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MUWS (Microbiology in Urban Water Systems) – an interdisciplinary approach to study microbial communities in urban water systems

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Abstract

Microbiology in **U**rban **W**ater **S**ystems (MUWS) is an integrated project, which aims to characterize the microorganisms found in both potable water distribution systems and sewer networks. These large infrastructure systems have a major impact on our quality of life, and despite the importance of these systems as major components of the water cycle, little is known about their microbial ecology. Potable water distribution systems are large, highly interconnected and dynamic, and difficult to control. Sewer systems are also large and subject to time varying inputs and demands. Their performance also faces increasing loading due to increasing urbanization and longer-term environmental changes. Therefore, understanding the link between microbial ecology and any potential impacts on short or long-term engineering performance is important. By combining the strengths and research expertise of civil-, biochemical engineers and molecular microbial ecologists, we aim to link the abundance and diversity of microorganisms to physical and engineering variables so that novel insights into the ecology of microorganisms within both water distribution systems and sewer networks can be explored. By presenting the details of this multidisciplinary approach, and the principals behind the molecular microbiological methods and techniques that we use, this paper will demonstrate the potential of an integrated approach to better understand urban water system function and so meet future challenges.

1 Introduction

1.1 The challenges

Urban water systems (e.g. drinking water distribution and sewers networks, wetlands and urban rivers) are important for millions of people living in urban areas. They are major components of the water cycle and present unique challenges; the systems are large, complex, highly interconnected and dynamic, with variable hydraulics, input

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sources and behaviour. These large infrastructure systems have a major impact on people's quality of life by preventing serious disease, protecting/enhancing the environment and reducing flood damage to other infrastructure, thus enabling economic and social development. Their overall performance can be controlled by different types of processes; physical (e.g. momentum and solid/solute transport in conduits), chemical (e.g. carbon based transformations) and biological (e.g. the molecular biochemistry and the social and mechanical interactions of organisms and cells).

Urban water systems throughout Europe face significant new challenges to continue to maintain the provision of safe water supplies, hygienic sanitation and good environmental management against the setting of increased urbanisation, ageing infrastructure and changing climate conditions. These changes are expected to have a negative impact on freshwater resources. The important role of urban water systems has been recognized by the EU with the provision of a series of directives (e.g. Urban Wastewater Treatment, Bathing Waters and Water Framework Directive (WFD)), which govern the use of water in order to provide equitable standards of service and improving environmental protection. Unlike earlier directives, which quantified environmental quality by simple physical and chemical parameters, the WFD aims to ensure that "good ecological status" is attained in all European water bodies. It is less prescriptive than previous directives and aims to address the management of the water bodies in a more holistic manner.

1.1.1 Water distribution systems

A technological challenge for the EU and worldwide water industry is the continuous delivery of high quality drinking water to customers' taps that meets increasingly stringent standards for aesthetic, bacteriological and chemical water quality. Most distribution systems comprise a complex network of pipes of different ages and material types (UKWIR, 2003). Frequently, the system is supplied from a number of different treatment works each with different source water and treatment systems. Despite the fact that modern water treatment works produce high quality water as it enters the distribution

system, the quality of the water is known to deteriorate during transportation within the system (e.g. Furtado et al. (1998) found 7 out of the 10 intestinal disease outbreaks reported in a 3 year study, arose due to contamination occurring within the distribution network). Changes in water quality are due to distribution systems acting as large bio-chemical reactors in which many complex, dynamic, and interrelated hydrodynamic and biochemical processes occur. Water distribution systems harbour microbial consortia, for example, anaerobic bacteria, protozoa, together with meio- and macro-fauna such as copepods and nematodes (Evins, 2004; Berry et al., 2006). Additionally the presence of pathogenic bacteria, that are normally undetectable by traditional culture based methods, can represent a potential reservoir for disease outbreaks or long-term illness (Szewzyk et al., 2000).

1.1.2 Sewer systems

A recent study in the UK indicated that if no remedial measures were adopted, the discharge of excess volumes from sewer systems during rain fall events to the environment could increase by up to 250% based on expected climate and urbanization changes (Evans et al., 2004), therefore increasing their future environment impact. Recent studies have also provided strong evidence that sewer flow quality can be strongly influenced by microbiological activity within sewer deposits (Tait et al., 2003). In cases of system failure, the discharge of untreated wastewater and sediments can degrade the water quality of the receiving water body, and may also be a risk to public health. In this respect the microorganisms released with the wastewater may be particularly important, due to the potential release of pathogens. In addition to these acute risks, the activity of the microorganisms in sewers changes the composition of the wastewater. This can, for example, lead to septic wastewater, which is associated with problems such as the formation of toxic gases and malodorous gases; the first posing an acute risk for sewer workers, the latter affecting the public perception of the sewers (Hvitved-Jacobsen, 2002).

