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Analysis and characterization of planctonic and biofilm-forming Pseudomonas aeruginosa in water systems

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Dedicated to my parents and those who always help me fulfil my dreams!

"Life, water, science; everything flows when the perfect conditions come together"

(Sonia)

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List of abbreviations

ACN	Acetonitrile
AHL	Acylhomoserine lactone
A. niger	*Aspergillus niger*
ASR	Aquifer storage and recovery
BCYE	Buffered charcoal-yeast extract
CA	Cetrimide agar
C. albicans	*Candida albicans*
CC	Combined chlorine
CF	Cystic fibrosis
CFU	Colony Forming Unit
CLSM	Confocal laser scanning microscopy
DBP	Disinfection By-Products
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide triphosphate
DWST	Drinking water storage tanks
EDTA	Ethylene diamine tetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
E. coli	*Escherichia coli*
EPS	Extracellular polymeric substances
EUCAST	European Committee on Antimicrobial Susceptibility Testing
FA	Formic acid
FAV	Free available chlorine
GVPC	Glycine vancomycin polymixin cyclohexamide
HAA	ß-hydroxydecanoyl-ß-hydroxydecanoate
HCCA	α-cyano-4-hydroxycinnamic acid

HOCl	Hypochlorous acid
HPC	Heterotrophic plate count
IC	Inorganic carbon
ICU	Intensive care unit
L. pneumophilla	*Legionella pneumophilla*
MALDI- TOF MS	Matrix Assisted Laser Desorption/Ionisation Time Of Flight mass spectrometry
MB	Methylene blue
MDR	Multidrug-resistant
MIC	Minimum inhibitory concentration
MRGN	Multiresistant Gram-negative
OCl^-	Hypochlorite ion
OD	Optical density
ORP	Oxidation Reduction Potential
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PFGE	Pulsed-field gel electrophoresis
P. aeruginosa	*Pseudomonas aeruginosa*
QS	Quorum sensing
RNA	Ribonucleic acid
SEM	Scanning Electron Microscopy
SJD	Sant Joan Despí
SFSW	Sand filtered surface water
S. aureus	*Staphylococcus aureus*
TFA	Trifluoroacetic acid
TFP	Type IV pili
TC	Total carbon
TOC	Total organic carbon

TSA	Tryptic soy agar
TSB	Tryptic soy broth
UBA	Umweltbundesamtes
VBNC	Viable but nonculturable
WHO	World Health Organization
WSH	Water of standardised hardness
WSP	Water Safety Plans
WTP	Water treatment plants
WWTP	Waste water treatment plants

1. Introduction

1.1 Waterborne microorganisms and diseases

Water is one of the absolutely essential prerequisites for all life. Development of life on earth was only possible because water was and is present in sufficient amounts and in a suitable physical condition. Development of life on earth has its origin in the water and the history of development of all living organisms on earth is traceable to its origins in the depth of the prehistoric sea. The first “real” organisms on earth have been the direct predecessors of modern bacteria and since then, all corners of the planet have been occupied by bacteria. Therefore, water is and has been a common and suitable habitat for microorganisms since ever. Today, all water bodies of the world are colonized by countless different species, and probably not all of them have been identified.

Whilst most bacteria in water are harmless, some of them may pose severe threats to human health. Illnesses like cholera, typhoid fever, hepatitis and others are known to spread mainly via water. However, it is only about 100 years ago that this insight was generated and since then, diverse strategies to fight waterborne diseases have developed.

Today, it is common knowledge that bacteria are the most abundant organisms on earth and that microbes are present in most habitats on the planet. Bacteria belong to the group of organisms called prokaryotes. The word prokaryote comes from the Greek πρό- (pro-) "before" and καρυόν (karyon) "nut or kernel", meaning that prokaryotes do not have a nucleus, mitochondria, or other organelles, unlike eukaryotic cells which possess all these characteristics.

At the end of the 19th century scientific evidence emerged that microorganisms may be the reason for a variety of diseases. The subsequent discovery of individual illness causing microorganisms was one of the great milestones in medical research. Based on this knowledge on individual bacteria, researchers relatively quickly discovered different pathways of transmission: infectious diseases may be transmitted by a variety of direct and indirect pathways. Soon science could proof that depending on the specific type of disease it may be caused by viruses, parasites, or bacteria. Whilst, for example, measles are caused by respiration of a certain virus, cholera is typically transmitted by drinking water. It was very early, that measures to fight waterborne diseases have been developed, and the application of chlorine was very early amongst them.

Water is an ideal environment for the growth and development of many microbes, as soon as the temperature is not too low or too high, and if at least a minimum concentration of organics serving as nutrients is present. However, specialized bacteria may even grow under very extreme conditions, and they may use very limited nutrition resources.

Whilst waterborne diseases have been fought successfully in western countries, they are still a part of daily life in many parts of the world. A great part of the human population does not only suffer of a general lack of water, but is also still affected by waterborne diseases that have been erased in other parts of the world.

One of the last large outbreaks of waterborne diseases in Germany was the Cholera epidemic in Hamburg, 1892 causing the infection of nearly 17000 people and the death of at least 8605. It was the famous German microbiologist Robert Koch who identified basic hygienic problems as being the reason for the spreading of cholera.

Typically, waterborne pathogens are of faecal origin. Contamination of drinking water may, for example, occur by access of waste water to drinking water from leaky waste water lines and may affect large parts of the population at the same time.

Faecal derived pathogens in drinking and other kinds of water usually do not proliferate significantly in the water and will disappear after a certain amount of time. However, this time-span may be long enough to infect many people – typically in places with low standards of hygiene (like in Hamburg as mentioned before) where many people are living closely together these illnesses may spread very fast via drinking water. From a hygienic point of view, lack of sanitation is the most important reason, why diarrheal diseases are amongst the most common reasons for the death of children under 5 years of age in developing countries. Lack of sanitation is the main reason for contamination problems in drinking water.

A basic scheme for the classification of waterborne microbes with relevance for human health is shown in Fig. 1. Faecal derived waterborne bacteria include different types of *Salmonella* (typhoid fever), as well as *Vibrio cholera* or different types of *Shigella* causing dysentery, only to mention a few of them. Typical water-transmitted viruses include hepatitis A as well as polio. Protozoa in drinking water may also be a major reason for waterborne epidemics: it was only in 1993 that a tremendous outbreak of diarrheal diseases caused by *Cryptosporidium parvum* occurred, with 300.000 people getting sick. However, as indicated in Fig. 1 some of the typical waterborne pathogens are essentially not of faecal origin. The organisms listed there may live, proliferate and grow in the water as long as certain minimum requirements are fulfilled. Typical examples for pathogens of non-faecal

origin are *Legionella* as well as different *Pseudomonas* species with *Pseudomonas aeruginosa* (*P. aeruginosa*) being most important to human health (de Bentzmann and Plesiat, 2011; Revelas, 2012).

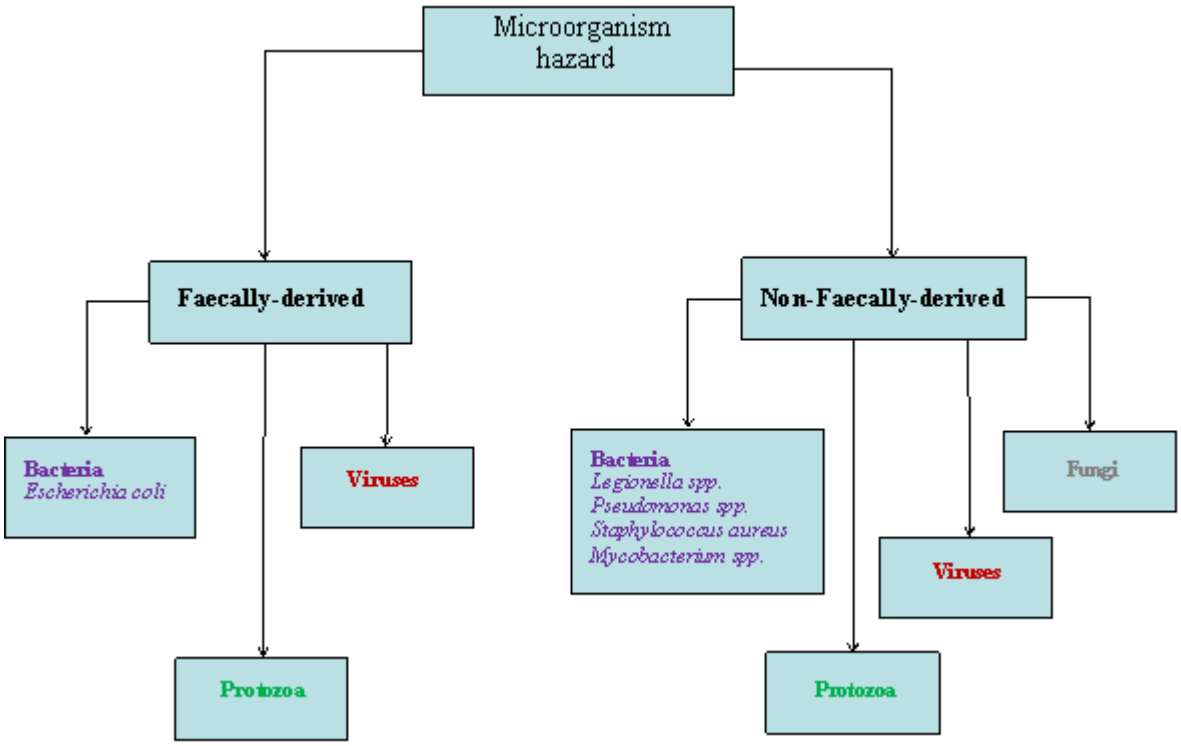


Fig. 1: Potential microbial hazards in pools and similar environments.
Waterborne microorganisms may be devided in faecally derived organisms and non-faecally derived species. The WHO Guidelines for safe recreational water environments, Volume 2: Swimming pools and similar enviroments, 2006

The bacterial pathogen *Legionella* usually infects amoeba in the water, and it may be transmitted to humans via fine aerosols and may cause severe cases of pneumonia (Leginellosis). *Pseudomonas* may be especially dangerous to patients with pre-existing lung diseases like cystic fibrosis (Hassett *et al.*, 2010), but may also be the reason for a variety of disagreeable and severe skin diseases (Lyczak *et al.*, 2000; Tena and Fernandez, 2015). *P. aeruginosa* will occupy suitable ecological niches in water systems and will preferable form biofilms. However, as will be shown in detail later, although biofilms are sessile structures adhering very firmly to a variety of surfaces, individual organisms or even parts of the biofilm may escape and show up as “planktonic” contaminants of the water. Unfortunately, biofilms of *P. aeruginosa* are relatively common and may show up in a

variety of wet environments like swimming pools, drinking water and plumbing systems of any kind.

This study tries to elucidate several aspects of *P. aeruginosa* biofilms in wet environments. The aim of this study is to broaden the knowledge on biofilms in ostensibly different environments and finally to avoid or minimize the risk associated to the occurrence of *P. aeruginosa* based biofilms.

1.2 *P. aeruginosa*

1.2.1 *P. aeruginosa* – A short introduction

In 1882 Carle Gessard, a chemist and bacteriologist from Paris, discovered a new organism causing a blue-green coloration of wound dressings. The bacterium was named *Bacillus pyocyaneus*, later the name has been changed to *P. aeruginosa* (Pirnay *et al.*, 2009).

P. aeruginosa is a Gram-negative environmental bacterium, aerobic, rod-shaped (1.5-5.0 µm long and 0.5-1.0 µm wide), monoflagellated and with unipolar motility (Ryan and Ray). Its optimum temperature for growth is 37°C, and in contrast to most other waterborne organisms it is able to grow at temperatures as high as 42°C (Tsuji *et al.*, 1982). This bacterium may secrete a variety of pigments, including pyoverdine (yellow-green and fluorescent), pyocyanin (blue-green) and pyorubin (red-brown).

P. aeruginosa has a large genome, with one of the largest regulatory networks of any bacteria sequenced up to now (Stover *et al.*, 2000). Part of the significance of this large genome is that it provides the bacterium with a tremendous diversity of metabolic pathways and physiological responses, which allows the bacteria to acclimatize to many diverse environments (Hewitt, 2010).

P. aeruginosa can be found in a wide variety of ecological environments ranging from fresh and salt water to the rhizosphere in which they may colonize the endemic fauna, flora and fungi (Glodberg, 2000). As soon as biofilms form, for example in water taps, in bottles, flower vases or in other places, growth of biofilms may be noticed by a slimy coating. It is an opportunistic pathogen that migrates from its natural environment and causes disease in animal and humans, infecting commonly immunocompromised patients (Oberhardt *et al.*, 2008). Specifically, *P. aeruginosa* infections of the eye, bloodstream, urinary tract, and burn wounds are common, and are frequently acquired in hospitals as nosocomial infections (Lyczak *et al.*, 2000). Population at increased risk of contamination with *P. aeruginosa* are people with immune system compromised, including elderly, young children, pregnant woman. (Flores-Mireles *et al.*, 2015; Mena and Gerba, 2009b).

As mentioned before, one of the most important pathogenicity factors of *P. aeruginosa* is its ability to form biofilms in virtually any environment, including living organisms. The structure and formation of biofilms will be detailed later.

In biofilms, many organisms of the same or different kinds live together in a highly organized form, making the whole of the biofilm much more resistant against attacks from outside. One of the most obvious strategies is the formation of slime-like compounds, known to everybody from daily experience.

Basically, *P. aeruginosa* strains may be subdivided according to their ability to form mucoidal compounds responsible for the slimy surface. "Mucoid strains" overproduce the exopolysaccharide called alginate, which consists of a variety of more or less specific compounds including, for example, polymers of partially acetylated β-d-mannuronic acid as well as its C5 epimer α-l-guluronic acid (Ertesvag, 2015; Jain and Ohman, 2005). The alginate-containing matrix of mucoid strains is an important factor for the formation of a highly structures and diverse biosystem, and, for example, allows the formation of microcolonies and increases the resistance to a variety of factors with negative impact as for example opsonisation, phagocytosis, and destruction by antibiotics (Kostakioti *et al.*, 2013).

Up to now, only few aspects of biofilm formation have been examined in detail. Regarding co-existence of different *P. aeruginosa* strains, many problems remain to be resolved. For example, some researches consider that different strains of bacteria could live in symbiosis within the biofilm, where mucoid strains may ensure survival of biofilm through alginate hyperproducing and non-mucoid strains may protect probably bacteria against antibiotics (Owlia *et al.*, 2014). However, many more aspects of biofilm occurrence, formation, structure and other details need to be investigated.

Although *P. aeruginosa* is a potential pathogen, no maximum contamination levels for drinking water are defined in German legislation. Nevertheless, according to Mena & Gerba (Mena and Gerba, 2009a), *P. aeruginosa* is found in 2% of all drinking water samples. In this context it is worth mentioning that *P. aeruginosa* outbreaks have been identified in drinking water (Bedard *et al.*, 2016).

However, the health relevance of *P. aeruginosa* based biofilms remains unclear, and additional information is urgently needed to broaden the base for evidence based regulations in the sector of drinking water hygiene:

- "Pseudomonas Hot-Foot Syndrome" consists of a skin eruption on the soles of the feet that usually occurs in children who are in contact with pools, which has been documented in several articles (Fiorillo *et al.*, 2001; Michl *et al.*, 2012).
- One of the major health effects associated to *P. aeruginosa* is otitis externa, an infection of the inner ear which may be painful and long-lasting (Orji *et al.*, 2014).
- Several reports have been published on the occurrence of folliculitis in swimmers and bathers and have been identified as being another pool water based disease being of major concern. Folliculitis is an infection of roots of hair.
- *P. aeruginosa* has also been associated with a range of other infections, including eye, urinary and respiratory tract infections (Mena and Gerba, 2009b).
- Hospitals: nosocomial infections occur worldwide and affect both developed and developing countries. Between 10% and 20% of infections in most hospitals are caused by *P. aeruginosa*. Pseudomonas infections occur mainly in patients with burn wounds, cystic fibrosis, acute leukemia, organ transplants, and intravenous-drug addiction (Sousa and Pereira, 2014). The organism is frequently found in moist areas such as sinks, antiseptic solutions, and urine receptacles. Some studies show that water fittings are a major source of *P. aeruginosa* in intensive care unit (ICU) and also other reports relate environmental and clinical strains, existing contributions of endogenous and exogenous sources to *P. aeruginosa* and infections may vary both between ICU and within ICU depending on the period considered (Bilinski *et al.*, 2012).

1.2.2 Infections caused by *P. aeruginosa*

P. aeruginosa is a common dweller in water containing systems. Most people will have no problems getting in contact with *P. aeruginosa*, even at elevated concentrations. *P. aeruginosa* may be a part of the gut flora and remain there without any trouble. However, in certain cases, especially if the immune system is not working as it should, *P. aeruginosa* may cause severe infections.

In swimming pools, certain forms of dermatitis may be transmitted, most probably via biofilms attached on surfaces. Prolonged stay in the water may macerate the upper layers of the skin thus facilitating the attachment of *P. aeruginosa*. Finally, an infection of the roots of hair, called "folliculitis" may result. Especially, infections occurring in hot-whirl-pools have been described several times.

P. aeruginosa plays a significant role as an organism causative for nosocomial infections. Nosocomial infections occur worldwide and affect both developed and developing countries

(Revelas, 2012). Between 10% and 20% of infections in most hospitals are caused by *P. aeruginosa*. The organism is frequently found in moist areas such as sinks, antiseptic solutions, and urine receptacles. Some studies show that water fittings are a major source of *P. aeruginosa* in ICU and also other reports relate environmental and clinical strains, existing contributions of endogenous and exogenous sources to *P. aeruginosa* and infections may vary both between ICU and within ICU depending on the period considered (Bilinski *et al.*, 2012).

1.2.3 Biofilm formation by *P. aeruginosa* in aquatic environments

Biofilms are highly organized communities of bacteria. Within biofilms, the behaviour of bacteria resembles to certain structures of somatic eukaryotes in living organisms. Microbes in biofilms do not only form channels for transport of water and nutrients but they also developed communication skills called "quorum sensing" (QS) and very sophisticated systems for the protection of the film to the outside. These protection systems include, for example, synthesis of certain compounds called "extracellular matrix".

Within this shelter, bacteria are also significantly more resistant to antibiotics and to most disinfection methods, including disinfection by chlorine, and therefore may pose serious problems to human health, depending on the quality and quantity of the biofilm.

Charles Darwin developed in the 19th century the modern theory of evolution. In his book *On the Origin of Species*, published in 1859, he proposed that all of the millions of species of organisms present today evolved over billions of years from a common ancestor by way of natural selection, where the individuals best adapted to their habitat increase the proportion of their genes leaving their traits to their offspring. Biofilms have been identified as being among the first organized living structures on earth and may be regarded as being the background for all further development (Donlan, 2002). Intensive research on biofilms over the last decades elucidated, that this early form of organized life appears very early in fossil record and dates back to ~3.25 billion years ago (Krumbein *et al.*, 2003). Biofilms contributed also to fossil preservation, as, for example, in the case of the Eocene Florissant Formation, Colorado, where insect and plants fossils were associated with extracellular polymeric substances (EPS) (Buskirk *et al.*, 2016).

1.2.3.1 The very beginning of a biofilm: bacterial communication by quorum sensing

Formation of a biofilm on a pristine surface is an extraordinary act of bacterial organisation (Dang and Lovell, 2016). As described later, biofilm formation can be described by a multi-stage process, including adherence, growth, maturation and dispersion of the film.

However, individual properties of bacteria are a necessary prerequisite for the formation of biofilms, and therefore, this chapter starts with a basic introduction into bacterial communication strategies and, at least equally important, with the capability of *P. aeruginosa* to move in aquatic environments.

Basically, bacterial communication depends greatly on QS. This terminus refers to a system of stimulus and response correlated to the density. QS is not limited to certain forms of living, but may be regarded as being a messaging tool and decision-making process in any decentralized community.

QS is based on signalling molecules emitted and received within bacterial communities (Gao *et al.*, 2016). Response depends on the concentration of the signalling molecules: as soon as certain concentrations are reached or passed, certain actions will be triggered by activation of defined genes. Depending on the quality and quantity of signalling molecules, QS will have significant influence on the behaviour and further fate of bacterial communities.

QS is mentioned at the beginning of the chapters on biofilm formation because QS is probably involved in many stages of biofilm development, and it plays a significant role in the very first onset of the mechanism leading to its formation. QS is not a concentration dependent reaction of the system, but a complex system of cell interaction and messaging.

As mentioned before, *P. aeruginosa* may be present in water as free-flowing or planktonic bacteria, or it may be present in form as firmly attached films consisting of a high quantity and density of microbes. The differentiation of planktonic *P. aeruginosa* cells into biofilms is triggered by QS (Flickinger *et al.*, 2011). In case of *P. aeruginosa*, cell-to-cell communication (de Kievit, 2009), and a probably distinct mechanism of regulated gene expression in bacterial communities in response to other cells and the physical constraints imposed by their environment (Boedicker *et al.*, 2009) will lead to the formation of biofilms. However, as mentioned before, QS is not limited to this aspect but it is a major regulatory instrument for a variety of processes within the biofilm. It is often linked to environmental as well as to metabolic and probably many other influences.

The majority of signalling molecules identified thus far in QS can be classified into three main groups: acylhomoserine lactones (AHLs), oligopeptides and the LuxS/autoinducer 2 (Keller and Surette, 2006; Zhang and Li, 2015). However, the types of chemicals associated with cell-to-cell signalling represent an ever-expanding collection of molecules that are structurally quite diverse (de Kievit, 2009). *P. aeruginosa* uses QS to coordinate the

formation of biofilms, to regulate its swarming motility as well as its exopolysaccharide production, and it will have impact on cell aggregation (Sauer *et al.*, 2002).

1.2.3.2 Abilities of *P. aeruginosa*: motility

Different and versatile ways of bacterial motility, that means ability to move, is an additional important feature of *P. aeruginosa* influencing biofilm formation (Rasamiravaka *et al.*, 2015). *P. aeruginosa* has several different possibilities to move in different environments depending on the particular situation: *P. aeruginosa* is able to move by swimming, swarming and so-called "twiching" (Fig. 2) (Tremblay and Deziel, 2010).

