s), Organisation(s), Contact details (name and email), Quality controller (name and organisation) and Date prepared. (The TKI administrator can enter Source (= TECHNEAU), Date submitted (TKI) and Date revised (TKI)).

TKI Categorisation

Classification									
Supply Chain		**Process Chain**		**Process Chain (cont'd)**		**Water Quality**		**Water Quantity (cont'd)**	
Source		**Raw water storage**		**Sludge treatment**		**Legislation/regulation**	X	- Leakage	X
- Catchment	X	- Supply reservoir		- Settlement		- Raw water (source)		- Recycle	
- Groundwater	X	- Bankside storage		- Thickening		- Treated water			
- Surface water	X	**Pretreatment**		- Dewatering		**Chemical**			
- Spring water	X	- Screening		- Disposal		- Organic compounds			
- Storm water		- Microstraining		**Chemical dosing**		- Inorganic compounds			
- Brackish/seawater		**Primary treatment**		- pH adjustment		- Disinfection by-products			
- Wastewater		- Sedimentation		- Coagulant		- Corrosion			
Raw water storage		- Rapid filtration		- Polyelectrolyte		- Scaling			
- Supply reservoir		- Slow sand filtration		- Disinfectant		- Chlorine decay			
- Bankside storage		- Bank filtration		- Lead/plumbosolvency		**Microbiological**	X		
Water treatment	X	- Dune infiltration		**Control/instrumentation**		- Viruses		**Consumers / Risk**	
- Pretreatment		**Secondary treatment**		- Flow		- Parasites			
- Primary treatment		- Coagulation/flocculation		- Pressure		- Bacteria	X	**Trust**	
- Secondary treatment		- Sedimentation		- pH		- Fungi		- In water safety/quality	X
- Sludge treatment		- Filtration		- Chlorine		**Aesthetic**		- In security of supply	
Treated water storage		- Dissolved air		- Dosing		- Hardness / alkalinity		- In suppliers	

		flotation(DAF)							
- Service reservoir		- Ion exchange		- Telemetry		- pH		- In regulations and regulators	
Distribution	X	- Membrane treatment		**Analysis**		- Turbidity		**Willingness-to-pay/acceptance**	
- Pumps		- Adsorption		- Chemical		- Colour		- For safety	
- Supply pipe / main		- Disinfection		- Microbiological	x	- Taste		- For improved taste/odour	
Tap (Customer)	X	- Dechlorination		- Physical		- Odour		- For infrastructure	
- Supply (service) pipe	x	**Treated water storage**						- For security of supply	
- Internal plumbing		- Service reservoir				**Water Quantity**		**Risk Communication**	
- Internal storage		**Distribution**						- Communication strategies	
		- Disinfection				**Source**		- Potential pitfalls	
		- Lead/plumbosolvency				- Source management		- Proven techniques	
		- Manganese control				- Alternative source(s)			
		- Biofilm control	x			**Management**			
		Tap (Customer)				- Water balance			
		- Point-of-entry (POE)				- Demand/supply trend(s)			
		- Point-of-use (POU)				- Demand reduction			

TKI Categorisation (continued)

Contains		Constraints		Meta data					
Report	x	Low cost	x	*Author(s)*	x				
Database		Simple technology	x	*Organisation(s)*	x				
Spreadsheet		No/low skill requirement		*Contact name*	x				
Model		No/low energy requirement		*Contact email*	x				
Research	x	No/low chemical requirement		*Quality controller name*	x				
Literature review		No/low sludge production		*Quality controlerl organisation*	x				
Trend analysis		Rural location		Source					
Case study / demonstration		Developing world location		*Date prepared*	x				
Financial / organisational				Date submitted (TKI)					
Methodology	x			Date revised (TKI)					
Legislation / regulation									
Benchmarking									

Summary

Untreated water contains many bacteria, protozoa, fungi and viruses. Some of them may pass into drinking water and become established in biofilm (a complex layer on the surface of the water distribution system). Attachment of organisms to surfaces has been shown to alter their physiology rendering them more active in absorbing nutrients as well as more resistant to environmental stress. The ideal aim is to bring to the consumer potable water of an identical quality to that leaving the treatment plant. However, it has been well documented that water, which reaches the consumer's tap, is often of inferior microbiological and sometimes chemical quality, when compared to that which left the waterworks. Thus in order to serve the consumer best the analytical systems used for monitoring of microflora in potable water/ water distribution system must be (i) rapid to perform, (ii) able to detect the major groups of concern, (iii) reliable, (iv) inexpensive and (v) preferably non distructive, *i.e.* such that they do not disturb the sample composition.

In this report the development of fluorescence-in-situ-hybridization (FISH) method is described. The method can be used not only to identify the microorganisms in bulk water but also to identify the bacteria directly on the pipes and other surfaces in water distribution systems without removing the sample. We selected *Escherichia coli* as a model organism for pathogenic bacteria and *Aeromonas hydrophila* as model organism for the opportunistic pathogens, capable of regrowth, for the development of the method. The major reasons for this choice are that *E. coli* is still used as indicator for drinking water pollution and their enterotoxigenic and enterohaemorrhagic forms are one of the major causes of water-related outbreaks. *Aeromonas*, in particular the species *Aeromonas hydrophila*, has been known to cause acute diarrhoea in children and traveller's diarrhoea in adults, gastroenteritis, septicemia and pancreatic abscess. The method of choice is FISH since the method is quick as identification on species level can be made within a couple of hours and the method is cheap enough to be used on a routine basis. FISH method does not require any expensive devices or reagents hence it is hoped that in the future also smaller and lesser-equipped laboratories all over the world could apply the method on a day-to-day basis. Finally, this method used both for the surface samples and for the bulk water.

After this summary the descriptions of terms and definitions (abbreviations and probe sequences) used in the report are listed (**Chapter 1**). The reasoning behind the selected two bacteria for probe development is disclosed in **Chapter 2**. The principle of the method is described in **Chapter 3** along with the description of current state-of-art in the field. Investigation of membrane biofouling using DNA probes is dealt with in **Chapter 4**.. Major operational challenges are reviewed in **Chapter 5** including such problems as low signal intensity, autofluorescence and unspecific binding. Furthermore, the developments regarding the protocol are described in this chapter. In the **Chapter 6** the basis of sequence selection is reviewed and, finally, **Chapter 7** provides the conclusions.

Contents

1 Terms and definitions

1.1 Terms

ABNC- active but not culturable cells. These cells do not grow on conventional media and are detectable by molecular methods only

AOC - assimilable Organic Carbon is part of organic carbon which is converted to biomass by specified bacteria or consortium of bacteria

ATP - adenosine triphosphate

DOC - Degradable Organic Carbon is the part of DOC which is consumed by a community of natural bacteria in favourable conditions during a certain period of time (normally less then one month)

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

DAPI- 4′,6-diamidino-2- phenylindole

DNA probe - an approximately 18-20 nt long dezoxyribonucleic acid fragment carrying a marker (fluorescent dye or hapten)

FISH- fluorescence *in situ* hybridization

NADH- nicotinamide adenine dinucleotide

PAH- polycyclic aromatic hydrocarbons

PNA probe - an approximately 15-16 nt long peptide nucleic acid fragment carrying a marker (fluorescent dye or hapten)

1.2 Probes used in this work

Table 1.1. DNA probes

Sequence	Marker	Target	Notes
TCA ATG AGC AAA GGT`	CY3	*E. coli*	COLIDNA
CGT TCG YTC TGA GCC AG	PET	α-proteobacteria	ALF1b
GCC TTC CCA CCT CGT TT	6-FAM	β-proteobacteria	BET24a-FAM
GCC TTC CCA CCT CGT TT	CY3	β-proteobacteria	BET24a
GCC TTC CCA CAT CGT TT	PET	γ-proteobacteria	GAM42a
TGA GGA TGC CCT CCG TCG	6-FAM	Eubacteria	EUB-FAM
TGA GGA TGC CCT CCG TCG	CY3	Eubacteria	EUB

Table 1.2. PNA probes

Sequence	Marker	Target	Notes
TCA ATG AGC AAA GGT	CY3	*E. coli*	ECOLIFILM
TCA ATG AGC AAA GGT	Biotin	*E. coli*	ECOLI-BIO
TCA ATG AGC AAA GGT	Alexa488	*E. coli*	ECOLI-Alexa
ACGTCACAGTTGATACG	CY5	*A. hydrophila*	AEROHYD

2 Selection of bacteria for development of the probe

2.1 Background

Untreated water contains many bacteria, protozoa, fungi and viruses. The most commonly encountered waterborne diseases are caused by pathogenic bacteria among which major pathologens are *Salmonella*, *Shigella*, *Escherichia coli*, *Campylobacter* spp. and *Vibrio cholerae*. However water contains opportunistic pathogens such as *Acinetobacter* spp. *Aeromonas* spp., *Flavobacterium* spp., *Moraxella* sp., *Corynobacterium* spp. *Arthrobacter* spp., and *Pseudomonas* spp. (for review see Percival, et al., 2000).

The growth of microorganisms in water distribution systems has been well documented throughout the world during the last century. Many cases of microbial growth in water distribution systems and sudden increases in coliform counts in the final drinking water have been reported (see *e.g.* Hudson, et al., 1983). Water treatment plants are designed to remove pathogenic but not opportunistic pathogens. Thus, some of them may pass into drinking water and become established in biofilm. The phenomenon of biofilm formation, or the attachment of microorganisms to the inner surfaces of the drinking water distribution system, has also been well documented (Allen, et al., 2004; Olson, 1981, Fig.1). Attachment of organisms to surfaces has been shown to alter their physiology. Attached organisms were found to be generally more active in absorbing nutrients as well as more resistant to environmental stress such as starvation, heavy metals and chlorine (Backer, 1984; LeChevallier, et al., 1984). Experiments have shown that bacteria attached to surfaces show greater resistance to disinfection (LeChevallier, et al., 1988).