1.2 The interdisciplinary approach

Answers to key questions, such as “which microorganisms are present?”, “what are they doing?”, and “how can we use their outputs and manage their activity to achieve better system outcomes?” are important for understanding the physical, chemical and biological interactions in urban water systems. The project Microbiology in Urban Water Systems (MUWS) therefore aims to assess the impact of microorganisms, due to their presence, diversity and response to various environmental conditions on aspects of system performance within drinking water distribution systems and sewer networks. The MUWS project aims to address these key questions through the use of research expertise of civil-, biochemical engineers and molecular microbial ecologists, across different length scales of the urban water systems. The aim of this paper therefore is to describe the work carried out in the MUWS project to permit the application of advanced microbial methods in urban water systems, thus highlighting the potential of our integrated approach.

2 Methods

In the MUWS project, variations in the microbial community of drinking water distribution systems and sewer networks are characterized by using molecular microbiological techniques, rather than culture based techniques. The molecular microbiological techniques, which have previously been successfully applied to freshwater and marine plankton samples, sediments and soil samples (Gelsomino et al., 1999; Moeseneder et al., 1999; Wagner et al., 2003; Smalla et al., 2007) are further developed specifically for the analysis of drinking water, wastewater, biofilms and sewer sediment microbial communities (Fig. 1). In this section we introduce two key methods. The data obtained from these methods has allowed us to gain insight into how a microbial community changes under different conditions at a variety of scales within urban water systems.

2.1 Denaturing gradient gel electrophoresis (DGGE)

DGGE analysis (Muyzer et al., 1993) can be used to investigate mixed microbial communities from various environments. The method is based on the molecular separation of DNA fragments when migrating through a DGGE gel, which results in a specific banding pattern. Each individual discrete band refers to a unique "sequence type" or phylotype (van Hannen et al., 1999) which can further be analyzed by sequencing for taxonomic identification. The similarities between banding patterns from different samples can then be analyzed using multivariate analysis such as cluster analysis (Fromin et al., 2002). To demonstrate the DGGE profiling technique, the method was applied to both planktonic samples from a drinking water distribution system and sewer biofilms. The key steps are outlined in Fig. 1 with specific detail relating to the individual samples listed below.

2.1.1 Drinking water

Planktonic samples were collected from a water distribution system (domestic cold water tap) in Sheffield. In brief, after a 1 min flush, water samples were collected in sterile bottles and transported to the laboratory on ice and processed immediately. Two liters of water was filtered through a 0.22 µm polycarbonate membrane filter (diameter 47 mm; Millipore Ltd., UK) and the filters were kept at −80 °C until further analysis. The membrane filters were cut in to halves under sterile conditions and placed directly into the bead solution tubes of the MoBio Ultra Clean Soil DNA Isolation Kit (Cambio Ltd., Cambridge, UK). The DNA was extracted as per the manufacturer's protocol and then used for polymerase chain reaction (PCR) amplification of 16S rRNA genes (Saiki et al., 1988). A direct-PCR, nested-PCR approach was used for comparative analysis of the culture independent drinking water samples. After performing all the DNA extractions, 16S rRNA gene fragments were amplified by direct-PCR, using the universal bacterial primers 338F with a GC-clamp and 530R (Whiteley and Bailey, 2001). For the same water samples, a first round PCR was performed with the bacterial primers 27F

and 1492R (Lane, 1991) followed by a second PCR amplification using the primers mentioned above. This approach is called nested-PCR and it can improve the sensitivity of PCR.

For the cultivation-dependent approach one half of individual membrane filters were sonicated in R2A medium (Reasoner and Geldreich, 1985) and 100 µl of those samples were used to inoculate 50 ml of R2A medium and cultured at 20 °C for 48 h. Two ml of the culture used for DNA extraction as described above. After performing all the extractions, 16S rRNA gene fragments were amplified by direct-PCR, using the universal bacterial primers 338F with a GC-clamp and 530R as mentioned above.

The PCR products were then loaded on a 8% polyacrylamide gel with a denaturant gradient ranging from 40 to 70%. DGGE analysis was performed using the Bio-Rad DCode System (Bio-Rad, Hertfordshire, UK). The gel was run at 100V at 60 °C for 16h in 1x TAE (Tris-Acetate-EDTA) running buffer and the gels were stained with SYBR Gold prior to image acquisition.