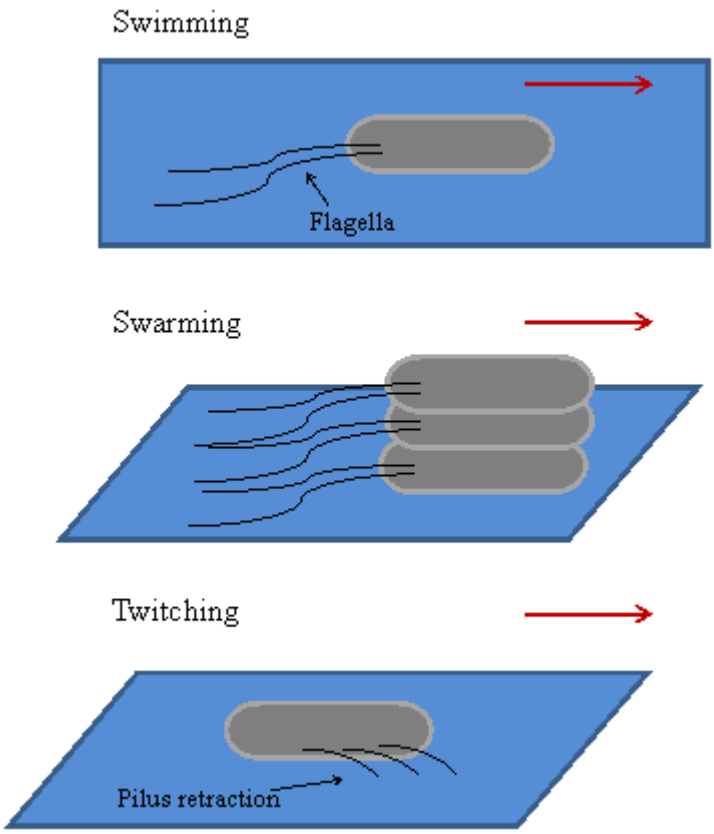


Fig. 2: Three types of motilities in *P. aeruginosa*. (Based on Kearns (Kearns, 2010))

- Swimming is performed by rotating a single polar flagella. It is the typical form of movement of individual cells in liquid environments (Kearns, 2010). Planktonic cells are freely moving through aquatic environments and may therefore be able to colonize whole water systems. However, during the "planktonic phase" *P. aeruginosa* is relatively vulnerable against any disinfectant in the water and may easily be eliminated.
- Swarming is a rapid multicellular movement of bacteria across a surface, also powered by rotating flagella (Kearns, 2010). *P. aeruginosa* has two flagellar stators necessary for

swarming motility, whilst only one is necessary for swimming (Toutain *et al.*, 2005). *P. aeruginosa* synthesizes ß-hydroxydecanoyl-ß-hydroxydecanoate (HAA), a complex fatty acid thought to contribute to swarming motility. Friction between the cell and the surface will be reduced and dendrite formation may be observed depending on secretion of multiple surfactants (Caiazza *et al.*, 2005).

- Twitching is another variation of surface motility enabled by the extension and retraction of type IV pili (TFP). Twitching is characterized by low cell movement, often with a jerky or "twitchy" appearance (Kearns, 2010). Twitching by TFP plays an important roles in such diverse biological phenomena as surface adhesion, motility and Deoxyribonucleic acid (DNA) transfer, with significant consequences for pathogenicity (Imam *et al.*, 2011). *P. aeruginosa* deploy TFP symmetrically or asymmetrically to modulate motility behaviours in different nutrient conditions and thereby form biofilms only where nutrients are sufficient, which greatly enhances their competitive capacity in diverse environments (Ni *et al.*, 2016).

1.2.3.3 A Short description of "Ab-initio" biofilm formation

Biofilm formation is a complex process depending on the microbial species, its communication and motility skills, the specialities of the surface where the biofilm will be attached, and, of course, the quality as well as chemical and physical properties of the surrounding aquatic environment.

Biofilms may develop in any wet environment: biofilms are present in waste water treatment plants (WWTP), where the main task of cleaning the water is performed by biofilms. They may grow as well in cooling towers, in fruit juice or beer lines, as well as in water storage tanks, swimming pools and drinking water lines. However, the structure, size and constitution of the particular biofilm will depend on the individual situation.

Our own laboratory studies to grow biofilms in water on glass surfaces showed, that movement of the liquid phase, concentration of nutrients and temperature do have significant impact on speed of growth. However, the process of biofilm attachment and development may be explained by a simple five step process (Fig. 3) taking into account, as mentioned before, that individual conditions will have significant impact on each of these steps.

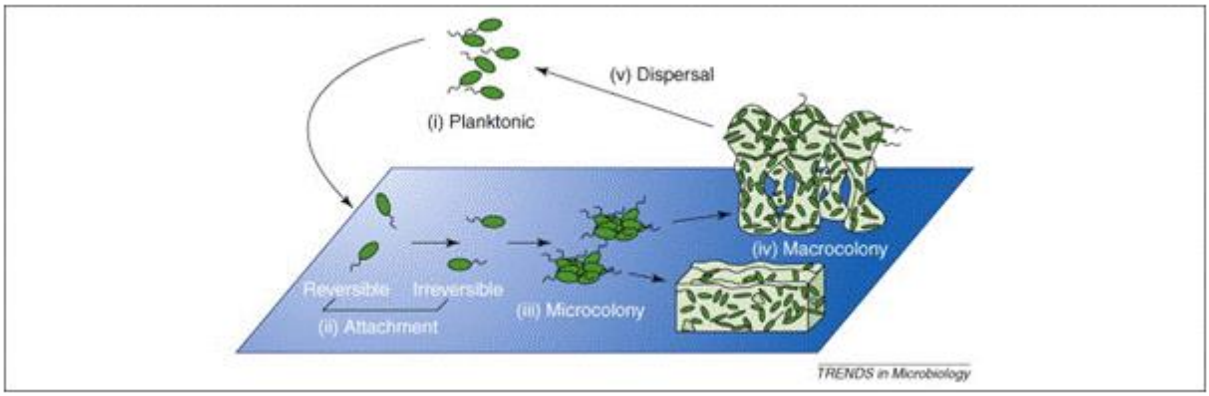


Fig. 3: Developmental model of biofilm formation (Monds and O'Toole, 2009)

1. Reversible attachment to a surface: at first, single bacteria will reach the surface through the liquid phase usually by swimming. Single bacteria will attach to surfaces using flagella and other surface appendages (Watnick *et al.*, 2001). This attachment is generally reversible and it is largely mediated by "van der Waals forces". However, early stages of biofilm development will depend on the specific strain. Research lately published in "Nature" reveals that at least non-mucoid strains of *P. aeruginosa* will attach on surfaces using two distinct types of exopolysaccharides (Psl and Pel).

2. Irreversible attachment: during the second stage bacteria will slowly but tightly adhere to the surface via pili, proteins, polysaccharides and fimbriae (Jenkins *et al.*, 2004). During this stage, *P. aeruginosa* may move across the surface and form aggregates using the above mentioned twitching motility (Conrad, 2012; Klausen *et al.*, 2003a; Klausen *et al.*, 2003b).

3. Microcolony formation: is a subsequent step characterized mainly by the proliferation and production of EPS. During this stage, cells lose their flagella-driven motility (O'Toole *et al.*, 2000) and the whole system turns to be immobilized. However, *P. aeruginosa* cells may remain fixed at the point of attachment or they may move. Knowledge about factors leading to the formation of microcolonies is very limited. New research indicates that individual *P. aeruginosa* cells probably create a system of Psl trails on surfaces necessary for the attachment of new cells and finally, for biofilm formation.

4. Biofilm maturation: during this step microorganisms continue to proliferate and will excrete larger amounts of hydrated EPS consisting of polysaccharides, proteins, nucleic acids and lipids (Flemming and Wingender, 2010; Mann and Wozniak, 2012), providing stability to the biofilm as a whole and additional shelter to individual bacteria.

5. Dispersion or detachment: during this stage single motile cells may disperse from the film; by diverse mechanisms (Li *et al.*, 2007). Cells from the biofilm will attach at other places and will promulgate the spreading of the film.

Thus, biofilms may colonize whole systems and may be very difficult do remove. Whilst in some cases, for example for waste water purification or certain technological processes biofilms are wanted and their growth is supported, there are many other situations where biofilms are unwanted and may be regarded as harmful contaminations. However, removing biofilms is a difficult task. Especially in drinking water lines or water storage tanks *P. aeruginosa* based biofilms need to be removed, as *P. aeruginosa* is a potential pathogen. However, whilst drinking water lines are subjected to microbiological investigations on a regular basis, occurrence of biofilms in drinking water storage tanks (DWST) is a more or less neglected subject.

1.3 Biofilms in aquatic environments

1.3.1 The case of DWST

Depending on the local situation, many drinking water distribution systems include storage tanks to balance different levels of water consumption and to store certain amounts of water as a reserve. In the past DWST have been an important and significant part of any supply system. However, modern systems are able to provide enough water for all without the use of water reservoirs because of higher pumping capacities and modern water distribution management. Nevertheless, many systems retained their storage tanks and still use them as an integrated part of the distribution system. Storage tanks are frequently constructed as open systems, and contaminations may take place through the water and from outside. Biofilm formation in water storage tanks is of special significance, because all drinking water will pass through the tanks and get in touch with the films.

In Germany, most of the drinking water is produced using ground water. Ground water is usually of good quality depending on the depth of the wells. In the Rhine-Neckar region around Heidelberg, depth of wells is usually more than 10m providing water of excellent microbiological quality. Our own routine analysis of drinking water from the area indicates that heterotrophic plate count (HPC) is usually in the area of 0-5 colonies/ml. As the water is not disinfected at all, natural purification processes in the vase zone, as well as water temperature may be the reason for this. Ground water usually shows the yearly average temperature of the area, and therefore water temperature in the Upper Rhine Valley is between 12 and 14 °C. At least pathogens will have difficulties to grow within this

temperature range; however, there are specialized members of the aquatic flora that may grow even at lower temperatures. Unfortunately, scientific information on growth of *P. aeruginosa* in this environment is very limited.

Biofilms may form to a significant extend if the water at least partially becomes too warm. Of course, in warmer countries the problem of microbial contamination in drinking water systems including DWST will increase with the water temperature.

Biofilms in DWST may be responsible for a wide range of water quality and operational problems. One of the aims of this study was to investigate the presence and composition of biofilms in local DWST. However, this task turned out to be relatively complicated; during normal operation, sampling of walls and floors in water storage tanks is usually not possible because of hygienic problems. However, tanks are usually emptied and cleaned about once a year, and during this time sampling should be possible. Unfortunately, an emptied tank may be contaminated from different sources, and temperature of the walls and floors will rise as soon as the water is drained, thus changing the situation fundamentally. Additionally, the interest of water works officials in analysis that may have a "negative" outcome is somewhat limited.

Usually, DWST are not cleaned because of microbiological problems but because of chemical reactions of iron and manganese turning the walls and floors of tanks brown. Ground water often contains soluble forms of iron and manganese, which will be oxidized as soon as oxygen is available. Therefore, over the course of time harmless coatings of iron and manganese compounds will cover all surfaces under water. Actually, these coatings may be a supporting factor during the formation of biofilms.

1.3.2 The case of biofilms in swimming pools

In public swimming pools, hygienic problems may emerge because many people with very different hygienic backgrounds come together and share the same resources. Necessarily, swimming pool water needs to be purified and disinfected to avoid the spreading of diseases. In contrast to drinking water, the situation is significantly more challenging because the water is continuously contaminated by the bathers. Additionally, water preparation systems like sand- or charcoal filtration do not really remove soil and parasites from the water, but concentrate them on the filters. Only as soon as the filters are backflushed, the dirt is really removed. In regular public swimming pools, backflushing is done at least once a week. This means, that microorganisms and dirt have left a significant span of time to concentrate, and in case of living microorganisms, to grow.

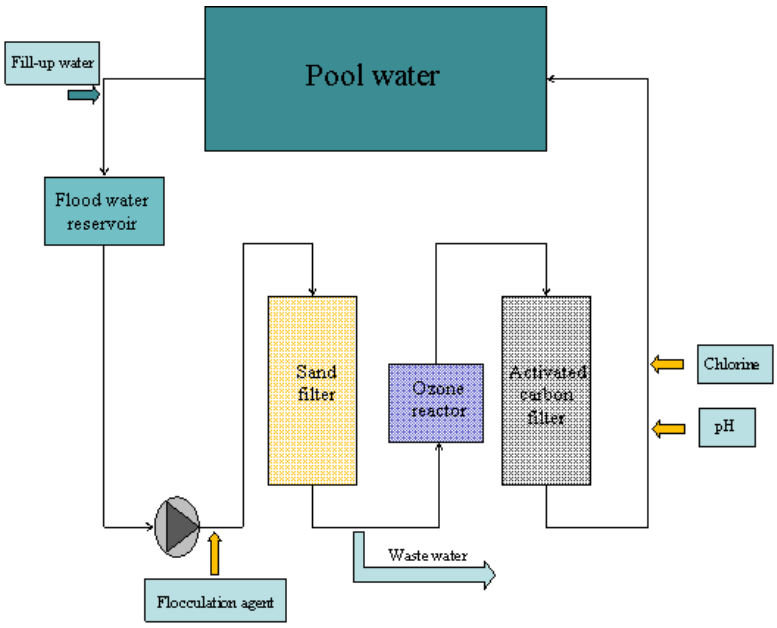


Fig. 4: Water purification system in a swimming pool

A schematic for water preparation in swimming pools is depicted in

Fig. 4. Pool water from the basin passes an equilibrium tank and is mixed with a flocculation agent. The flocculants together with adsorbed organics is later filtrated on a sand filter. In most cases, water is redirected from here to the pool, however, chlorine is added and pH is stabilized before the water enters the basin. In some cases, ozone is employed to augment the water purification capacity of the system. To this end, ozone is produced from air on site and mixed with the water. After a couple of minutes of reaction time, however, the ozone needs to be removed from the water because of its high toxic potential. This is usually done using activated charcoal filtration where the ozone reacts chemically with the carbon forming harmless carbon dioxide. The rest of the water preparation is the same as in non-ozone pools. In contrast to public belief, all “ozone pools” are disinfected using chlorine; because ozone is simply too toxic and it would be too dangerous to leave this compound in the basin at concentrations necessary to kill microorganisms.

As swimming pools are used by many different users it is not astonishing, that a variety of microorganisms may be found in swimming pool water in certain cases (for example as soon as the continuous disinfection ceases by any reason) as well as in the environment of swimming pools (in case of inadequate cleaning measures) and in similar recreational water environments.

Like in drinking water, microbial contaminations in swimming pools may be categorized as being of faecal and/or non-faecal origin. Faecal contaminations may usually be due to

deliberate or accidental faeces released by bathers or, in outdoor pools, may additionally be the result of contaminations by animals (e.g. birds and rodents).

Non-faecal human shedding (e.g. from vomit, mucus, saliva or skin) in the swimming pool or similar recreational water environment is another potential source of pathogenic organisms (World Health Organization. Water, 2006). Furthermore, infected users can directly contaminate pool waters and the surfaces of objects or materials with pathogens (viruses or fungi) and with opportunistic pathogens (bacteria). This is of course a significant difference to drinking water, where contamination sources are much more limited.

In comparison to swimming pool water, microbiological analysis of drinking water is essentially limited to organisms indicating faecal contaminations. In many countries, microbial regulations for swimming pool water include a variety of other bacteria of non-faecal origin, with *P. aeruginosa* being probably the most important, next to *Legionella (Guida et al., 2016).* In Germany, regulations stipulate that *P. aeruginosa* must not be higher than "not detectable in 100 ml of pool water". However, low concentrations of *P. aeruginosa* in the filtrate may be acceptable if *P. aeruginosa* does not show up in the pool; according to the Hygiene Recommendations of the Umweltbundesamtes (UBA). In contrast to faecal contaminations, *P. aeruginosa* may colonize technical systems in swimming pools and may form difficult to remove biofilms with high significance to hygiene.

Despite ongoing improvements in water quality and disinfectant monitoring, swimming pools experience frequently problems with *P. aeruginosa*, being an important factor for public health and for the pool management. *P. aeruginosa* is one of the most frequently isolated opportunistic pathogens in pools and hot tubs (Rice *et al.*, 2012). Although the pools are cleaned according to the rules, in the analyses that are carried out routinely, the presence of *P. aeruginosa* has been observed in some cases.

Although *P. aeruginosa* is able to form biofilms virtually on all surfaces, particularly swimming pool environments including all pool structures with damp or wet surfaces (such as decks, drain and benches), pool carpets, shower floors (Leoni *et al.*, 1999), pool tools and toys are addressed. Furthermore, the water purification and recycling system may be an ideal place for harbouring *P. aeruginosa* persistently in hard-to-access parts of the system, as in the case of filters (sand filter and activated carbon filter) (Uhl and Hartmann, 2005), where *P. aeruginosa* may grow intensively. Concerning biofilm growth on filters, it has been demonstrated that *P. aeruginosa* can reach up to 10^7 CFU/g on packed glass bead columns confirming that filtration media, such as sand filters, can harbour high numbers of bacteria (Liu and Li, 2008). Within a recent study, Uhl & Hartmann (Uhl and Hartmann,

2005) observed accumulation of nutrients on activated carbon surface, encouraging and even intensifying biofilm growth.

Furthermore, a recent study (Lutz and Lee, 2011) done on the prevalence and antimicrobial-resistance *of P. aeruginosa* in swimming pools and hot tubs, confirmed that elevated chlorine levels are not sufficient to prevent *P. aeruginosa* biofilm growth. As described for water installation systems, notable levels of antimicrobial resistant *P. aeruginosa* may be endemic in hot tubs and swimming pools.

Therefore, these observations suggest that *P. aeruginosa* biofilms may not be adequately managed under current pool management regimes. Nevertheless, knowledge on *P. aeruginosa* contaminations in swimming pools is poorly developed and fighting *Pseudomonas* biofilms in contaminated water preparation systems of swimming pools is an ambitious task. To control biofilm formation and *P. aeruginosa* colonization in swimming pool settings, some products has been applied with encouraging preliminary results (Guida *et al.*, 2016).

1.3.3 Water installation systems

In drinking water distribution systems (pipes, water taps, toilet tanks…), all surfaces in contact with water may be colonized by microorganisms (Wingender and Flemming, 2004). It has been estimated that about 95% of all microbial cells present in drinking water distribution systems exist as biofilms on pipe surfaces and only 5% occur in the water phase (Flemming *et al.*, 2002).

Drinking water biofilms are formed predominantly by microorganisms of the autochthonous aquatic microflora without any relevance to human health. However, drinking water biofilms have the potential to harbour opportunistic pathogens, which can harm human health. *P. aeruginosa* is one of the most important opportunistic pathogens which can be involved in biofilm-associated contamination of domestic plumbing systems (Eboigbodin *et al.*, 2008) as outlined before for DWST and swimming pools. Although the situation is comparable, there are remarkable differences between these three examples, and each has its own individual hygienic background: whilst DWST are individual components of water distribution systems before the water is entering the individual water installation, water installations are much more complex and may be influenced by a variety of different factors. The situation may become critical as soon as people are concerned who already suffer from pre-existing illnesses like patients in hospitals. However, people in homes for the aged,

nursing homes or other facilities for people suffering from weak health are concerned as well.

In hospital environments, *P. aeruginosa* may cause so-called nosocomial infections, that means, infections that patients get after being hospitalized. Hospital-acquired infections are a problem of fundamental significance in any hospital environment. Whilst most of these infections are transmitted by the hands of the personnel, others may be caused by a variety of reasons, including contaminated tap water. The reason of getting ill due to facultative pathogens in hospitals is naturally much higher than in "private" environments at home: patients in hospitals usually suffer from certain illnesses, and their immune system is in a worse condition as compared to none-patients.

However, many efforts are undertaken in hospital environments to avoid or at least to minimize the danger of exposing patients to bacterial contaminations: cleaning the hands and disinfecting all the equipment and environments in hospitals can help prevent infections. Unfortunately, formation of biofilms in water installation systems is commonly not among the factors to be screened on a regular basis although it may occur very easily. Additionally, water based medical devices like anaesthesia apparatuses, mechanical ventilators, and humidifiers are some of the equipment of hospitals where *P. aeruginosa* may be present and contribute to this problem.

A major problem in hospital hygiene is the presence and occurrence of multi-resistant bacteria, rendering common antibiotic therapy ineffective. Whilst *Staphylococcus aureus (S.aureus)* is amongst the most important multi-resistant organisms in hospitals, multi-resistant *P. aeruginosa* are known to a much lesser extent, although it is resistant to many antibiotics commonly used for therapy. *P. aeruginosa* demonstrates resistance to multiple antibiotics, and may be a serious threat to patients already suffering from other illnesses. The presence of multidrug-resistant *P. aeruginosa* in an aquatic milieus of hospitals or other health related institutions may be important for immune-suppressed or other at-risk individuals, for whom treatment difficulties have great implications (Obritsch *et al.*, 2004). However, the source of *P. aeruginosa* infections in hospitalized patients is often unclear, although there are some reports of hospital outbreaks of *P. aeruginosa* related to environmental contamination, including tap water (Breathnach *et al.*, 2012). Some recent publications on *P. aeruginosa* outbreaks in hospitals have shown that the origin of these outbreaks may be associated with water systems: with sinks used for hand washing (Hota *et al.*, 2009) and with the contamination of waste-water systems (Breathnach *et al.*, 2012). Therefore, *P. aeruginosa* biofilms in hospitals may be identified as being of special

relevance, and were included in this study. In some countries like the United Kingdom, the Public Health England has established a reproducible and controllable water distribution test rig in a laboratory setting to further understand the contamination of water systems by *P. aeruginosa* and to identify vulnerable sites for microbial colonization in hospital water systems (Walker and Moore, 2015).

