Microbiological detection for sustainable water use in chemical, paper, textile and food industries

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Executive summary

In this report an overview is given about the up-to-date status on currently available microbiological data in the 4 different industries. The molecular-based methods used in the project for microbial analysis are summarized and the benefits and limitations of the technologies are discussed.

The literature based information on micro-organisms supposed to be relevant in the four sectors is constantly updated and is substituted with new experimental information that has been achieved by applying suitable cultivation-dependent as well as cultivation-independent methods on samples from the paper and textile industry.

Literature and experimental data were combined and a total of 184 micro-organisms were selected so far as being considered relevant for water streams within the four sectors.

Based on the gained knowledge a prioritisation on further probe development can be performed. An update is provided on the current status of the gene probe development detecting pathogens and other micro-organisms supposed to be relevant for processes as well as in hygiene, biofilm and biofouling aspects. So far 62 gene probes are available for cultivation-independent monitoring of important micro-organisms directly in water samples of the four industries.

As early biofilm forming bacteria in paper mills harboring neutral to alkaline conditions the first two bacteria groups are likely to be identified. The presence of bacteria related to Tepidimonas aridensis and to Cloacibacterium as stable micro-flora in paper mill samples of SAP could be confirmed by using specific gene probes. Research is ongoing and if relevance can be confirmed the first easy-to-use monitoring kits for these organisms will be developed for general use.

A first look into the black box of microbial diversity present in water samples from textile industry could be performed. By applying VIT-profiling and bio-digital approaches on samples from textile industry a surprising diversity of microbial populations could be analysed. All in all 79 isolates were identified and 205 clone sequences were analysed.
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1. Introduction

The concept of water being a never-ending resource with a limitless renewable capacity belongs to the past (Beekman et al., 1998). Especially the sustainable use of drinking water for processes which can be run with a minor quality of water is an important task for the next decade. Therefore, the development and evaluation of adapted re-use of water strategies for selected process applications in the four different industries paper, chemical, food and textile is under investigation in Aquafit4Use.

Besides the successful technical implementation of new treatment technologies and processes another key aspect within the project is the assessment and definition of the real microbiological water quality required for each process. For a successful combination of knowledge about required water quality and the development of treatment technologies suitable microbiological methods need to be available for process evaluation and variance comparison. Moreover, a comprising knowledge regarding the potential process relevant micro-organisms per industry needs to the established per industry. Based on the established knowledge adapted monitoring tools are to be developed to verify the microbiological quality in the different industries.

Within Aquafit4Use not only cultivation-based methods are applied, but a strong focus is given to molecular-methods for microbial analysis in order to reveal the complete microbial composition of relevant water streams and processes. Thus, it is expected to have a more detailed and comprising overview on microbiological parameters influencing water quality, safety and process stabilities.

1.1. Objectives

Determination of microbiological quality standards adapted to different industries and to different processes/products within industries but with the aim to give cross-sectorial input (learning from each other).
2. **State of the art: Current industrial microbiology – Differences among the industries**

In the four different industrial sectors of the project (paper, chemical, food and textile) current microbiological knowledge and routine quality control is mainly obtained by traditional cultivation-based methods. However, it is also widespread knowledge that cultivation-based microbiology is restricted by slowness and inadequacy to describing actual population communities (Wagner et al., 1993, Amann, et al., 1995, Casani et al., 2005, Lahtinen et al., 2006).

Cultivation-based monitoring is suitable for analysis of known pathogens and indicator organisms which are thought to represent a certain “trend” in a monitoring area. For particular industries like food industry the absence of pathogenic micro-organisms in food and food related processes is a clear pre-requisite for food safety and public health. However, it is well accepted that cultivation-based methods can only show up a maximum of 0.1 % - 15 % of all micro-organisms in a certain environment (Amann et al., 1995). Thus, it can be concluded that the main part of the present populations remains undetected. Main reasons for this deviation are the inadequacy to prepare and offer artificially the right conditions for all micro-organisms to grow but also the ability of certain micro-organisms to enter a non-cultivable status (“viable but non-culturable” - VBNC) (Barer and Harwood, 1999). The formation of VBNC cells has been proposed as a survival strategy in order to response to mild environmental stress (Yamamoto et al., 1996, Pommepuy et al., 1996, Besnard, et al., 2002, Ganesan et al., 2007).

Moreover, the formation of agglomerations of many micro-organisms as biofilms represents another survival strategy of the micro-organisms to protect themselves against unfavourable conditions and toxic substances, e.g. antibiotics, chlorine and detergents (Costerton et al., 1987). This often inhibits the ability to grow the bacteria on artificial media, too. Furthermore, in complex samples a high diversity of micro-organisms can be expected which underline a certain competition about available substrates. By transferring such consortia to artificial agar plates the “slow-growers” are out-competed by the “fast-growers” under the supplied living conditions (Wagner et al., 2003). Thus, only a small fraction of all present organisms will show (grow) up. Hence, for the actual description of an environment including all relevant micro-organisms (not only the small cultivable fraction), cultivation-independent methods need to be applied.

During the last two decades new molecular-biological methods were developed which enable the analysis of micro-organisms without prior cultivation. One of the most important and successfully applied techniques is the Fluorescence in situ Hybridisation (FISH) which uses specific gene probes in a simple diffusion process for the detection of the micro-organisms and for establishing comprising population profiles (for review see Amann et al., 1995).

PCR (Polymerase Chain reaction) is another powerful method which enables the detection of DNA traces of micro-organisms in a given sample by an automated enzyme driven process. Several derived PCR-based methods, e.g. LH-PCR (Tiirola et al., 2009) or combined methods like T-RLFP (Restriction Length Fragment Polymorphism (Granhall et al., 2010) and DGGE (Denaturing gradient gel electrophoresis) (de Clerck et al., 2004) are currently applied to establish population profile patterns to elucidate and compare microbial community structures.

Within Aquafit4Use cultivation-based methods are applied where suitable (e.g. confirmation of the safety of a certain process by using Standard DIN methods). But molecular-based methods are favoured for microbiological monitoring of different processes in order to

a) describe various population communities completely not only partial. Improve the current microbiological knowledge in the industries.

b) increase the speed of analysis in order to quickly evaluate newly implemented processes
c) develop new tools to monitor process relevant, but difficult to cultivate or non-cultivable micro-organisms

Among the different industries, process complexities, process conditions and potential toxicities of substrates of investigated processes as well as of water treatment management vary enormously. As a consequence, the microbiology and/or potential microbiological problems may differ. However, development of water re-use options are handled in each industry and strategies which will lead to success in terms of microbiological quality in a certain industry could thus also be helpful in another industry sector. Successful monitoring of certain relevant organisms by using fast, sensitive and cultivation-independent methods can improve processes not only in one sector but distributed knowledge concerning the developed strategies can help solve problems also in other industries - learning from each other.

In order to develop cultivation-independent microbiological monitoring tools a comprising catalogue of micro-organisms supposed to be relevant in terms of biofilm/biofouling, hygiene and pathogenicity per industry sector is constantly updated by literature research.

In parallel to the establishment of the micro-organisms catalogue corresponding 16S rDNA sequences are collected and added to a 16S database which serves as basis for the development of specific gene probes. Therefore, 16S rDNA sequences are added from literature but also by application of advanced molecular-biological approaches which are carried out on samples from relevant processes of the 4 industries. Also from these studies valuable sequences are retrieved and added to the database. Data are collected and processed. Processed data are used for the development of specific gene probes for relevant micro-organisms. Relevance of the micro-organisms will be confirmed and assessed by application of the newly developed gene probes on industry samples. And finally, based upon these results a collection of easy-to-use test-kits for the monitoring of relevant micro-organisms will be established and marketed for general use (for overview see Figure 1).
So far, the abundance of certain micro-organisms is mainly monitored by cultivation of sample material on different media and the resulting frequencies and numbers of grown bacteria are given as colony forming units (cfu) /sample volume.

Generally, in order to simplify microbiological monitoring it is widespread practice to monitor so-called indicator organisms in water samples instead of all possible pathogens. Indicator organisms are normally present in the feces of human and other warm-blooded animals. If present they indicate that water was polluted by fecal-related substances. The most monitored indicator organisms are *E. coli* and coliform bacteria. By analysing these bacteria the microbiological quality of drinking water and other types of water is assessed (see Table 1). According to drinking water guidelines *E. coli* and coliform bacteria may not be present in 100 ml of drinking water, EU Drinking Water Directive 98/83/EC (EU-DWD). By this monitoring strategy microbial analysis is easy to perform as suitable cultivation media or easy rapid tests (like e.g. Colilert, IDEXX) for the targeted bacteria are available. Since several years it is discussed to replace coliform bacteria as fecal indicator for water by Gram-positive enterococci. These bacteria
are more resistant to environmental stress and thus can indicate fecal contaminations which originate over longer periods than those indicated by Gram-negative coliforms. However, in many water laboratories enterococci are still only analysed on a periodic basis and not as standard parameter as specified by EU-DWD (pers. commun.).

In Table 1 the typical analysis parameters are summarized which are mandatory or recommended for monitoring of different types of water. In addition to the above mentioned indicator bacteria the aerobic heterotrophic colony count at 22°C and 36°C is used as an additional indicator parameter for monitoring purposes (prEN ISO 6222). By this approach trends of the microbial quality can easily be observed and also the effectiveness of cleaning processes can be assessed without much effort. In drinking water certain pathogens like Clostridium perfringens, Pseudomonas aeruginosa or Legionella pneumophila are only analysed on a periodic basis or upon request (presumed biofilm formation, control of house installations, bottling of water). Cooling towers also need to be analysed periodically regarding the amount of pathogenic Legionella pneumophila. Depending on the amount of L. pneumophila detected, different counteractive measure activities are pre-scribed and also shorter analytical periods.