2.1.2 Sewer biofilms

Sewer biofilms were collected from two different sewer systems, one in Nantes (France) and the other in Frejlev (Denmark). Two sampling sites were used in Nantes and one site in Frejlev. All three sites were located in catchments predominantly served by combined sewers and all samples were collected during dry weather. The biofilm samples were scraped off the sewer pipe surfaces directly above the waterline using sterile 15 ml Falcon tubes. The samples were stored at −20 °C until further processing. DNA was extracted from 0.2 g (wet weight) of biofilms using the same DNA isolation kit as mentioned above for drinking water. To minimize interference with humic substances DNA extracts were diluted (1:10) before performing PCR amplifications. For the amplification of 16S rRNA gene fragments, the same primer pair with the GC-clamp as mentioned above was used (direct-PCR approach). PCR products were visualized by gel electrophoresis followed by ethidium bromide staining to ensure that the correct

size fragment was amplified. DGGE analysis of the PCR fragments was performed as described above for the drinking water samples.

2.2 Fluorescent in situ hybridization (FISH)

FISH with rRNA-targeted oligonucleotide probes is used for detection and quantification of microorganisms without prior cultivation (Amann et al., 1995). The method has been applied widely to different environmental samples including drinking water systems (Manz et al., 1993). To overcome problems of low detection limit and fluorescence intensity faced in oligotrophic environmental samples (such as drinking water), we have optimized and applied the CARD-FISH (catalysed reported deposition-fluorescence in situ hybridization) method. This method was originally developed for studying bacterioplankton in marine samples (Pernthaler et al., 2002). Again to demonstrate the technique the planktonic samples from a drinking water distribution system and sewer biofilms were analysed using CARD-FISH (Fig. 1).

2.2.1 Drinking water

The water samples (50 ml) were fixed in 2% (v/v) final concentration of formalin for less than 24 h. The samples were then filtered on to 0.22 µm pore size white polycarbonate membrane filters (diameter 47 mm, Millipore Ltd., UK) and stored at −20 °C until further processing. The samples were permeabilised with lysozyme and achromopeptidase as described previously (Pernthaler et al., 2002; Sekar et al., 2003). The hybridization was done with the HRP labeled eubacterial oligonucleotide probes (EUB338). The hybridization, washing and tyramide signal amplification with FITC-labeled tyramides were done as per the protocol described in Pernthaler et al. (2002). The preparations were counter-stained with the DNA specific fluorescent stain, DAPI, and observed under an Olympus BX51 epifluorescence microscope (Olympus Ltd., UK). The images were captured using CellB imaging software (Olympus Ltd., UK). Quantification of cell

numbers of the drinking water samples was performed by imaging and counting the DAPI stained cells.

2.2.2 Sewer biofilms

The sewer samples were fixed either with 2% (v/v) formalin or 1:1 PBS/Ethanol on the day of sampling. The water samples were filtered on to 0.22 µm membrane filters and used for the hybridization. The sewer biofilm samples collected from France and Denmark were gently vortexed, mixed with low gelling agarose (0.2% w/vol) and 10 µl of the samples were pipetted into the wells of a standard type multi-well Epoxy slides (Carl Roth GmbH + Co, Karlsruhe, Germany). The permeabilization, hybridization with the eubacterial probes (EUB338) and the tyramide signal amplification was done as described above for the water samples. The preparations were observed under an epifluorescence microscope as described above.

3 Results and discussion

3.1 Bacterial community profiling of drinking water samples

Figure 2 shows the DGGE patterns for drinking water samples based on a independent ("Drinking water") and culture-dependent ("DW culture") approach. The differences in DGGE patterns of the direct-PCR approach for both samples, show that with the culture-dependent approach we target a specific bacterial community that responds to the imposed enrichment. One would conclude from Fig. 2, that by using the culture-dependent approach, two phylotypes dominate the bacterial community in the drinking water collected. (NB: Often the brightest bands in the profile represent the dominant members of the community, but be aware of potential biases (Forney et al., 2004)).