The formation of biofilms in drinking water systems produce undesirable effects, such as contamination potential, resistance against disinfection and harboring of pathogens. The transfer of antibiotic-resistance genes (Obst, et al., 2006) provides an additional problem.

The ideal aim is to bring to the consumer potable water of an identical quality to that leaving the treatment plant. However, it has been well documented that water, which reaches the consumer's tap, is often of inferior microbiological and sometimes chemical quality, when compared to that which left the waterworks. Therefore the analytical systems used for monitoring of microflora in potable water must be quick to perform and able to detect the major groups of concern. Additional requirements are that they must be reliable, inexpensive and preferably such that they do not disturb the sample composition.

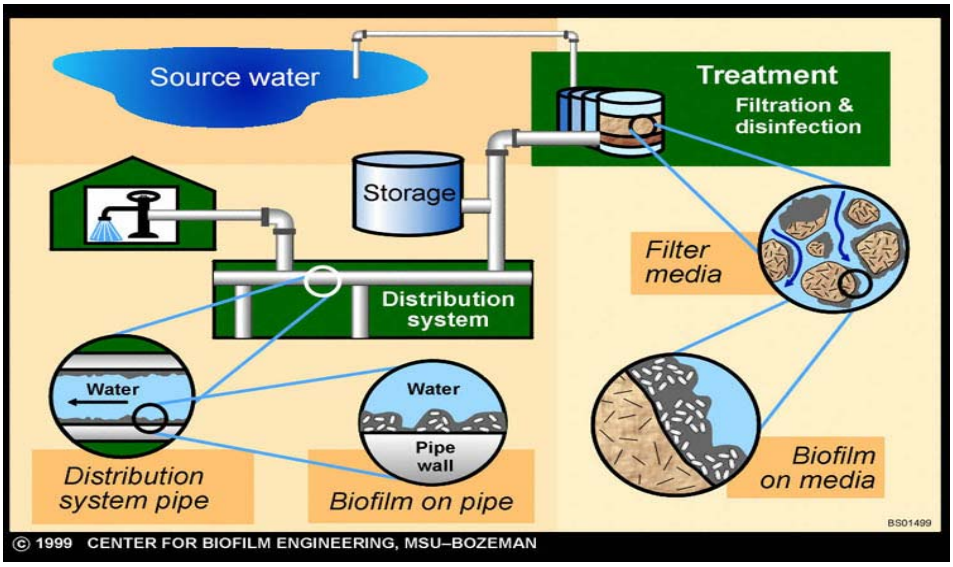

Figure 1. Biofilms in drinking water supply systems (Source: www.erc.montana.edu)

In this study we have selected one pathogenic bacterium *Escherichia coli* and one opportunistic pathogen *Aeromonas hydrophila* as model organisms for the development of a fast and reliable analysis method. The major reasons for this choice are that *E. coli* is still used as indicator for drinking water pollution and their enterotoxigenic and enterohaemorrhagic forms are one of the major causes of water-related outbreaks (Percival, et al., 2000). *Aeromonas*, especially the species *A. hydrophila*, has long been known to cause acute diarrhoea in children and traveller's diarrhoea in adults (Moyer, 1987), gastroenteritis (Pavlov, et al., 2004), septicemia (Merino, et al., 1995) and recently has been shown to cause even pancreatic abscess (De Gascun, et al., 2007). The method of choice is FISH or fluorescence *in situ* hybridization since the method is quick as identification on species level can be made within a couple of hours and cheap enough to be used on a routine basis. The method does not require any expensive devices or reagents hence it is hoped that in the future also smaller and lesser-equipped laboratories could apply the method on a day to day basis.

2.1.1 Escherichia coli.

Several pathogenic bacteria as candidates for development of probes were investigated including *Helicobacter pylori*, *Mycobacterium* spp. and *Campylobacter* spp. It appears that more and more of pathogens are found to be associated with drinking water, thus the list of emerging pathogens will continually be upgraded in future. It was therefore decided to develop probe for *E. coli* using this bacteria as a model of pathogenic bacteria in drinking water systems. *E. coli* are Gram-negative, non-spore-forming, rod-shaped, facultatively anaerobic bacteria that normally live in the intestines of humans and animals. Although, most strains of this bacteria are harmless, several are

known to produce toxins that can cause diarrhoea and/or kidney damage (for review see Hunter, 2003).

2.1.2 Aeromonas spp.

Species of *Aeromonas* are Gram-negative, non-spore-forming, rod-shaped, facultatively anaerobic bacteria that occur ubiquitously and autochthonously in aquatic environments.

***Table 2. Relative frequency of occurrence of human infections associated with mesophilic* Aeromonas[a]**

Type of infection	Characteristics	Relative frequency[b]
Diarrhoea		
Secretory	Acute watery diarrhoea, vomiting	Very common
Dysenteric	Acute diarrhoea with blood and mucus	Common
Chronic	Diarrhoea lasting more than 10 days	Common
Choleraic	"Rice water" stools	Rare
Systemic		
Cellulitis	Inflammation of connective tissue	Common
Myonecrosis	Haemorrhage, necrosis with/without gas gangrene	Rare
Erythema gangrenosum	Skin lesions with necrotic centre, sepsis	Uncommon
Septicaemia	Fever, chills, hypotension, high mortality	Fairly common
Peritonitis	Inflammation of peritoneum	Uncommon
Pneumonia	Pneumonia with septicaemia, sometimes necrosis	Rare
Osteomyelitis	Bone infection following soft-tissue infection	Rare
Cholecystitis	Acute infection of gallbladder	Rare
Eye infections	Conjunctivitis, corneal ulcer, endophthalmitis	Rare

[a] Modified from Janda & Duffey, 1988, and Nichols et al., 1996.
[b] Frequency of occurrence relative to all cases of *Aeromonas* infection.

The genus includes at least 13 genospecies, among which are the mesophilic *A. hydrophila*, *A. caviae*, *A. sobria*, *A. veronii*, and *A. schubertii*, and the non-motile, psychrophilic *A. salmonicida*. *A. salmonicida* is a fish pathogen and has not been associated with human infection. By contrast, the mesophilic species have been associated with a wide range of infections in humans (Merino, et al., 1995, Janda, et al., 1996), see also Table 2 (source: WHO, http://www.who.int/water_sanitation_health/dwq/en/admicrob2.pdf).

Aeromonas spp. are prevalent within potable water and have been implicated as causative agents in waterborne outbreaks (Havelaar, et al., 1990). Aeromonas bacteraemia is not a common infectious disease, but can cause a grave outcome in infected cases. The infections usually occurred in the patients with hepatic cirrhosis or malignancy, heralded a poor prognosis and were community-acquired (Ko, et al., 2000). In a recent study it was elucidated that 62% of *Aeromonas* isolates (out of 116 in total) caused clinically evident infections, of which the major clinical manifestations were primary bacteremia (40%), followed by soft tissue infections (27%), and hepatobiliary tract infections (15%). The crude fatality rate reported for *Aeromonas* infections

was 30% (Ko, et al., 2000). In another study it was estimated to 26% and 52% of these were found to be *A. hydrophila* complex (Wu, et al., 2007).

Membrane filtration is the procedure most commonly used for the enumeration of *Aeromonas* from treated water; it employs a variety of culture media, most of which contain ampicillin. For drinking-water, the most widely used medium is ampicillin–dextrin agar (ADA) (Havelaar, et al., 1987). The incubation time is typically 24–48 hours. In addition, these media, contain selective agents and are nutrient-rich, and their use may result in low recovery of some aeromonads from low-nutrient or chlorinated waters, weighting any data in favour of the more robust, rapidly growing strains (Gavriel, et al., 1998). Thus a rapid, reliable and growth rate-independent analysis method for the detection of *A. hydrophila* in potable water is needed.

Unlike other many true pathogens *Aeromonas hydrophila* may display aftergrowth (microbial growth in the water distribution system). This aftergrowth is a result of *Aeromonas* growth within biofilms (Holmes and Niccolls, 1995). Thus, this microorganism can be used as indicator for biological stability of drinking water.

3 Principle of FISH method and current state of the art

Fluorescence *in situ* hybridization (FISH) is a type of hybridization in which a probe is labeled with fluorescent molecules so that it can be seen with a microscope. The word "*in situ*" means that the hybridization occurs "in place", within the nucleus of specimen cells that have been fixed to a microscope slide, a coupon or a pipe segment. Hybridization is a process where a DNA sequence of interest is identified among other DNA sequences by pairing it with a complementary sequence used as a probe. The process depends upon the biophysical properties of DNA nucleotide chains, which will unwind from a double helix at elevated temperatures and will rewind (pair or hybridize) with complementary sequences at lower temperatures. FISH is a multistep procedure that involves the following general sequence of events:

1. Fixation of biological samples and preparation of microscopic specimens.
2. Pretreatments of microscopic preparations, where necessary.
3. Probe addition.
4. Denaturation of in situ target DNA.
5. In situ hybridization and post-hybridization washing.
6. Microscopy.

Labeling of probes for FISH is generally achieved by enzymatic incorporation of hapten- (biotin and digoxigenin) or fluorochrome-labeled deoxyribonucleoside triphosphates. Today the most popular probes are either DNA probes or PNA probes. Peptide nucleic acid (PNA) molecules are DNA mimics in which the negatively charged sugar-phosphate backbone of DNA is replaced with a non-charged polyamide backbone. PNA probes contain the same nucleotide bases and follow standard Watson-Crick base-pairing rules while hybridizing to complementary nucleic acid sequences. The synthetic backbone provides PNA probes with unique hybridization characteristics compared to DNA probes (Karkare and Bhatnagar, 2006).

It is worthwhile to remember that there is no universal FISH protocol. Variations in time, temperature and buffers should be tried in order to find the best conditions for a given application. Furthermore, DNA and PNA hybridization protocols differ so these should not be substituted.