### Table 1: Overview of mandatory and recommended microbiological quality parameters for different types of water

<table>
<thead>
<tr>
<th></th>
<th>drinking (tap) water (routine)</th>
<th>drinking (tap) water (periodic)</th>
<th>surface water (freshwater)</th>
<th>ground water</th>
<th>process water food (routine)</th>
<th>cooling water</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Indicators</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>aerobic heterotrophic counts (22°C, 36°C)</td>
<td>+</td>
<td>+</td>
<td>(+)</td>
<td>(+)</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>coliform bacteria</td>
<td>+</td>
<td>+</td>
<td>(+)</td>
<td>(+)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Escherichia coli</td>
<td>+</td>
<td>+</td>
<td>(+)</td>
<td>(+)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Enterobacteriaceae</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Enterococci</td>
<td>+1,3,5)</td>
<td>+</td>
<td>(+)</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><strong>Pathogens</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Clostridium perfringens</td>
<td>+4,5)</td>
<td>+</td>
<td>(+)</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Pseudomonas aeruginosa</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>(+)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Legionella/ L. pneumophila</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Salmonella</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Campylobacter</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>(+)</td>
<td>-</td>
</tr>
<tr>
<td>Listeria</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>(+)</td>
<td>-</td>
</tr>
<tr>
<td>Staphylococcus</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>(+)</td>
<td>-</td>
</tr>
<tr>
<td>Bacillus cereus</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>(+)</td>
<td>-</td>
</tr>
<tr>
<td>Enterobacter sakazakii (Chronobacter)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>(+)</td>
<td>-</td>
</tr>
</tbody>
</table>

1) parameter according to EU-DWD, some countries use the parameter coliforms instead of enterococci for routine monitoring; 2) for special application: e.g. house installations; 3) needs to be monitored for water used for bottling; 4) needs to be monitored if water is received from surface water; 5) not yet regulated, but recommended parameter; 6) monitoring of these parameters varies among different food sub-sectors

Unfortunately, up to now for all other water types than drinking water and cooling water no defined microbial monitoring criteria are established. Therefore, European water experts highly recommend the implementation of parameters for other water types as they are indispensable for early-warning systems, surveillance and control of treatment processes and thus safeguard the
high quality of (drinking) water. For reference see reports of Expert Working Group Microbial Quality, EU-funded project WEKNOW (no.: EVK1-CT-2002-2004) and reports water expert consortium EU project TECHNEAU (no.: 018320).

In the food sector with its various sub-sectors (e.g. meat, cheese, milk, beverage, water) the microbiological knowledge and awareness has a high priority in comparison to the other three industries which are covered in this project. This is due to requested and regulated health, safety and hygienic standards for raw material, production, product and distribution of products within food industry. Compared to drinking water the number of analysed microbiological parameters for water in contact with food or packaging material are more comprising and more focused on pathogens (see Table 1). Also here total heterotrophic counts are measured as universal hygiene status and E. coli as fecal indicator. But for safety reasons other pathogens like Campylobacter or Staphylococci are analysed additionally, dependent on the type of food industry. In general, the analysis of coliform bacteria is replaced by the analysis of the much bigger group of Enterobacteriaceae, which includes important pathogens like Salmonella and Shigella. Other pathogens like Enterobacter sakazakii (Cronobacter) are only analysed if water is used for manufacturing products related to the nutrition of babies and infants.

However, also in the food sector with established process technologies hygienic or process-related problems can result from presence of non-cultivable micro-organisms which can only be analysed and traced by molecular methods (e.g. Loy et al., 2005). Moreover, new process technologies are going to be established or innovative water re-use options are to be evaluated which can create new microbiological problems in terms of sustainable water management which might not be traced accurately enough by using solely cultivation-based approaches (Casani et al., 2005).

Safety or process-related problems due to the presence of biofilm formation or biofouling can only be monitored to a minor extent by analysing current water indicator organisms like e.g. coliform bacteria. This is because biofilm-forming and biofouling-related bacteria can show totally different behaviors in terms of resistance against toxic substances or cleaning agents compared to certain indicator bacteria (Watnick and Kolter, 2000, Costerton et al., 1987, Flemming, 2002). Thus, in many publications the analysis of other actually relevant indicators or pathogens which are much more related to the real conditions of the processes are requested (Casani et al., 2005, Lahtinen et al., 2006).

Knowledge about valuable indicator organisms, pathogens or biofilm/biofouling micro-organisms varies among the four industry sectors of the projects:

Microbiology in the textile industry was almost no issue before the establishment of Aquafit4Use project. This is mainly due to the toxicity of some of the used process chemicals, the partly high process temperatures and to the poor results, which microbiological analysis by cultivation assays has created so far. Only few publications summarizing microbiological issues in the textile or related industries are available (Lacey and Lacey, 1987). However, several unsolved problems (e.g. spotting on produced fabrics) do exist in the textile industry with a high impact on costs and quality but with no answers regarding: What is the real reason for the problem (could it eventually also be related to microbiology? And correspondingly how can the problem be eliminated and solved.

Processes in the chemical industry sector seem not to be affected to a wide extent by microbiological problems. At least related literature is almost impossible to find. We conclude that the toxicity of chemicals and processes limit microbial growth but also possible proprietary data
about production processes might be the reasons for non-disclosure of microbiological problems to the public. However, within Aquafit4Use new wastewater treatment processes are developed, implemented and tested for water re-use options in chemical industry sector. That means microbiological issues related to produced water quality but also to wastewater treatment are relevant to analyse. The latter were treated by various molecular biological approaches in the past which led to complete new insights into the microbial composition of wastewater treatment processes and the corresponding real microbial players (Snaidr et al., 1997, Juretschko et al., 1998, Schmid et al., 2003.)

In the paper industry a comprising knowledge based on cultivation has been gained about microbiology and microbiological problems (Väisanen et al., 1998, Desjardins and Beaulieu, 2003, Oppong et al., 2000, Verhoef et al., 2005, Suihko and Skyttä, 2009). However, as biofilm and biofouling related problems influence strongly the paper making process and thus also the quality of the final product, classical indicators and corresponding cultivation-based methods for drinking water are not relevant in these processes (Lahtinen et al., 2006). Based on such conclusions research in the paper industry had a strong focus in the last years to analyse related problems with the best suitable cultivation-independent methods. As a consequence of this research activity in the paper sector the highest number of potentially relevant organisms was found for this industry (see also data displayed in Table A1, ANNEX)

Also former EU-funded projects (e.g. BIOTECH CONTROL (No.: QLRT-1999-01389), ODOUR CONTROL (FP6-2003-SME-1, project no.:016730) which used molecular methods for microbial analysis in the paper industry could show that the uncultivable fraction of micro-organisms within production processes can represent an important part of the biofilm forming community (Granhall et al., 2010). Recent studies (Kolari et al., 2001, Ekman et al., 2007, Tiirola et al., 2009) focused on the biofilm-forming organisms in the paper industry by molecular methods and found different organisms to be responsible as early slime formers. It was concluded that pH could be a principal process parameter which triggers the growth of different micro-organisms and correspondingly biofilm formation. (Tiirola et al., 2009). Very surprisingly, 2 of 3 supposed to represent early slime formers (relatives of Tepidimonas arfidensis and Cloacibacterium) from the latter study were also detected within ODOUR CONTROL project. They were considered to have possibly high impact on biofilm and odour problems in paper mills. Specific gene probes were developed by VER and the high abundance of these bacteria could also be confirmed in process samples from SAP (see results examples under 4.2).
3. Methods

3.1. Basics: Molecular-biological methods for microbial analysis

As cultivation-dependent methods are limited to elucidate the complete population structure of complex environmental samples a set of different molecular-biological methods, partly in combination with cultivation methods is applied during Aquafit4Use. Beforehand an overview on the necessary principles and findings is given which led to the development of new powerful methods for community analysis in environmental samples.

The work of Carl Woese and colleagues with their successful approach to reconstruct microbial evolution using comparative sequences analysis of rRNA molecules represented the starting point for modern identification methods (Fox et al., 1977, Woese, 1987). Nowadays, comparative sequence analysis of 16S rRNA has become an integral part of the species description of bacteria and archaea, and 16S rRNA sequences have been determined for virtually all of the ca. 8.000 validly described species.

![Figure 2: The tree of life (TOL) based on comparative analysis of rRNA sequences (domain Bacteria = blue, domain = Archaea = green, domain Eukarya = red) (Letunic and Bork, 2007).](image)

rRNA molecules are well suited for the identification of bacteria and archaea for several reasons.
First, all cells require ribosomes for translation. Each prokaryotic ribosome contains one 16S rRNA with ~1,600 nucleotides, one 23S rRNA with ~3,000 nucleotides and one 5S rRNA with ~120 nucleotides. As each cell contains many ribosomes, these target molecules are naturally amplified in relation to the physiological condition of the cell to numbers that can range from a few hundred to 100,000 per cell (Amann and Fuchs, 2008).

Second, the evolutionary conservation of rRNA sequences is patchy, that means some parts of this molecule are very conserved among all bacteria or certain groups of bacteria and other parts are very variable and thus specifically present only in a certain species. This fact enables the design of oligonucleotide gene probes (mostly 18–25 bases) with different specificities: for large taxonomic entities, such as domains, phyla or genera but also for distinct species.

Third, targeting rRNA for identification purposes links microbial ecology and microbial evolution, a concept that was first promoted by Norman Pace and colleagues (Olsen et al., 1986). In this system, categorization is no longer based on morphology (e.g. rods versus cocci) or physiology (e.g. aerobes versus facultative anaerobes, versus strict anaerobes), but rather is based on the three domains, Archaea, Bacteria and Eukarya, and phyla and classes, such as the Alpha-, Beta-, and Gammaproteobacteria (see Figure 2).

3.1.1. Fluorescence in situ hybridisation (FISH)

Based on the findings of Carl Woese Fluorescence in situ hybridisation (FISH) was developed during the late 1980s and has led to completely new insights into the microbial world. Since that time it has become a widely used method for the identification, quantification and – in combination with other techniques – characterisation of phylogenetically defined viable microbial populations in complex environments (for review: Wagner et al., 2003).

The basic principle of FISH consists of 4 main steps:

Fixation. By using ethanol or formaldehyde the microbial cells are fixed directly (in situ) in the samples. This not only stabilises cell morphology, but also permeabilises the cell membrane for subsequent hybridisation. In case that certain Gram-positive bacteria from the Firmicutes phylum are to be analysed an additional enzyme treatment of the fixed cells needs to be carried out prior to hybridisation (Beimfohr et al., 1993).

Hybridisation. By incubation (only 1-2 hours) of the fixed cells with specific gene probes (made of DNA) labelled with a fluorescent dye, the small gene probes diffuse to its intracellular rRNA targets and form stable hybrids.

Washing. In a final washing step excess probe material is washed out of the cells. In that way only specifically bound gene probes remain within the cells and the dye labelled to the probes can be excited by high energetic light e.g. from a fluorescence microscope.