This is significant as one method that is widely used to assess the general microbial water quality of drinking water is the cultivation-dependent method of heterotrophic plate counts (HPCs) (see review Sartory, 2004). The method is highly variable since the cultivation medium, incubation temperature, incubation time, origin, season of the year, and age of the water sample have a significant effect on the fraction of the total bacterial cells that grow and hence will be detected (Allen et al., 2004). Despite the discrepancy between total bacterial concentrations and cultivable cell concentrations (HPCs) in aquatic samples as shown by Staley and Konopka (1985), HPCs are still used for routine monitoring applications in a quantitative way (*Standard Methods for the Examination of Water and Wastewater*, 20th Edition). Pepper et al. (2004), for example, quantified the concentration of HPC bacteria within water from the source to the consumer's tap. Their study showed that the number of HPC bacteria increased dramatically from the distribution system to the consumers tap, but they did not quantify whether the bacterial community changed as well. There are only a few studies providing qualitative data about the HPC community composition and/or population dynamics (e.g. Kalmbach et al., 1997; Norton and LeChevallier, 2000).

For the enumeration of bacteria in drinking water, low-nutrient media are commonly used such as R2A (Reasoner and Geldreich, 1985). It was designed specifically as a low-nutrient, low-ionic strength formulation to isolate bacteria that have a water-based lifestyle (Reasoner, 1990). Our results support previous observations that media used for HPC are selective for those bacteria that can grow under the specific conditions used. Comparing these results to the DGGE profiles of either direct- or nested-PCR approach (no cultivation step involved) reveals considerable differences in the banding pattern observed. Several unique bands that were not visible in the culture dependent approach were seen, suggesting that the culture dependent method underestimated the number of phylotypes present in the sample.

Figure 2 shows that differences can be seen between the nested-PCR approach, which includes an additional PCR step, and the direct-PCR approach. Bands that appear at the same position have changed their intensity and new bands become visible.

Therefore, in certain cases the nested-PCR approach may need to be applied to increase sensitivity and to complete the overall presentation of the taxonomic diversity present in the sample.

3.2 Bacterial community profiling of sewer biofilm samples

Figure 3 shows the DGGE profiling for a sampling site from Denmark and two sites from France. A total of 26 discrete bands were detected on the gel. A total of 18 bands were present in the samples from Denmark where as 19 and 9 bands were found in France sampling site 1 and 2, respectively. This analysis suggests similar levels of diversity (although not necessary the same composition) between sewer biofilms from Denmark and France sampling site 1; and that sampling site 2 in France showed lower diversity. The overall results indicate that 4 bands were common to all three sites and 12 were found in two sites and 10 were unique to one site. Identification of common bands (and further taxonomic identification) could be used to identify indicator microorganisms that are present across diverse spatial distributions within the sewer networks. Unique bands can also be used for fingerprinting microorganisms related to distinctive networks, or spatial distribution within the network if comparison between effluent, biofilm and sediments samples is made.

Hence the DGGE technique can be used to monitor changes in bacterial communities within urban water systems, taking spatial and temporal variation into account (see Fig. 1). This variability can be investigated in terms of differences in the presence or absence of specific bands and/or in changes in their relative abundance (band intensity). Clustering techniques can be applied to identify samples, which generate similar patterns (Boon et al., 2002). Multivariate analysis also allows the interpretation of DGGE patterns in relation to environmental variables (e.g. McCaig et al., 2001). Hence, when conducted in combination with relevant physical and chemical measurements, DGGE provides the opportunity to study the changes in microbial diversity relevant to conditions within the urban water system.

3.3 FISH of microorganisms in drinking water and sewer biofilms

In this study we have applied CARD-FISH methods to detect and quantify the microorganisms in drinking water (Fig. 4a), and sewer biofilms (Fig. 4b). The images from the CARD-FISH method, with the eubacterial probes, provide an opportunity to quantify the number of bacteria within the water samples (Fig. 4a). Unlike HPC however, this technique is not based on a prior basis of culturable bacteria. Counter staining the samples with a DNA stain like DAPI (results not shown), confirms that CARD-FISH is able to detect, and therefore confidently account for, more than 90 to 93% of the bacteria present in drinking water sample. Hence the CARD-FISH method provides more confidence in enumerating the number of bacteria in water samples over the routinely used HPC method.

Figure 4b demonstrates the application of CARD-FISH for sewer biofilms collected from France. Whilst it is not possible to easily count the number of cells that are present in the biofilm, as found with Fig. 4a, the CARD-FISH method with eubacterial probes enables the morphology of the biofilm to be visualized. Comparison of CARD-FISH images across spatial and or temporal samples could therefore provide insight into the morphological development of biofilms within urban water systems.

An added functionality of CARD-FISH is the ability to target specific microorganisms (see Fig. 1) which will reveal the abundance of different phylogenetic or functional bacterial and other microbial groups within biofilms or water samples.