3.1 DNA FISH

DNA probes have been extensively used since the eighties (see *e.g.* Barker, 1989). Double-stranded target DNA has to be denatured prior to in situ hybridization using a DNA probe. This can be achieved by treatment with extremes of pH or heat. Such treatments generally lead to loss of morphology, therefore, a compromise has to be found between intensity of hybridization signal and preservation of morphology. While hybridizing to complementary nucleic acid sequences DNA probes must overcome a destabilizing electrostatic repulsion between negatively charged backbones resulting in slower and weaker binding. In practice this means that hybridization

procedure is longer when DNA probes are used and the obtained signal is usually weaker (Zwirglmaier, 2005).
DNA probes are often not able to penetrate the dense layer of biofilm (Wilks and Keevil, 2006) which occurs on the pipes and coupons so that samples need to be sonicated. This has the disadvantage of disturbing the investigations of the spatial ecology in the biofilm.
Their main advantage, however, remains in the fact that these probes are cheaper to manufacture compared to PNA probes. Thus DNA oligos are useful for basic experiments, such as positive/negative identification in non-complex samples.

3.2 PNA FISH

PNA probes do not encounter the electrostatic repulsion because of non-charged backbone hence they hybridize to the targets rapidly and tightly. PNA probes, having a synthetic backbone are resistant to both nuclease and protease degradation (Demidov, et al., 1993) and they hybridize independently of the salt concentration (Pellestor, et al., 2005). Thus, the lifetime of PNA probes will be longer than that of DNA probes, both *in vivo* and *in vitro*. An additional advantage when investigating drinking water biofilms is that PNA probes can penetrate the thick layers (Wilks and Keevil, 2006) and can give valuable information on the location of different cells of interest and their possible interaction, *e.g.* synergism or antagonicity.
To conclude, PNA technology is more on the cutting edge of pathogen research because it is more specific, gives brighter signal, allows simpler protocol and penetrates biofilm, where necessary. As with all relatively new technologies it is very likely that the manufacturing prices of PNA probes, which is their only disadvantage, compared to DNA probes, will decrease.

3.3 Drawbacks of current FISH protocol

There are, however, problems that have been observed using both PNA and DNA FISH in environmental samples, such as (i) low intensity of signal and (ii) autofluorescence. Both decrease the detectability and the degree of positive identification. Of these autofluorescence is the most complex issue as there are many possible causes such as (i) so-called natural fluorescence, (ii) fixative-induced fluorescence and (iii) unspecific binding. Fixative-induced fluorescence, once understood (aldehyde in combination with amines/proteins creates fluorescent products) can be worked upon by using other fixatives and will not be discussed further in this report. Other possible causes such as low intensity of signal, natural fluorescence and unspecific binding will be described more in detail.

Another important drawback when probes, based on rRNA are used is the inability to distinguish between live cells, active but not culturable (ABNC) cells and dead but not disintegrated cells. The latter ususally do not pose danger to the consumer while the ABNC cells do (Desnues, et al., 2003, Vora, et al., 2005).

4 Investigation of membrane biofouling

As fouling is a fundamental problem in membrane processes, biofouling (*i.e.* fouling which is caused by microorganisms) control is considered as a major challenge in operating membrane systems. The formation of a fouling layer affects the filtration properties of the membranes which results in a decrease of flux and an increase of the differential pressure (Flemming, et al., 1997). There have been studies dealing with prevention of biofouling, *e.g.* Hu *et al.* (Hu, et al., 2005) studied the feasibility of using biofiltration as a pretreatment process to control membrane biofouling. Biofiltration was found to be a viable way of assimilable organic carbon (AOC) and dissolved organic carbon (DOC) removal, with removal efficiencies of 40-49% and 35-45%. It was also found that biofiltration as a pretreatment step reduced the rate of biofouling. Our interest, however, lies within development of fast assays which indicate the occurrence of biofouling and for this it is necessary to study the groups of microorganisms involved in different stages of membrane biofouling. Cooperation with NTNU (Norway) has been initiated within the TECHNEAU project.

The compositions of planktonic and biofilm microbial communities have recently been analyzed using culture independent molecular-based methods (Horsch, et al., 2005, Miura, et al., 2007). The SEM and LIVE/DEAD staining analyses clearly showed that the biofilm gradually developed on the membrane surfaces with time, which had a strong positive correlation with the increase in trans-membrane pressure. This indicates that the biofilm formation induced the membrane fouling. The examination of biofouling layer, however has given somewhat dissimilar results, as Horsch *et al*. showed that the bacterial composition of the primary fouling layer (after 5 days) displayed a dominance of the γ-subclass of proteobacteria, which have attachment mechanisms to form the primary biofilm, which is then colonized by other bacteria (Horsch, et al., 2005).

In contrast, according to Miura *et al*. (Miura, et al., 2007) FISH and 16S rRNA gene sequence analyses revealed that β-proteobacteria, probably played a major role in development of the biofilms. The mature fouling layer was dominated by bacteria of the α- and β-subclass, which was similar to the population structure of the raw water (Horsch, et al., 2005) whereas in the more recent study it was found that the microbial communities on membrane surfaces were quite different from those in the planktonic biomass (Miura, et al., 2007).

The work is carried out using DNA probes specific for α–, β– and γ– subclass of proteobacteria as well as a probe for Eubacteria. As model bacteria *S. paucimobilis* (α-proteobacteria), *Burkholderia cepacia* (β-proteobaceria) and *Legionella pneumophila* (γ-proteobacteria) were used as these bacteria are common in water distribution systems. It was verified that the specific probes bind to corresponding bacteria (Fig. 2) and that these specific probes do not bind to unspecific bacteria (data not shown). These probes have been applied earlier (*e.g.* (Horsch, et al., 2005)).

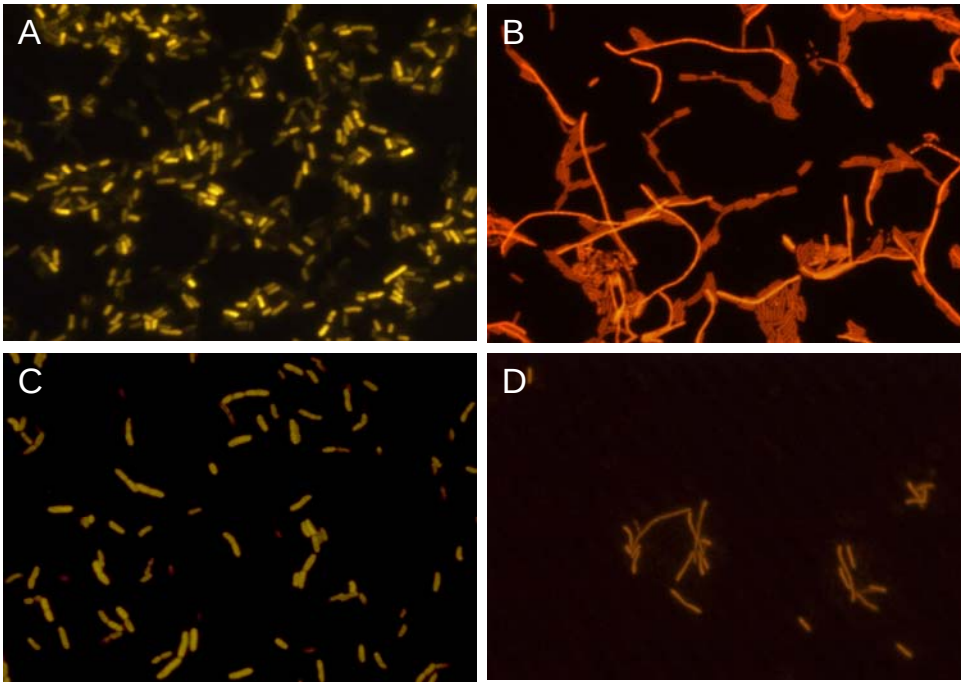

Figure 2. *E. coli* hybridized with EUB probe (A), *Sphingomonas paucimobilis* hybridized with ALF1b probe (B), *Burkholderia cepacia* hybridized with BET42a probe (C) and *Legionella pneumophila* hybridized with GAM42A probe (D).

The hybridization procedure is as follows:
Pure culture suspensions of 3 different proteobacteria were placed on microscope slides and air dried, fixed with 4% formaldehyde solution for 20 minutes, washed once with sterile distilled water and covered with 1% agarose. Then the samples were air dried and covered with 40 µl of hybridization buffer (40% v/v formamide; 0,9M NaCl; 0,01% SDS; 20mM Tris-HCl; pH 7,2) with 100 ng of ALF1b (or BET42a; GAM42a; EUB) probe. The slides were incubated for 2 h in a humidified chamber at 43°C. After the incubation the slides were gently washed with washing buffer (5mM EDTA, 40mM NaCl, 0,01% SDS, 20mM Tris-HCl, pH 7,2) and left in the washing buffer for 20 minutes (in the dark at 43°C).
Prior to visualizing the slides, counterstaining with DAPI was performed. Air dried slides were covered with 0,5ml of 0,1% TritonX-100, then with 10µg/ml DAPI and left for 5 minutes at room temperature. After 5 minutes the slides were washed with sterile distilled water, air dried and visualized.
Microscopy examinations were conducted using an epifluorescence microscope (Leica DMLB) equipped with a 50-W power supply, mercury lamp, and several filter sets and a camera (CoolSNAP Pro, Media Cybernetics, Inc, USA). For detection of CY3 labeled probes, a narrow range Y3 filter (Ex: 545 ± 30; Em. 610 ± 75, dichromatic mirror 565 nm) was used, for DAPI stained cells A filter (Ex: 340-380;. Em: >425 nm, dichromatic mirror 400 nm) was used. Samples were examined using a 1000× oil immersion objective. Images were analyzed using Image-Pro Plus version 4.5 (Media Cybernetics, Inc, USA) for Windows software.

It was discovered that (i) FAM labeled probes give low signal intensity compared to the PET labeled probes and (ii) DAPI emits in the green filter which interferes with FAM (or any green fluorescent) signal thereby creating false positive results. It was also carefully checked that DAPI does not interfere with CY3 signal. Thus the probes labeled with FAM (BET42a and EUB) were not used further and CY3 labeled probes were purchased instead.