Evaluation. The sample is then ready for single cell identification and quantification by either epifluorescence microscopy or flow cytometry.
3.1.2. From FISH to VIT – transfer of a powerful scientific method to a suitable standard method for routine use in different industries

Over the past 12 years vermicon has transferred the powerful scientifically-applied FISH method into an industrialised standardised format, the vermicon identification technology (VIT™). VIT is based on the same working principle as FISH, but all steps were simplified, shortened and standardised so that a very handy and effective molecular-biological method was developed for fast and specific analysis of micro-organisms. Figure 3 shows the single steps of the VIT-Analysis.

![Figure 3: Overview on the steps of the VIT-Analysis](image)

The VIT-technology is applied as service but also as ready-to-use test kits. VIT-kits represent a very stable, robust and easy-to-use method which is applied for specific analysis of pathogens, process relevant bacteria and spoiling bacteria in food, beverages, water, and process water. The VIT-kits contain specific gene probes in combination with all required buffers and hardware so additionally only a fluorescence microscope is required (see Figure 4). The complete molecular-biological VIT method can be carried out and evaluated within 3 hours and no molecular-biological skills are required from the operator. Customers world-wide are wastewater treatment plants, food producers, water laboratories, biogas producers, automotive industry, universities and others. For an overview on available VIT-kits and services for microbiological quality control visit also www.vermicon.com.
3.1.3. VIT – fast method applicable for qualitative and/or quantitative analysis

VIT-kits are suitable for qualitative analysis of e.g. *Salmonella* in food samples or beer-spoiling bacteria in beer samples (e.g. Thelen et al., 2004). With these kits all *Salmonella* species are detected within 30 hours in food stuff. In comparison to that the classical cultivation-based detection has a 5-7 days time-to-result. In cases where quantitative results are relevant, e.g. monitoring of filamentous bacteria or nitrifying bacteria in wastewater treatment plants a standardised semi-quantitative approach is followed comparable to the well-known Eikelboom quantification key.

For applications which require distinct quantitative results per volume (e.g. detection of *Legionella pneumophila* in water from cooling towers or water from house installations), the so-called ScanVIT™-approach was developed. With ScanVIT the sample is filtrated on a filter membrane, then the filter is cultivated for only 72 h on a suitable agar-plate until micro-colonies of the bacteria are grown. Then the filter is treated with specific labelled VIT gene probes, washed and analysed using a low-budget fluorescent microscope or a stereo-microscope. Each grown micro-colony which emits a green (*Legionella* genus) or red (*Legionella pneumophila*) fluorescent signal under the microscope can be easily quantified. An exact quantitative result per volume is obtained with a sensitivity of 1 cell per filtrated volume. The time saving is about 7-10 days in comparison to conventional method.

Extensive validation trials comparing ScanVIT-Legionella with conventional method proved applicability and correctness of obtained results (e.g. Christino et al., 2007).

3.1.4. Comparison of VIT with other molecular methods – advantages and disadvantages

Besides FISH or the standardised format VIT other alternative molecular methods exist and are applied as routinely used methods or for research purposes.
The most interesting method in this context for the analysis of process water samples like in Aquafit4Use is represented by polymerase chain reaction (PCR). This method was developed during the early 1980s by Kary Mullis (Saiki et al., 1988). With this technique traces of DNA can be amplified in an automatic procedure. It is widely used in genetic diagnostics, forensics and as basic method in current scientific-based molecular-biology. Moreover, PCR method is also distributed as diagnostic test-kits for mainly medical, food, water and veterinary industry.

The principle of this method is that after total DNA extraction from a given sample two so-called primers (oligonucleotide probes), nucleotides and an enzyme (Polymerase), are added to the DNA. By an automated cyclic assay with 3 constantly changing temperatures the DNA of interest (= target DNA) is amplified by the activity of the Polymerase. Only traces of DNA are sufficient to act as template which makes this method very efficient and sensitive. In the classic format the amplified DNA (PCR product) is analysed by gelelectrophoresis and if the target PCR product is detected, the analysis is called positive. In case of microbiology it means: the organisms DNA was present in the sample. The analysis is very fast, time-to-result takes only a few hours. However, it is important to note, that in most cases the target molecule is the very stable DNA (e.g. DNA can be isolated from ancient mummies), so that a positive finding normally does not indicate whether the detected organism was alive at sampling time.

An advanced format of PCR is represented by the so-called q-PCR. With this approach a very fast quantitative result is obtained by automated simultaneous measuring of the amount of target PCR product and a corresponding standard DNA. A big advantage of q-PCR is that many samples (e.g. 96) can be analysed simultaneously, thus increasing the speed and decreasing the costs of the analysis. The delivered result is not displayed as “colony forming units” (cfu) but as “DNA copies” which can be recalculated as cfu’s.

Another modification of PCR is represented by RT-PCR (RT = Reverse Transcriptase) assays, which should circumvent the disadvantage that no real life/dead differentiation can be carried out by PCR. In this case also RNA is used as target and transcribed by the enzyme reverse transcriptase into DNA before the already described PCR cyclic approach with polymerase and primers is started. Many publications exist with successful applications of a very sensitive method in scientific laboratories. However, so far routine use for applications in industrial microbiology labs seems far from being performable due to the high risk of contamination and the real existing sensitivity levels measured in environmental samples.

### 3.1.5. Why VIT is chosen for many applications in Aquafit4Use?

**Specificity:** In comparison to PCR VIT shows a similar specificity and speed. Both methods are very specific (discrimination up until down to the species level) and have the advantage that by using higher order specific primers and probes, bacterial groups on almost every hierarchical level can be analysed (see also VIT-Analysis 2.2.1) and thus an overview of population structures in complex sample materials can easily be given but also distinct species can be traced. The only exception is that VIT is not suitable for distinguishing organisms on the subspecies or strain level, e.g. different strains of *E. coli*. That means e.g. the very pathogenic *E. coli* strain EHEC can not be differentiated from the normal *E. coli* strains via FISH but by PCR.

**Sensitivity:** VIT shows a very similar sensitivity in comparison to PCR. Both methods have a detection limit of 1,000 cells / ml. Although many publications exist which report PCR sensitivity levels up until half a bacterial cell this is unrealistic for routine use in standard industrial laboratories and especially for environmental samples (Bergmann et al., 2010, Burtscher and
Wuertz, 2003, Cankar et al., 2006, v. Wintzingerode et al., 1997). Moreover, environmental samples tend to contain substances (e.g. humic acids, phenols) which can inhibit partly or totally the activity of the polymerase so that enough target molecules need to be present in order to produce a positive result or the inhibiting substances need to be completely removed from the sample. Otherwise false-negative results by PCR are foreseen. In contrast to this the sensitivity of ScanVIT applications is even lower. Up to 1 cell / filtrated volume can be detected by ScanVIT.

**Life/dead differentiation.** The biggest advantage of FISH/VIT versus PCR is represented by the fact that only viable cells are detected by the FISH/VIT method. FISH uses rRNA as target versus rDNA which is mainly used by PCR for microbial identification. This advantage is even more important if environmental samples are to be analysed *in situ* like in Aquafit4Use where pre-cultivation of the target cells are excluded in order to prevent cultivation-based population shifts.

**Handling and output.** PCR is a very sensitive method with a high risk of cross-contamination. DNA is ubiquitous (e.g. air, equipment, hands etc). Therefore, high-skilled personnel and also high-level laboratory equipment as well as separate rooms for DNA extraction and DNA amplification are required in order to produce constantly valuable results. In contrast, VIT is a very robust method without the need to have experienced personnel or very clean (and extra) rooms for performing the analysis. The evaluation of the results by using a cheap fluorescence microscope allows to analyse not only presence/absence or the amount of the analysed organisms but enables a visualization of the detected micro-organisms directly in the sample material. In contrast to PCR cell morphologies, species diversity, microbial interactions, biofilms or symbiotic living conditions can be analysed and visualised very easily.

See Table 2 for overview of features of a selection of different methods.

**Table 2: Overview of a selection of different methods**

<table>
<thead>
<tr>
<th></th>
<th>VIT</th>
<th>ScanVIT</th>
<th>Q-PCR</th>
<th>Cultivation</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Specificity</strong></td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td><strong>Sensitivity</strong></td>
<td>1000 cells/ml</td>
<td>1 cell/volume</td>
<td>1000 cells/ml</td>
<td>1 cell/volume</td>
</tr>
<tr>
<td><strong>Life/dead discrimination</strong></td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td><strong>Robustness/Handling</strong></td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td><strong>Time to result</strong></td>
<td>3 h</td>
<td>8 h - 72 h</td>
<td>1-3 hours</td>
<td>several days</td>
</tr>
<tr>
<td><strong>Real quantitative results</strong></td>
<td>(+)</td>
<td>+</td>
<td>(+)</td>
<td>+</td>
</tr>
<tr>
<td><strong>Automation</strong></td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td><strong>Differentiation up until subspecies/strain level</strong></td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
</tbody>
</table>

+) very good; -) negative; (+) limited possible
3.2. Applied molecular-biological approaches in the project to assess complex community structures

Environmental samples like biofilms or process waters (wastewater, cooling water etc.) harbour complex community structures of micro-organisms. Up to 99% of these micro-organisms are hard to cultivate or even uncultivable (Amann et al., 1995). To assess the actual microbial abundance, diversity, dynamics and community structure of complex consortia of micro-organisms molecular-biological techniques need to be applied. The following paragraphs will show applicable molecular-biological methods and the degree of information output they can deliver for Aquafit4Use. To understand how the applied technologies as single components are integrated in the big picture of understanding the microbiology in the 4 different industry within Aquafit4Use see Figure 1.

The following Table 3 provides an overview of the applied molecular-biological methods and the degree of information output which can be expected from the different approaches. They are explained in the following sectors.