1.3.4 Evaluation of clogging during sand filtered surface water injection for aquifer storage and recovery: pilot experiment in the Llobregat Delta (Barcelona, Spain)

The aquifer storage and recovery system of Sant Joan Despí (SJD) in the Llobregat basin (Barcelona) has been injecting potable water since its construction in 1969. In order to increase the environmental and economic sustainability of the process, the substitution of potable water by sand filtered surface water (SFSW) has been considered. This study aimed at assessing the clogging potential of SFSW by reproducing the aquifer storage and recovery (ASR) system in a column-type pilot system. This study was carried out within the project Demonstrate Ecosystem Services Enabling Innovation in the Water Sector (DESSIN), funded by EU FP7 programme.

1.4 Prevention, disinfection and removal of biofilms

Control of microbial growth in water may be accomplished in two ways: either by killing of microorganisms or by inhibiting their growth. Sterilization is any process that kills all forms of microbial life whilst disinfection will only reduce the colony count to a non-infectious number. Whilst sterilization processes usually employ physical methods, most disinfection methods are based on the action of certain chemical compounds. The choice of a disinfectant for a given task depends on a variety of factors.

Disinfection as well as sterilisation methods are available for a wide variety of different purposes. The choice of chemical compounds for disinfection depends on a number of aspects. For example, whilst alcohol may be used for the disinfection for hands and skin, it is not suitable for the disinfection of water. Actually most disinfecting compounds are toxic and not suitable for water.

Therefore, disinfectants for water need to be both effective and non-toxic to humans, and not too many disinfection methods are currently in use. Additionally, all disinfection methods may be effective as long as *P. aeruginosa* is present as planktonic organism, but real problems may arise when biofilm formation has taken place. For example, biofilms of *P. aeruginosa* were up to 10.000-fold more tolerant to the treatment with certain biocides

like quaternary ammonium chloride or even treatment with chlorine compared to planktonic cells (Buckingham-Meyer *et al.*, 2007).

Whilst growth and dispersal of biofilms in water installation system is a natural process taking place on its own, removal of biofilms and clearance of any kind of water system may be a challenging task. Depending on the local situation, biofilms may grow and infiltrate whole building installations and even complete water distribution systems, they may be present in swimming pools and other places where they are not only unwanted but may pose a significant threat to human health.

Prevention, disinfection and removal of biofilms are therefore a common topic in the field of water hygiene. However, although this problem is very common, no sustainable not to mention universal strategies for the removal of *P. aeruginosa* have been developed up to now.

One of the oldest and most common ways to fight at least planktonic bacteria in nearly any kind of water is the use of water disinfectants with chlorination being one of the oldest methods that is still used on a regular basis in many countries.

Chlorine may be employed using either chlorine gas or salts of its disproportionation products, the hypochlorites.

Chlorine gas is added to the water and breaks down into hypochlorous acid (HOCl) containing the hypochlorite ion (OCl^-). Disinfection relies only on the concentration of the hypochlorous acid and is strongly dependent on the pH of the water. As soon as the pH rises, concentration of hypochlorous acid will fall and disinfection capacity will be diminished. Therefore, the levels of HOCl and OCl^- as well as the interconnected disinfection capacity strongly vary with the pH level. Ideally, the level of pH should be between 7 and 7.5. If the pH is too high, not enough HOCl is present and disinfection is not successful.

The mode of action of hypochlorous acid on bacteria is still not completely understood however, it seems to be clear that the disinfection capacity is based on the oxidizing properties of hypochlorite. But, in comparison to other oxidizing compounds, the disinfection capacity of hypochlorous acid is much higher.

Basically, it is known that chlorine is able to disintegrate lipids of the cell wall and react with intracellular enzymes and proteins, making them non-functional. Thus, microorganisms then either die or are no longer able to multiply (Kleijnen, 2011).

Actually, there is no scientific valid information on chlorine concentrations necessary to kill microorganisms in water and to prevent growth of biofilms. In most European countries,

chlorine concentrations applied for the sanitation of drinking- and swimming pool water are below 1 mg/l. Other countries rely on much higher concentrations in both drinking and swimming pool water. In the United States of America, for example, free chlorine concentrations of 4 mg/l and more are applied on a regular basis.

Depending on the quality and temperature of the water, an adjustment of chlorine concentrations necessary for disinfection should be made; however, as mentioned before, there is no reliable scientific background for the estimation of these concentrations and applied concentrations are mainly justified by the experience present on site.

Basically, killing of microorganisms in biofilms by chlorine or hypochlorous acid usually requires relatively high concentrations. As microbes in the biofilms are well protected by EPS and others, even chlorine concentrations of 20 mg/l are occasionally not enough to remove biofilms completely. Chlorine will react with the organic matrix of the biofilm and produce oxidized and chlorinated compounds. These side-reactions are unavoidable and they are the reason for the formation of so-called “Disinfection By-Products” (DBP). By these reactions chlorine concentrations will be lowered within the biofilm, and final concentrations at the bottom of the film are frequently not high enough for significant disinfection. That means that there is a gradient of the disinfectant concentration within the biofilm which allows individual bacteria to employ the film as a shelter and survive any disinfection measure.

Therefore, in most cases biofilms cannot be removed by the action of chlorine only. Usually additional action of, for example, tensides and other compounds dissolving the EPS matrix is necessary and only mechanical removal of surface films will guarantee successful biofilm elimination. Unfortunately, the efficiency of chemical cleaning products on the market is only limited resulting in high persistence of the problem and tedious and repeated attempts. New approaches for the removal of biofilms, and especially of biofilms caused by *P. aeruginosa* are urgently necessary and are addressed here.

Other methods for water disinfection exclusively address planktonic bacteria in the water and application for the fight against biofilms is not included.

For example, UV disinfection involves the irradiation of waterborne bacteria using UV-lamps. Depending on the technology used, low-, medium-, or high-pressure lamps may be employed. In any case the UV light will provide enough energy to break C-C bonds and thus to kill or at least inactivate most bacteria. However, UV irradiation will only address planktonic bacteria and therefore will be ineffective against biofilms. All available data from literature for the inactivation of bacteria using UV-light are for experimental

laboratory systems, there are apparently no data available for the action of UV light under natural solar conditions (Rice *et al.*, 2012). However, Dejung observed that the time required for 1 log10 inactivation of *P. aeruginosa,* at 1.69 mW/cm^2 of UV-A (320-405 nm), was 1.2 h (Dejung *et al.*, 2007). This is, at least, a hint in the direction of quantitative assessment of the activity of UV-based water disinfection. On the other hand, the relative tolerance of *P. aeruginosa* suggests that UV disinfection may not be adequate for its elimination. This reduced efficacy becomes even smaller when *P. aeruginosa* grows as a biofilm, considering that bacteria are protected in the EPS from exposure to UV-A, UV-B and UV-C.

Another way to remove bacteria from water is filtration: as mentioned before, at least swimming pool water is cleaned and prepared by sand filtration using a flocculant. However, this method is frequently used in drinking water preparation, if necessary, and may be used as a "second barrier" against microbial contaminations. Filtration may improve water quality significantly by removing planktonic bacteria including *P. aeruginosa*. Rapid sand filters and diatomaceous earth being frequently used in the field of water preparation have been shown to remove > 99% of Pseudomonas (Leoni *et al.*, 1999). However, as mentioned before, regarding the efficiency of chemical disinfection methods, filtration as a single step is not sufficient as a lone measure of water disinfection. Technical water filtration can therefore only support water preparation systems and may augment its affectivity.

However, use of 0.1 µm microfiltration allows production of water free from microorganisms such as *P. aeruginosa* and *Legionella* species. This technology ("Ultra-filtration") is a common method at the laboratory scale; however, this method is already in use on a technological scale for the filtration of large drinking water supply systems as well as for the disinfection of swimming pool water.

However, none of the disinfection methods, even if combined with state-of-the-art cleaning methods, is suitable for the fight against biofilms and especially against *P. aeruginosa* based biofilms.

1.5 Project aims

Biofilms caused by *P. aeruginosa* are ubiquitous in water distribution systems, as well as in swimming pools and any other surface being in contact with water over a prolonged period of time. Practical aspects of growths and removal of *P. aeruginosa* based biofilms are in the centre of this dissertation (Fig. 5).

To this end, a robust and reproducible method for the production of *P. aeruginosa* biofilms should be established on a laboratory scale.

P. aeruginosa biofilms should be analyzed and characterized using adequate methods, like confocal laser scanning microscopy (CLSM) for the analysis of their special constitution as well as for the judgement of the efficiency of disinfection measures.

Another task was to look for new analytical methods able to characterize biofilms by the presence and amounts of certain chemical elements and components. The basic idea was to find out, whether a common chemical parameter used in water analysis, total organic carbon (TOC) may be used to describe growth and volume of biofilm formation.

Additionally, behaviour and growths of *P. aeruginosa* biofilms should be observed and analysed in their natural environment. To this end, biofilms from DWST, from swimming pools and from water installations in hospitals should be screened for biofilms and *P. aeruginosa* specific films should be investigated in detail.

In cooperation with other project partners, new and innovative tools for disinfection of *P. aeruginosa* biofilms in water systems should be developed at least on a laboratory scale and new methods for the evaluation of these methods should be established.

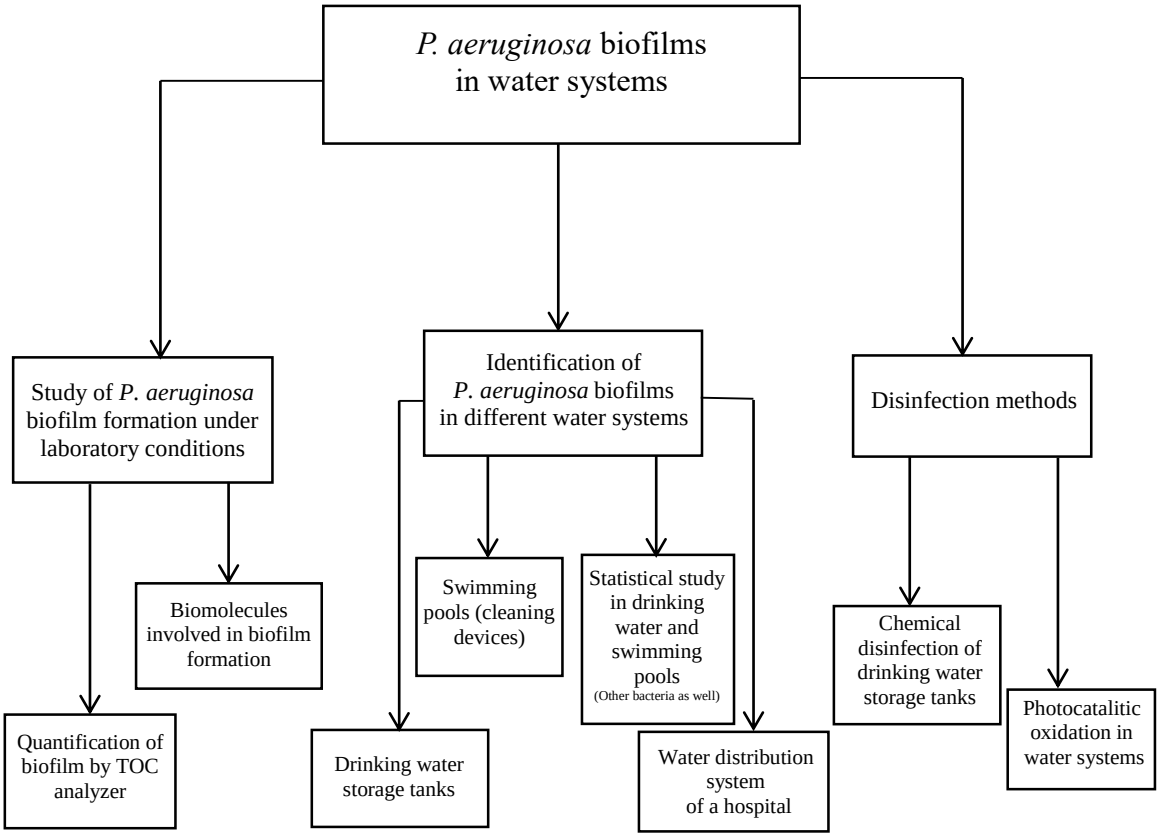


Fig. 5: Outline graphic of the project aim

2. Materials and methods

2.1 *P. aeruginosa* cultivation

For experiments described here, bacterial strains *P. aeruginosa* ATCC 15442, *E. coli* NCTC 10538 and *S. aureus* ATCC 6538 were selected.

Bacterial cultures were prepared according to standard laboratory methods. Shortly, bacteria were inoculated in Erlenmeyer flasks with 30 ml of Tryptic Soy Broth (TSB) (Merck, Germany) and incubated in a shaking water bath at 37°C for 12 h. This incubation time was sufficient to guarantee that bacteria arrived at the beginning of the stationary phase of growth. Then 15 ml of the bacterial suspensions were transferred to centrifuge tubes and 30 ml of sterile water of standardised hardness (WSH) was added. WSH instead of "real" tap water was used for standardization reasons. Composition of WSH: 17,5 ml of the solution (10g CaCl2 *2H2O in 100ml distilled water) and 5 ml of the solution (10g MgSO4*7H2O in 100ml distilled water) are added in 3.300 ml of deionized water. Then 15 min in the autoclave at 121ºC. Then bacterial suspensions were centrifuged for 10 min at 4000 rpm. The supernatant was discarded and the washing/centrifugation step was repeated three times.

2.2 Determination of colony count

Colony count was determined according to standard methods. Pellets containing bacteria were re-suspended in 30 ml WSH and were well agitated by vortexing. WSH was added to an optical density (OD) of 0.5 MacFarland units at OD_{600nm} (U-2000, Hitachi, Japan). Finally, colony count was evaluated by serial dilution using 0.9% sodium chloride solution. Diluted samples were plated onto Tryptic soy agar (TSA) plates (BD, Germany) and incubated overnight at 37°C.

2.3 Identification by MALDI-TOF mass spectrometry

Identification of microbes in samples of any kind is usually done using cultivation methods. However, since a couple of years, substantially different methods are available and already routinely applied in the field of medical microbiology. Matrix Assisted Laser Desorption/Ionisation Time Of Flight mass spectrometry (MALDI-TOF MS), is a rapid bacteria identification method, where bacteria are reproducibly degraded to well defined fragments by energy delivered by laser pulses. Subsequently, ionized fragments are analyzed using an adequate mass spectroscopic method. For the examination of bacteria, uniform colonies grown on a conventional culture medium are necessary.

For the preparation of the analysis, visible amounts of biological material are prepared as smear preparation. For critical samples a short extraction protocol is recommended as cell wall structures or cell wall components can hamper unambiguous identification of smear preparations.

For the extraction, 1-2 bacterial colonies were placed in Eppendorf tubes (1.5 ml) with 300 µl of sterile distilled water and were well mixed to disintegrate the colonies. Then, 700 µl of 70% Ethanol was added to the samples and vortexed. Samples were centrifuged for 3 min at 13000 rpm (Biofuge pico, Heraeus, Germany) and the supernatant was drained off. The last step was repeated, leaving Eppendorf tube lids open for 10-15 min to allow evaporation of any residual ethanol. Depending on the size of the cell pellets between 25 µl and 50 µl of 70% formic acid (FA) was added to the samples and the samples were stirred. Subsequently, samples were allowed to stand for 10 min, after that 25 µl - 50 µl of pure acetonitrile (ACN) was added into the samples and stirred. Finally, samples were centrifuged for 3 min at 13000 rpm.

Smear preparations were spotted on a MSP 96 polished steel target (Bruker Daltonics, Germany) and 1 µl of the almost clear supernatants were placed on it. Samples were allowed to dry at room temperature. A solution of α-cyano-4-hydroxycinnamic acid (HCCA) matrix (20 mg/ml) was prepared in 500 µl 50% acetonitril (ACN) and 2.5% trifluoroacetic acid (TFA). Then, the matrix was vortexed and allowed to stand for 15 min. Samples were overlaid with 1 µl matrix solution and allowed to dry at room temperature. Mass spectrometry was performed on Microflex LT spectrometer (Bruker Daltonics GmbH, Germany) and analysed by BioTyper version 3.0.

2.4 *P. aeruginosa* – Pulsed-field gel electrophoresis (PFGE)

Samples were processed on Cetrimide agar (CA) plates and incubated for 48h at 37°C as described before. Probable *P. aeruginosa* colonies were plated onto CA plates (BD, Germany) and incubated for 24h at 42°C. Finally, the bacterial identity was confirmed by MALDI-TOF MS.

Cell suspensions were washed with 300 µl of cell suspension buffer [100 mM Tris pH 7.5, 100 mM ethylenediaminetetraacetate (EDTA), 150 mM NaCl] and they were mixed well by vortexing. According to standard methods published elsewhere, Agarose (2% agarose in distilled water) was melted and 340 µl were added into new Eppendorfs with 20 µl of the samples. The mix was inserted in disposable plug molds and was stored for 15 min at 4°C, until the samples were solid. Plugs were removed and each set of 5 was placed into a 24-well cell culture plate (Greiner Bio-One, Germany), containing 1ml lysis buffer per well [6

mM Tris, 1 M NaCl, 100mM EDTA, 0.5% Brij 58, 0.2% deoxycholate, 0.5% sarcosyl] and were incubated at 37°C with agitation for 5 h. The plugs were then washed briefly with 1 ml wash buffer [100mM Tris pH 7.5, 100mM EDTA pH 8] and incubated overnight in a shaking water bath (Köttermann 3047, Gemini BV, Netherlands) at 50°C with 1 ml of proteinase K buffer [1% Sarcosyl, 0.5 M EDTA pH 8, proteinase K (20mg/ml, Merck, Germany)]. The plugs were then washed with wash buffer five times per hour during three hours and stored at 4°C for later enzymatic treatment.

Plugs were cut to size (5 × 1.5 × 2.5 mm) and digested in 50 µl of restriction buffer [100 mM Tris pH 8, 5 mM $MgCl_2$] with 30 U *Spe*I (Fermentas, Thermo Scientific, Germany) overnight at 24°C. The plugs were loaded into a 1% pulsed-field certified agarose gel (Bio-Rad, Germany) with the samples flanked by the lambda phage cI857S7 (Lonza Bioscience, Rockland, USA). Gel was electrophoresed in 0.5 × TBE buffer at 6 V/cm for 24 hours at 13°C with a pulse duration of 5 to 90 seconds ramped linearly in a CHEF-DR system (Bio-Rad, Germany).

Gel was stained with ethidium bromide, bleached in distilled water, and photographed with a Gel Doc 2000 (Bio-Rad, Germany). Data were obtained from the digital image using the Quantity One software (version 4.4.0, Bio-Rad).

2.5 Universal bacterial Polymerase Chain Reaction (PCR): 16S rDNA PCR plus sequencing

Studies of universal genes, especially the small-subunit rRNA may provide a phylogenetic picture of the communities. DNA sequencing of bacterial strains was done after PCR amplification. All samples mentioned here were sequenced by a commercial company. Sample preparation, however, was done in our laboratory.

From CA plates 1-2 colonies of the samples were transferred to 1.5 ml microcentrifuge tubes with sterile glass beads (0.1 mm; BioSpec, Germany) and DNA extraction buffer (3 mL Tris EDTA buffer concentrate, 97 mL distilled water). Samples were vortexed and incubated in a heating block at 95°C for 10 min. Samples were shaken using an agitator for 10 min and were centrifuged at 13000 rpm for 5 min. Supernatant was used for the 16S PCR amplification step.

Bacterial universal PCR was performed on all samples with primers 27f (5´-AGA GTT TGA TCM TGG CTC AG-3´) and 907r (5´-CCG TCA ATT CMT TTR AGT TT-3´), producing an amplicon of approximately 900 base pairs.

The PCR reaction was made using up to 50 µL with nuclease-free water and 1 µL of primer 27f (50 pmol), 1 µL of primer 907r (50 pmol), 1.25 mmol of deoxyribonucleotide

triphosphate (dNTP) (Roche Diagnostics GmbH, Germany), 25mM of $MgCl_2$, 5 µL of 10x Taq buffer and 2.5 U/µL of Taq DNA polymerase (Roche Diagnostics GmbH, Germany). We used 10 µl of purified DNA.

16S PCR was performed in a PCR System PTC 150 thermocycler (Bio-Rad, Germany). The cycling conditions were as follows: 94°C for 5 min, followed by 31 cycles at 94°C for 45 seconds, 53°C for 1 min, and 72°C for 1.5 min, with a final elongation at 72°C for 10 min. In each run, a negative control with nuclease-free water was included every 5 test samples and a positive DNA control was prepared with 10^4 CFU/mL of *Escherichia coli (E. coli)* strain and extracted in the same way as the samples. 16S PCR products were analysed by electrophoresis through a 2% agarose gel, which was run at 130 volts for 30-45 min in 1x Tris-acetate-EDTA buffer. PCR results were considered valid if all controls were positive or negative. Amplicons were detected by ethidium bromide staining and UV transillumination.

16S PCR products were purified using a commercial system (Qiagen PCR purification kit, Germany) and samples were shipped for sequencing (GATC Biotech, Germany). Sequencing was performed using primers 357f (5´-ACT CCT ACG GGA GGC AGC AG-3´) and 519r (5´-GWA TTA CCG CGG CKG CTG-3´). The sequences obtained were compared using BIBI software (Bioinformatic Bacterial Identification).

2.6 Antibiotic susceptibility tests with the VITEK 2

Suspensions of *P. aeruginosa* strains were made in 0.9% sodium chloride, adjusted to 0.5 McFarland units, and loaded on test cards for the VITEK 2 system (BioMérieux SA, France). During the automatic analysis, a turbidity signal is automatically measured every 15 min for up to 18 h for every single antibiotic-containing test well. These data are used to generate growth curves and, by comparison with a control; the MIC (Minimum inhibitory concentration) of each antibiotic is estimated. Calculation of the result is done with an algorithm specific for each antibiotic but independent of the species (Livermore *et al.*, 2002).

P. aeruginosa ATCC 27853 was used as control strain for the susceptibility test. The susceptibilities of the isolates were tested for the following antimicrobial agents:

- Amoxicilline/clavulanate
- Piperacilline/tazobactam
- Ceftriaxone
- Ceftazidim

- Imipenem
- Meroponem
- Ciprofloxacin
- Moxifloxacin.