All samples were from the same suspension of 17 day old biofouling on hollow fibre membrane. The test cell is fed with humic enriched tap water that is ozonated and bio-oxidated (Kaldnes filter) before it enters the membrane filtration test cell.

The water inlet characteristics (average values) were:

Conductivity ($\mu s.cm^{-2}$)	288
T (^oC)	14,4
pH	7,25
Turbidity (NTU)	0,393
Color (mg $Pt.L^{-1}$)	11,9
UV_{254}	0,075
DOC ($mg.L^{-1}$)	5,0
P ($mg.L^{-1}$)	
N ($mg.L^{-1}$)	

The membrane biofouling samples were prepared as follows:

1. Membrane samples were transferred to 0.01 M PBS (pH 7.2) into an Eppendorf tube
2. Membrane samples in PBS were treated with ultrasound, 3 min, on ice.
3. After removal of the membrane sample, the suspension was centrifuged at 4^oC, 5000 x g
4. After discarding the supernatant, the pellet was resuspended in 0.2 mL PBS by vortexing
5. Suspended cells were fixed by adding 3 volumes paraformaldehyde solution to 1 volume sample, and held at 4^oC for 1-3 hours
6. The fixed cells were pelleted by centrifugation (4^oC, 5000 x g), and the fixative was removed
7. The cells were washed with 1 mL PBS, pelleted by centrifugation (4^oC, 5000 x g), and the supernatant was removed
8. The pellet was resuspended in PBS, one volume of ice cold ethanol (96 %) was added and the sample was mixed
9. 10-30 uL fixed cells were spotted onto slides and air dried

10. The cell material was dehydrated in an ethanol series (3 min each) : 50%, 80%, and 96% ethanol

11. Slides with fixed cells were then sent to RTU for analysis

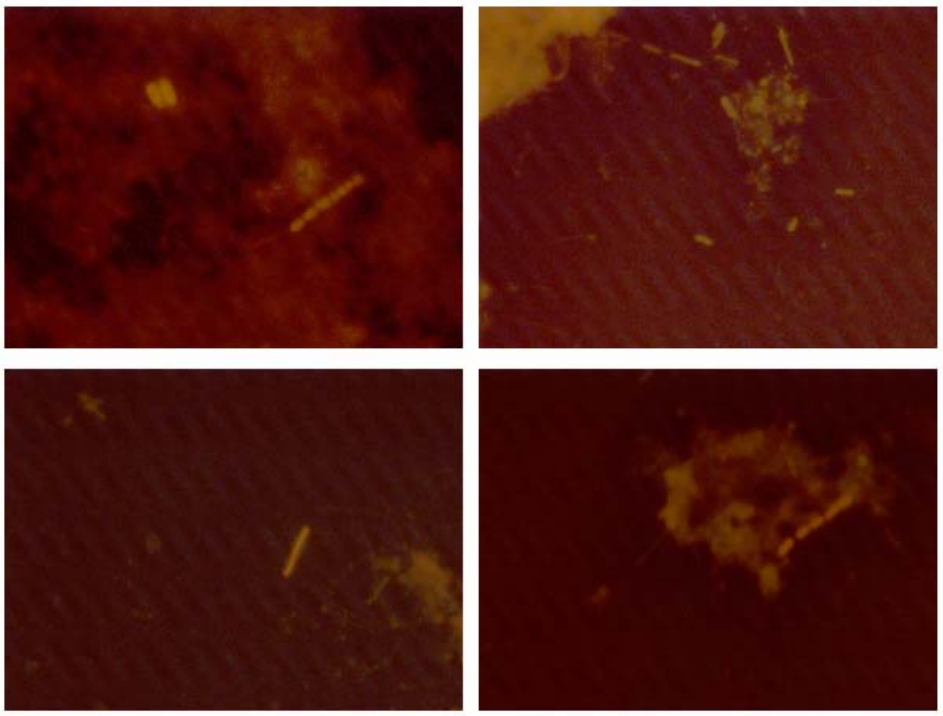

Figure 3. Sonicated membrane samples (NTNU) analyzed using GAM42A probe.

Agarose coating of the samples was introduced as repulsion between sample, probe and microscope slide was observed. As a result initial hybridization experiments were not successful, probably due to negative charge, displayed by DNA probe. As shown in Fig. 3, the coating allows efficient hybridization. The work is in its initial stage yet and β-proteobacteria probe containing CY3 label has not yet been tried for membrane biofouling experiments. However, it can be concluded from the initial experiments using probes for α- and γ-subclass, α-proteobacteria could not be found in the samples (data not shown) however cells belonging to γ-proteobacteria subclass were detected (Fig. 3).

5 Improving the protocol

5.1 Factors, affecting the signal intensity

In this section the main factors on which the signal intensity depends will be briefly reviewed, such as autofluorescence, low signal intensity and unspecific binding.

5.1.1 Natural fluorescence

Natural fluorescence may be due to cell-related substances like flavins, porphyrins and chlorophyll, including the enzymes containing any of these compounds as well as NADH. There are also reports indicating environment-related causes of autofluorescence, such as various particles/ compounds. Specifically, metallic sulfide (Chevaldonné and Godfroy, 1997) and PAH (*e.g* fluoroanthrene, (Azevedo, 2005)) have been mentioned.
Other causes of "natural fluorescence" include collagen, lipofuscin and elastin but these are not relevant in microbial cells.

5.1.2 Low signal intensity

Single-cell detection methods are severely limited when applied to the enumeration of cells present in low concentrations (such as microorganisms in potable water) –direct analytical devices (*e.g*. epifluorescence microscope or flow cytometer) are quantitatively limited to count highly diluted cells.

Figure 4. An environmental biofilm sample contains microbial cells (seen in blue) and *E. coli* among them (seen in red). The analysis was done using ECOLIFILM probe (Juhna et al, submitted).

We have found that PNA probes provide significantly (50%-75%) higher signal intensity compared to DNA probes and can be used for analyzing complex samples, such as biofilm on coupons even when the concentration of target cells on them is scarce (see Fig 4, earlier results from FP5 SAFER project). In addition, signal strength may also depend on a successful choice of the label and this is reviewed below, in the protocol development section.

5.1.3 Unspecific binding

During in situ hybridization, perfect hybrids form between probe and target, but imperfect ones may also form with less homologous target sequences, leading to nonspecific hybridization. Perfect hybrids are more stable than imperfect ones. To prevent the latter hybrids from forming, the stringency of the hybridization can be increased by lowering salt and raising formamide concentrations as well as by raising temperature. Remaining nonspecific hybrids can be removed by stringent post-hybridization washes.
Non-specific binding was not observed using PNA probes and it was found that washing step decreases signal intensity and causes false positive signals (see subsection 5.2.1.2. below).

5.2 Development of a new PNA hybridization protocol

Our approach is to perform the hybridization on the surface of coupon (in combination with signal amplification where appropriate) with the aim to detect viable cells. It is important to note that a generalized protocol for all cell types and probes does not exist. For many FISH applications, however, the multitude of experimental variables have been documented fairly accurately, so that with optimization studies, an operational in situ hybridization protocol can be designed rapidly. We chose to investigate the possibility to increase the signal by optimizing PNA hybridization protocol as this is a more novel technology and the more promissing one for biofilm research (see advantages described in Section 3.2).

5.2.1 Signal intensification

The basic protocol was as follows:
E. coli (ATCC 25922) strains were grown on R2A agar (Eaton, 1995), picked and suspended in 1 ml of PBS (7 mM Na_2HPO_4, 3 mM NaH_2PO_4, 130 mM NaCl, pH 7.2) at a concentration of 10^7 –10^8 cells/ml and vortexed. Cell suspensions were pelleted by centrifugation at 6,000 rpm for 3 min, supernatant was removed and the cells were then resuspended in PBS. This washing procedure was repeated two more times. Following the last wash, about 200 µl of cell suspension was spread onto a clean microscope slide and allowed to dry. To the dried cells 3-4% (v/v) formaldehyde was applied and the cells were fixed for 20 min. After fixation the microscope slide was rinsed with water and allowed to dry.
50 - 500 µL of PNA hybridization mix consisting of hybridization buffer (50 mM Tris-HCl, 10% w/v 50% dextran sulphate, 0,1mM of NaCl, 30% v/v formamide, 30% v/v tetra-sodium pyrophosphate, 0.2% w/v polyvinylpyrrolidone, 0.2% w/v Ficoll 400, 5mM Na_2EDTA, 0.1% v/v Triton X-100) containing 200 nM fluorescently labeled PNA probe was applied to the

slide or dry coupon and covered with cover glass. The sample was incubated at 57°C for 30, 60 or 90 min in a tight vessel containing water vapor to avoid concentration effects due to evaporation. The samples were immersed in a vessel containing pre-warmed (57°C) washing buffer (5mM Tris, 15 mM NaCl, 0,1% Triton X 100, pH 10), and incubated for 30 minutes. After that the samples were removed from the vessel, rinsed with water and allowed to dry.
Finally, TBC number was determined immediately after FISH analyses. For this 4',6-diamidino-2- phenylindole (DAPI) was applied as counter-stain. On the surface the 1:1 mixture of DAPI (10 µg/ml) and Triton X-100 (0.1 %), was applied so that the final concentration of DAPI was 5 µg/ml and incubated for 15-20 minutes, then excess liquid removed, rinsed and the surface was air-dried.
Microscopy examinations were conducted using an epifluorescence microscope (Leica DMLB) equipped with a 50-W power supply, mercury lamp, and several filter sets and a camera (CoolSNAP Pro, Media Cybernetics, Inc, USA). For detection of *E. coli* with ECOLIFILM probe, a narrow range Y3 filter (Ex: 545 ± 30; Em. 610 ± 75, dichromatic mirror 565 nm) was used, for DAPI stained cells A filter (Ex: 340/380;. Em: >425 nm, dichromatic mirror 400 nm) was used. Samples were examined using a 1000× oil immersion or 400× dry objective. Images were analyzed using Image-Pro Plus version 4.5 (Media Cybernetics, Inc, USA) for Windows software.