Table 3: Overview of the applied molecular-biological methods for the investigation of environmental samples

<table>
<thead>
<tr>
<th>Type of analysis</th>
<th>Cultivation</th>
<th>FISH</th>
<th>Clone library</th>
<th>general</th>
<th>detailed</th>
<th>very detailed</th>
<th>not possible</th>
<th>limited possible</th>
<th>unlimited possible</th>
</tr>
</thead>
<tbody>
<tr>
<td>VIT-Analysis</td>
<td>X</td>
<td>x</td>
<td>x</td>
<td></td>
<td></td>
<td>x</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>VIT-Profiling</td>
<td>x</td>
<td>x</td>
<td></td>
<td>x</td>
<td></td>
<td>x</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bio-Digital approach</td>
<td>x</td>
<td>x</td>
<td>x</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
3.2.1. VIT-Analysis

The most basic analysis for environmental samples is the VIT-Analysis. Here specific gene probes on every hierarchical level (from species to phylum-level) to identify and quantify certain micro-organisms (e.g., *Burkholderia cepacia*) or wider groups like e.g., *Enterobacteriaceae* or all ammonia oxidizing bacteria are applied (as an example of application on Aquafit4Use samples see Figure 5).

Directly in the sample and within 3 hours the micro-organisms are detected and can be quantified in relation to all viable bacteria present in the sample. Results are given as % share of all viable bacteria, because a second gene probe specific for all bacteria is always used in the same assay but labelled with a different dye. The cells detected by this bacteria-specific gene probe represent 100% of all viable bacteria in the sample.

VIT-Analysis of ammonia oxidising bacteria (AOB) in WWTP Perstorp

![Figure 5](image)

**Figure 5**: Specific VIT-Analysis of ammonia-oxidising (AOB) bacteria in WWTP Perstorp. Figures demonstrate that in sample of 2009 the amount of AOB in the WWTP is apparently much lower than in 2008. This corresponds to the measured nitrification rates (data not shown).

VIT-Analysis is often combined with the analysis of the total cell count/total viable count. Here an aliquot of the sample is filtrated on a filter membrane. Subsequently, all cells (viable and dead) are stained with a DNA stain (DAPI: 4',6-Diamidino-2-phenylindol-dihydrochloride) and are hybridised with the general probe for all bacteria (or also in combination with the probe for all archaea). Cells stained by these probes are acknowledged as the viable cell count. Stained cells are analysed and quantified manually by fluorescence microscopy. Output is given as cells/analysed volume. Principally obtained result (viable count) is comparable to the heterotrophic cell count, but normally amounts are higher by VIT because also uncultivable organism are stained.
3.2.2. VIT-Profiling

The VIT-Profiling is based on the generation of comprising profiles or patterns of the microbial community at defined sampling points in a process. A general very useful overview on the present bacteria groups in a given sample is obtained and population profiles can easily be compared. An applied set of 10 specific gene probes on phylum and subphylum level covers approximately 95% of all bacteria present in industrial samples (see Figure 6).

![Figure 6: Simplified Tree of Life including the 10 gene probes for the main bacteria groups on phylum- and subphylum-level (marked green)](image)

Micro-organisms targeted by these profiling probes present in the samples can be grouped according to their affiliation to the analysed groups. Results are given as % share of all viable bacteria. The advantage of this approach is to quickly assess and compare the microbial community structure of different samples without knowing the exact names of each detected organism. (Figure 7, Part 2).

The applied set of probes can be extended depending on demand. In this case more detailed information within the 10 different bacteria groups can be assessed by application of further existing gene probes on species- or genus-level which are directly subordinated to the 10 screening gene probes.

Further detailed data on the community structure of the samples can be collected by the sequencing-based identification of cultivable micro-organisms which are grown in parallel on standard media from the samples (Figure 7, Part 1). Furthermore, the micro-organisms identified via sequence analysis are also grouped in the 10 profiling groups. In this case first detailed information of present organisms is established. This can help to assess quickly first specific information on species level and provides the possibility e.g. to design specific gene probes for the cultivated bacteria.
This approach is applicable as one time analysis, monitoring analysis over a period of time or for comparison of different samples in the same or different processes.

### Figure 7: VIT-Profiling approach

#### 3.2.3. Bio-Digital approach

The Bio-Digital approach is a very powerful tool for assessing microbiology. By applying this approach the highest degree of information of a unique sample can be retrieved. By connecting all analyses as "closed loop" it is the most complex and time-consuming analysis performed within Aquafit4Use. The information about community composition is retrieved from the sample and is used in a subsequent step to gain new information from the same sample (Figure 8).

For samples which contain a huge amount of micro-organisms which are not covered by the pool of gene probes or are mostly unknown the Bio-Digital approach can be applied to get a detailed cultivation-independent insight in the abundance of micro-organisms and total community structure of the sample. The Bio-Digital approach will address the questions "who's actually out there - known or unknown – cultivable or uncultivable" and how many of them are present in the sample? Figure 8 displays the main steps of the Bio-Digital approach.
The Bio-Digital approach comprises the following main steps:

DNA extraction. Total DNA of all micro-organisms is extracted from the sample.

rDNA PCR. By applying specific primer sets comprising the rDNA genes of all micro-organisms those genes are amplified by polymerase chain reaction (PCR) from the bulk DNA.

Cloning. The pool of rDNA genes is separated by cloning of single rDNA genes into *E. coli* cells. Thus, each clone in the established clone library contains the rDNA of an individual bacterium of the source sample.

Clone sequencing. The clones are sequenced to retrieve the rDNA gene sequences to elucidate the diversity of the micro-organisms present in the samples.

Phylogenetic analysis of retrieved sequences. Comparative sequence analysis with the rRNA gene ARB databases enables the phylogenetic analysis and identification of the retrieved species. A phylogenetic overview on all retrieved sequences is obtained on the DNA level (viable and dead organisms). Based on this information, real abundance and relevance of the identified sequences will be further investigated by VIT-Analysis.
Specific gene probe design. By using the software package ARB gene probes are designed specifically either for the distinct retrieved sequence or for a group of sequences (including the newly found sequence). This group can be on genus, group or phylum level.

Probe development. The designed gene probes will be tested for their specificity on selected target and non-target micro-organisms and after this applied on the environmental sample.

VIT-Analysis. By applying the newly developed gene probe on the original sample a qualitative and quantitative assessment of the corresponding organism(s) is obtained. This investigation will deliver the information of the abundance -is the organism alive present in the sample and to what extent? - and thus, the relevance of the identified micro-organism in the sample can be revealed.

3.3. From sequences to specific probes

Data collection, compilation of 16S rDNA sequence database, phylogenetic analysis and development of gene probes

Data collection. In order to develop monitoring tools for relevant micro-organisms in the different industries, comprising databases of relevant micro-organisms need to be established. On the one hand information of the micro-organisms detected in the industries are collected. On the other hand corresponding 16S rDNA sequence information are collected and combined with existing qualified sequences from public as well as vermicon databases. Thus, a new Aquafit4Use sequence database is established. Sequences from literature research are processed (aligned) and phylogenetically analysed. Using these sequence information new gene probes can be developed which serve as monitoring tools for the detection of the micro-organisms in situ.

Compilation of a 16S rDNA sequence database of micro-organisms supposed to be relevant.

16S rDNA sequence information regarding the selected micro-organisms are collected and compiled in a sequence database of micro-organisms supposed to be relevant. “ARB-Silva-2007” 16S rDNs sequence database, comprising more than 200,000 sequences from described species as well as from unknown micro-organisms (mainly retrieved by molecular approaches) served as a basis for compilation of the database of relevant micro-organisms. For quality reasons of the new sequence database to be established only full (> 1.400 bp) and almost full length sequences (> 1.300 bp) were exported from “ARB-Silva-2007”. Thereby, a number of approximately 50,000 sequences was exported and imported into the new database. This high amount of sequences is a prerequisite to work effectively with 16S rDNA sequences. The sequences cover almost all the hitherto known and described genera and species. Furthermore, sequences were retrieved not only from described micro-organisms but also from hitherto unknown micro-organisms with a relevant relationship to known species.

To these sequences an amount of 2,000 full lengths sequences from vermicon`s own database is added. These sequences are part of the results of more than 12 years of molecular work performed for the food, paper and chemical industry. It comprises known species but also sequences from established clone libraries.

Third source for sequences of relevant micro-organisms is represented by ongoing subsequent molecular work on samples from industry partners in Aquafit4Use. So far comprising VIT-
profilings including cultivation approaches on process samples from textile industry (TXT and SVI) were established. Obtained sequence information of retrieved isolates from the textile samples is added to the sequence database. Furthermore, a 16S rDNA clone library was established for a selected sample from textile industry (TXT4) which showed the highest microbial diversity in the total analysed process.

Besides that additional 16S rDNA clone libraries are currently under establishment in the paper sector. Here samples from 2 different wastewater treatment plants representing a good and a bad performing plant in terms of degrading processes and biogas formation are analysed. Results will be compared accordingly in order to assess the relevant populations in the two treatment plants. All retrieved 16S rDNA sequence data is added to the sequence database.

Alignment, re-alignment and phylogenetic analysis of all compiled 16S rDNA sequence information of relevant micro-organisms is performed using software package ARB (Ludwig et al., 2004, Pruesse et., 2007).

The software package ARB has a graphically-oriented interface and a fully-integrated package of cooperating software tools for handling and analysis of sequence and other relevant information, for example literature reference. It enables e.g. import/export of sequences, aligning of sequences, calculation of sequence similarities, design and matching of oligonucleotide probes for FISH analysis. It is a linux based software. Due to the huge database it runs only on adapted and optimized linux workstations with high amount of RAM. Figure 9 describes the structure of the software package and the corresponding database.

**Gene probe development.** Gene probe development is carried out by the function PROBE DESIGN of the software package ARB. The specificity of the designed probes is further confirmed by using the Check Probe program in RDP ([http://rdp.cme.msu](http://rdp.cme.msu)). Specificities of the designed gene probes are further checked by increasing formamide concentrations in the hybridization buffer during *in situ* testing on fixed target and non-target strains (if available). If such strains do not exist because they are difficult to cultivate or even non-cultivable designed probes are tested on the original fixed samples.
**Figure 9**: Structure of ARB software package
4. Results and achievements

4.1. Major results and achievements covering all sectors

4.1.1. Establishment of literature-based database for micro-organisms supposed to be relevant in the 4 industry sectors

Based on continuous literature research a comprising microbiological database for micro-organisms supposed to be relevant in the following context is established (see Table A1, ANNEX).

Relevant in this context means: described
- in combination with hygienic and safety aspects,
- in combination with biofilm formation,
- in combination with biofouling
- in combination with spoilage of end product
- in combination with spoilage of raw material or water inflow

Up to now to a number of 87 micro-organisms is supposed to be relevant for the water in the different industries. Following data per selected micro-organism were collected.