4 Future interdisciplinary approach

Better quantification of bacterial communities and their temporal or spatial changes in urban water systems, will lead to further understanding of their associated biological processes. This will provide the fundamental knowledge to ultimately develop “biological” management tools that will aid system operators to achieve improved levels of environmental and public health protection without resorting to the need for additional in-

frastructure or energy intensive treatment processes. Molecular microbiological analysis of water and biofilm/sediment samples, as presented here, should therefore be conducted at the same time as the measurement of the physical and chemical properties of the urban water systems. To achieve this, the MUWS project specifically operates across the length scales from laboratory to pilot and field studies (Fig. 5) and draws on the expertise of civil-, biochemical engineers and molecular microbial ecologists to address key challenges. This interdisciplinary and multi-scale approach provides a unique opportunity to develop and understand relationships between the presence and behaviour of microbial assemblages and their potential release into the environment, with asset characteristics, operation (hydraulics and cleaning/disinfection regimes) and water quality (i.e. linking biological function with engineering performance).

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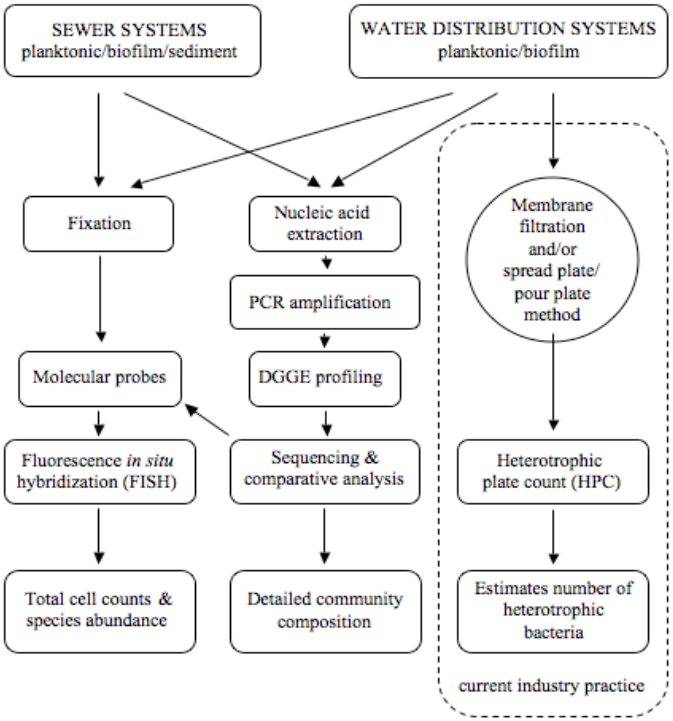

Fig. 1. Scheme for the major steps in the molecular analysis of bacterial communities from water distribution and sewer systems and the respective results obtained. In comparison, the right hand side shows the current procedure for monitoring water quality.

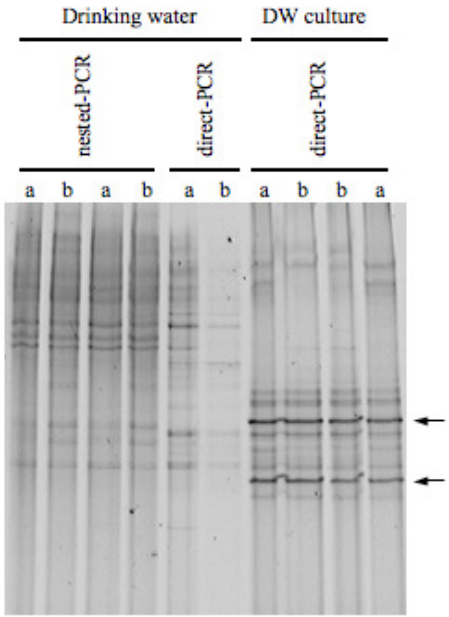

Fig. 2. DGGE profiles of PCR-amplified 16S rRNA gene fragments derived from direct-, nested- PCR approach for drinking water (DW) samples and a direct-PCR approach for culture-dependent samples. Arrows indicate the two dominant phylotypes present using the culture-dependent approach.