5.2.1.1 Influence of hybridization time

The apparent start is the optimization of the hybridization protocol with respect to the hybridization time. The standard hybridization time is 90 minutes (see *e.g.* Oliveira, et al., 2002, Azevedo, et al., 2003, Lehtola, et al., 2005, Poppert, et al., 2005, Lehtola, et al., 2006, Wilks and Keevil, 2006). Our protocol has now been optimized in this respect.
Hybridization experiments were done using *E. coli* (ATCC 25922) cells and ECOLIFILM probe. ECOLIFILM probe is a PNA probe labelled with CY3 (Ex: 550, Em: 570), flanked with solubility enhancers and has a following sequence: 5` TCA ATG AGC AAA GGT- 3`, published earlier by O'Keefe *et al.* (Perry-O'Keefe, et al., 2001).
Samples were treated in an identical manner with only the hybridization time as a variable. Hybridization was followed by rinsing for 30 minutes with a buffer containing 5 mM Tris-HCl, 15 mM NaCl and 0.1% (v/v) Triton X-100, pH 10. The mean values of at least 3 experiments are shown. As minimum, 300 cells or 20 viewing fields were examined for each sample.
We found that a period of 30 minutes is sufficient to ensure optimal specific hybridization (Fig 5) but for biofilm samples it is probably best to use a hybridization time of 60 minutes in order to insure sufficient penetration.

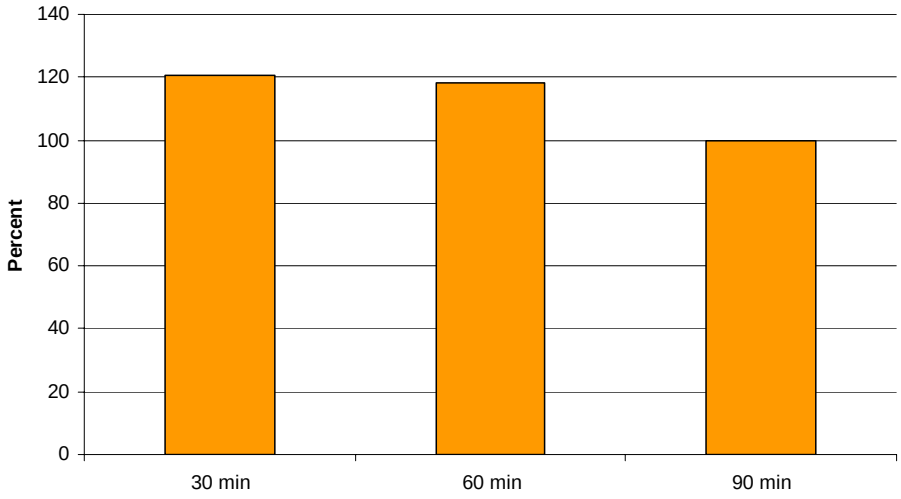

Figure 5. The influence of hybridization length on the intensity of the signal. The hybridization intensity is taken as 100% at 90 minutes as it is the most common hybridization time found in the literature.

5.2.1.2 *Influence of the washing step*

Next we investigated the time/intensity of rinsing and the choice of buffers. The buffer composition had no major effect on the signal intensity. The two known buffer compositions tried (10 mM Tris pH 9.0, 1 mM EDTA, (Perry-O'Keefe, et al., 2001) and 5 mM Tris-HCl, 15 mM NaCl and 0.1% (v/v) Triton X-100, pH 10, (Wilks and Keevil, 2006) had no effect on the signal intensity therefore all further work was continued with the latter buffer.

Table 3. Experimental design

Microorganism	Washing time (min)	Probe
E. coli	none	+
	none	-
	5	+
	10	+
	20	+
	30	+
	30	-
S. paucimobilis	none	+
	none	-
	5	+
	10	+
	20	+

	30	+
	30	-
P. fluorescens	none	+
	none	-
	5	+
	10	+
	20	+
	30	+
	30	-

The experiments were done using ECOLIFILM probe and cells of *E. coli*, *Pseudomonas fluorescens* and *Sphyngomonas paucimobilis*. *S. paucimobilis* was chosen as a non-target organism, ubiquitous in drinking water systems in order to check the extent of possible unspecific binding which then the washing step is generally claimed to remove. *P. fluorescens* is not only ubiquitous but also displays autofluorescence therefore is was included in these experiments as well.
The experimental design is shown in the Table 3. All experiments were repeated 3 times. In addition, the experiments without the probe with all the cultures were done without washing and applying 30 minutes washing step.

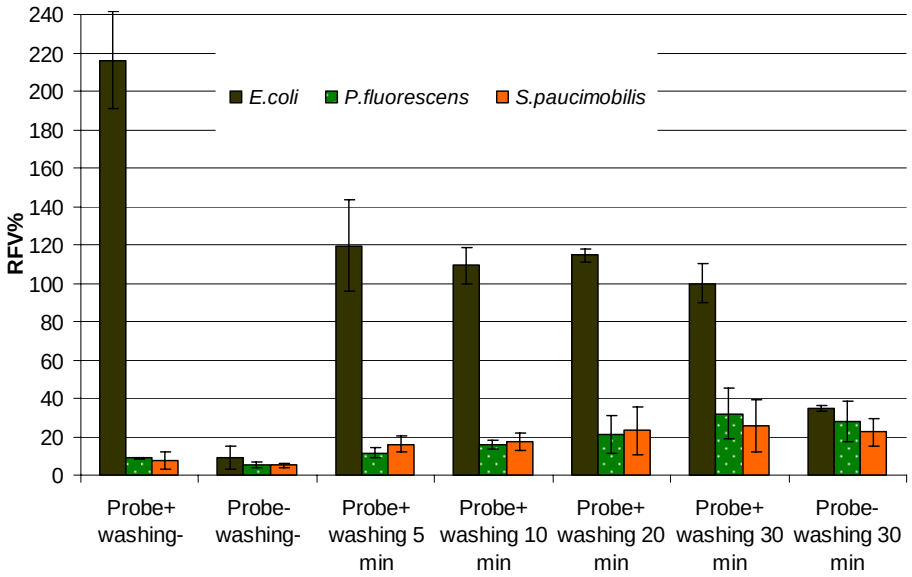

Figure 6. The influence of the washing step on the signal intensity. "+" /"-" denotes the presence/absence of the probe or washing. When washing was present, the time is indicated.

The intensity of the signal, expressed as RFU (relative fluorescence intensity) using following experimental conditions: 60 minutes hybridization followed by 30 minutes washing was assumed as 100% intensity (Fig 6) and the rest of the results were calculated from that. The reason for this assumption is that (i) we have shown that 60 minutes hybridization time is sufficient (see above) and (ii) 30 minutes washing time is included in the protocols most often described in the literature (Oliveira, et al., 2002, Azevedo, et al., 2003, Lehtola, et al., 2005, Poppert, et al., 2005, Lehtola, et al., 2006, Wilks and Keevil, 2006).

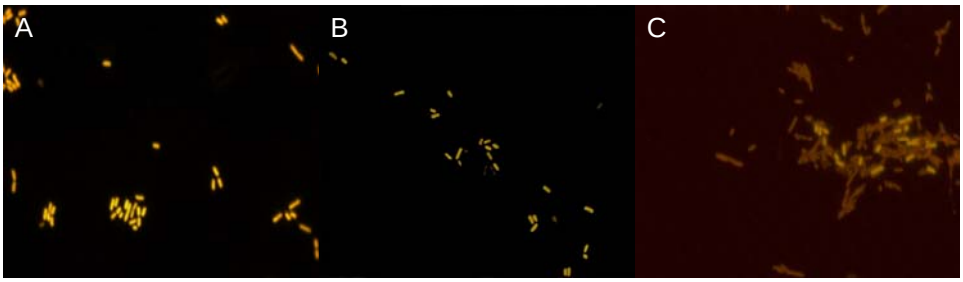

Figure 7. *E. coli* hybridized with ECOLIFILM probe using the improved protocol without washing (A) a mixed sample of *E. coli* and *S. paucimobilis* (B) and a mixed sample of *E. coli* and *S. paucimobilis* after 30 minutes washing (C).

There was no significant signal where no probe was used for all the microorganisms where washing was not present. Washing significantly decreased (by about 50%) the signal intensity of the labeled *E. coli* cells as showed in Fig 6. Fig 7 illustrates the experiment where no washing was performed (A) and the experiment where a mixed sample of *E. coli* and *S. paucimobils* was used with no washing step included in the protocol (B). *E. coli* is seen as bright yellow fluorescent cells whereas *S. paucimobilis* cannot be detected. There was a clear trend of increase in fluorescence for the non-target cells with increase of the washing time observed. It was present also in the case of the absence of the probe (Fig 6). It can be observed arbitrarily (comparing Fig 7 A and C) as well as by comparing the percentages shown in Fig 6 that the washing step has decreased the fluorescence of *E. coli* (target organism) and increased that of *S. paucimobilis* (non-target organism). The two cultures are however still clearly discernible (Fig 7 C). Both these factors are undesirable. In addition, the same trend can be seen for *P. fluorescens* (Fig 6).
The decrease in the fluorescence intensity in the case of washing step applied to the target organism is most probably removal of bound probe however it is more difficult to determine with certainty why the fluorescence increases with washing. It could be some interaction of hybridization and washing buffer or an interaction of washing buffer with cell components. Further work is needed to elucidate what is behind this interesting finding.
It should be pointed out that this observation is perhaps not clouding the evaluation when pure cultures are used however it is definitely undesirable with field experiments where the target cells might have different rRNA content and therefore display a different degree of brightness when labeled compared to pure cultures.

The new approach, i.e. hybridization without washing was also tested on coupons from the river Daugava, inserted after the first filters and removed after 6 weeks. Quite many *E. coli* cells are seen on the spiked coupon, but there were single cells detected on original coupons as well (Fig 8 B). DAPI channel confirmed the identity of the cell seen in Fig 8 B (data not shown).