- Industry sector, where organism was described
- phylogeny of the micro-organism
- relevance in hygienic indicator/pathogen/biofilm context
- process step where MO was analysed/described
- frequency of analysis
- analytical method
- reference

Table A1 (ANNEX) summarizes the collected data.

30% of described micro-organisms were collected from studies concerning the food industry. 70% were affiliated to the paper industry, 10% to the chemical industry and 8% were affiliated with topics of the textile industry. (Note that the sum of all given percentages is higher than 100%. This is due to micro-organisms which were described in more than one industry.

In the food sector it became apparent that a high amount of different hygienic indicators and pathogens are described depending on the production process and used raw materials. However, published studies on real biofilm or biofouling cases including distinct identified populations in the food industry are rare. In the paper industry knowledge about biofilms and description of concrete biofilm populations is much more widespread than in the food industry. In the textile industry microbiology data are very rare so far, probably due to the toxicity of the used chemicals and processes. Here ongoing research on textile samples will elucidate the current picture. For the chemical industry also relatively few information is shared publicly. It is estimated that due to secrecy policies of companies problems concerning above mentioned problems are treated more or less internally.
4.1.2. Selection of micro-organisms for probe development

The following part of this report combines literature data on micro-organisms considered as relevant (in terms of biofilm/biofouling formation, hygiene, pathogen or process relevant) for Aquafit4Use with data obtained until now by experimental analyses during Aquafit4Use (see also component data collection in Figure 1).

Table A2 (ANNEX) lists all 184 so far as relevant considered species or genera. This table contains the 48 previously selected organisms (report IR2_1_3_1) plus up-to-date information from the sources literature as well as analysis output from industry samples (food, chemistry, paper and textile). In addition the progress on probe development and thus probe availability per organism is displayed.

158 of 184 selected micro-organisms were assigned to the domain Bacteria, 22 to the domain Archaea and 4 to the domain Eukarya. Table 4 and Figure 10 display an overview about the phylogenetic allocation of the entire group of selected micro-organisms.

<table>
<thead>
<tr>
<th>Phylum</th>
<th>Short Name</th>
<th>Identified micro-organisms per Phylum</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alphaproteobacteria</td>
<td>ALF</td>
<td>25</td>
</tr>
<tr>
<td>Betaproteobacteria</td>
<td>BET</td>
<td>23</td>
</tr>
<tr>
<td>unclassified bacteria</td>
<td>Caldithrix</td>
<td>1</td>
</tr>
<tr>
<td>Cytophaga-Flexibacter Subphylum</td>
<td>CF</td>
<td>9</td>
</tr>
<tr>
<td>Green-Non-Sulfur bacteria</td>
<td>Chloroflexi</td>
<td>2</td>
</tr>
<tr>
<td>unclassified bacteria</td>
<td>Cladiserica</td>
<td>1</td>
</tr>
<tr>
<td>Crenarcheota, Archaea</td>
<td>Cren</td>
<td>1</td>
</tr>
<tr>
<td>Deinococcus/Thermus</td>
<td>Dein/Thermus</td>
<td>5</td>
</tr>
<tr>
<td>Deltaproteobacteria</td>
<td>Delta</td>
<td>1</td>
</tr>
<tr>
<td>Entoplasmatales</td>
<td>Entplas</td>
<td>1</td>
</tr>
<tr>
<td>Epsilonproteobacteria</td>
<td>Epsilon</td>
<td>5</td>
</tr>
<tr>
<td>Eukarya</td>
<td>EUK</td>
<td>4</td>
</tr>
<tr>
<td>Euryarchaeota, Archaea</td>
<td>Eury</td>
<td>21</td>
</tr>
<tr>
<td>Fibrobacteres</td>
<td>Fibrobacteres</td>
<td>1</td>
</tr>
<tr>
<td>Gammaproteobacteria</td>
<td>GAM</td>
<td>32</td>
</tr>
<tr>
<td>Actinobacteria</td>
<td>HGC</td>
<td>12</td>
</tr>
<tr>
<td>Firmicutes</td>
<td>LGC</td>
<td>37</td>
</tr>
<tr>
<td>Planctomycetales</td>
<td>Pla</td>
<td>1</td>
</tr>
<tr>
<td>Spirochaetes</td>
<td>Spiro</td>
<td>1</td>
</tr>
<tr>
<td>unclassified bacteria</td>
<td>unclassified</td>
<td>1</td>
</tr>
</tbody>
</table>

**Table 4:** Phylogenetic allocation of the 184 so far as relevant considered micro-organisms

As shown in Table 4 high numbers (> 20) of as relevant considered micro-organisms were phylogenetically assigned to the Alphaproteobacteria (ALF), Betaproteobacteria (BET), Gammaproteobacteria (GAM), Euryarchaeota (Eury) and Firmicutes (LGC).

Literature is constantly searched for valuable information on potentially process relevant micro-organisms in the 4 industry sectors. This data is substituted with actual results from molecular-biological analyses performed on Aquafit4Use samples. Experimental data on potentially relevant micro-organisms is currently generated by Bio-Digital approaches (see Bio-Digital approach: 2.2.3) on samples from paper and textile industry. A 16S rDNA clone library was established for a selected sample from textile industry (TXT4) which showed the highest microbial diversity in the total analysed process. And from the paper industry 2 samples from different wastewater treatment plants representing good and bad performing plants in terms of degrading processes and biogas formation are under investigation.

Furthermore, experimental data is included from species information which was retrieved by using VIT-Profiling approaches (see VIT-Profiling: 2.2.2) on two different sampling rounds in textile sector (SVI, TXT).

Data from literature and results on samples from the different industries are combined in order to investigate whether organisms described as potentially relevant in literature are actually present in industrial samples. This data comparison is very important, because it allows a preliminary qualification of certain organisms as relevant. Micro-organisms which are found in literature as well as in actual samples were prioritized higher than micro-organisms only present in industrial samples or described in literature. Probe design and later application of this specific probe in industry samples will complete the qualification of relevant micro-organisms as quantitative information and thus actual abundance can be displayed.

Of 184 micro-organisms 37 revealed highest priority as they were described in literature as well as in industrial samples from the project. They are summarized in Table 5 as extract of all so far selected 184 micro-organisms. Furthermore, 93 micro-organisms were present solely in industry samples and 54 micro-organisms were retrieved from literature research, only.
4.1.4. Status on probe development:

Specific probe development is constantly continued. Table A2 (ANNEX) displays the up-to-date progress of probe development. Priority (high, medium, low) for probe design is determined by following criteria:

**High priority probe design** is performed for micro-organisms which were retrieved from literature and could also be identified in Aquafit4Use samples by applying molecular analysis. Furthermore, known process relevant organisms (e.g. bacteria with a proven beneficial or negative impact on industrial processes (*Rhodobacter, Cloacibacterium*) and organisms from literature which were identified by cultivation-independent approaches are prioritized as high, too.

**Medium priority probe design** is performed for micro-organisms identified in at least two industry sectors, or assumed to have lower process impact. Plus micro-organisms from literature which were identified by cultivation-dependent approaches.

**Low priority probe design** is done for all other micro-organisms which are not covered by the above mentioned criteria. Thus micro-organisms are categorized low priority, if they are identified in just one sector or only described in literature and minor information is available on their possible impact on the different industries.

Up to now 62 specific gene probes are available (see Table A2, ANNEX). Probe design and probe testing is in progress. These developed probes are used to identify and analyse the different relevant micro-organisms in industry samples in order to confirm their actual abundance (see also Figure 1).
4.2. Major results and achievements covering Paper sector

First successful application of developed specific gene probes for monitoring early biofilm forming bacteria in paper mill samples

4.2.1. Executive summary

By cultivation-independent specific gene probes screening two bacteria groups were analysed as dominant bacteria in paper mill samples. Detected bacteria groups were related to *Tepidimonas arfidensis* (member of the *Betaproteobacteria*) and *Cloacibacterium* (member of the *Cytophaga-Flexibacter* subphylum), respectively. In paper mill samples from SAP both groups together represented between 56% and 86% of all viable bacteria. Retrieved results not only confirm our findings in an earlier EU-project ("ODOUR CONTROL"- No.: 016730) but confirm findings of recent studies considering these two bacteria groups together with *Rhodobacter* species as the early biofilm forming bacteria in samples of paper mill samples with neutral to alkaline process conditions.

According to these preliminary results it is likely that at least *Tepidimonas arfidensis* and *Cloacibacterium* may have a high potential to act as indicator organisms for biofilm formation in paper mill samples with neutral to alkaline process conditions. Developed specific probes will be used as monitoring tools for screening further paper mill samples. In case results can be confirmed developed probes will be transferred into easy-to-use test kits for general applications in paper mills.

Apart from that in samples of wastewater treatment plant high risk potential for sludge bulking was observed due to the identified and quantified filamentous bacteria.

4.2.2. Results

Cultivation-independent analysis of water samples from SAP. Establishment of comprising population profiles by VIT-Profiling.

Water samples originating from the same spots along the process flow of SAP (fresh water, clear water after filtration at PM6, inflow wastewater treatment plant (WWTP), aeration tank, effluent from WWTP) but from different years (autumn 2008 and autumn 2009) were analysed regarding total cell counts and total viable counts per ml to assess the general microbial impact within the samples (see Table 6). Moreover, based on high load of bacteria within the samples “clear water after filtration at PM6”, inflow WWTP and aeration tank, these samples were screened using a set of phylum- and subphylum-specific gene probes (VIT-Profiling). Cultivation-independent population profiles were established and compared.