MICs were interpreted as being in susceptible, intermediate or resistant categories according to European Committee on Antimicrobial Susceptibility Testing (EUCAST) breakpoints. According to the EUCAST breakpoint table version 2.0, MIC breakpoints are:

susceptible S ≤ 1 mg/L

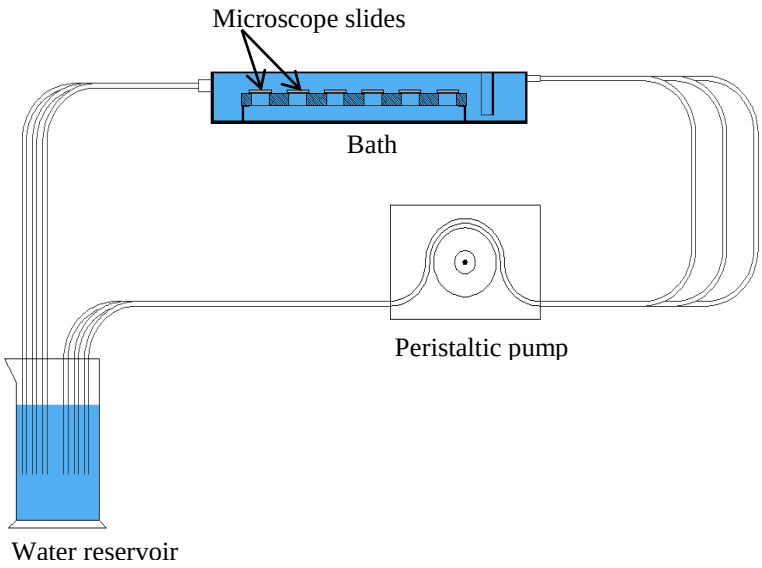
intermediate 2-8

resistant R > 8

2.7 Lab method growth of *P. aeruginosa* biofilms

OD_{600} of the inoculums prepared as described above was adjusted to 0.1 MacFarland units (1–1.6 × 10^8 CFU/ml). Biofilms were grown using this bacterial suspension on microscope slides (app. 75 × 25 mm, thickness app. 1mm, Marienfeld, Germany). Biofilm growth experiments were performed using a stainless steel container designed and made for the purposes of this work (Fig. 6). The entire volume of the stainless steel container, where the microscope slides were deposited was about 2,6 l. The water level was approximately 3 mm and the distance from microscope slides to the ground was approximately 14 mm. The reservoir (test tube of 5 l) contained about 1.4 l. Water entered throw three tubes and left the container through six tubes through a peristaltic pump (Model 131900, Desaga, Germany), being the water in continuous circulation, like in drinking water storage tanks. The water flow was 4.5 to 6 l/h. The initial inoculum was prepared as described before.

a)



b)

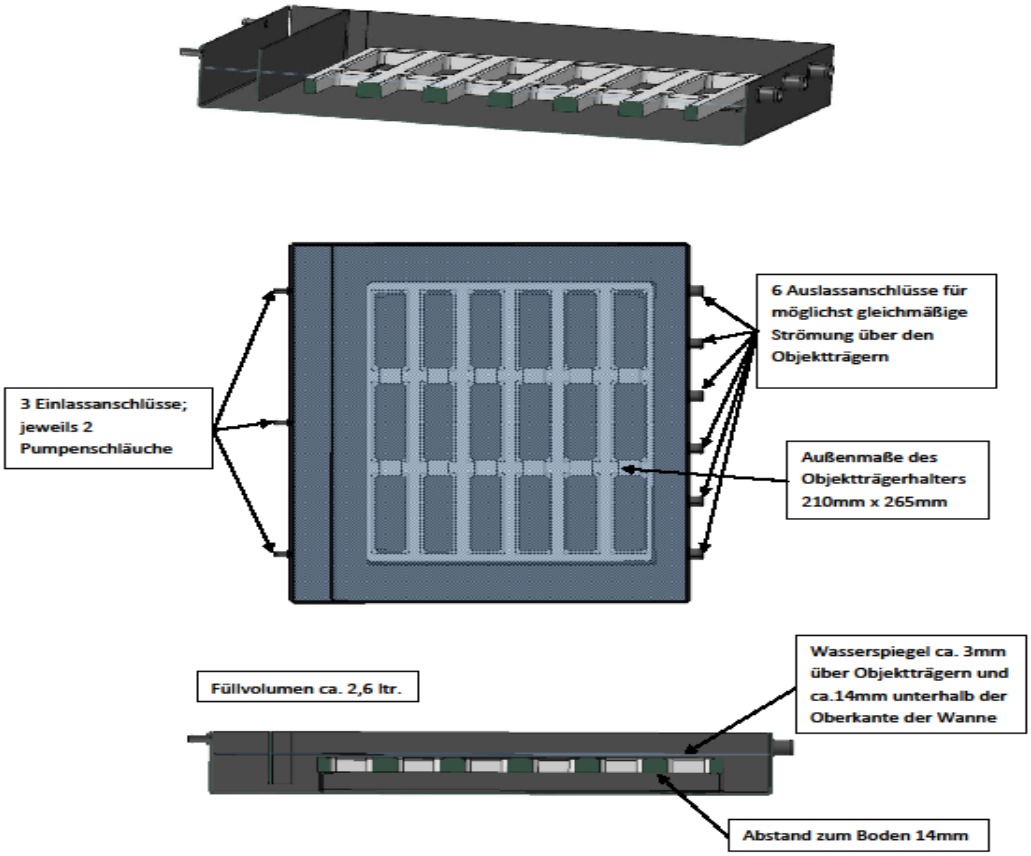


Fig. 6: a) Scheme of experimental setup for biofilm formation experiment. b) Construction drawing of the bath of the experimental setup for biofilm

2.8 Microtiter plate biofilm assay

Biofilm formation capacity was tested using a simple microtiter assay. The assay procedure followed a slightly modified version of the protocol described by Christensen (Christensen *et al.*, 1985).

Samples were incubated at 37°C for 24 h to obtain cultures. Bacterial suspensions were prepared in 3 ml of 0.9% sodium chloride using a Densimat photometer (BioMérieux, France). The bacterial suspension was diluted to 10^5 CFU/well. Microtiter plates (96 well; Greiner, Germany) were incubated at 37°C for 24 h. Samples were prepared in

quadruplicate to allow statistical evaluation of the assay precision. As positive control *P. aeruginosa* ATCC 15442 was used and TSA without bacteria as negative control.

After incubation, the medium was removed carefully and the rest of planktonic bacteria were removed by washing three times with 200 µL sterile phosphate buffered saline (PBS). Then the attached biofilm was immediately fixed with 200 µL of 100% ethanol and the microtiter plate was allowed to dry for 10 min with the lid off in a laminar flow sterile bench. Biofilms were subsequently stained with 200 µL 0.25% crystal violet in each well for 2 min and washed three times with PBS. Thereafter the plate was allowed to dry overnight at room temperature.

2.8.1 Microtiter plate biofilm assay: Quantitative biofilm analysis

For quantitative evaluation of the biofilm, 100 µL ethanol (100%) were added to each well and allowed to interact for 10 min to elute the colour adsorbed by the bacterial membrane. Subsequently, samples were transferred to a new plate and OD_{570nm} was measured using an Enzyme-linked immunosorbent assay (ELISA) plate reader (Tecan Sunrise, Switzerland). Biofilm formation was categorized according to Table 1. The criteria used to choose the OD_{570nm} ranges for the biofilm formation were taken from previous works carried out by other researchers (Hou *et al.*, 2012).

Table 1: Classification biofilm formation

Biofilm formation	**OD_{570nm}**	
Strong biofilm productive	Over 0.24	
Weak biofilm productive	0.12	0.24
Not biofilm productive	Below 0.12	

2.9 Biofilm formation characteristics of different materials

Basically, using the same test system as described above, material samples of virtually any kind could be analysed for their biofilm forming characteristics. The method was individually adapted to the materials under research. Usually, 40 × 40 mm sample plates were used to test the growth characteristics of *P. aeruginosa* biofilms. Pre-treatment of surfaces was done as necessary. For example, because of the high pH of cement plates, samples were treated and neutralized using 0.2 M phosphate buffer (pH 7.2). All plates were autoclaved prior to use.

For the analysis, samples were placed in 6-well cell culture plates (Sarstedt, Germany). Each well was filled with 9 ml of 1:10 diluted TSB in WSH and with 1.6 µl of bacterial suspension (inoculum: 10^8 bacteria/mL). Samples were incubated for 9 days in duplicate at room temperature (22±1°C) and at one temperature level similar to drinking water storage tanks (11±1°C). Samples were subjected to slight orbital shaking of 70 rpm during incubation (Pos-300, Grant-bio, UK).

2.10 Staining-based method for cell quantification using CLSM

Biofilm samples were tested at different stages for growth using the Live/Dead Baclight L-7007 assay (Invitrogen, Germany). To this end, 1 ml of PBS was mixed with 1.5 µl of SYTO9 and 1.5 µl of propidium iodide. Subsequently, this mixture was applied to the biofilm surface and incubated in darkness for 3 min at room temperature. After the incubation period, the biofilm surface was rinsed with H_2O_{dest}. Finally, the stained biofilm was observed at randomly chosen locations by CLSM (TCS SP5, Leica Microsystems, Germany). The LIVE/DEAD BacLight Bacterial Viability Kits utilize mixtures of SYTO9 green-fluorescent nucleic acid stain and the red-fluorescent nucleic acid stain, propidium iodide. These stains differ both in their spectral characteristics and in their ability to penetrate healthy bacterial cells. When used alone, the SYTO 9 stain generally labels all bacteria in a population, those with intact membranes and those with damaged membranes. In contrast, propidium iodide penetrates only bacteria with damaged membranes, causing a reduction in the SYTO 9 stain fluorescence when both dyes are present. Thus, with an appropriate mixture of the SYTO 9 and propidium iodide stains, bacteria with intact cell membranes stain fluorescent green, whereas bacteria with damaged membranes stain fluorescent red. The excitation/emission maxima for these dyes are about 480/500 nm for SYTO 9 stain and 490/635 nm for propidium iodide (from Manual & Protocols LIVE/DEAD BacLight Bacterial Viability Kits).

Live bacteria with intact membranes show green fluorescence and dead bacteria with compromised membranes appear with red fluorescence.

2.11 Analysis of biomolecules involved in biofilm formation

This experiment is based on the differentiation between polysaccharide- and protein-mediated biofilms by sodium metaperiodate ($NaIO_4$) and proteinase K treatment, respectively.

Proteinase K is used for the destruction of proteins in cell lysates (tissue, cell culture cells) and for the release of nucleic acids, since it very effectively inactivates DNases and RNases.

Periodates can cleave carbon-carbon bonds on a variety of 1,2-difunctionalised alkanes. The most common example of this is diol cleavage, which was also the first to be discovered. In addition to diols, periodates can cleave 1,2-hydroxy ketones, 1,2-diketones, α-keto acids, α-hydroxy acids, amino acids, 1,2-amino alcohols, 1,2-diamines, and epoxides to give aldehydes, ketones and carboxylic acids (Clamp and Hough, 1965).

A triple approach was performed in three separate 96-well microtiter plates (one 96-well microtiter plate was used for the proteinase K, the other one for $NaIO_4$ and the last one as a control). The protocol for performing this study was provided by Prof. Wilma Ziebuhr (Institut für Molekulare Infektionsbiologie, Würzburg, Germany), and has been described in detail elsewhere (Mack *et al.*, 1996; Wang *et al.*, 2004).

Biofilm formation in microtiter plates for the experiments described here was described in detail in the section 2.8.

Samples from each well were prepared in quadruplicate. The negative control sample was TSA without bacteria under addition of Penicillin-Streptomycin (1:1000).

For sodium metaperiodate treatment, the medium was removed carefully after incubation and remaining planktonic bacteria were removed by washing three times with 200 µL PBS. Subsequently, each well was filled with 200 µl of 40mM $NaIO_4$ and incubated for another 24 h at 4°C. Then the sodium metaperiodate solution was discarded.

For Proteinase K-treatment, the plate was treated as described before. However, now each well was filled with 200 µl of proteinase K (AppliChem, Germany) solution (1mg/ml in 100mM Tris-HCl pH 7.5) and incubated for 4 h at 37°C. Afterwards proteinase K solution was discarded.

For control samples, after incubation, the medium of bacteria was simply removed carefully. Biofilms were subsequently stained with 200 µL 0.25% crystal violet in each well for 2 min and washed three times with PBS. Thereafter the plate was allowed to dry overnight at room temperature.

100 µL 100% ethanol were added in each well for 10 min to elute the colour and samples were transferred into a new plate. Finally, the OD_{570nm} was measured in an ELISA plate reader (Tecan Sunrise, Germany). Biofilm formation was categorized as in Table 1.

2.12 TOC intro

Biofilms and biofilm formation may be characterized by TOC, a standard surrogate chemical parameter. To this end, correlation between TOC and colony count of bacterial suspensions needs to be established. Experimental conditions of biofilm formation were closely adapted to conditions in real water systems.

2.12.1 Analysis of TOC of planktonic samples

Bacterial samples were diluted in WSH to the same colony count for all bacterial strains. Samples were then further diluted to obtain six standardized concentrations for measurement. Plate count and TOC of the samples was measured simultaneously. For measurement of TOC, the instrument (TOC-V CPH, Shimadzu, Japan) was calibrated using two calibration curves, one for total carbon (TC) (Fig. 7) and the other curve for inorganic carbon (IC) (Fig. 8). Substances for the calibration curves were prepared as follows:

- TOC: 212,5mg $C_8H_5KO_4$ to 100ml Millipore water = 1000mg C/L
- TIC: 350mg $NaHCO_3$ and 441,5mg Na_2CO_3 to 100ml Millipore water =1000mg C/L

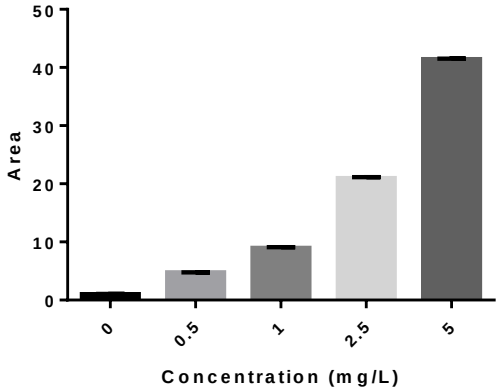


Fig. 7: TC calibration curve

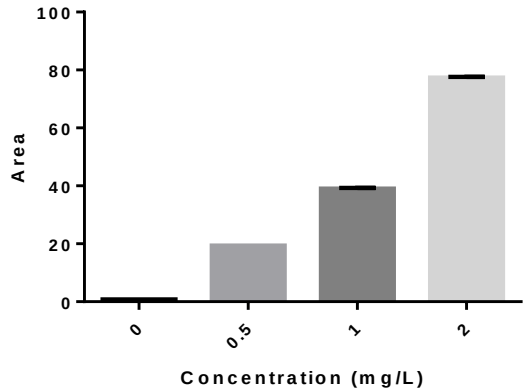


Fig. 8: IC calibration curve

2.12.2 Quantification of TOC concentration in biofilms

For the analysis of TOC build-up in biofilms over time, over 34 days approximately every three to four days single microscope slides were taken out for analysis of bacterial plate count of the biofilm as well as for TOC analysis.

Slides were rinsed using 1.5 ml sterile WSH and biofilms were scraped off using a commercially available sterile cell scraper (Greiner bio-one, Germany). From the bacterial solution, 500 µl were transferred into a test tube and filled up to 10 ml with sterile WSH. Then, samples were sonicated for 10 min in an ultrasonic bath (Sonorex, Bandelin, Germany), ensuring detachment of bacteria adhered to the glass surface. After this step, samples were analysed for TOC and viable bacteria were quantified using the serial dilution method.

2.13 *P. aeruginosa* in water systems

Water samples were taken in sterile plastic 250-mL bottles dosed with sodium thiosulfate (Stein, Germany). At the same time biofilm samples were collected from the internal surfaces by scraping using sterile cotton swabs with transport media (Deltalab, Spain). Sterile cotton swabs with transport media were inoculated onto CA.

Water analysis: 100 mL of each water sample were filtered through a 0.45-µm pore size glucose nitrate filter (Sartorius, Germany) and incubated on CA plates for 24 h at 37°C.

Fluorescent colonies with blue-green or yellow-green halos were counted as *P. aeruginosa* isolates. Furthermore, when the bacteria on CA plates were white and UV fluorescent an acetamide test (Sifin, Germany) was carried out to confirm presence of *P. aeruginosa*. For this test, 2-3 colonies were inoculated into the acetamide solution and incubated for 48 h at 37°C. 1-2 drops of Nessler´s Reagent were added. Appearance of a colour between yellow and brick-red proves presence of *P. aeruginosa*.

2.14 Biofilm sampling in DWST

Samples were taken while the tanks were emptied for cleaning purposes and before the respective cleaning took place (Fig. 9).

Biofilm samples were collected in different parts of the DWST using sterile cotton swabs. The swabs were immediately placed into 10 ml sterile TSB tubes. Samples were transported to the laboratory and were incubated for 48 h at 37°C.

Fig. 9: Empty DWST

The samples were inoculated onto CA plates and incubated for 24 h at 37°C. Bacteria were isolated from the plates and inoculated again onto CA plates to ensure the purity of the samples.

2.15 Clogging analysis

2.15.1 Column set-up and operation

The configuration and operation of the column were selected to mimic the ASR in Sant Joan Despí as closely as possible.

The column consisted of a transparent metacrylate cylinder (length of 85 cm and inner diameter of 39.2 cm) packed with 20 cm height of Llobregat aquifer sediments (approx. 35 kg) placed on a 20 cm layer of spherical glass beads (average diameter of 7 mm) to hold the aquifer material (90 % between 1 - 10 mm) in place. The size of glass beads was small enough (7 mm) to prevent losses of soil particles. The cylinder material of the column was transparent methacrylate in order to facilitate visual observations of events occurring within it.

Flow of injected water through a well screen in the ASR system in Sant Joan Despí was simulated by horizontally placing a metallic screen on the top of the aquifer material bed. This metallic screen was made of 16 perforated, rectangular, ST52 steel pieces that had exactly the same dimensions (4 cm x 4cm x 0,6 cm) that those used in the ASR wells. The 16 pieces were arranged in a 4x4 array (covering an area of the column section of 16 cm x 16 cm) (Fig. 10). The design of the metallic screen allowed to withdraw each piece

separately for sampling and analysis purposes during the experiment. An additional 1cm2 steel piece was put beside as a sample for SEM analysis.

SFSW was pumped from the sand filter unit of the Sant Joan Despí plant to the top of the column, from which it flowed by gravity in a downflow mode.

The filter operated at room temperature (20±5 ºC) comparable to Llobregat aquifer temperature. The pilot was operated manually during 201 days with a continuous injection during working days (Monday to Friday) and being stopped during the weekends. The injection was also stopped during the experiment for other maintenance issues like not having SFSW available or system pumps retrofit. Therefore, the calculated continuous injection time was 80 days.

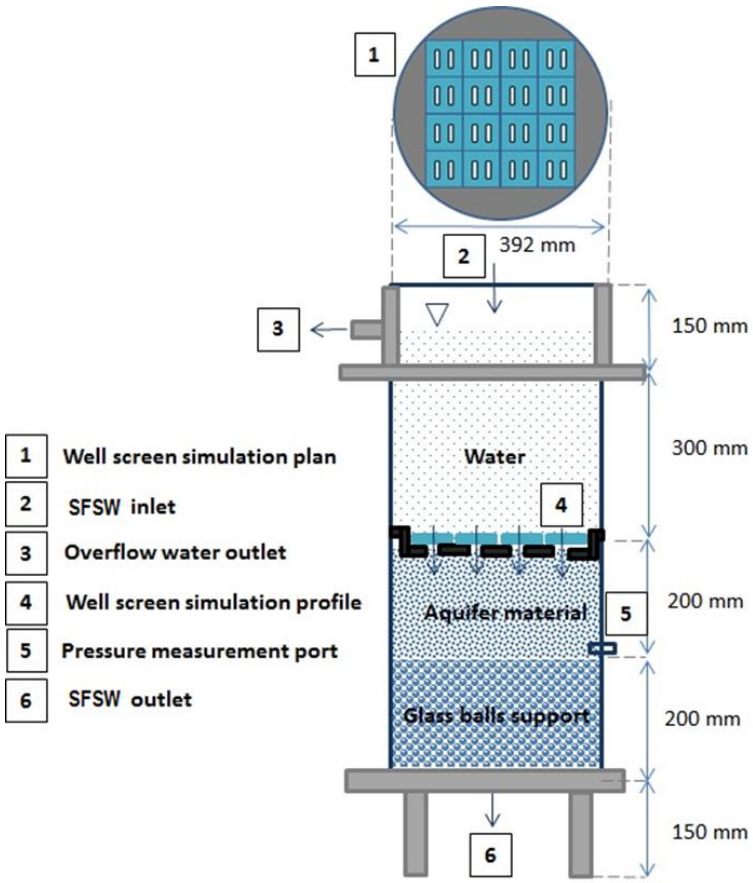


Fig. 10: Pilot column scheme simulating ASR

2.15.2 Biofilm growth evolution determination

During the experiment, pieces of the metallic screen were periodically taken out for EPS analysis (on days 12, 56, 106, 139, 154, 175, 182 and 201).

EPS present in a 1 cm2 sample was removed using a phosphate tampon solution, stored in an Eppendorf safe–lock tube and analysed following the method described in (Romani *et al.*, 2008). The analysis results of the EPS content in the biofilm of the different samples are given in micrograms of glucose equivalents per sampled area (1 cm2). In this case, duplicates (taken from either a different 1 cm2 of the same piece or from another piece) were analysed to gain in representativity (giving rise to a total of 16 samples). On the completion of the analysis, the piece analysed was placed back into the column.

Photographs were taken regularly during the experiments in order to capture images of the metallic screen pieces and document changes, if any, in their appearance. Prior to take photograph, water in the column was removed. Once emptied, photographs were taken from above using a digital camera.