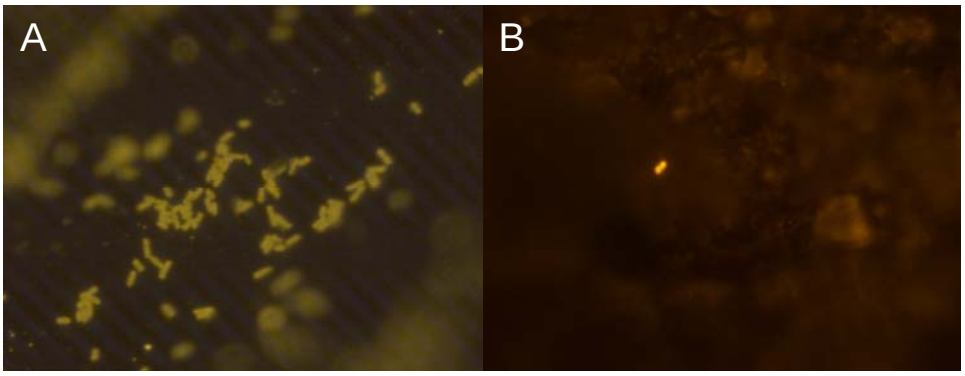

Figure 8. A coupon from river Daugava, spiked with *E. coli* cells (A), not spiked (B) and examined in the Y3 channel.

Thus, to sum up, if the composition of buffers did not have any significant effect on the signal intensity, the washing step as such, had.
In conclusion, before the optimization the protocol took more than 2 hours to complete (90 minutes for hybridization and 30 minutes for washing but now it is possible to obtain up to 50% better signal intensity in half the time necessary. This protocol was validated for field samples as well.

5.2.1.3 The influence of the label

The same sequence was used to design ECOLIFILM probes labeled with CY3 or Alexa488. These were compared and it was found that apparently the latter is far less intensive as a marker than the former.

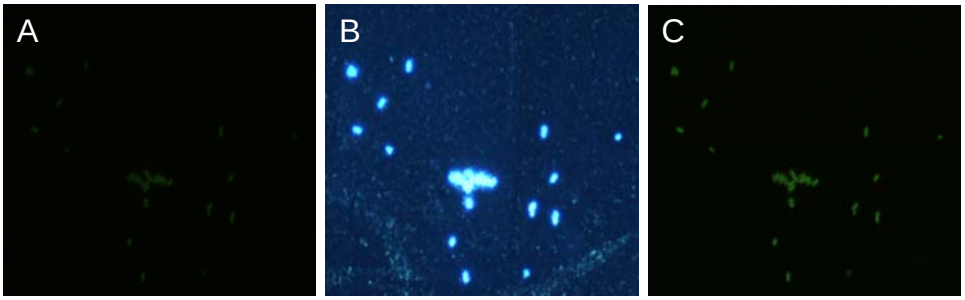

Figure 9. *E. coli* sample labeled with ECOLI-Alexa probe and examined in, at first, I3 filter (A), then DAPI filter (B) and finally, in I3 filter again.

Using CY3 the relative fluorescence intensity was around 2000 units, with slightly over 3000 as a maximum while Alexa488 gave a signal around 300 with about 500 as a maximum (data not shown). In addition, Alexa488 is a green fluorescent dye and should not be used together with DAPI stain (see

illustration in Fig 9 and also Chapter 4) which somewhat decreases its possible contribution for biofilm experiments.The fluorescence intensity of ECOLI-Alexa probe using the specific filter I3 (Ex. 450/490, Em. >515) is much lower (Fig 9, A) than when the cells are labeled with ECOLIFILM probe in the Y3 filter (see e.g Fig 7, A). After examination of the sample in the DAPI channel (Fig 9, B) using A filter (Ex. 340/380, Em. >425) and immediate repeated examination in I3 channel again (Fig 9, C) it can be seen that the intensity is considerably higher (over 50% higher according to RFU measurements, data not shown) due to excitation of DAPI. DAPI then is seen in the green channel.

5.2.1.4 Signal amplification

Another approach is to amplify the specific binding signal which may be attempted with a several approaches, *e.g*. two-pass TSA- FISH.
The simplified principle of TSA-FISH involves specific binding of a probe labeled by HRP (horseradish peroxidase), followed by a dinitrophenyl-labeled tyramide, resulting in localized deposition of the activated tyramide derivative. Further dye deposition, and therefore higher levels of signal amplification, can be generated by detecting dye deposited in stage 1 with a horseradish peroxidase–labeled anti-DNP antibody in conjunction with a fluorophore-labeled tyramide. This has been recently done using a DNA probe (Kubota, et al., 2006).
As our approach is the use of PNA probes a considerable effort was put into researching the options of producing a PNA-HRP construct. Unfortunately none of known providers (*e.g*. Applied Biosystems, Eurogentec) nor less known companies (e.g. Bionucleon., Panagene) nor University laboratories (*e.g.* Prof. Brown (Southampton University, Dept. of Chemistry)) were able to provide such a construct. The reason for this is the very expensive and laborious synthesis of such construct.
Therefore more conventional approach was considered instead using the commercially available labels and ready-made kits. GreenStar*™ labeled probes (Genedetect, USA) are prepared using a labeling technology which chemically attaches a novel cross-linked structure to the 3' end of the probe. This cross-linked structure incorporates multiple molecules of biotin, FITC, rhodamine or digoxigenin (DIG). Unfortunately the company works with DNA probes only.
Various amplification systems using a variety of labels are available from *e.g.* Roche, PerkinElmer and Molecular Probes/Invitrogen. After careful consideration it was decided to label the probes with biotin and apply two different signal amplification kits from two different providers which are suitable for this type of construct. Figure 10 shows the principle of the assay. Samples are prepared and probed with haptenylated molecules according to standard techniques. Hapten- recognizers are then applied to the sample, generally as alkaline phosphatase or horseradish peroxidase conjugates. Examples of hapten-recognizers include streptavidin and antibodies directed against fluorescein, dinitrophenyl and digoxigenin. The sample is then incubated with substrate working solution. Once the substrate is

enzymatically cleaved, the resulting product forms an intensely fluorescent precipitate at the site of enzymatic activity.

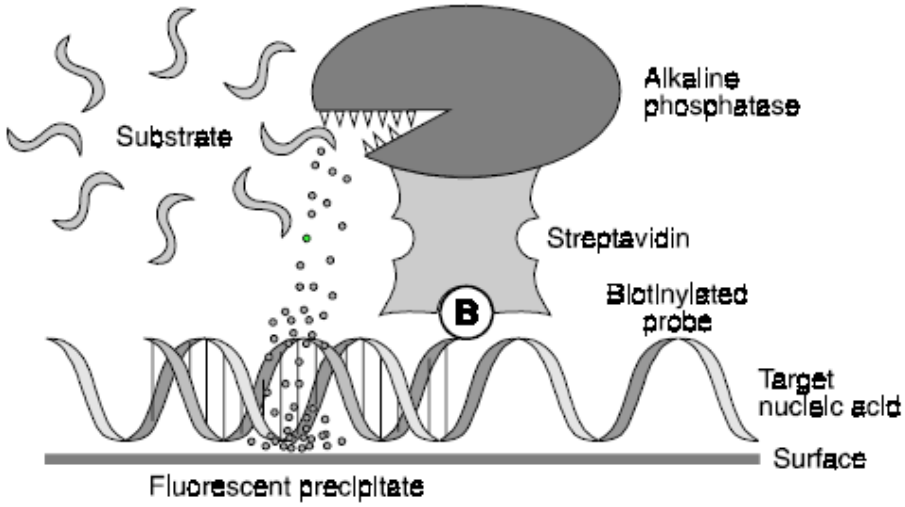

Figure 10. Schematic diagram of the methods employed in the mRNA In Situ Hybridization Kits.

So far the results of signal amplification with ECOLI-BIO probe have not been remarkable, possibly due to that the final fluorescent product is FITC (green fluorescence). Thus the signal intensity is weaker that using CY3 marker and it, again, should not be used together with DAPI. However, more experiments need to be done before making final conclusions on usefulness of this approach for biofilm samples.

5.3 Viability assays

Depending on environmental conditions (starvation, stress etc.) bacteria can be spread in water in "viable but non-culturable" state (Colwell and Grimes, 2000, Oliver, 2005) thus not detectable with culture-based methods. These bacteria, however do retain their virulence factors (Desnues, et al., 2003, Vora, et al., 2005). Methods able to detect bacteria in VBNC state include measuring the efflux pump activity (using SYTO-9 plus ethidium bromide), membrane potential (using [bis-(1,3-dibutylbarbituric acid)trimethine oxonol; $DiBAC_4(3)$]), membrane integrity (using *e.g.* LIVE/DEAD BacLight), cellular respiration (using CTC reduction), glucose uptake activity (using 2-[*N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino]-2-deoxy-D-glucose; 2-NBDG), total ATP concentration (determined with BacTiter-Glo) (Berney, et al., 2006) and direct viable count (DVC) in combination with FISH (Baudart, et al., 2002), The principle of DVC is application of a substance inhibiting cell division but not growth (synthesis processes). After staining viable cells can be observed as larger and more elongated compared to non-viable cells.
Using this approach we have found that drinking water samples which do not display the presence of *E. coli* when analyzed with traditional methods actually contain *E. coli* cells. This was confirmed by PCR analysis as well (manuscript entitled "Detection of *Escherichia coli* in biofilms from pipe

samples and coupons inserted in European drinking water distribution networks", Juhna *et al*, submitted).