Table 6 displays results and Figure 11 illustrates displayed results.
Table 6: Summary of results obtained by VIT-Profiling and additional specific gene probes on some selected samples from paper mill SAP

<table>
<thead>
<tr>
<th>Analysed target organism(s)</th>
<th>Share of each group in relation to the overall bacteria population; Amounts in %</th>
<th>clear water after filtration at PM6</th>
<th>inflow WWTP</th>
<th>aeration tank WWTP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alphaproteobacteria</td>
<td></td>
<td>&lt;1 1</td>
<td>7 16</td>
<td>32 40</td>
</tr>
<tr>
<td>Alysiosphaera</td>
<td></td>
<td>n. a. n. a.</td>
<td>n. a. n. a.</td>
<td>10 15</td>
</tr>
<tr>
<td>Betaproteobacteria</td>
<td></td>
<td>45 47</td>
<td>67 67</td>
<td>14 17</td>
</tr>
<tr>
<td>“Tepidimonas arfdensis”-related clones</td>
<td></td>
<td>40 12</td>
<td>n. a. n. a.</td>
<td>n. a. n. a.</td>
</tr>
<tr>
<td>Gammaproteobacteria</td>
<td></td>
<td>n. d. n. d.</td>
<td>1 1</td>
<td>&lt;1 &lt;1</td>
</tr>
<tr>
<td>Deltaproteobacteria</td>
<td></td>
<td>&lt;1 &lt;1</td>
<td>&lt;1 &lt;1</td>
<td>&lt;1 &lt;1</td>
</tr>
<tr>
<td>Chloroflexi</td>
<td></td>
<td>n. d. n. d.</td>
<td>&lt;1 &lt;1</td>
<td>35 23</td>
</tr>
<tr>
<td>Eikelboom Type 1851</td>
<td></td>
<td>n. a. n. a.</td>
<td>n. a. n. a.</td>
<td>22 21</td>
</tr>
<tr>
<td>unknown filament(s)</td>
<td></td>
<td>n. a. n. a.</td>
<td>n. a. n. a.</td>
<td>11 &lt;1</td>
</tr>
<tr>
<td>Planctomycetes</td>
<td></td>
<td>n. d. n. d.</td>
<td>n. d. n. d.</td>
<td>3 3</td>
</tr>
<tr>
<td>Firmicutes</td>
<td></td>
<td>&lt;1 &lt;1</td>
<td>&lt;1 &lt;1</td>
<td>n. d. n. d.</td>
</tr>
<tr>
<td>Actinobacteria</td>
<td></td>
<td>n. d. n. d.</td>
<td>n. d. n. d.</td>
<td>5 2</td>
</tr>
<tr>
<td>Nocardioform actinomycetes</td>
<td></td>
<td>n. a. n. a.</td>
<td>n. a. n. a.</td>
<td>&lt;1 &lt;1</td>
</tr>
<tr>
<td>Nostocoida limicola Type II</td>
<td></td>
<td>n. a. n. a.</td>
<td>n. a. n. a.</td>
<td>3 n. d.</td>
</tr>
<tr>
<td>Cytophaga-Flexibacter-Subphylum</td>
<td></td>
<td>47 45</td>
<td>21 13</td>
<td>4 4</td>
</tr>
<tr>
<td>“Cloacibacterium”</td>
<td></td>
<td>46 44</td>
<td>n. a. n. a.</td>
<td>n. a. n. a.</td>
</tr>
<tr>
<td>total cell counts / ml</td>
<td></td>
<td>3.47 x 10^7</td>
<td>5.29 x 10^7</td>
<td>8.70 x 10^5</td>
</tr>
<tr>
<td>total viable cells / ml</td>
<td></td>
<td>2.83 x 10^7</td>
<td>4.29 x 10^7</td>
<td>6.33 x 10^5</td>
</tr>
</tbody>
</table>

Figure 11: Diagram of obtained gene probe results. Graphical comparison of data on same spots in paper mill SAP but from different years.
Detection of early biofilm forming bacteria in samples from production process using specific gene probes.

As expected all three sample types showed different population profiles but each sample compared with the same sample from the other year showed similar population patterns with the lowest differences on the process samples within the mill. Most interesting population profiles were revealed in samples “clear water after filtration at PM6”. After VIT-Profiling both samples were dominated by bacteria of the Betaproteobacteria and Cytophaga-Flexibacter subphylum. Both groups together represented more than 90% of all viable bacteria cells within these samples. All other analysed bacteria groups were underrepresented in these samples.

Interestingly, revealed population profiles were similar to population profiles obtained in a German paper mill which was investigated in a former EU-project ("Odour Control"- No.: 016730). The same bacteria groups Betaproteobacteria and Cytophaga-Flexibacter subphylum) were found to represent the stable dominant micro-flora in samples from the production site of that mill. These bacteria groups were supposed to contribute to odour problems in this mill but a final conclusion could not be drawn. During that time both bacteria groups represented unknown uncultivable organisms but by allying the Bio-Digital approach on samples from that mill 16S rDNS sequences could be retrieved, phylogenetically analysed and specific gene probes were developed. The Betaproteobacteria were mainly represented by bacteria related to Tepidimonas arfidensis and the Cytophaga-Flexibacter bacteria were mainly represented by bacteria related to Cloacibacterium. Further Identification and monitoring of the bacteria was performed using newly developed gene probes for the retrieved sequences. Both bacteria groups were constantly dominant in periodically analysed samples but with changing quantities. The output of that project comparing 3 different paper mills harbouring biofilm and odour problems was that each paper mill seemed to be unique, harbouring “it’s own” completely different micro-flora populations. During that study none of the analysed microbiological population profiles of different paper mills were comparable (Granhall et al., 2010).

However, and very interestingly, the more specific analysis of the SAP process samples with the earlier developed probes revealed that the same bacteria populations were also present and partly dominant here. In the sample of 2008 Tepidimonas arfidensis was measured with an approximate share of 25% of all Betaproteobacteria which had a share of 47% of all viable bacteria. In the sample of 2009 the share of Tepidimonas arfidensis was even higher and represented more than 90% of all Betaproteobacteria which in this sample had a share of 45% of all viable bacteria.

Similar to that the major part of all Cytophaga-Flexibacter bacteria which had shares of 45% and 47% of all viable bacteria, respectively, could be assigned to bacteria being positive with the specific probe developed for sequences related to Cloacibacterium. In sample of 2008 they represented 45% of all viable bacteria and in 2009 they represented 46% of all viable bacteria.

Thus, taking together shares of Tepidimonas arfidensis and Cloacibacterium, in paper mill samples from SAP represented between 56% and 86% of all viable bacteria which by itself indicates a certain relevance for the process (see also Figures 12 and 13).
Figure 12: Detection of bacteria related to *Tepidimonas arfidensis* in sample “clear water after filtration at PM6 (2009). They represented one of two dominating groups in the sample.

Figure 13: Detection of bacteria related to *Cloacibacterium* in sample “clear water after filtration at PM6 (2009). They represented one of two dominating groups in the sample.
Independent from our findings a recent publication with results based on cultivation-independent PCR approaches described members of *Tepidimonas* and *Cloacibacterium* as well as *Rhodobacter* species (member of the *Alphaproteobacteria*) as the early biofilm forming microorganisms in paper mill samples (Tiirola et al., 2009). According to the authors these three bacteria groups seem to represent the “early growers”. By first colonizing surfaces they act as basis for subsequent other micro-organisms which follow and create the macroscopically visible biofilms in paper mills. They concluded that successful biofilm fighting measures should be concentrated on these three bacteria groups in order to inhibit the successive formation of biofilms in paper mills with neutral to alkaline process conditions.

Our findings support their conclusions by detection of the same bacteria in major amounts directly within samples of two different paper mills harbouring also neutral to alkaline process conditions. We think that *Tepidimonas arfidensis* and *Cloacibacterium* may have a high potential to act as biofilm indicator organisms in such paper mills.

Besides, we have doubts about *Rhodobacter* species playing a major role as early biofilm former in paper mill samples. Reasons are that so far no related sequences were retrieved by Bio-Digital approaches on paper mill samples by us. Second, also with the comprising gene probe for *Alphaproteobacteria* no major shares of bacteria were detected in the analysed paper mill samples so far. However, this needs to be confirmed by developing a specific gene probe for *Rhodobacter* and applying it on further samples of selected paper mills.

On the other hand specific analysis with developed specific gene probes for *Deinococcus geothermalis* and *Meiothermus* supposed to be relevant as early biofilm forming bacteria by other studies (Kolari et al., 2001, Ekman et al., 2007) could not be confirmed by our studies. These bacteria were never detectable in all paper mill samples analysed so far (data not shown). This result is also in concordance with the findings of Tiirola et al., 2009. They also could never detect these organisms by the used LH-PCR method. However, these discrepancies were explained by different pH values in the production processes of the analysed mills. So far *Deinococcus geothermalis* and *Meiothermus* were detected in mills with acidic production conditions whereas *Tepidimonas*, *Cloacibacterium* and *Rhodobacter* were detected in paper mills with neutral to alkaline conditions.

In any case it seems likely that at least *Tepidimonas* and *Cloacibacterium* have a high potential to act as indicator organisms for early biofilm formation. Developed gene probes might deliver valuable support to analyse the biofilm formation and succession in situ. Moreover, possible effects of strategies for inhibiting biofilm formation by the organisms can now easily be monitored by application of the gene probes. If relevance of these bacteria can be confirmed in further studies first easy-to-use testkits for monitoring biofilm formation in paper mills targeting *Tepidimonas* and *Cloacibacterium* will be developed for general use.

**Assessment of high risk potential for sludge bulking in wastewater treatment plant.**

VIT-Profiling of the aeration tank of the wastewater treatment (WWTP) of SAP revealed that WWTP flora was dominated by members of the *Alphaproteobacteria* and bacteria of the *Chloroflexi*. Both groups contain important filamentous bacteria representing risk factors for settling problems of the sludge flocs.

A more detailed analysis with specific gene probes for filamentous bacteria revealed that the dominant filament of the sludge was Eikelboom Type 1851 (member of the Chloroflexi). It represented a share of 21% of the total viable cell count. In such high numbers the filament represents a risk factor for the WWTP. Eikelboom Type 1851 contributes to sludge bulking and settling problems in many industrial WWTP. Eikelboom Type 1851 is Gram-variable and often
present in nitrifying plants with low inflow and high sludge age. The presence of this filament is an indicator for low molecular compounds.

The other main filament was Alysiosphaera. It had a share of 15% of the total viable count. It is a member of the Alphaproteobacteria and if present in high numbers can lead to major sludge bulking problems in the WWTP. Gram-negative Alysiosphaera is morphologically very similar to Gram-positive Nostocoida limicola II and in former times was not distinguishable from this filament. However, recent molecular analyses revealed that both filaments are phylogenetically different and supposed to have different substrate requirements (Levantesi et al., 2004, 2006). Alysiosphaera is mainly present in industrial WWTPs and very often in WWTPs of paper industry where it supports bulking sludge events. Alysiosphaera grows mainly within the sludge flocs and if present in higher amounts can lead to severe settling problems in the aeration tank and clarifier.