2.15.3 Examination of sediment morphology by SEM-EDX

In order to have a clearer idea of the morphology of the bioclogging layer and to identify biofilm formation in the well screen it was performed scanning electron microscope (SEM) observations. SEM is a very useful technique for the investigation of surface structure of biological samples. Much of the current knowledge about biofilms is due to the advances in imaging studies, especially the SEM (Soumya El Abed, 2012). The analysis of characteristic energy dispersive X-rays (EDX) emitted from the sample gives more quantitative elemental information. Furthermore, SEM/EDX is the best known and most widely-used of the surface analytical techniques. High resolution images of surface topography, with excellent depth of field, are produced using a highly-focused, scanning (primary) electron beam. At the final of the experiment (day 182) the 1 cm2 piece was also analysed by SEM.

Sample preparation was carried out using a standardised methodology (John J. Bozzola, 1999). Samples of biofilm were fixed in 2.5% glutaraldehyde in phosphate buffer at 4°C (0.1M and pH7.4) for 2 hours and washed four times (10 minutes each wash) in the same buffer. Samples were immediately postfixed in a mixture of 1% osmium tetroxide and 0.8% potassium ferricyanide in phosphate buffer at 4°C. After washing samples six times in ultrapure water (first wash quick and another 5 washes every 10 minutes, at 4°C), samples were then dehydrated at 4°C in successively increasing gradient concentrations of ethanol (50% ethanol for 10 minutes, 70% ethanol overnight, 80% ethanol for 10 minutes, three changes of 90% ethanol for 10 minutes each, three changes of 96% ethanol for 10 minutes

each, three changes of 100% ethanol for 10 minutes each) and dried by critical point, where ethanol was replaced by liquid CO2 and changed to gas without changing its density. Microscopic examination was performed using a SEM (JEOL LTD, Tokyo, Japan) equipped with an EDX analyser (Oxford Instruments, Bucks, UK, INCA-250 model) operated at 20 kV. For the visual characterization of the morphology, the solid samples were sputter-coated with carbon to facilitate electrical conduction in the SEM.

3. Results

3.1 Occurrence of *P. aeruginosa* in drinking- and in swimming pool water

According to legal regulations in many countries as well as to World Health Organization (WHO) recommendations, drinking water needs to be free of pathogens. As mentioned before, pathogens may be of faecal origin or because this environment is their usual habitat. Whilst faecal contaminations may be detected by certain indicators, non-faecal contaminations and pathogens need to be analyzed individually.

However, there is only little information about the results of microbiological water examinations and the prevalence of indicators and pathogens in non-chlorinated water as well as in chlorinated swimming pool water. Therefore, this work was initiated by an overview of results of microbiological routine water analysis. All results evaluated here have been analysed in the laboratories of the Department of Infectious Diseases if not stated otherwise. The laboratory maintains a quality assurance system and is accredited according to DIN EN ISO 17025 and participates on a regular basis in interlaboratory comparisons.

Sampling and analysis of water specimen is done according to standardized method. However, it should be mentioned that analysis for *E. coli* and Coliforms is done using a commercial test kit (IDEXX Colilert).

3.2 Analysis of routine monitoring data

A statistical study of all water samples analysed in our laboratory was carried out from January 2010 to December 2012. As indicated in Table 2, a total of approximately 12125 water samples were analysed from drinking water samples (different hospitals, private springs, storage tanks, water supplies, private houses…) and from swimming pools samples (outdoor and indoor swimming pools) from Heidelberg and its surroundings. The microbiological analyzes carried out on the water samples were *P. aeruginosa, L. pneumophila, E. coli, Coliforms,* HPC at 22ºC and at 36ºC.

Table 2: Number of water samples analysed during the statistical study

	Nº samples in 2010	Nº samples in 2011	Nº samples in 2012
Drinking water	2841	1839	2512
Swimming pools	1580	2229	1124
Total	4421	4068	3636

3.2.1 Bacteriological analysis

P. aeruginosa

100 mL of each sample was filtered through a 0.45 μm cellulose membrane filter, placed on Pseudomonas CA, and plates were incubated at 36°C for 48 hours.

Blue/green colonies, pyocyanin-producing bacteria were considered as confirmed *P. aeruginosa* colonies. The membrane filter was then examined under UV light. All non-pyocyanin-producing colonies that fluoresce, counted as suspicious *P. aeruginosa* and were confirmed in Acetamide Broth. At the same time, suspicious colonies were inoculated onto blood plates and incubated 20-24 hours at 42± 1°C.

All other red/brown pigmented colonies that do not fluoresce were counted as suspicious *P. aeruginosa* and confirmed by oxidase test, Acetamide Broth and King's B medium.

L. pneumophila

First, 2 x 0.5 ml of the original water sample was plated on Glycine Vancomycin Polymixin Cyclohexamide (GVPC) agar (Oxoid, Wesel, Germany). The inoculated plates were incubated at 37°C for 7 to 10 days. Greyish-white, greenish-white, or lilac-white, shiny colonies were suspected to be *Legionellae* and were subcultured on buffered charcoal-yeast extract (BCYE) agar with cysteine and sheep blood agar for verification at 37°C for 2 days. If the isolate could grow with a typical colony morphology on BCYE agar but not on sheep blood agar and stained as a gram-negative rod it was regarded as *Legionella*.

E. coli/Coliforms

Detection of coliforms and *E. coli* by using the Colilert-18 (Quanti-Tray/2000) method was done according to the manufacturer's instructions. Yellow wells indicated *Coliform* bacteria, and wells that were yellow and fluorescent when exposed to UV light indicated *E. coli*.

HPC at 22ºC and 36ºC

1 ml of the water sample was pipetted into each of the petri dishes and added 18-20 ml DEV nutrient agar. After 40-48h incubation at 22°C and 36°C, the individual colonies were counted by placing the agar plate on a counting device with a magnifying glass (6-8-fold magnification).

3.2.2 Statistical analysis

In the following tables are represented the percentage of the number of times the different microorganisms were detected in the analyses performed.

Analysis of variance for one factor (one-way ANOVA) and two factors (two-way ANOVA) were used to test:

- If there is a significant seasonal difference in the occurrence of bacteria
- If there is a significant difference between the annual monitoring campaigns
- If the water matrix has significant effect on the occurrence of bacteria species:
  - Drinking water versus swimming pool water
  - Outdoor versus indoor swimming pool water

P. aeruginosa

Significant seasonal difference has been observed for *P. aeruginosa* in drinking waters, as the higher bacterial counts were observed in summer and autumn seasons, while for difference in swimming pool this factor was not found to play significant role. Closer look on data for *P. aeruginosa* in Table 3 for indoor swimming pools, reveals that from May to September the bacterial counts were significantly higher than from October till April, pointing out that the temperature plays significant role also for the growth of *P. aeruginosa*, but the temperatures in May already affected *P. aeruginosa* proliferation.

It was observed a significant difference in the annual monitoring campaigns, as 2011 being the year when the most *P. aeruginosa* bacterial counts (give numbers on average or max) were observed either in swimming pools or in drinking waters. Possible explanation of this fact should be searched in the average temperature (2011 was hotter on average compared to 2010 and 2012) or in changes in the operational procedures.

One of the hypotheses tested in this study was to evaluate if the occurrence of bacteria species depends on the water matrix. With other words does the *P. aeruginosa* occur more frequently in drinking water or in swimming pools? To do so, we compared the bacteria counts of *P. aeruginosa* in drinking water versus the bacteria counts in swimming pool water on annual basis. The results from ANOVA revealed that it was not observed a significant difference at 5% when comparing these two data matrices over the investigated periods. *P. aeruginosa* has not had a preference to one of the matrices, proliferating with equal possibility in both, when other factors such as temperature, chlorine or other factors related to the disinfection probably favoured the growing. Moreover, it was not observed

also a significant difference between outdoor and indoor swimming pool waters matrices (for 6 months when data coincided in time).

L. pneumophila

It was not observed in Table 4 significant differences for the three years of monitoring, neither that the seasonality played a significant role for Legionella occurrence in swimming pools water or in drinking waters. The significant difference in the *Legionella* occurrence was observed when *L pneumophila* counts from drinking water matrix were compared to counts from swimming pool matrix. It was observed more percentage detectable *Legionella* in drinking water than in swimming pool water at ($P<0.05$). These results reveal that the temperature or seasonality did not play a significant role, but the matrix and other factors such as disinfection managements, closed systems and etc. play much more significant role in the *L. pneumophila* occurrence.

E. coli

As we can see in Table 5, it was not observed significant statistical difference for all tested hypothesis. These results underline that the appearance of *E. coli* depends more on other factors than on the temperature, monitoring campaign or water matrix.

Coliforms

As observed in Table 6, for drinking water matrix, it was not observed significant difference for the three years of investigation or to exist a strong seasonal dependence ($P>0.05$).For swimming pools water matrix it was observed a significant difference in the Coliforms counts. The summer time appeared to account for more percentage detectable. When compared the monitoring campaigns from 2010 -2012, the results from ANOVA did not show a significant difference. Also, it was not observed significant difference in the bacterial counts when compared the swimming pool matrices outdoor vs indoor swimming pools at 5%.

More Coliforms counts were observed in drinking water matrix than in swimming pool waters during 3 years with significant difference at 5%.

HPC at 22ºC

As we can see in Table 7 for drinking water there were observed significant difference for the three years of investigation ($P<0.05$) and also showing a strong seasonal dependence. Again for 2011 it was observed more percentage detectable bacteria during summer time

(up to 17% detectable) while for 2010 and 2012 the maximum percentage detectable for summer time accounted to 5% and 6% respectively than usual ($P<0.05$). These facts were contributing to the importance of the temperature as factor.

There were observed also a significant difference for the seasonality as factor ($P<0.05$) with summer time favoring a strong growing of bacteria colonies in swimming pools. However, it was not observed significant difference when the monitoring campaigns for 2010-2012 were examined.

When bacteria counts from outdoor swimming pool water matrix was compared to indoor swimming pool water matrix for the three years, it was not observed a significant difference at 5%.

When comparing bacteria counts from drinking water matrix to bacterial counts from swimming pools water matrix for the 3 years, it was observed more colonies in drinking water than in swimming pool water with significant difference at 5%.

HPC at 36ºC

As observed in Table 8 for drinking water a significant difference for the three years of investigation ($P>0.05$) was not observed, while data showed the strong effect of seasonality ($P<0.05$) on the bacterial occurrence. More counts were being observed for summer and autumn seasons than for spring and winter, pointing out the strong temperature effect on bacteria growth ($P<0.05$).

It was not observed significant differences in the bacteria counts for the three years of monitoring campaigns in swimming pools neither it was observed a statistical significance for the bacterial counts during different seasons or in outdoor or indoor swimming pool water matrices.

For drinking water vs swimming pools during 3 years, it was observed more colonies in drinking water than in swimming pool water, significant difference at 5%. No difference during these 3 years ($P>0.05$).

For drinking water there were observed significant difference for the three years of investigation ($P<0.05$) and also showing a strong seasonal dependence. Again for 2011 it was observed more percentage detectable bacteria during summer time (up to 17% detectable) while for 2010 and 2012 the maximum percentage detectable for summer time accounted to 5% and 6% respectively than usual ($P<0.05$). These facts were contributing to the importance of the temperature as factor.

There were observed also a significant difference for the seasonality as factor ($P<0.05$) with summer time favouring a strong growing of bacteria colonies in swimming pools. However it was not observed significant difference when the monitoring campaigns for 2010-2012 were examined.

When bacteria counts from the outdoor swimming pool water matrix was compared to the indoor swimming pool water matrix during these three years, it was not observed a significant difference at 5% showing no preference for growth in one or another of the swimming pools water matrices.

When compared bacteria counts from drinking water matrix to bacterial counts from swimming pools water matrix for the 3 years of monitoring, it was observed more colonies in drinking water samples than in swimming pool water samples underlying the importance of water matrix for colonies occurrence.

Table 3: Detectable *P. aeruginosa* from 2010 to 2012

Detectable *P. aeruginosa* (%)												
	Year 2010				Year 2011				Year 2012			
	Drinking water	Swimming pool	Outdoor swimming pools	Indoor swimming pools	Drinking water	Swimming pool	Outdoor swimming pools	Indoor swimming pools	Drinking water	Swimming pool	Outdoor swimming pools	Indoor swimming pools
January	1,7	1.3	-	-	4,3	1.2	-	-	2,8	9.5	-	-
February	0,8	0.0	-	-	3,8	1.2	-	-	5,7	5.1	-	-
March	2,1	1.2	-	-	0,0	6.5	-	-	3,8	5.6	-	-
April	5,0	0.0	-	-	3,8	2.9	-	-	2,7	2.4	-	-
May	1,5	1.1	0,0	1,2	4,5	9.1	12,2	8,9	5,6	0.0	0,0	0,0
June	4,7	2.1	0,0	3,3	26,4	6.4	4,6	10,5	0,0	5.1	16,2	0,0
July	6,6	1.6	7,2	0,0	9,0	29.1	38,2	32,3	9,5	9.6	14,7	0,0
August	8,6	7.3	7,8	8,2	20,8	10.1	5,1	7,5	10,8	7.4	8,5	3,7
September	7,5	5.5	7,1	4,2	18,7	4.9	0,0	4,8	15,5	4.1	11,1	3,4
October	5,0	1.0	-	-	11,7	16.9	-	-	0,5	1.0	-	-
November	0,0	2.5	-	-	2,2	5.5	-	-	0,0	1.2	-	-
December	0,8	5.6	-	-	0,0	10.1	-	-	5,3	0.0	-	-

Table 4: Detectable *L.pneumophila* from 2010 to 2012

Detectable *L. pneumophila* (%)												
	Year 2010				Year 2011				Year 2012			
	Drinking water	Swimming pool	Outdoor swimming pools	Indoor swimming pools	Drinking water	Swimming pool	Outdoor swimming pools	Indoor swimming pools	Drinking water	Swimming pool	Outdoor swimming pools	Indoor swimming pools
January	29,4	4.3	-	-	25,0	0.0	-	-	32,7	0.0	-	-
February	18,8	4.9	-	-	25,5	2.6	-	-	18,2	2.9	-	-
March	22,1	6.3	-	-	31,5	7.1	-	-	17,1	2.2	-	-
April	26,8	4.8	-	-	17,0	8.2	-	-	15,3	8.9	-	-
May	25,8	9.3	-	12.7	14,8	4.2	0,0	9.8	10,6	7.4	12,5	13.1
June	18,8	0.0	10,0	0.0	46,2	5.3	0,0	4.3	36,8	0.0	0,0	4.8
July	19,8	3.2	6,7	3.8	9,0	4.8	9,1	8.3	34,9	1.6	0,0	0.0
August	35,4	12.0	0,0	7.7	26,0	7.4	0,0	8.6	16,2	3.2	0,0	4.9
September	40,5	8.3	0,0	22.6	13,4	0.0	0,0	7.7	25,2	4.5	-	13.2
October	28,7	0.0	-	-	27,4	0.0	-	-	26,4	5.4	-	-
November	12,6	2.1	-	-	12,2	1.7	-	-	19,8	7.7	-	-
December	33,9	5.9	-	-	18,8	6.3	-	-	42,2	15.1	-	-

Table 5: Detectable *E.coli* from 2010 to 2012

Detectable *E. colli* (%)												
	Year 2010				Year 2011				Year 2012			
	Drinking water	Swimming pools	Outdoor swimming pools	Indoor swimming pools	Drinking water	Swimming pools	Outdoor swimming pools	Indoor swimming pools	Drinking water	Swimming pools	Outdoor swimming pools	Indoor swimming pools
January	0,9	1.3	-	-	2,7	2.5	-	-	2,1	2.7	-	-
February	0,0	0.0	-	-	0,5	0.0	-	-	0,6	0.0	-	-
March	0,0	1.2	-	-	0,0	0.0	-	-	0,0	2.7	-	-
April	0,4	0.0	-	-	0,5	0.0	-	-	0,7	0.0	-	-
May	1,2	0.0	0,0	0,0	0,4	0.0	0,0	0,0	0,4	0.8	0,0	0,0
June	0,7	0.0	0,0	0,0	1,0	0.9	1,5	0,0	0,5	0.8	0,0	0,0
July	0,0	0.8	0,0	0,0	0,7	0.0	0,0	0,0	2,2	2.6	3,2	0,0
August	0,4	0.0	0,0	0,0	0,9	1.2	0,0	0,0	0,3	2.0	1,2	0,0
September	1,5	1.1	0,0	2,1	0,4	1.2	0,0	2,4	1,5	2.1	0,0	1,7
October	1,0	0.0	-	-	0,0	1.3	-	-	0,0	0.0	-	-
November	0,0	0.0	-	-	0,0	0.0	-	-	0,0	1.2	-	-
December	0,5	0.0	-	-	0,0	0.0	-	-	0,0	0.0	-	-

Table 6: Detectable *Coliforms* from 2010 to 2012

Detectable *Coliforms* (%)												
	Year 2010				Year 2011				Year 2012			
	Drinking water	Swimming pool	Outdoor swimming pools	Indoor swimming pools	Drinking water	Swimming pool	Outdoor swimming pools	Indoor swimming pools	Drinking water	Swimming pool	Outdoor swimming pools	Indoor swimming pools
January	3,5	1.3	-	-	4,0	1.2	-	-	2,1	0.0	-	-
February	1,1	0.0	-	-	2,0	0.0	-	-	5,7	0.0	-	-
March	2,3	2.4	-	-	2,4	0.0	-	-	2,8	1.4	-	-
April	4,0	0.0	-	-	4,9	0.0	-	-	3,0	0.0	-	-
May	3,6	0.0	0,0	0,0	1,5	0.0	0,0	0,0	1,7	0.0	0,0	0,0
June	2,0	1.4	0,0	3,3	4,4	3.6	4,6	2,6	3,2	2.5	5,4	0,0
July	5,6	0.8	0,0	0,0	4,6	0.6	0,0	0,0	5,3	2.6	4,3	0,0
August	3,0	0.9	0,0	2,0	2,6	1.2	2,6	0,0	2,4	2.7	2,4	0,0
September	11,2	1.1	0,0	2,1	5,9	1.2	7,1	0,0	5,7	4.1	10,0	3,4
October	9,4	0.0	-	-	8,3	2.6	-	-	2,5	1.0	-	-
November	2,4	0.0	-	-	1,7	0.0	-	-	1,6	1.2	-	-
December	3,1	1.1	-	-	6,0	0.0	-	-	3,5	1.3	-	-

Table 7: Detectable HPC at 22ºC from 2010 to 2012

Detectable HPC at 22ºC (%)												
	Year 2010				Year 2011				Year 2012			
	Drinking water	Swimming pool	Outdoor swimming pools	Indoor swimming pools	Drinking water	Swimming pool	Outdoor swimming pools	Indoor swimming pools	Drinking water	Swimming pool	Outdoor swimming pools	Indoor swimming pools
January	3,1	0.0	-	-	2,4	1.2	-	-	2,0	0.0	-	-
February	0,4	0.0	-	-	0,0	0.0	-	-	1,5	0.0	-	-
March	1,6	0.0	-	-	1,9	0.0	-	-	4,1	1.4	-	-
April	3,7	0.0	-	-	3,9	0.0	-	-	2,9	0.0	-	-
May	1,3	0.0	0,0	36,0	3,8	0.0	36,6	35,7	1,4	0.8	26,7	22,7
June	1,8	2.8	26,4	23,0	17,0	0.0	29,2	2,6	1,9	2.5	40,5	21,9
July	1,9	0.0	36,8	28,6	11,2	1.9	40,5	26,5	1,7	0.0	11,7	15,8
August	5,1	0.9	56,9	26,5	17,9	1.2	20,5	37,5	6,5	2.0	24,4	22,2
September	2,3	1.1	14,3	20,8	4,5	0.0	0,0	4,9	6,9	2.1	20,0	20,3
October	2,9	0.0	-	-	5,8	0.0	-	-	3,0	0.0	-	-
November	1,4	0.0	-	-	1,6	0.0	-	-	1,3	2.4	-	-
December	1,9	0.0	-	-	1,2	0.0	-	-	1,8	0.0	-	-

Table 8: Detectable HPC at 36ºC from 2010 to 2012

Detectable HPC at 36ºC (%)												
	Year 2010				Year 2011				Year 2012			
	Drinking water	Swimming pool	Outdoor swimming pools	Indoor swimming pools	Drinking water	Swimming pool	Outdoor swimming pools	Indoor swimming pools	Drinking water	Swimming pool	Outdoor swimming pools	Indoor swimming pools
January	4,4	0.0	-	-	6,3	1.3	-	-	5,1	2.7	-	-
February	2,2	0.0	-	-	2,4	0.0	-	-	7,5	0.0	-	-
March	3,7	0.0	-	-	4,7	2.3	-	-	4,9	2.8	-	-
April	5,6	0.0	-	-	3,9	0.0	-	-	4,3	0.0	-	-
May	2,8	0.0	0,0	67,4	5,8	0.8	58,5	46,4	7,5	0.8	40,0	33,3
June	19,2	0.7	62,3	55,7	13,9	1.8	44,6	42,1	2,6	2.5	54,1	48,4
July	10,1	0.8	54,4	45,7	13,3	3.1	57,1	48,5	4,0	1.3	40,4	15,8
August	16,1	2.8	41,2	57,1	17,6	0.0	33,3	47,5	12,1	8.1	52,4	53,7
September	3,8	2.2	64,3	35,4	4,7	1.2	14,3	33,3	4,9	2.1	70,0	40,7
October	7,4	0.0	-	-	8,6	0.0	-	-	5,4	2.0	-	-
November	3,2	0.0	-	-	3,7	0.0	-	-	5,3	2.4	-	-
December	4,2	4.4	-	-	4,0	0.0	-	-	3,7	0.0	-	-

3.3 Characterization of strains isolated from DWST

In many areas DWST are an important part of the water distribution system. Drinking water is usually stored only over a very limited period of time, however, microbial growth may take place in these tanks and may be a source for the occurrence of *P. aeruginosa* in drinking water lines. For the establishment of Water Safety Plans (WSP), microbiological burden and probable influence of storage tanks on drinking water quality is crucial.

DWST contain large amounts of water for a limited amount of time. Although the water is usually relatively cold (11ºC in winter and around 14ºC in summer °C), certain bacteria may grow over the course of time, and may form biofilms on surfaces. However, not much is known about the kind of bacteria and their biofilm formation capacity. Therefore, swab samples as described before were taken from four different DWST and analyzed for bacterial composition using cultivation methods and MALDI-TOF/MS.