The identification, as such, can be performed using DNA or, preferably, PNA probes designed to hybridize with rRNA. However, rRNA is not a direct indicator of viability thus, in future, it preferably should be combined with other methods or other approaches, *e.g*. mRNA hybridization, a more direct viability detection method. On this approach very little literature data is available, probably due to the fact that mRNA content in the cell is much lower than rRNA content. It has been shown that 16S rRNA and tRNA alone are not suitable for assessing the viability of cells as rRNA remains intact for more than 16 hours after cell death (Sheridan, et al., 1998) but tRNA survives even longer (Davis, et al., 1986). This leaves mRNA as a possible candidate for direct viability assays. This approach will be tested after the evaluation of possibilities to increase the signal by protocol modification and evaluation of signal amplification strategies.

6 Search for rRNA sequences

Growing cells produce an abundance of ribosomal RNAs that contain regions of highly conserved, species-specific sequences and are therefore good targets for identification assays, such as FISH. However the target sequences are frequently located in highly structured regions of the rRNA which are virtually inaccessible to DNA probes. PNA probes due to their characteristics can access these regions resulting in a simpler yet highly sensitive and specific assay. Therefore it was decided to consider only PNA probes. The probe search was done using NCBI Blast (http://www.ncbi.nih.gov).
After comparison of several sequences published in literature (Prescott and Fricker, 1999), (Regnault, et al., 2000) and searching the NCBI Blast database (http://www.ncbi.nlm.nih.gov/blast/) the following sequence: 5` TCA ATG AGC AAA GGT- 3`, published earlier by O'Keefe *et al.* (Perry-O'Keefe, et al., 2001), was selected as the most specific and appropriate for PNA probe synthesis. Based on this sequence a 15-mer PNA probe (ECOLIFILM) was designed, labeled with cyanine dye CY3 (Ex: 550, Em: 570) and flanked with solubility enhancers.

For *Aeromonas hydrophila* 16S rRNA a sequence GGAAAGGTTGATGCC was found to be suitable. The results of BLAST analysis are shown in the Table 4. Non-relevant hits are not shown (*e.g.* plants, mammals and uncultured bacterial isolates).

Table 4.. Sequences producing significant alignments with GGAAAGGTTGATGCC:

Accession	Description	Max ident
AB182082.2	Aeromonas hydrophila gene for 16S rRNA, partial sequence, strain:88F	100%
AY987774.1	Aeromonas sp. CCRC 13881 16S ribosomal RNA gene, complete sequence	100%
AY987772.1	Aeromonas molluscorum strain LMG 22214 16S ribosomal RNA gene, complete sequence	100%
AY987770.1	Aeromonas sp. 17m 16S ribosomal RNA gene, complete sequence	100%
AY987768.1	Aeromonas sp. 13m 16S ribosomal RNA gene, complete sequence	100%
AY987765.1	Aeromonas sp. RK 70363 16S ribosomal RNA gene, complete sequence	100%
AY987754.1	Aeromonas hydrophila strain ATCC 49140 16S ribosomal RNA gene, complete sequence	100%
AY987746.1	Aeromonas veronii strain 211c 16S ribosomal RNA gene, complete sequence	100%
DQ460782.1	Gamma proteobacterium T25 16S ribosomal RNA gene, partial sequence	100%
DQ460758.1	Gamma proteobacterium T1 16S ribosomal RNA gene, partial sequence	100%
DQ207728.2	Aeromonas hydrophila strain CCM 7232 16S ribosomal RNA gene, complete sequence	100%
EF077527.1	Aeromonas hydrophila 16S ribosomal RNA gene, partial sequence	100%
CP000462.1	Aeromonas hydrophila subsp. hydrophila ATCC 7966, complete genome	100%
DQ985285.1	Aeromonas sp. PW1 16S ribosomal RNA gene, partial sequence	100%
DQ990069.1	Bacterium 9-gw3-10 16S ribosomal RNA gene, partial sequence	100%
DQ916110.1	Bacillus subtilis strain CTA817-4 16S ribosomal RNA gene, partial sequence	100%
DQ539497.1	Aeromonas hydrophila 16S ribosomal RNA gene, partial sequence	100%
DQ837036.1	Aeromonas hydrophila 16S ribosomal RNA gene, partial sequence	100%
DQ837035.1	Aeromonas sp. IB-l 16S ribosomal RNA gene, partial sequence	100%
DQ837026.1	Aeromonas sp. Hunan-BM 16S ribosomal RNA gene, partial sequence	100%

Accession	Description	Max ident
DQ837025.1	Aeromonas sp. Hunan-BL 16S ribosomal RNA gene, partial sequence	100%
AM184306.1	Aeromonas hydrophila partial 16S rRNA gene, strain WAB1968	100%
AM184287.1	Aeromonas hydrophila partial 16S rRNA gene, strain WAB1948	100%
AM184282.1	Aeromonas hydrophila partial 16S rRNA gene, strain WAB1943	100%
AM184262.1	Aeromonas hydrophila partial 16S rRNA gene, strain WAB1922	100%
AM184260.1	Aeromonas hydrophila partial 16S rRNA gene, strain WAB1920	100%
AM184246.1	Aeromonas hydrophila partial 16S rRNA gene, strain WAB1905	100%
AM184242.1	Aeromonas hydrophila partial 16S rRNA gene, strain WAB1901	100%
AM184219.1	Aeromonas hydrophila partial 16S rRNA gene, strain WAB1877	100%
AM184217.1	Aeromonas hydrophila partial 16S rRNA gene, strain WAB1875	100%
AM262149.1	Aeromonas media partial 16S rRNA gene, strain V47	100%
AY745743.1	Aeromonas sp. D6 16S ribosomal RNA gene, partial sequence	100%
AY689044.1	Aeromonas sp. 6B_1 16S ribosomal RNA gene, partial sequence	100%
AY689038.1	Aeromonas sp. 6A_1 16S ribosomal RNA gene, partial sequence	100%
AY686711.1	Aeromonas hydrophila subsp. decolorationis 16S ribosomal RNA gene, partial sequence	100%
AY576722.1	Aeromonas sp. 18III/A01/071 16S ribosomal RNA gene, partial sequence	100%
DQ190302.1	Bacterium UASWS0089 16S ribosomal RNA gene, partial sequence	100%
DQ190287.1	Bacterium UASWS0074 16S ribosomal RNA gene, partial sequence	100%
DQ190281.1	Bacterium UASWS0068 16S ribosomal RNA gene, partial sequence	100%
DQ188941.1	Aeromonas sp. IIPON2 16S ribosomal RNA gene, partial sequence	100%
DQ166817.1	Aeromonas hydrophila strain FN100 16S ribosomal RNA gene, partial sequence	100%
AY880208.1	Aeromonas media isolate H3 16S ribosomal RNA gene, partial sequence	100%
AY880192.1	Aeromonas hydrophila isolate EW4 16S ribosomal RNA gene, partial sequence	100%
DQ029351.1	Aeromonas veronii strain HQ010516C-1 16S ribosomal RNA gene, partial sequence	100%

The sequence produced an alignment with 100% similarity with *Aeromonas veronii* as well, however this species is also known to cause diarrhoea in humans. Unfortunately it also produces a significant lignment with *Bacillus subtilis* (Table 4.)

The best sequence, however was CGTATCAACTGTGACGT as it provided distinction between *A. hydrophyla* and other related species which are not a threat to humans, *e.g. A. salmonicida* (a fish pathogen) or *A. molluscorum* (isolated from moluscs), Table 5. In addition, this sequence corresponded best to the standards for successful probe design (G/C percentage).

Table 5. Sequences producing significant alignments to the sequence CGTATCAACTGTGACGT

Accession	Description	Max ident
AY987774.1	Aeromonas sp. CCRC 13881 16S ribosomal RNA gene, complete sequence	100%
AY987770.1	Aeromonas sp. 17m 16S ribosomal RNA gene, complete sequence	100%
AY987768.1	Aeromonas sp. 13m 16S ribosomal RNA gene, complete sequence	100%
AY987754.1	Aeromonas hydrophila strain ATCC 49140 16S ribosomal RNA gene, complete sequence	100%
EF140713.1	Aeromonas hydrophila 16S ribosomal RNA gene, partial sequence	100%
DQ207728.2	Aeromonas hydrophila strain CCM 7232 16S ribosomal RNA gene, complete sequence	100%
CP000462.1	Aeromonas hydrophila subsp. hydrophila ATCC 7966, complete genome	100%
DQ837036.1	Aeromonas hydrophila 16S ribosomal RNA gene, partial sequence	100%
AM184306.1	Aeromonas hydrophila partial 16S rRNA gene, strain WAB1968	100%