To monitor the populations of filamentous bacteria further analysis with VIT-kits specific for the detected filaments is recommended.

**Summary:**

- Bacteria related to Tepidimonas arfidensis and to Cloacibacterium were identified by specific gene probes as dominant bacteria groups in paper mill samples from SAP as well as in samples from a German paper mill (both paper mills with neutral to alkaline process conditions)

- For the first time similar population profiles with two major bacteria groups could be detected in two different paper mills over time, indicating that the detected groups could play a relevant role in the paper mill processes.

- Results obtained support findings in recent publications which described these species as important early biofilm forming bacteria in paper mills with neutral to alkaline process conditions

- Both bacteria groups might be relevant as early biofilm forming bacteria and thus would show a great potential to act as indicator bacteria for biofilm formation in paper mills

- If results with developed gene probes can be confirmed on further samples from paper industry, first easy-to-use monitoring test kits will be developed for general application

- The role of Rhodobacter as considered to be a third early biofilm forming bacterium needs to be assessed

- Filamentous bacteria with high numbers in WWTP of SAP detected representing major risk factors for settling problems.
4.3. Major results and achievements covering textile sector

First look into the black box of microbial diversity present in water samples from textile industry

4.3.1. Executive summary

Water samples from textile industry were analyzed using conventional cultivation-based methods and cultivation-independent technologies. It could clearly be shown that conventional techniques are not sufficient to get an overview of the microbial diversity in textile industry water streams which hampers the assessment of microbial quality in this industry. However, by using molecular VIT-technology and bio-digital approach we were able to obtain the real diversity in these samples.

2 sampling rounds were carried out in TXT and one sampling round in SVI. An extensive set of data including 79 16S rDNA sequences from isolates and 205 16S rDNA sequences retrieved from clones based on one sample from TXT was obtained. This dataset will be used in a second step to correlate it with the technological data from the textile industries. A first risk assessment of the detected microbes was performed and will be supported by more cultivation-independent data in the following months. Moreover, it could be shown that there is a high possibility that the so-called "spot-problem" in textile industry might be correlated with micro-organisms.

4.3.2. Introduction: Microbiology in textile industry

The microbiology of water streams in textile industry is still a black box. This is due to the harsh conditions of the textile industry processes where growth of micro-organisms is limited and only the most adapted micro-organisms can survive. On the other hand it is also known that a huge part of micro-organisms appearing in samples is undetectable by standard approaches based on cultivation solely. As a consequence the gained knowledge so far obtained by such methods might be limited.

The aim of vermicon's work in WP 5.4 is to obtain a better understanding of the microbial populations in water streams of textile industry to correlate the micro-organisms with quality of water streams and problems during the textile processes.

4.3.3. Applied methods

For analysis of the micro-organisms we used different approaches which are described in detail in part 3 (Methods) of this report.

4.3.4. Results and major achievements

Samples were received from two textile companies. Table 7 gives an overview of performed sampling rounds and subsequent applied approaches. The bio-digital approach was used only on the most interesting sample from TXT sampling round 1 (TXT 1-4). For subsequent analysis three main types of sampling points were chosen: Discharge after different processes, input water and output water (= wastewater).
Table 7: Performed samplings and subsequent analysis

<table>
<thead>
<tr>
<th>Sampling period</th>
<th>VIT-profiling</th>
<th>Bio-digital approach</th>
</tr>
</thead>
<tbody>
<tr>
<td>TXT sampling round 1</td>
<td>March 2009</td>
<td>X</td>
</tr>
<tr>
<td>TXT sampling round 2</td>
<td>October 2009</td>
<td>X</td>
</tr>
<tr>
<td>SVI sampling round 1</td>
<td>March 2009</td>
<td>X</td>
</tr>
</tbody>
</table>

4.3.5. Samples from SVI

Table 8: Description of samples obtained from SVI

<table>
<thead>
<tr>
<th>Sample ID</th>
<th>Type of water</th>
<th>Type of process</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>SVT 1</td>
<td>Discharge</td>
<td>Yarn dyeing</td>
<td>Same sampling point as SVT 4; First discharge after dark reactive yarn dyeing</td>
</tr>
<tr>
<td>SVT 2</td>
<td>Discharge</td>
<td>Fabric dyeing</td>
<td>Same sampling point as SVT 3,5; First discharge after light reactive fabric dyeing</td>
</tr>
<tr>
<td>SVT 3</td>
<td>Discharge</td>
<td>Fabric dyeing</td>
<td>Same sampling point as SVT 2,5; First discharge after light reductive fabric dyeing</td>
</tr>
<tr>
<td>SVT 4</td>
<td>Discharge</td>
<td>Yarn dyeing</td>
<td>Same sampling point as SVT 1; First discharge after yarn bleaching</td>
</tr>
<tr>
<td>SVT 5</td>
<td>Discharge</td>
<td>Fabric dyeing</td>
<td>Same sampling point as SVT2,3; First discharge after fabric bleaching</td>
</tr>
<tr>
<td>SVT 6A</td>
<td>Wastewater</td>
<td>Yarn dyeing</td>
<td>Wastewater after yarn dyeing processes</td>
</tr>
<tr>
<td>SVT 6B</td>
<td>Wastewater</td>
<td>Fabric dyeing</td>
<td>Wastewater after fabric dyeing processes</td>
</tr>
<tr>
<td>SVT 7</td>
<td>Input water</td>
<td>-</td>
<td>Water used for different process before softening</td>
</tr>
</tbody>
</table>

4.3.6. Samples from TXT

First sampling round TXT

Table 9: Description of samples obtained from TXT (first sampling round):

<table>
<thead>
<tr>
<th>Sample ID</th>
<th>Type of water</th>
<th>Type of process</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>TXT 1-1</td>
<td>Discharge</td>
<td>Fabric dyeing</td>
<td>Washing after cold bleaching</td>
</tr>
</tbody>
</table>
In the first sampling round not all relevant processes could be analysed properly so that a second sampling round was performed in October 2009. (See Figure 16 for overview on sampling points and process).

**Table 10:** Description of samples obtained from TXT (second sampling round):

<table>
<thead>
<tr>
<th>Sample ID</th>
<th>Type of water</th>
<th>Type of process</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>TXT 2-1</td>
<td>Input water</td>
<td>-</td>
<td>Input water before softening</td>
</tr>
<tr>
<td>TXT 2-2</td>
<td>Input water</td>
<td>-</td>
<td>Input water after softening</td>
</tr>
<tr>
<td>TXT 2-3</td>
<td>Discharge</td>
<td>Printing</td>
<td>Discharge after printing</td>
</tr>
<tr>
<td>TXT 2-4</td>
<td>Discharge</td>
<td>Washing</td>
<td>Discharge after washing</td>
</tr>
<tr>
<td>TXT 2-5</td>
<td>Discharge</td>
<td>Impregnation</td>
<td>Discharge after impregnation</td>
</tr>
<tr>
<td>TXT 2-6</td>
<td>Discharge</td>
<td>Stenter</td>
<td>Discharge after stenter (same as TXT 2-7)</td>
</tr>
<tr>
<td>TXT 2-7</td>
<td>Discharge</td>
<td>Stenter</td>
<td>Discharge after stenter (same as TXT 2-6)</td>
</tr>
<tr>
<td>TXT 2-8</td>
<td>Wastewater</td>
<td>End of streams</td>
<td>Wastewater after homogenization</td>
</tr>
<tr>
<td>TXT 2-9</td>
<td>Wastewater</td>
<td>Yarn dyeing</td>
<td>Wastewater before homogenization</td>
</tr>
<tr>
<td>TXT 2-10</td>
<td>Discharge</td>
<td>Yarn dyeing</td>
<td>Discharge after yarn dyeing</td>
</tr>
<tr>
<td>TXT 2-11</td>
<td>not received</td>
<td>not received</td>
<td>not received</td>
</tr>
<tr>
<td>TXT 2-12</td>
<td>Discharge</td>
<td>F1</td>
<td>Discharge after F1 process</td>
</tr>
</tbody>
</table>

4.3.7. **Understanding microbial diversity: VIT-profiling**

The VIT-profiling approach was applied on all samples from the first and second sampling round. The aims of the VIT-profiling were as follows:
• profiling of the microbial populations
• phylogenetic analysis of the cultivable micro-organisms
• risk assessment of the micro-organisms
• knowledge of cultivation-independent cell numbers

4.3.8. Analysed total cell numbers and vital cell numbers

Sampling round 1 SVI and TXT: Total cell numbers could not be measured. Vital cell numbers were measured by cultivation methods.
Sampling round 2 TXT: Total cell numbers and vital cell numbers were measured by cultivation-independent methods.
Tables 11-13 summarise the measured cell numbers in the different water types.

Table 11: Total cell numbers (vital and dead) input water

<table>
<thead>
<tr>
<th>Factory</th>
<th>Sample ID</th>
<th>Vital bacteria per ml</th>
<th>Total cell number per ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>SVI</td>
<td>SVT 7</td>
<td>2,10E+05</td>
<td>n.m.</td>
</tr>
<tr>
<td>TXT (1st)</td>
<td>TXT 1-6</td>
<td>7,00E+03</td>
<td>n.m.</td>
</tr>
<tr>
<td>TXT (2nd)</td>
<td>TXT 2-1</td>
<td>1,26E+06</td>
<td>2,23E+06</td>
</tr>
<tr>
<td></td>
<td>TXT 2-2</td>
<td>9,08E+05</td>
<td>2,21E+06</td>
</tr>
</tbody>
</table>

Table 12: Total cell numbers (vital and dead) wastewater

<table>
<thead>
<tr>
<th>Factory</th>
<th>Sample ID</th>
<th>Vital bacteria per ml</th>
<th>Total cell number per ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>SVI</td>
<td>SVT 6A</td>
<td>2,75E+06</td>
<td>n.m.</td>
</tr>
<tr>
<td></td>
<td>SVT 6B</td>
<td>6</td>
<td>n.m.</td>
</tr>
<tr>
<td>TXT (1st)</td>
<td>TXT 1-7</td>
<td>3,30E+06</td>
<td>n.m.</td>
</tr>
<tr>
<td>TXT (2nd)</td>
<td>TXT 2-8</td>
<td>1,03E+07</td>
<td>1,70E+07</td>
</tr>
<tr>
<td></td>
<td>TXT 2-9</td>
<td>1,66E+07</td>
<td>3,53E+07</td>
</tr>
</tbody>
</table>

Table 13: Total cell numbers (vital and dead) discharges

<table>
<thead>
<tr>
<th>Factory</th>
<th>Sample ID</th>
<th>Vital bacteria per ml</th>
<th>Total cell number per ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>SVI</td>
<td>SVT 1</td>
<td>4</td>
<td>n.m.</td>
</tr>
<tr>
<td></td>
<td>SVT 2</td>
<td>1</td>
<td>n.m.</td>
</tr>
<tr>
<td></td>
<td>SVT 3</td>
<td>0</td>
<td>n.m.</td>
</tr>
</tbody>
</table>
4.3.9. Phylogenetic analysis of retrieved isolates

By cultivation of the textile water samples on plate count agar 79 isolates were obtained which were identified via 16S rDNA sequencing. Moreover, for comparison with results obtained by gene probe screening the isolates were affiliated to taxonomic groups. Table 14 summarises the relevant taxonomic (phylogenetic) groups. Table 15 summarises all obtained isolates. The major part of all isolates belonged to the Bacteria. 3 of 79 isolates belonged to the Eucarya. One interesting colony with violet colour on plate count agar (see violet row in Table 15) could not be affiliated to any other known bacterium and thus is completely unknown. However, it seems to be relatively relevant because it was found in SVI and TXT (first sampling round). Further analysis is ongoing.