Fig. 11 shows a typical mass spectrum of a *P. aeruginosa* isolate. Every peak contains information on specific fragments that form during the laser desorption/ionization process. The total pattern of peaks therefore is indicative for certain bacterial species.

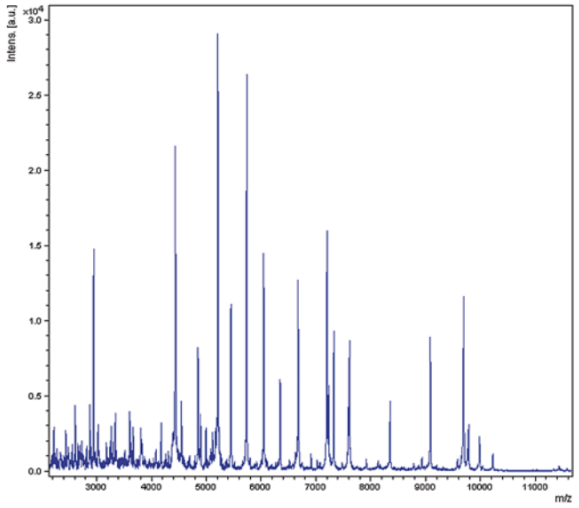


Fig. 11: Typical mass spectrum of a *P. aeruginosa* isolate

However, quality of the mass spectra depends on several factors, like purity of the sample and others. Therefore, evaluation of mass spectra is done by software running certain algorithms comparing individual peaks from reference spectra to those found in the sample under analysis. The quality of this analysis is expressed by the "MALDI score". This score is a simple number calculated by the algorithms mentioned before. As soon as the score is higher than 2.00, identification of the bacterial identity is sufficient and reliable.

According to the manufacturer´s instructions and some publications (Croxatto *et al.*, 2012; Klein *et al.*, 2012), a result was considered valid at the species level whenever the score value attributed by BioTyper was $x \geq 2.0$, moderate identification at the genus level when the score was $x \geq 1.7$, and no reliable identification when the score was $x < 1.7$. It is for that reason that only values higher than 2.0 were represented. DWST not only have benefits, they may also bring diseases to the users if the operation and maintenance of the tanks are not adequate. For these reasons, it is necessary a thorough control of the installations as well as a proper sanitation periodically of all surfaces of the tanks. It must take into account that the average temperature of the tanks in Germany is around 11ºC in winter and around 14ºC in summer. Despite the low temperatures at which water is found in tanks, bacterial growth has been observed in different surfaces of the tanks (walls and floors). The origin of these bacteria can be either from groundwater or from human pollution when operators are working on the drinking water storage tanks.

Results for MALDI examination of 20 strains found in drinking water reservoirs are listed together with their MALDI scores in Table 9.

For each reservoir, identified bacterial strains are listed according to their score. In total, 13 different strains were identified. Whilst eight of the strains were only detectable in one sample each, five other strains were detectable in two reservoirs.

In three of the four reservoirs where the samples were taken, two strains were detectable in other reservoirs as well. In all reservoirs, there was at least one strain which did not show up in other drinking water tanks.

Interestingly, *P. aeruginosa* does not appear in any of the samples analyzed here, although other *Pseudomonas* strains may be identified.

Pseudomonas strains detected here include *P. mendocina*, *P. oleovorans*, *P. koreensis* as well as *P. stutzeri*. This means, that about one third of identified strains belong to the genus of *Pseudomonas*.

A look into the taxonomy shows, that at least *P. mendocina* and *P. oleovorans* are closely related to *P. aeruginosa* and belong to the *P. aeruginosa* group. *P. stutzeri* is a member of the *P. stutzeri* group and finally *P. koreensis* is of “incerta sedis” what means that it is not definitely classified. Details of these findings will be discussed later.

Table 9: Identification of bacterial strains from 4 different drinking water reservoirs

	Identification	MALDI score
Reservoir 1	*Staphylococcus epidermidis*	2,30
	Micrococcus luteus	2,17
	Kocuria rhizophila	2,10
	Bacillus subtilis	2,00
	Brevibacillus parabrevis	2,00
Reservoir 2	*Staphylococcus vitulinus*	2,33
	Staphylococcus epidermidis	2,27
	Pseudomonas mendocina	2,16
	Pseudomonas oleovorans	2,15
	Staphylococcus warneri	2,15
Reservoir 3	*Pseudomonas koreensis*	2,43
	Staphylococcus aureus	2,43
	Micrococcus luteus	2,36
	Aeromonas bestiarum	2,11
	Staphylococcus warneri	2,10
Reservoir 4	*Pseudomonas oleovorans*	2,43
	Pseudomonas mendocina	2,36
	Pseudomonas stutzeri	2,10
	Acinetobacter lwoffii	2,11

All species and strains of *Pseudomonas* are Gram-negative rods and have historically been classified as strict aerobes. *Pseudomonas* specie may be able to form biofilms and strictly adhere to certain surfaces. However, in DWST the situation for biofilm formation is non-optimal at least because of the low temperature. However, information about biofilm formation capacity of strains identified in the reservoir will lead to a better understanding of basic properties of these waterborne microbes.

For the evaluation of biofilm formation capacity of bacterial strains from DWST all bacterial strains listed in Fig. 12 were examined using a simple method based on the measurement of biofilm growth in microtiter wells. Basically, microbes having biofilm formation capacity will grow and form biofilms on the walls of plastic microtiter plates under optimized

conditions. After incubation, biofilm formation (or absence) is confirmed by dying the bacterial membranes in the film with crystal violet, then extracting the dye to ethanol and measuring absorbance using a spectrophotometer. Crystal violet is used because the dye has a blue-violet colour with an absorbance maximum at 590 nm and an extinction coefficient of 87,000 M−1cm−1. Occasionally the dye is called “gentian violet”. Because of its affinity to cell membrane, the dye shows antibacterial, antifungal, and antihelmintic properties.
Results depicted indicate that 19 of 20 strains show biofilm formation capacity.
On the y-axis of the diagrams absorption at 570 nm of the dye extracted from treated bacterial membranes is indicated. On the x-Axis the names of bacterial strains isolated from different reservoirs are given. In each series of analysis, a positive control (*P. aeruginosa*) and a negative control has been examined in parallel. A red line shows the threshold limit value for biofilm formation: strains delivering a lower value are considered to be negative whilst strains with an absorption measurement above the line are deemed to be positive.

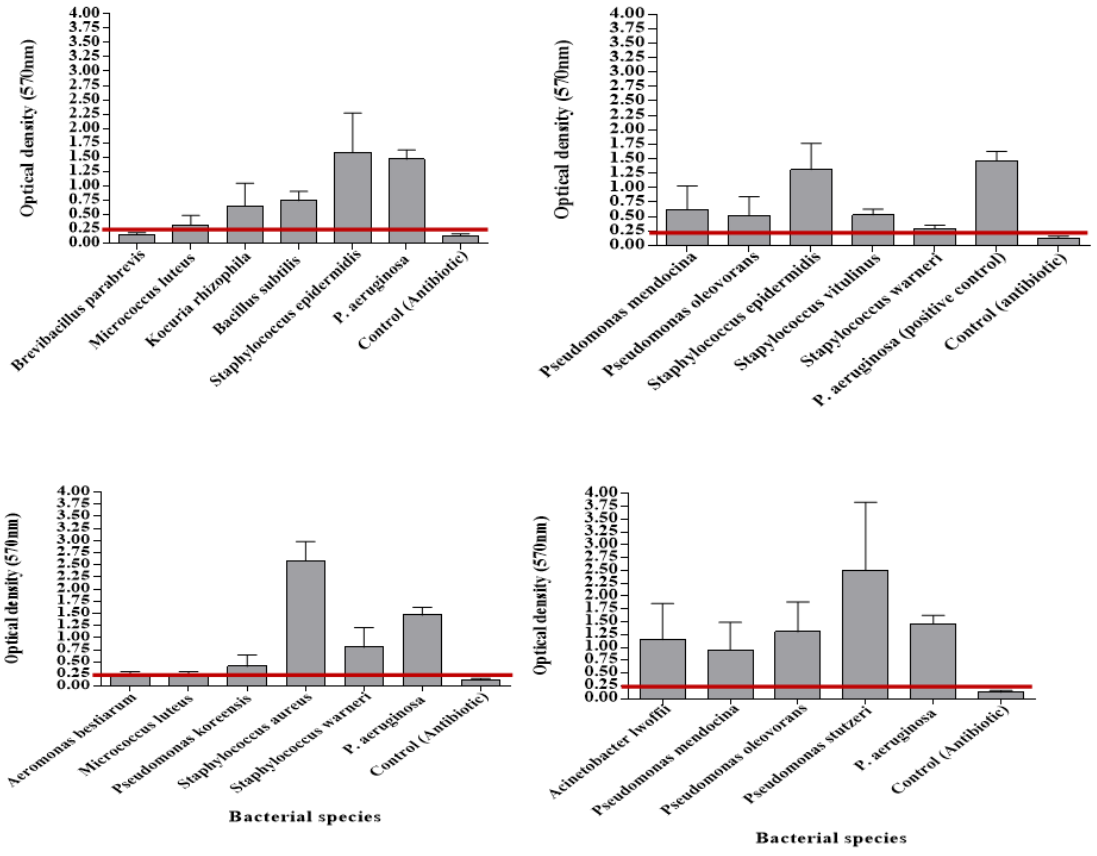


Fig. 12: Comparison of biofilm formation capacity of bacterial strains recovered from drinking water reservoirs (R). Upper left: R1, right: R2; lower left: R3, lower right: R4.

All *Pseudomonas* strains identified so far are above the “biofilm-line”. This means that all *Pseudomonas* strains isolated from DWST may be able to form biofilms. However, on average their biofilm formation capacity is significantly lower compared to *P. aeruginosa* being used as positive control. Of all *Pseudomonas* strains listed here, highest absorption was measured for *P. stutzeri*. Interestingly, *S. epidermidis* and *S. aureus* show similar activities, whilst other *Staphylococci* identified here show much lower activity.

However, presence of biofilm potential does not necessarily mean that biofilms will really grow in DWST. Actually, drinking water contains only limited amounts of nutrients, and the temperature is probably too cold for significant growth. Unfortunately, data from literature are only available for growth of planktonic *Pseudomonas* species. Therefore, growth of biofilm has been investigated at different temperatures.

3.4 Growth of planktonic *P. aeruginosa* and *P. aeruginosa* biofilms at different temperatures

Again, the microtiter plate assay has been used for the purposes of this experiment; however, some modifications have been made.

Microtiter plates have been covered with a lid with pegs fitting into the wells of the microtiter plate allowing growth of the biofilm directly on the pegs. After certain incubation time the lid with the biofilm was removed and rinsed in a new plate containing rinsing solution. Thereafter, a number of pegs was pinched off the lid and was transferred to a test tube containing a defined amount of physiological sodium chloride solution. Subsequently, the test tube was ultrasonicated to disintegrate the biofilm on the lids and the colony count in the medium was analyzed using the dilution method described before.

Fig. 13 shows the results of this investigation. The y-axis is divided by a logarithmic scale, whilst the temperature is indicated on the x-axis with a linear scale. Between 8 und 22°C speed of growth of *P. aeruginosa* significantly increases and reaches a maximum at temperatures between 30 and 35°C.

Basically, growth of the biofilm is similar to the growth of planktonic *P. aeruginosa*. For the data shown in Fig. 14 bacteria were cultivated in 100 millilitre of tap water in dependence of the temperature. Initial colony count was adjusted to 100/ml by dilution of cultures grown in nutrient broth. Additionally, 500µl of nutrient broth were added to each sample to guarantee bacterial growth.

Compared to the experimental biofilm data shown above, results indicate a maximum of grown colonies at about 30°C.

Obviously, there is a certain growth even at lower temperatures. Fig. 15 shows the growth kinetic of *P. aeruginosa* at three distinct temperatures, additional data are depicted in Table 10.

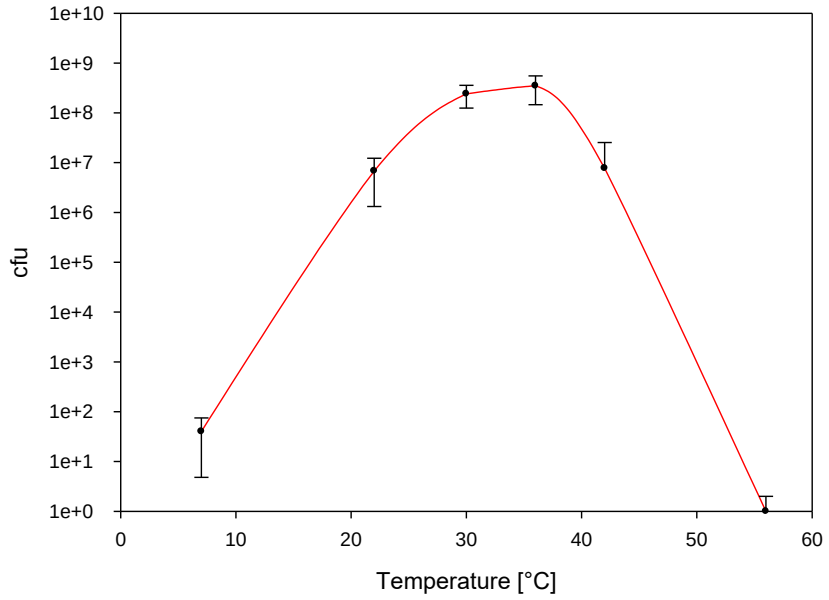


Fig. 13: Growth of *P. aeruginosa* biofilms at different temperatures

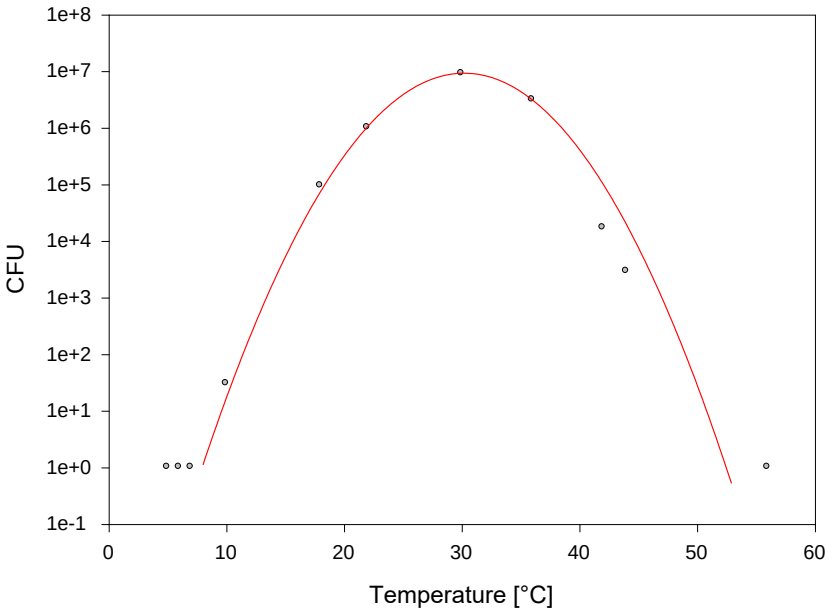


Fig. 14: Growth of planktonic *P. aeruginosa* at different temperatures

Below 10°C there is no measurable growth of *P. aeruginosa*, however, at 10°C there is a measurable build-up of colony count over time. Whilst at lower temperatures colonies

completely disappear, there is colony count at 10°C increases up to 2800 colonies per milliliter of broth after 7 days.

Table 10: Temperature dependency of growth of *P. aeruginosa*

T [°C]	0h	24h	48h	72h	144h	168h
4	28	0	0	1	1	8
5	123	0	0	0	14	1
6	34	0	0	0	1	0
7	122	6	0	0	15	0
10	126	20	8	30	2000	2800
18	120	1600	2,56E+03	9,40E+04	5,30E+06	1,80E+07
22	64		4,20E+04	1,00E+06	2,45E+07	2,00E+08
30	73		1,42E+05	9,00E+06	1,90E+08	1,70E+08
36	100			3,10E+06	1,80E+07	2,00E+08
42	91		1,54E+04	1,70E+04	2,10E+05	1,50E+07
44	90	2160	6320	2890	370	16
56	92	0	0	0	0	0

Further increase of the temperature to 18°C shows a tremendous push and speed of progeny increases so much that after two days of breeding at 18°C there is almost the same colony count at after 7 days at 10°C.

As mentioned before, *P. aeruginosa* is a typical mesophilic strain. However, the aim of the examinations described here was to find out specific temperature dependency of growth of *P. aeruginosa* because this parameter may have major impact on growth of these bacteria in DWST and, for example, in cold water lines.

Results in Fig. 15 however indicate the possibility of growth even in water being as cold as 10°C. Another aspect of bacterial growth is the structure and composition of the surface, were the biofilm may attach. Therefore, this aspect of biofilm growth was examined in another set of experiments.

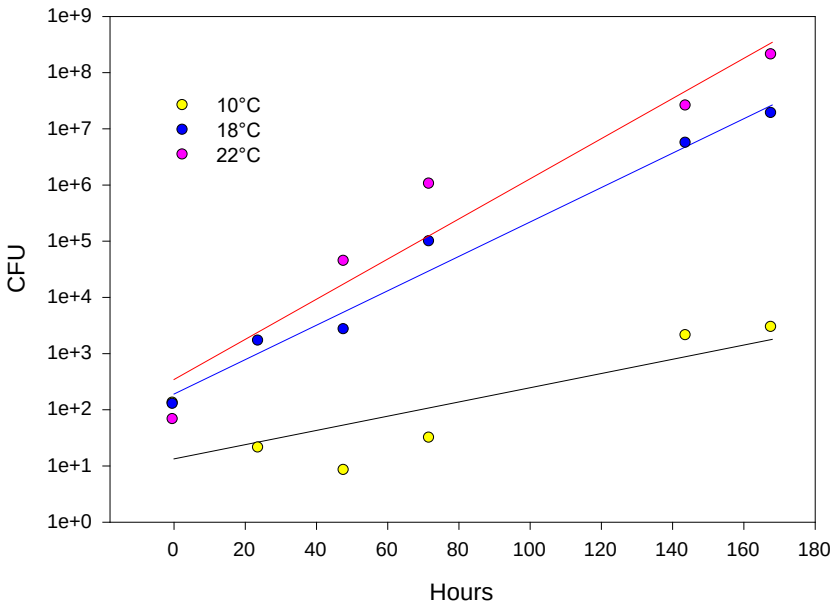


Fig. 15: Growth kinetic of *P. aeruginosa* at different breeding temperatures

3.4.1 Ultrasonic sound treatment of *P. aeruginosa*

As mentioned before, *P. aeruginosa* may form biofilms on surfaces embarking on a variety of strategies. For the analysis of biofilms and effects on biofilms, as much of the biofilm should be recovered from experimental setups. To this end disintegration of the film will be necessary. One of the possible means is mechanical work using sterile scrapers; another way of disintegration employs treatment with ultrasonic waves. However, ultrasonic equipment used for this purpose may differ in a variety of parameters, including wavelength, intensity and transfer medium. Therefore, experiments were performed to analyze whether or not the ultrasonic equipment available in the lab may be a convenient tool for the disintegration of *P. aeruginosa* from surfaces.

All experiments were carried out using a high-power ultrasonic cleaning unit with an ultrasonic peak output of 850 W (Bandelin Sonorex super RK512H, Germany). Applied frequency was 35 kHz.

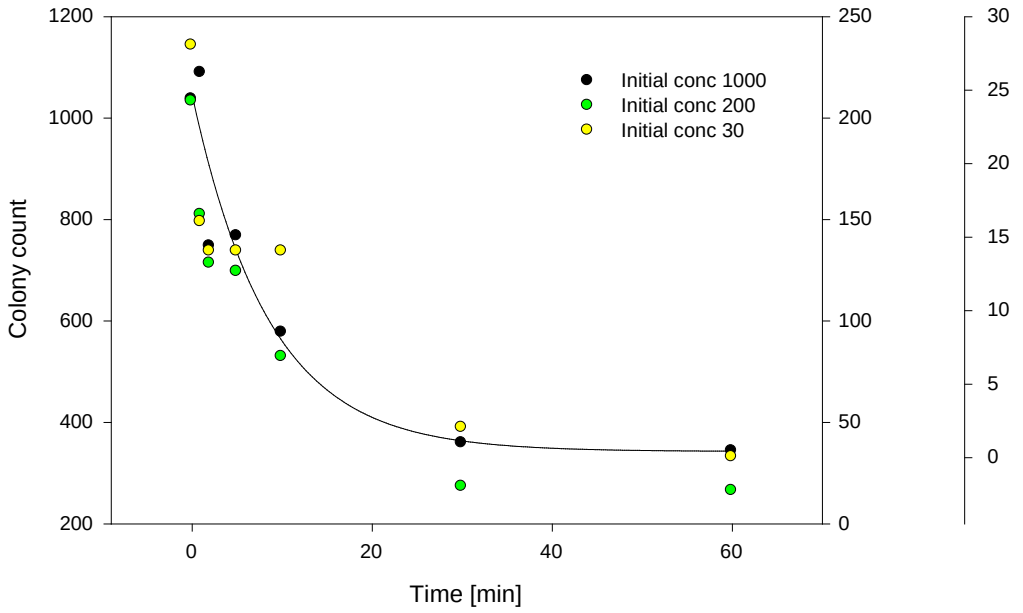


Fig. 16: Decay of microorganisms during application of ultrasound (35 MHz)

Three experiments with three different initial concentrations. Results are depicted in Fig. 16. Independent of the initial colony count concentration of colonies is reduced with a half-time value of about 12 min.

3.5 Formation of *P. aeruginosa* biofilm on different materials from DWST

Water storage tanks may be constructed using a wide variety of different materials. Among these, cement based materials as well as ceramic tiles and plastic materials like polyethylene play a major role.

However, there are no data available for the estimation of biofilm formation on these construction materials.