Accession	Description	Max ident
AM184287.1	Aeromonas hydrophila partial 16S rRNA gene, strain WAB1948	100%
AM184282.1	Aeromonas hydrophila partial 16S rRNA gene, strain WAB1943	100%
AM184262.1	Aeromonas hydrophila partial 16S rRNA gene, strain WAB1922	100%
AM184246.1	Aeromonas hydrophila partial 16S rRNA gene, strain WAB1905	100%
AM184242.1	Aeromonas hydrophila partial 16S rRNA gene, strain WAB1901	100%
AM184219.1	Aeromonas hydrophila partial 16S rRNA gene, strain WAB1877	100%
AM184217.1	Aeromonas hydrophila partial 16S rRNA gene, strain WAB1875	100%
AM262151.1	Aeromonas hydrophila subsp. ranae partial 16S rRNA gene, type strain CIP 107985T	100%
DQ219814.1	Aeromonas sp. m22 16S ribosomal RNA gene, partial sequence	100%
AY689044.1	Aeromonas sp. 6B_1 16S ribosomal RNA gene, partial sequence	100%
AY689038.1	Aeromonas sp. 6A_1 16S ribosomal RNA gene, partial sequence	100%
AY686711.1	Aeromonas hydrophila subsp. decolorationis 16S ribosomal RNA gene, partial sequence	100%
DQ190287.1	Bacterium UASWS0074 16S ribosomal RNA gene, partial sequence	100%
DQ190281.1	Bacterium UASWS0068 16S ribosomal RNA gene, partial sequence	100%
DQ188941.1	Aeromonas sp. IIPON2 16S ribosomal RNA gene, partial sequence	100%
AB192409.1	Aeromonas sp. W35 gene for 16S rRNA, partial sequence	100%
AB192407.1	Aeromonas sp. S7 gene for 16S rRNA, partial sequence	100%
AB192397.1	Aeromonas sp. Hi13 gene for 16S rRNA, partial sequence	100%
AB182089.1	Aeromonas hydrophila gene for 16S rRNA, partial sequence, strain:95F	100%
AB182086.1	Aeromonas hydrophila gene for 16S rRNA, partial sequence, strain:92F	100%
AB182081.1	Aeromonas hydrophila gene for 16S rRNA, partial sequence, strain:87F	100%
AB182055.1	Aeromonas hydrophila gene for 16S rRNA, partial sequence, strain:61F	100%
AB182054.1	Aeromonas hydrophila gene for 16S rRNA, partial sequence, strain:60F	100%
AB182039.1	Aeromonas hydrophila gene for 16S rRNA, partial sequence, strain:45F	100%
AB182038.1	Aeromonas hydrophila gene for 16S rRNA, partial sequence, strain:44F	100%
AB182034.1	Aeromonas hydrophila gene for 16S rRNA, partial sequence, strain:40F	100%
AB182024.1	Aeromonas hydrophila gene for 16S rRNA, partial sequence, strain:30F	100%
AB182021.1	Aeromonas hydrophila gene for 16S rRNA, partial sequence, strain:27F	100%
AB182018.1	Aeromonas hydrophila gene for 16S rRNA, partial sequence, strain:24F	100%
AB182017.1	Aeromonas hydrophila gene for 16S rRNA, partial sequence, strain:23F	100%
AB182012.1	Aeromonas hydrophila gene for 16S rRNA, partial sequence, strain:18F	100%
AB182008.1	Aeromonas hydrophila gene for 16S rRNA, partial sequence, strain:14F	100%
AB182007.1	Aeromonas hydrophila gene for 16S rRNA, partial sequence, strain:13F	100%
AB182003.1	Aeromonas hydrophila gene for 16S rRNA, partial sequence, strain:09F	100%
AB182002.1	Aeromonas hydrophila gene for 16S rRNA, partial sequence, strain:08F	100%
AB182000.1	Aeromonas hydrophila gene for 16S rRNA, partial sequence, strain:06F	100%
AB181999.1	Aeromonas hydrophila gene for 16S rRNA, partial sequence, strain:05F	100%
AB181998.1	Aeromonas hydrophila gene for 16S rRNA, partial sequence, strain:04F	100%
AB181997.1	Aeromonas hydrophila gene for 16S rRNA, partial sequence, strain:03F	100%
AB181995.1	Aeromonas hydrophila gene for 16S rRNA, partial sequence, strain:01F	100%
AB120347.1	Aeromonas sp. 12M17 gene for 16S rRNA, partial sequence	100%
DQ308551.1	Bacterium OrSF3 16S ribosomal RNA gene, partial sequence	94%
AB182053.1	Aeromonas hydrophila gene for 16S rRNA, partial sequence, strain:59F	94%
AB182043.1	Aeromonas hydrophila gene for 16S rRNA, partial sequence, strain:49F	94%
AB182041.1	Aeromonas hydrophila gene for 16S rRNA, partial sequence, strain:47F	94%

The latter sequence was chosen for the probe design. he probe was labeled with CY5 dye (Ex. 649 Em. 666) and flanked with solubility enhancers. The experiments were done as described above (optimized PNA probe protocol, 60 minutes hybridization, no washing) and the sample was vizualised using CY5 filter (Ex. 640/20, Em. 680/30) The cells are clearly labeled (Fig. 11). It has also been ascertained that the probe does not bind to *E. coli* (data not shown).

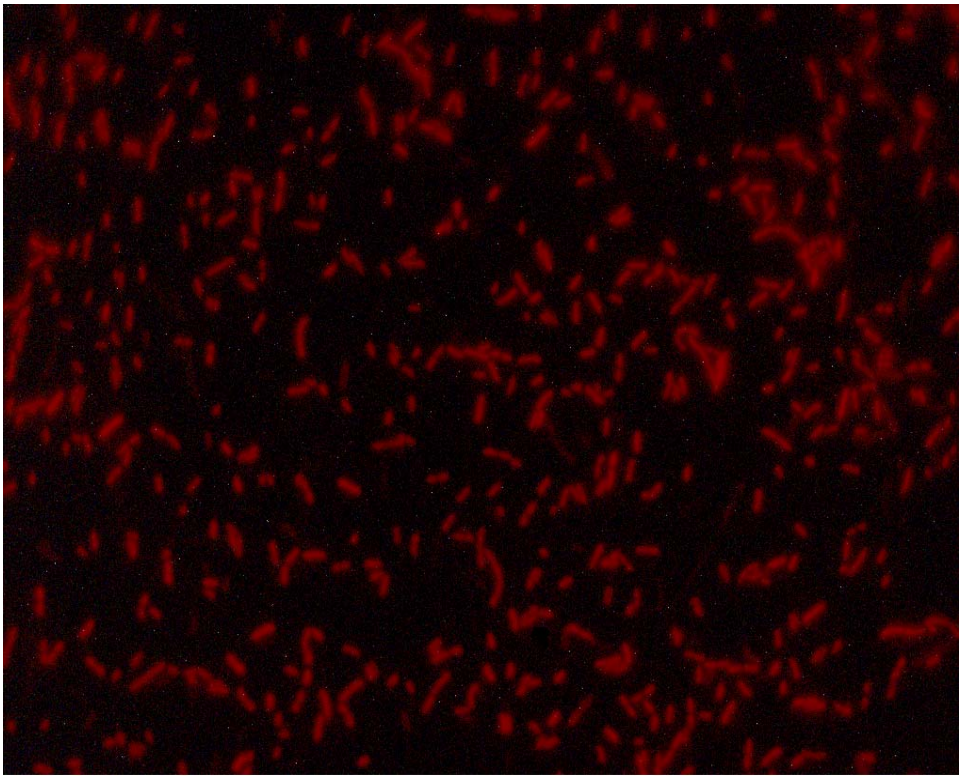

Figure 11. *A. hydrophila* hybridized using AEROHYD probe and visualized using CY5 filter.

7 Future work

7.1 Search for mRNA

As discussed above, if the viability needs to be assessed, rRNA must be analyzed along with viability indicators, such as DVC, respiration or membrane potential among others. Another approach is to target mRNA which could be possible if the probe design and experimental setup ensures maximum signal due to very low concentrations of mRNA in the cells. In order to select a suitable sequence for mRNA analysis one must consider several problems. First, mRNA content in the cell is low, compared to rRNA. Second, some genes are expressed more so their corresponding mRNAs will be found in higher concentrations compared to other genes. Third, the selected gene must be expressed not only under optimal growth conditions but also during sub-optimal growth conditions, *e.g*. starvation and oxidative stress as usually observed in drinking water biofilm cells. The expression profile can be very different from that in stationary phase or exponential phase of growth as it has been shown using *Enterococcus faecalis*. (Heim, et al., 2002). Thus a clear candidate for universal detection of all kinds of *e.g. E. coli* cells does not exist.

Therefore we chose to approach the problem from another angle. The heat shock response is a mechanism by which organisms react to a sudden increase in the ambient temperature. The consequence of such an unmediated temperature increase at the cellular level is the unfolding, misfolding, or aggregation of cell proteins, which threatens the life of the cell. This increase in damaged proteins is usually counteracted by a simultaneous increase in the level of heat tolerant proteins, known as chaperones and proteases, which either refold or degrade unfolded proteins. The heat shock proteins are necessary for protein folding.

The archetypical Stress70 chaperone machine was defined in *E. coli*, and consists of the products of dnaK , dnaJ and grpE. DnaK is the central, ATP-dependent component of the machine, and functions as a chaperone in association with DnaJ, an activating protein, and GrpE, a nucleotide exchange factor (Liberek, et al., 1991). DnaK is about 70 kDa protein and one of the most abundant in the cell upon heat shock (Neidhardt and VanBogelen, 1987) and is overexpressed when *E. coli* grows in biofilm (Beloin, et al., 2004).

Heat shock triggers an increase in chaperone dnaK mRNA. This response has been observed in wide variety of microorganisms, *e.g E. coli* (Richmond, et al., 1999), *Streptococcus pyogenes* (Woodbury and Haldenwang, 2003), *Lactococcus lactis* (Whitaker and Batt, 1991), *Campylobacter jejuni* (Stintzi, 2003) and *Chlamydia trachomatis* (Engel, et al., 1990). We have chosen to design a probe based on heat shock protein, DnaK next.

Two approaches will be used, (i) the new protocol, which provides intensive signal and therefore it is possible that CY3 labeled mRNA can be seen and (ii) signal amplification using biotin labeled probe.

Less information is available on *A. hydrophila* heat shock protein mRNA sequences but as the stress response is universal the approach could very well be suitable for *A. hydrophila* and other microorganisms of interest.
Depending on the results obtained from *E. coli* mRNA detection experiments a mRNA probe for *A. hydrophila* will be designed.

8 Conclusions

Improvements have been made for analyses of *E. coli* cells in (i) in lab-scale samples (water and biofilm) and (ii) environmental samples (water and biofilm) , namely,

- better signal intensity has been obtained
- hybridization time has been shortened
- washing step (consumes time and reagents as well as decreases signal) has been eliminated

As a result, it is possible to apply this protocol and detect *E. coli* directly on the surface using PNA probes and new protocol which ensures maximum signal intensity. The new protocol was also used for hybridization of *A. hydrophyla* with its corresponding probe.

A novel PNA probe for *A. hydrophila* rRNA has been designed and verified. Such probe to our best knowledge has not been designed and published previously.

The DNA probes for detection of α-, β- and γ- proteobacteria have been verified for pure cultures, spiked samples and real biofouling samples.

CY3 has so far proved to be the best fluorescent label for analysis of environmental samples, such as biofilm.

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