Table 14: Overview of phylogenetic groups and used abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Taxonomic group</th>
</tr>
</thead>
<tbody>
<tr>
<td>ALF</td>
<td>Alphaproteobacteria</td>
</tr>
<tr>
<td>BET</td>
<td>Betaproteobacteria</td>
</tr>
<tr>
<td>GAM</td>
<td>Gammaproteobacteria</td>
</tr>
<tr>
<td>SRB</td>
<td>Deltaproteobacteria including sulphate-reducing bacteria</td>
</tr>
<tr>
<td>LGC</td>
<td>Firmicutes</td>
</tr>
<tr>
<td>HGC</td>
<td>Actinobacteria</td>
</tr>
<tr>
<td>CF</td>
<td>Cytophaga-Flavobacterium subphylum</td>
</tr>
<tr>
<td>PLA</td>
<td>Planctomycetes</td>
</tr>
<tr>
<td>GNS</td>
<td>Green, non-sulfur bacteria (Chloroflexi)</td>
</tr>
<tr>
<td>TM7</td>
<td>Candidate division TM7</td>
</tr>
<tr>
<td>-------</td>
<td>---------------------------------</td>
</tr>
<tr>
<td>DEIN</td>
<td>Deinococcus-Thermus</td>
</tr>
<tr>
<td>CREN</td>
<td>Kingdom Crenarchaeota of domain Archaea</td>
</tr>
<tr>
<td>EURY</td>
<td>Kingdom Euryarchaeota of domain Archaea</td>
</tr>
<tr>
<td>EUK</td>
<td>Eukarya</td>
</tr>
</tbody>
</table>

**Table 15:** Summary of all 79 retrieved isolates from textile water samples sorted according to phylogenetic groups.

### 4.3.10. Affiliation of retrieved isolates to textile processes

Following Tables 16 - 18 affiliate isolates to the textile processes and factories from which they were isolated. Table 19 summarises phylogenetic affiliation of isolates.
Table 19: Summary of all isolates with phylogenetic affiliation to bacteria groups

<table>
<thead>
<tr>
<th>Sample</th>
<th>ALF</th>
<th>BET</th>
<th>GAM</th>
<th>SRB</th>
<th>LGC</th>
<th>HGC</th>
<th>CFC</th>
<th>PLA</th>
<th>GNS</th>
<th>TM7</th>
<th>n.c.</th>
</tr>
</thead>
<tbody>
<tr>
<td>SVT 1</td>
<td></td>
<td></td>
<td></td>
<td>X</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SVT 2</td>
<td></td>
<td>X</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SVT 3</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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4.3.11. Cultivation-independent in situ analysis of water samples from textile samples

All samples from textile industry were also analysed using a cultivation-independent VIT-gene probes screening (see 3.2.1). With this approach the direct analysis of the microbial populations is
carried out. Following diagrams show the retrieved results. Rows without data indicate negative gene probe results. After adapting VIT-technology to textile industry samples the best results were obtained on TXT samples from second sampling round.

**Fig. 17**: VIT-analysis of samples from SVI

**Fig. 18**: VIT-analysis of samples from TXT (1st sampling round)
4.3.12. Exploring the unknown: The bio-digital approach

One sample from TXT (TXT 1-4; discharge from process 'printing with reactive dyestuff') showed a very interesting high microbial diversity using VIT-profiling. This was still more interesting since it is known that textile factories generally are struggling with the so-called "spot-problem", where spots appear on the textile thus damaging the product. The high microbial diversity in the distinct sample from TXT led to the assumption that this well-known and economical important phenomenon might be correlated with micro-organisms.

Therefore, the bio-digital-approach was applied on sample TXT 1-4. A total of 651 clones were analyzed from which 205 correct inserts were retrieved and phylogenetically identified. Table A3 in the ANNEX summarises obtained results (phylogenetic affiliation, % similarities to next relative etc.) on all 205 clones analysed.

Figure 20 displays the diversity of all retrieved clone sequences. Not only bacterial sequences were obtained but also sequences from *Archaea* were identified (several *Crenarchaeota* sequences and two *Euryarchaeota* sequences - marked yellow in Table 20).

Table 20 shows the amount of each analysed sequence and Table 21 displays the analysed frequency of major bacterial genera or domains. Figure 21 illustrates the distribution and abundance of retrieved clone sequences.
Fig. 20: Diversity of all 205 analysed clone sequences according to affiliation to major groups

4.3.13. Major achievements

Extensive pool of data

The combination of cultivation-dependent with cultivation-independent technologies allowed the generation of a significant database consisting of 79 16S rDNA sequences retrieved from isolates and 205 16S rDNA sequences retrieved from clones. These data will allow in a next step the successful combination of technical process data with microbial data and result in a deeper understanding of microbiology in textile industries.

Comparison of techniques for monitoring microbiology in textile industry

While applying the VIT-profiling approach we were able to conclude that conventional and cultivation-dependent methods are not sufficient to describe the real microbiology in textile water
streams. There were considerable differences regarding the microbial diversity in the different samples. However, the performance of the VIT-technology needed be adapted to the requirements of the textile industry samples which led to significant better results in the second sampling round.

Improving VIT-technology for monitoring microbiology in textile industry

Because of the harsh conditions in discharges our cultivation-independent method VIT-analysis suffered during the first sampling round and could not fully reveal the complete biodiversity of micro-organisms. However, we were able to adapt our technologies to the conditions in discharges from textile industry thus improving the results during the second sampling round significantly.

Obtaining the "whole picture"

In contrast to other studies which investigated microbiology in textile industry and focused only on selected topics or sample origins which have been mainly wastewater (Amoozegar et al., 2008, Boon et al., 2002, Georgiou et al., 2004) we were able to analyze the complete process flows in textile industry – from input water to discharges of the process to wastewater. By continuing the studies we will retrieve the first comprehensive study of micro-organisms in textile industries.

Risk assessment of detected bacteria

By analyzing the 16S rDNA sequences of the retrieved isolates we were able to establish a first risk assessment of the bacteria in different processes. This is important if the analysis of the bacteria in textile industry should lead to an assessment of the microbial quality of re-used water.

Potential correlation of spot problems with micro-organisms

By detailed analysis of one sample applying the bio-digital approach we are in the process of correlating a common quality problem in textile industries ("spots") with micro-organisms. By analyzing every single bacterium directly in the sample we were able to establish a detailed profile of the micro-organisms (work ongoing). By comparing this profile with real textile samples which are damaged by the "spot-problem" we might be able to link the presence of spots with the presence of certain micro-organisms. This will result in the design of specific monitoring tools.

Summary

The analyses of the samples from SVI and TXT revealed an astonishing view into the microbiology of process streams in textile factories. The combination of VIT-profiling together with the bio-digital-approach allowed a deep look into the black box of unknown and non-cultivable bacteria and presented an exciting microbial diversity. To our present knowledge there exist until now only minor investigations regarding microbiology in textile factories but never has anyone succeeded in retrieving the "whole picture". Within this work-package we are able to present the first step of a complete approach for understanding microbiology in textile factories. That means not only in distinct steps of the different processes but from input water to process water to wastewater. Only with this approach we will be able to understand the full picture of the textile-
correlated microbiology. The AquaFit4Use project will without doubts present the worldwide deepest look into microbiology of textile companies and their process streams.

At this stage of our work within WP 5.4 we are able to conclude:

- there is an astonishing microbial diversity in the complete process flow from input water to discharges of the process to wastewater
- there are significant differences regarding microbiology within the process streams
- the application of innovative techniques like VIT-profiling and bio-digital-approach led to completely new insights regarding microbial diversity in samples of textile factories
- the comparison of the cultivation-independent technologies with cultivation-dependent techniques revealed the limitation of traditional techniques thus explaining the minor insights into real textile microbiology in the past
- in spite of the harsh conditions of the different process streams there survive lot of microorganisms – much more than expected and according to conventional methods
- the "spot problem" in textile industries might be correlated with microbiology, thus allowing subsequent tools to be developed in order to monitor and fight it successfully

Next steps

A large amount of data has been generated for this report which shows significantly differences in microbiology regarding the origin of the sample. We will continue this work by correlating the microbial data with the technical data of the processes to obtain a better understanding of the microbiology. It will enable us to evaluate the microbial quality in textile process streams and to develop adapted monitoring tools for textile industries. These data are new to an extend which no one has expected before. A surprising discovery has been the suspected cause of the so-called spot problems by micro-organisms. This has to be investigated in more detail.

The further interpretation of the retrieved data and the combination of the microbiology and technical data during the next steps will allow us to exploit the results thus serving the textile factories in improving the microbial quality of the process streams and to avoid typical microbial induced problems during production.

Since these results are completely new and innovative we will start in the next months preparing a paper for publication. This should strengthen the outstanding impact and competence of the AquaFit4Use consortium for water related questions and underline the innovative output of the project.
5. Literature