Focusing on the formation of *P. aeruginosa* biofilm on different materials, Fig. 17 presents a quantitative analysis of biomass fixed on cement, ceramic and polyethylene after 9 days at different temperatures.

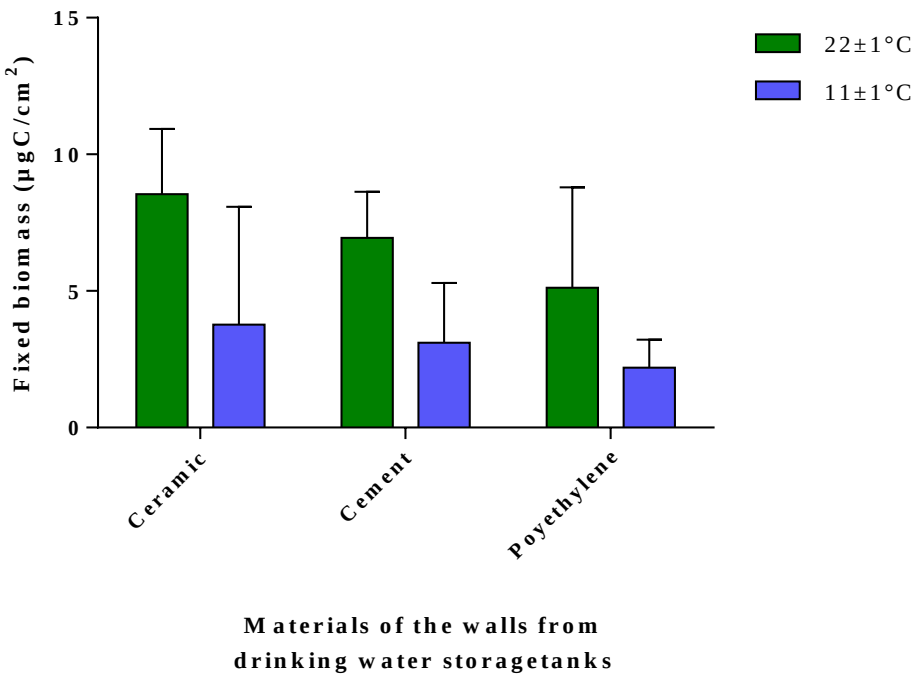


Fig. 17: Density of fixed bacterial biomass on different materials from drinking water storage tanks at 22±1°C and 11±1°C. Error bars represent standard deviations of the average.

Although results are obvious, it is interesting to note that the total number of fixed biomass at room temperature (22±1°C) was significantly higher than at low temperature (11±1°C). These results are in accordance with previous reports in the literature (Villanueva *et al.*, 2011), (whereby river biofilms composed of bacteria, algae, cyanobacteria and protozoa were tested for biofilm formation at low and high temperature) indicating that biofilm formation at the higher temperature was faster. It is noteworthy that average ground water temperature in Germany, as well as in drinking water storage tanks in Germany is low and biofilm formation might be hindered, but results have shown that biofilm growth is still possible.

Despite the assertion that roughness of the materials used during water distribution has been identified as an important factor affecting bacterial attachment (Pedersen, 1990; Percival *et al.*, 1998), in our research we obtained different results, being the highest bacterial accumulation on ceramic plates, with no roughness. Ceramic plates have a smooth surface, allowing an easy scraping of biofilm on its surface. Meanwhile cement plates could accumulate large amount of bacteria in its pores, without being scratched and therefore not

accounted. These could be some of the reasons why most bacterial accumulation was found on ceramic plates than on cement plates.

The lowest densities of fixed bacteria were measured on plastic-based materials (polyethylene) and the highest densities were measured on ceramic. Cement-based surfaces had intermediate levels of fixed bacterial biomass. As already was observed by Niquette et al. (Niquette *et al.*, 2000), densities of biomass fixed on cement was higher than biomass fixed on polyethylene, confirming our results.

3.6 TOC concentration per colony forming unity of planktonic samples

TOC concentrations in the bacteria *P. aeruginosa, E. coli* and *S. aureus* were measured through the correlation between TOC (mg/ml) and colony count (CFU/ml). The results are showed in Fig. 18, Fig. 19 and Fig. 20. The slope of the line is the TOC concentration per colony forming unity [m = 7.05 × 10-11 (± 0.05 × 10-11) for *P. aeruginosa*, m = 2.09 × 10-10 (± 0.01 × 10-10) for *E. coli* and m = 3.78 × 10-10 (± 0.04 × 10-10) for *S. aureus*]. The coefficient b of the regression line for the three strains was b = 0, showing there is no other organic material in the samples. Significant correlations (R2 = 0.999 for *P. aeruginosa*, R2 = 1 for *E. coli* and R2 = 0.999 and *S. aureus*) were observed between TOC concentration and colony count, providing an easy and fast method for the quantification of TOC concentration in microorganisms.

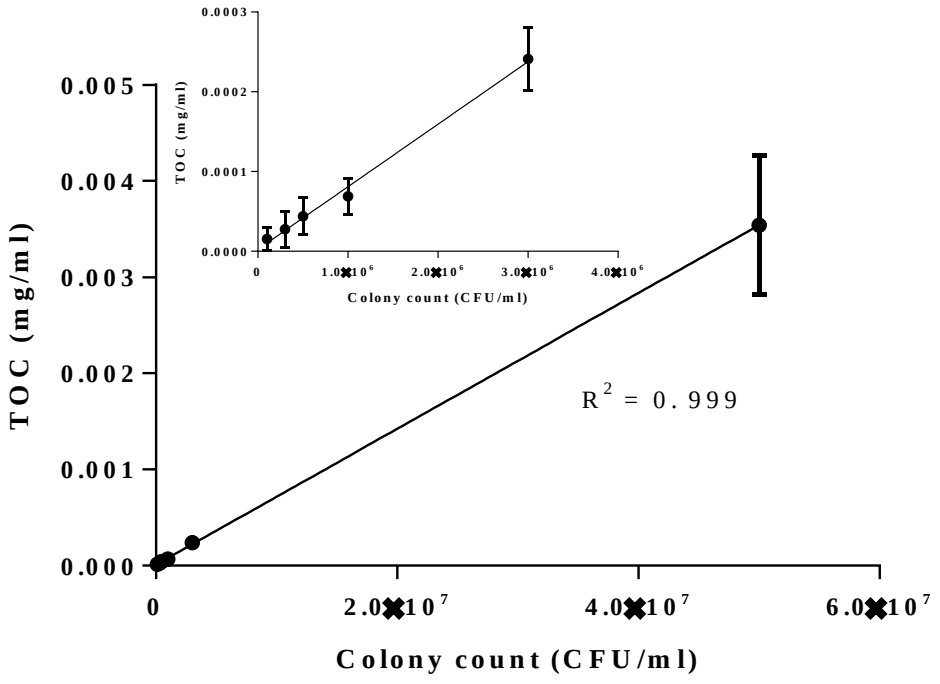


Fig. 18: TOC concentration per CFU of *P. aeruginosa*. The line drawn is the calculated linear regression. Small graphic represents the accumulated points at the beginning of the large graphic

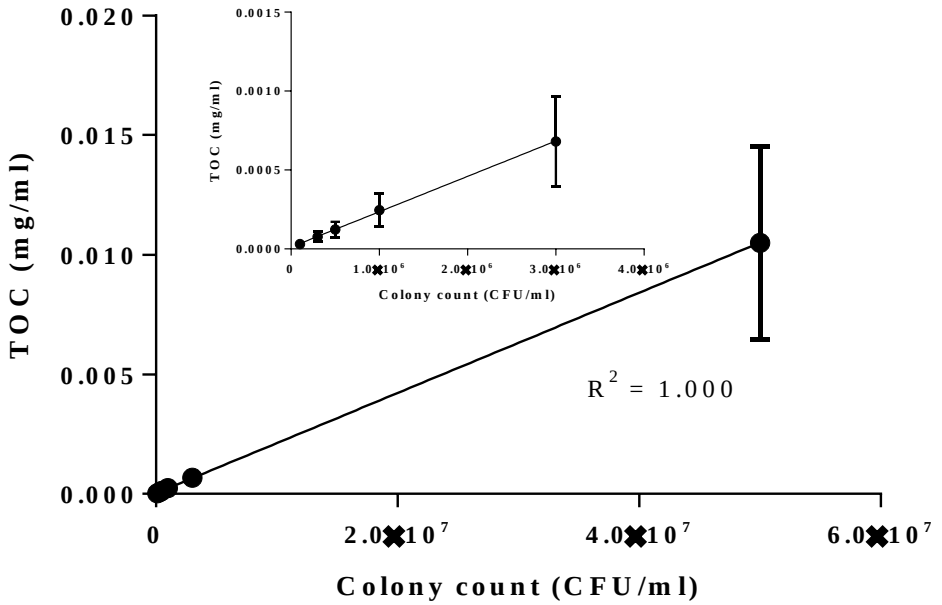


Fig. 19: TOC concentration per CFU of *E. coli*. The line drawn is the calculated linear regression. Small graphic represents the accumulated points at the beginning of the large graphic

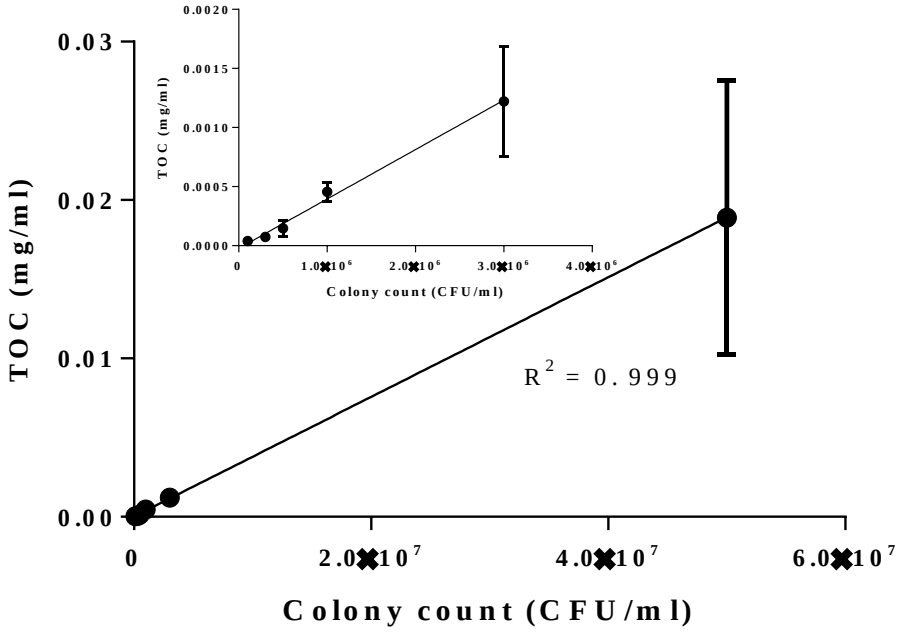


Fig. 20: TOC concentration per CFU of *S. aureus*. The line drawn is the calculated linear regression. Small graphic represents the accumulated points at the beginning of the large graphic

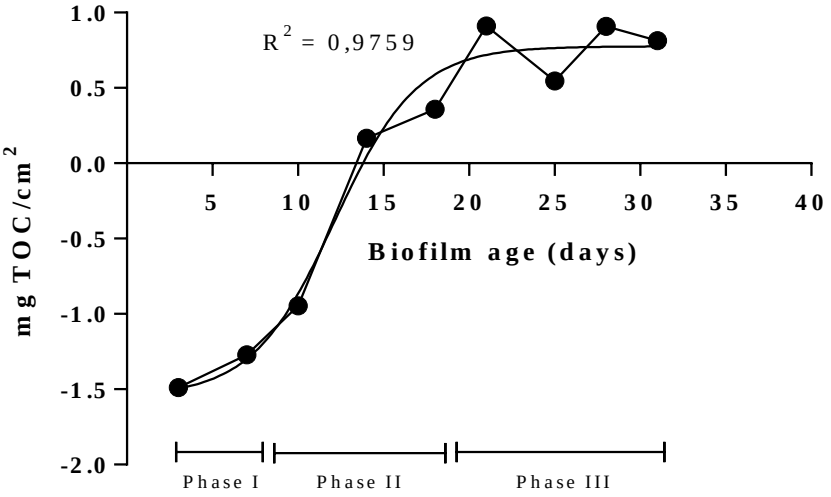
3.6.1 TOC concentration of *P. aeruginosa* biofilm

After the good results obtained from the correlation between TOC concentration and colony count in planktonic samples, was measured the TOC concentration in sessile samples (biofilms). All components of the biofilm, bacteria and biomolecules present in the biofilm matrix (polysaccharides, proteins, nucleic acids, lipids), are formed by carbon, hence the biofilm concentration can be estimated through total organic carbon.

The results are presented in Fig. 21a. At first glance, the most interesting observation is that the biofilm growth with the pass of time shows a sigmoidal tendency. Similar results were observed in studies carried out by Ward et al. (Ward and King, 2012), where was developed a thin-film modelling of biofilm growth and quorum sensing. Results clearly reveal the expected three stages biofilm growth as defined by Bryers et al. (Bryers and Characklis, 1982). Firstly, the phase I corresponds to the initial and irreversible attachment, where the solid-liquid interface between a surface and an aqueous medium (e.g., water) provides an ideal environment for the attachment and growth of microorganisms (Donlan, 2002). Secondly, the phase II correlates to biofilm maturation, where the content of carbohydrates, proteins, nucleic acids and lipids in the biofilm matrix increased significantly in the biofilm (Andrews *et al.*, 2010; Chao and Zhang, 2012; Ivleva *et al.*, 2009). Finally, the phase III takes place with the corresponding dispersion stage. There is a detachment of cells from biofilm colony with the following translocation of the cells to a new location (Kaplan, 2010). The detachment process can be through erosion or sloughing (Li *et al.*, 2007). Statistical comparisons of the two measurements were made using normalized values.

Parallel to this research the three different stages of *P. aeruginosa* biofilm growth were observed by CLSM (Fig. 21b), confirming the results obtained previously. The images were acquired at random locations in each of the biofilm stages, using the 63X oil lens with a 2X zoom.

a



b

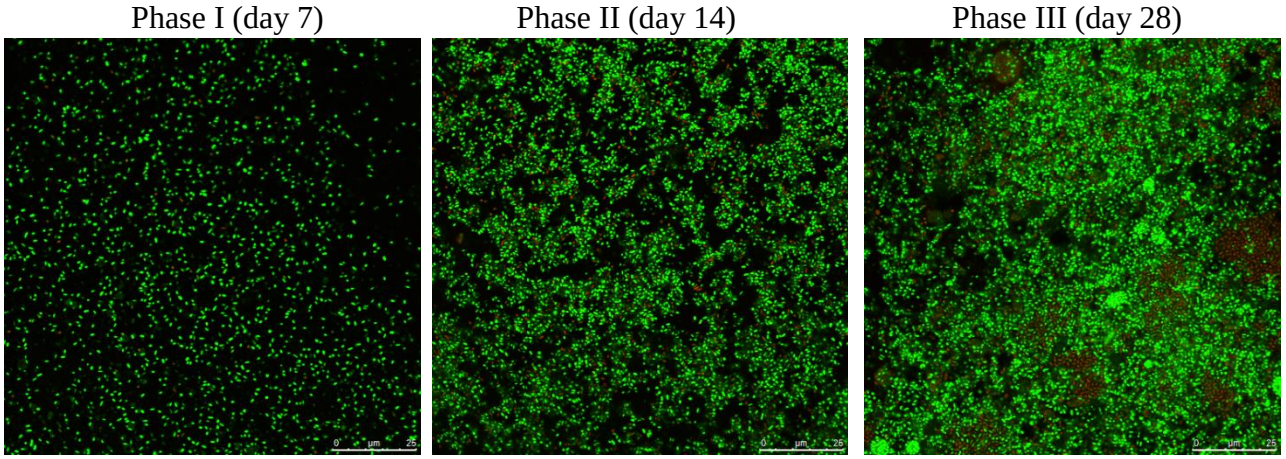


Fig. 21: (a) Growth curve of *P. aeruginosa* biofilm at room temperature (22±1°C). (b) Confocal scanning laser microscopic analyses of the three stages of *P.aeruginosa* biofilm growth. (Scale bars represent 25 µm in all the images)

3.7 *P. aeruginosa* biofilm adhesion on surfaces

In order to determine the mechanism of biofilm formation of *P. aeruginosa*, we used sodium metaperiodate and proteinase K to disperse adhered biofilm. Treatment with sodium metaperiodate should disperse polysaccharide-mediated biofilm and treatment with proteinase K should disperse protein-mediated biofilm.

Results showed (Fig. 22) a strong biofilm formation (OD570nm > 0.24) for the two treatments, confirming the mediation of both biomolecules in the formation of biofilm by this strain. Recent researches have discussed the role of polysaccharides and proteins in biofilm formation. Irie et al. (Irie *et al.*, 2012) demonstrated that Psl, a major biofilm matrix polysaccharide for this species, acts as a signal that stimulates different biomolecules involved in its own synthesis and in the production of components of the biofilm, promoting biofilm growth. Southey-Pillig et al. (Southey-Pillig *et al.*, 2005) also identified a number of key-biofilm proteins which control the transition to late stage biofilm development.

Interestingly, optical density values for the proteinase K treatment were significantly higher, suggesting a greater role for polysaccharides than for proteins in biofilm production by *P. aeruginosa*.

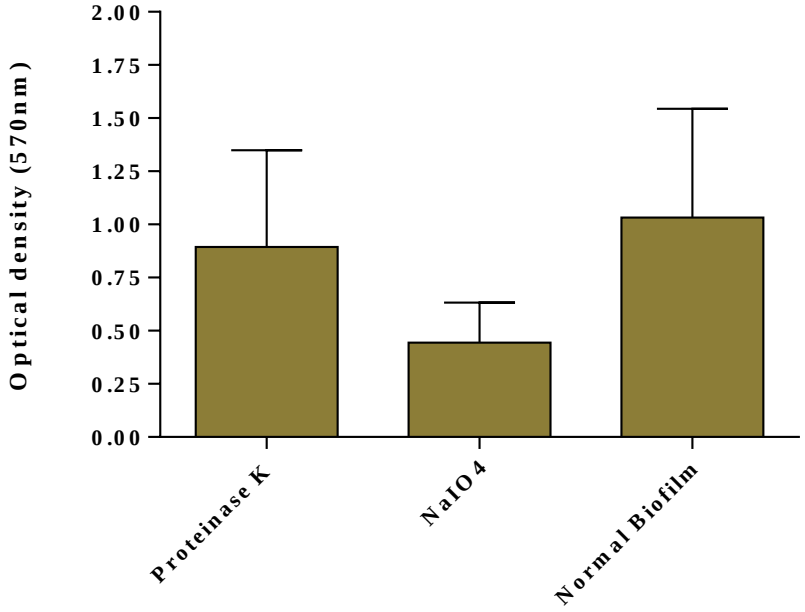


Fig. 22: Identifying the mechanism of biofilm production by *P. aeruginosa*: polysaccharides versus proteins.

3.8 Biofilm samples collected in DWST

3.8.1 Universal bacterial PCR: 16S rDNA PCR plus sequencing

Results obtained from the amplification of fragments of the bacterial ribosome 16S from bacteria founded in the first DWST sampled (Stadtwasser-Überlage Nord) in Table 9, are represented in Fig. 23.