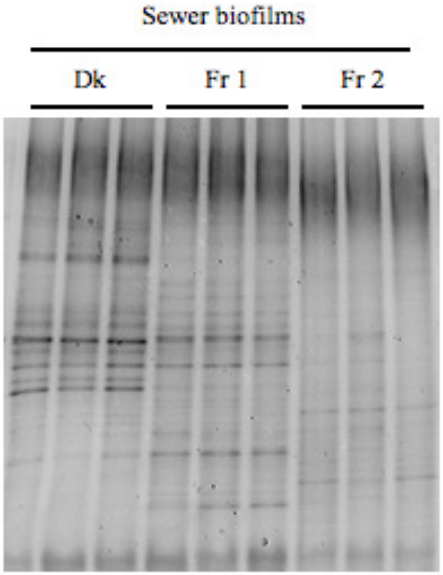

Fig. 3. DGGE profiles of PCR-amplified 16S rRNA gene fragments derived from sewer biofilm samples from one site in Denmark (Dk) and two sampling sites in France (Fr 1, Fr 2) – direct PCR approach.

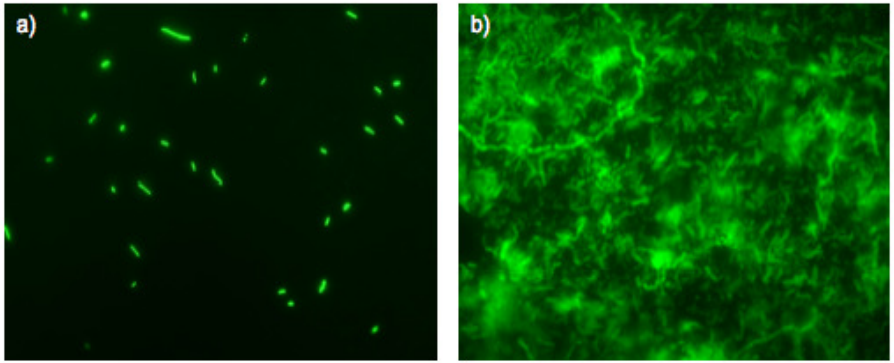

Fig. 4. Photomicrographs of drinking water bacterioplankton **(a)** and sewer biofilm bacteria **(b)** hybridized with eubacterial probes.

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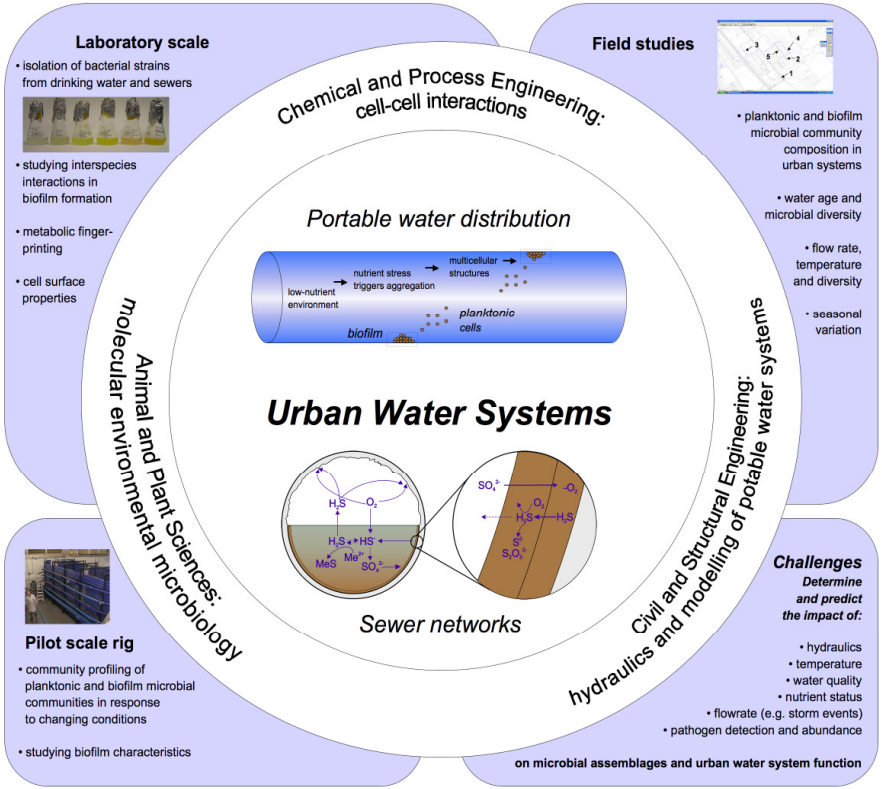

Fig. 5. Overview of the MUWS (Microbiology in Urban Water Systems) project showing the challenges, the approaches and the different departments involved.