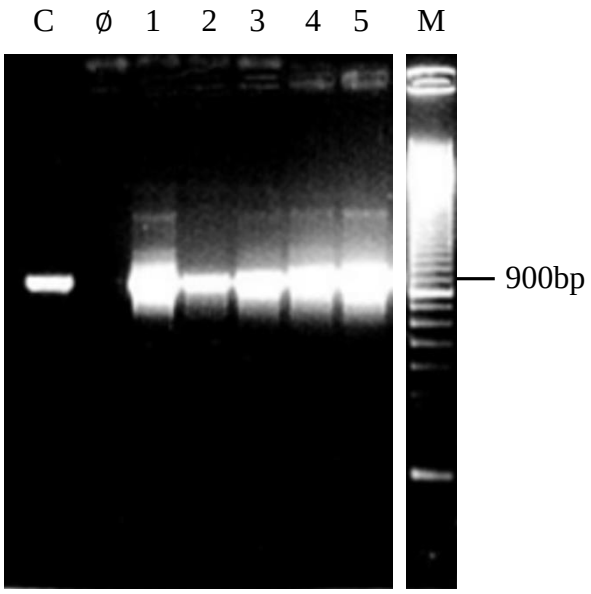
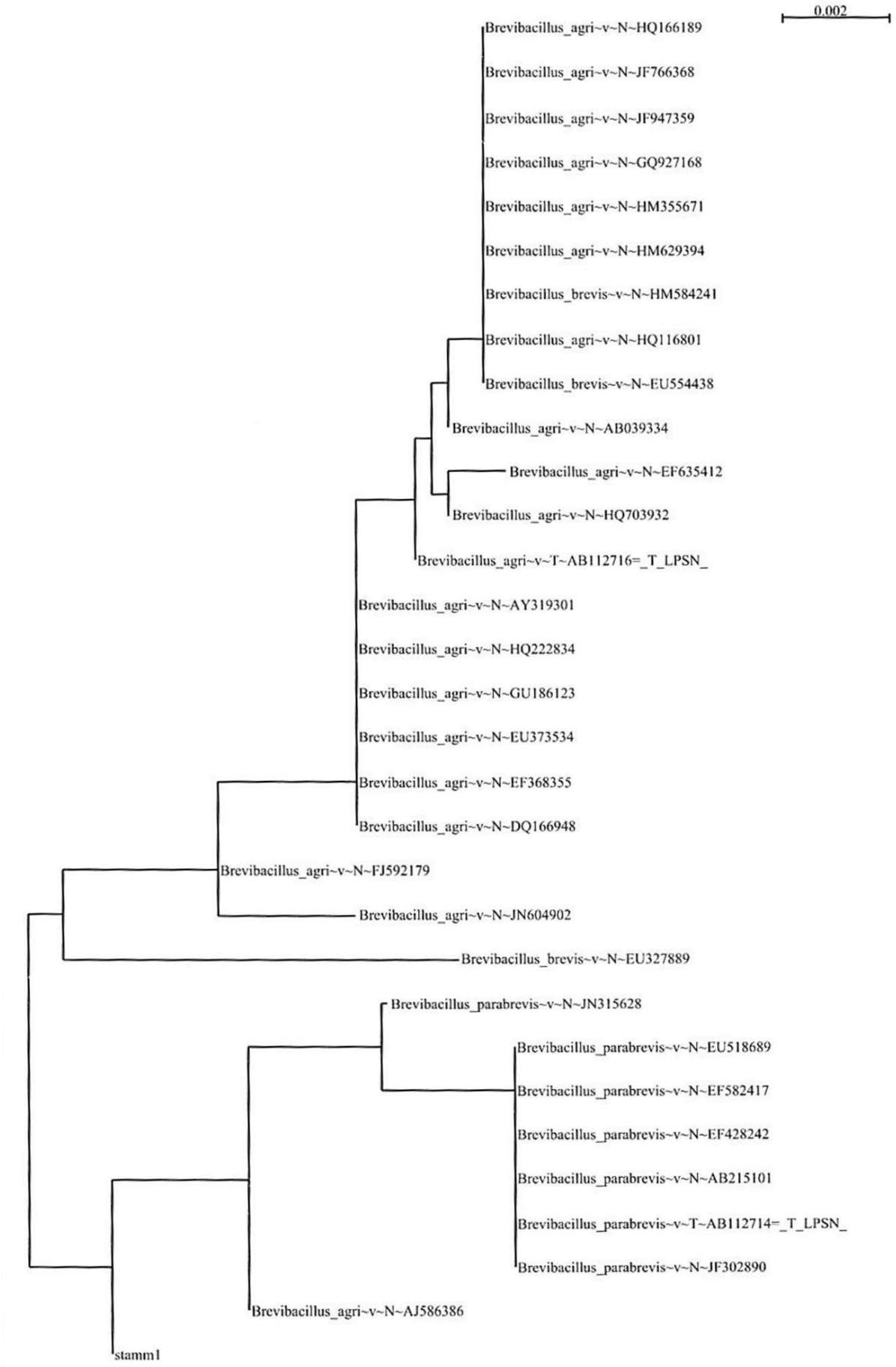


Fig. 23: Results for PCR Assay with bacteria founded in DWST I. Lane C- positive control with *Escherichia coli*. Lane ø- negative control with distilled water. Lane 1- *Brevibacillus parabrevis*. Lane 2- *Micrococcus luteus.* Lane 3- *Kocuria rhizophila*. Lane 4- *Bacillus subtilis*. Lane 5- *Staphylococcus epidermidis*. Lane M- 100 bp molecular DNA marker

After sequencing of amplicons were studied the phylogenetic relationships of the samples (Fig. 24). The phylogenetic tree was explained using the neighbour-joining method (Saitou and Nei, 1987). Results show that strain 1 was *Brevibacillus agri*, strain 2 was *Micrococcus luteus*, strain 3 was *Kocuria rhizophila*, strain 4 was *Bacillus subtilis* and strain 5 was *Staphylococcus epidermidis*. In a comparative analysis, we observed that MALDI-TOF MS assay and PCR assay results were quite similar, it was found only a difference at the species level in the strain 1. Thus, MALDI-TOF MS can be used to analyse bacterial samples from drinking water storage tanks, being a current investigative technique, which is easy and fast to use.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Brevibacillus_parabrevis~v~N~EU518689
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Brevibacillus_agri~v~N~AJ586386
stamm1


0.005
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Micrococcus_luteus~v~N~AM931275
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stamm2
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Micrococcus_luteus~v~N~EF187229
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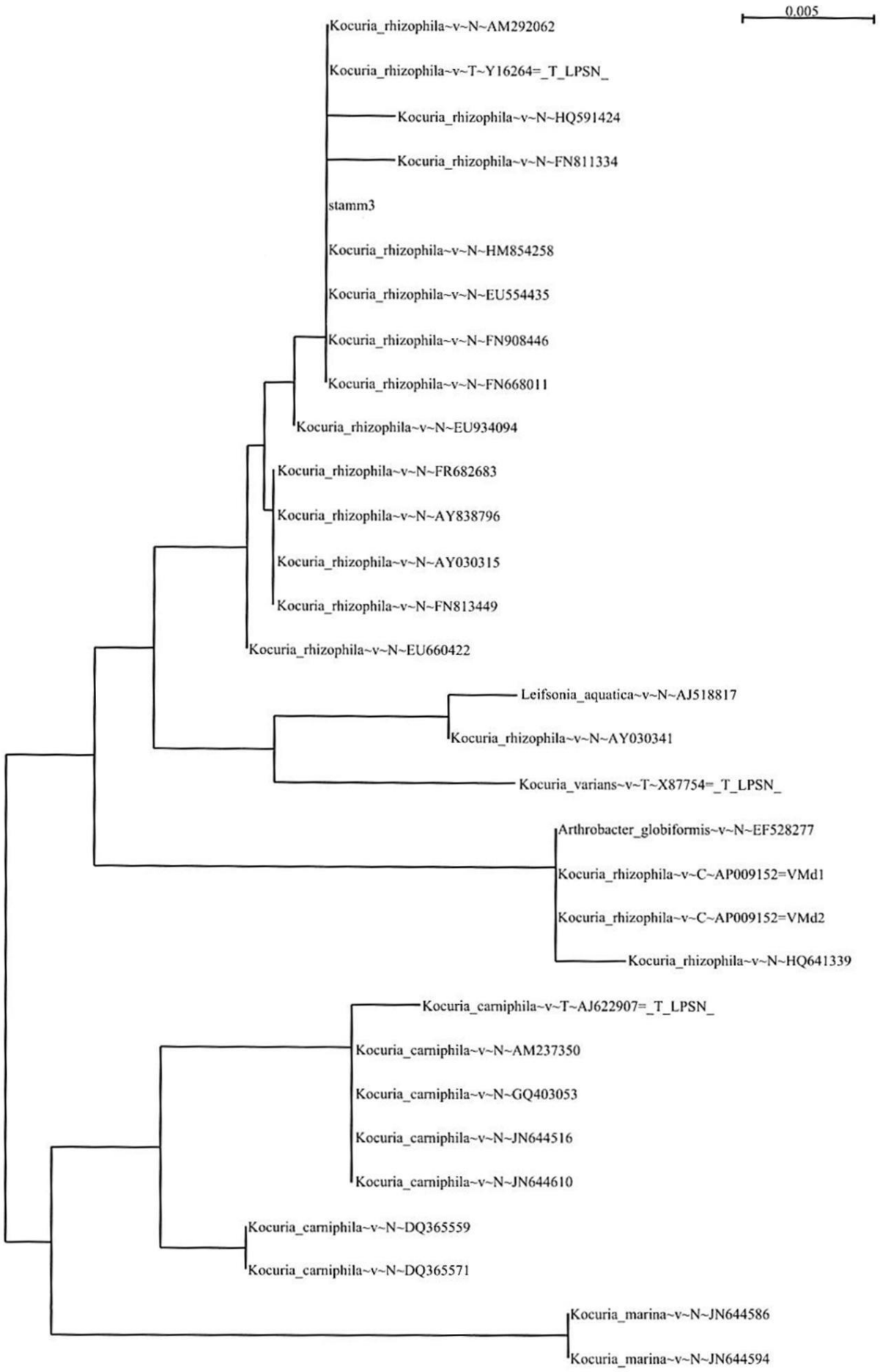
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Kocuria_rhizophila~v~N~FR682683
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Kocuria_rhizophila~v~N~FN813449
Kocuria_rhizophila~v~N~EU660422
Leifsonia_aquatica~v~N~AJ518817
Kocuria_rhizophila~v~N~AY030341
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Arthrobacter_globiformis~v~N~EF528277
Kocuria_rhizophila~v~C~AP009152=VMd1
Kocuria_rhizophila~v~C~AP009152=VMd2
Kocuria_rhizophila~v~N~HQ641339
Kocuria_carniphila~v~T~AJ622907=_T_LPSN_
Kocuria_carniphila~v~N~AM237350
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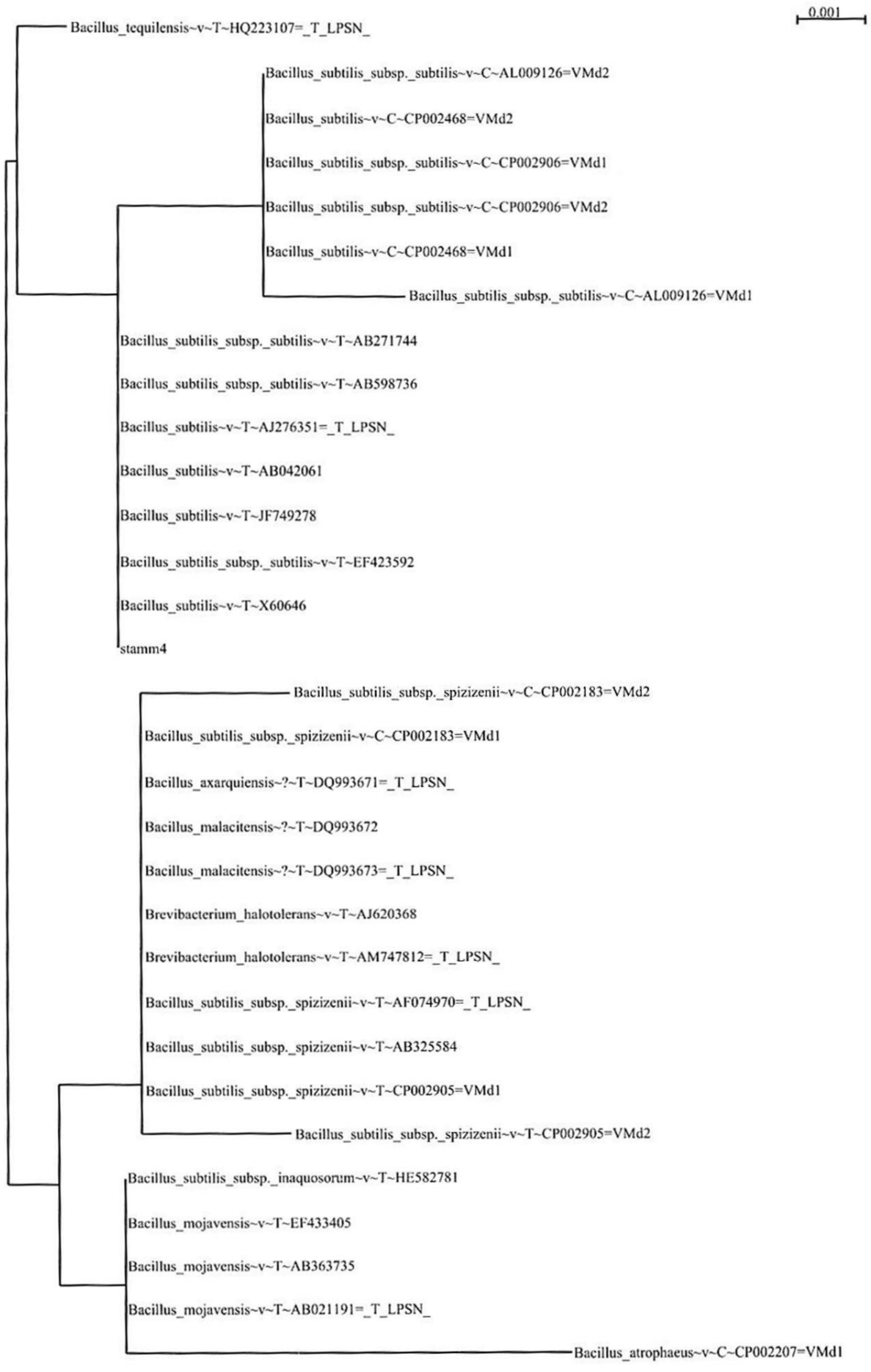
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Bacillus_subtilis_subsp._subtilis~v~C~CP002906=VMd1
Bacillus_subtilis_subsp._subtilis~v~C~CP002906=VMd2
Bacillus_subtilis~v~C~CP002468=VMd1
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Bacillus_subtilis~v~T~X60646
stamm4
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Bacillus_subtilis_subsp._spizizenii~v~C~CP002183=VMd1
Bacillus_axarquiensis~?~T~DQ993671=_T_LPSN_
Bacillus_malacitensis~?~T~DQ993672
Bacillus_malacitensis~?~T~DQ993673=_T_LPSN_
Brevibacterium_halotolerans~v~T~AJ620368
Brevibacterium_halotolerans~v~T~AM747812=_T_LPSN_
Bacillus_subtilis_subsp._spizizenii~v~T~AF074970=_T_LPSN_
Bacillus_subtilis_subsp._spizizenii~v~T~AB325584
Bacillus_subtilis_subsp._spizizenii~v~T~CP002905=VMd1
Bacillus_subtilis_subsp._spizizenii~v~T~CP002905=VMd2
Bacillus_subtilis_subsp._inaquosorum~v~T~HE582781
Bacillus_mojavensis~v~T~EF433405
Bacillus_mojavensis~v~T~AB363735
Bacillus_mojavensis~v~T~AB021191=_T_LPSN_
Bacillus_atrophaeus~v~C~CP002207=VMd1

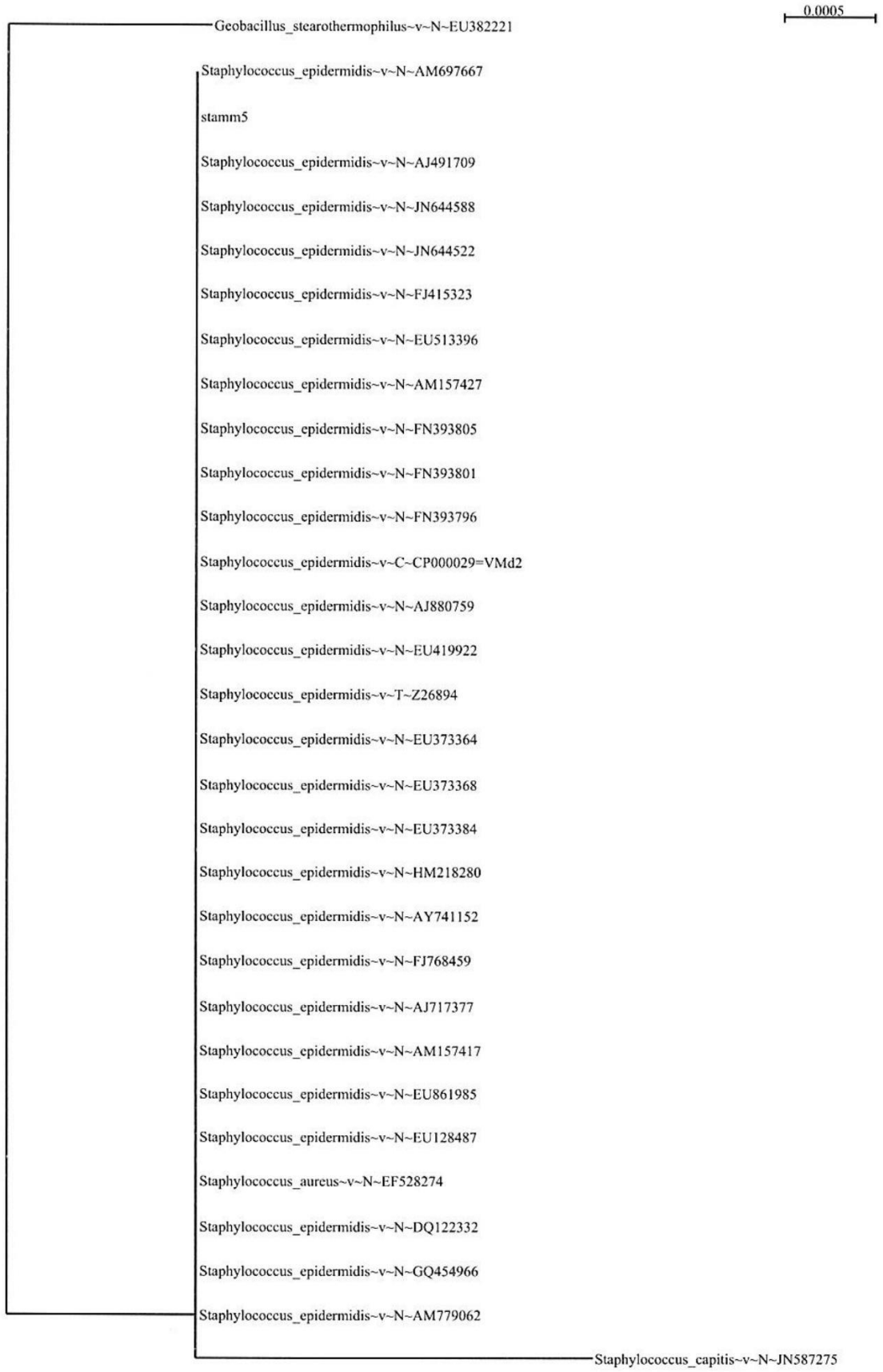

Fig. 24: Phylogenetic relationships based on 16S rDNA gene sequences obtained from bacteria founded in DWST I. The scale bars indicates the evolutionary distance between sequences determined by measuring the lengths of the horizontal lines connecting two organisms.

3.9 Statistical data from swimming pools

It was carried out a statistical study of all data collected on swimming pools sampled from July 2011 to December 2012. In the present study was examined the data related to the number of *P. aeruginosa* detectable, free available chlorine > 0.6 mg/l and combined chlorine > 0.2 mg/l in the two pools with *P. aeruginosa* problems and in all other pools (approximately 100) examined during that period of time. These data are summarized in Fig. 25, where the y-axis is represented with a logarithmic scale. It is interesting to note that the percentage of *P. aeruginosa* detectable in swimming pools was quite high in general (5.02%), but for the pools with *P. aeruginosa* problems was even higher (15.49 % for pool 1 and 12.50% for pool 2). It is worth to mention that free available chlorine > 0.6 mg/l in pool 1 (48.45%), pool 2 (65.52%) and in rest of pools (42.25%) were more or less the same, indicating the existence of a problem with *P. aeruginosa* in pool 1 and in pool 2. Finally, combined chlorine level in both swimming pools were always less than or equal to 0.2 mg/l, while in the rest of pools was obtained a 2% of combined chlorine with values higher than 0.2 mg/l.

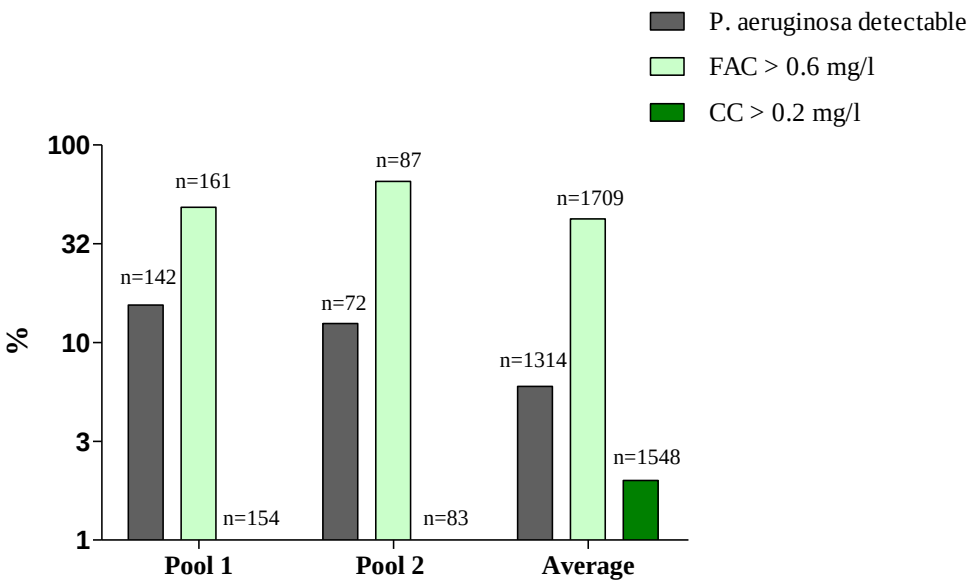

Fig. 25: Statistical data on the percentage of *P. aeruginosa* detectable, free available chlorine (FAC) and combined chlorine (CC) in swimming pools from July 2011 to December 2012. (n= total number of data obtained during the samplings)

3.10 Biofilm formation in an artificial house installation system

Examination of biofilm formation in real life installations is a challenging task primarily because of the wide variety of possible installation materials. Additionally, growth of biofilms depends on the water quality as well as on residence time of the water in the lines, and speed of water running through the lines as soon as taps are opened.

Taking into account the experiences of lab scale biofilm formation reactors, an artificial system was built consisting of stainless steel lines and two independent circulation pumps to ensure hydraulic conditions comparable to conventional drinking water installation systems.

Altogether, the system allowed the simultaneous installation of six sample tubes. However, for practical reasons only three pairs of tubes of the same material where employed in each experiment.

Test tubes installed in the system were made of PVC, copper and zinc-plated steel pipes.

The system was build up at the Steinbeis-Centre for Building Technology, an institution connected to the University of Applied Sciences, Esslingen, Germany.

However, the system turned out to have some major shortcomings.

Table 11: Results for the first test period Jan 2014

Day	Date	ORP	Fe	Zn	Pb	Cu	Cr	Ni	Mn	Al	Cl
		mV	mg/L	mg/L	µg/L	mg/L	µg/L	µg/L	µg/L	µg/L	mg/L
0	29.01.14	92,3	2	1,3	70	1,4	300	184	15	50	284
1	30.01.14	47,8	4,2	2,4	60	1,9	431	174	26	49	280
2	31.01.14	-66,3	5,6	3,8	65	2,2	468	197	38	70	277
5	03.02.14	-172,7	12,8	7,2	170	1,9	487	459	70	57	275

Fig. 26 displays the development of the Oxidation Reduction Potential (ORP) in the system, Obviously, ORP decreases dramatically over this time period. The only reasonable explanation for this finding is decrease of oxygen in the system. Consequently, metal ion concentrations increase over the same period of time due to corrosion processes. Fig. 26 also shows the linear increase of the iron concentration in the water. All other metal ion concentrations measured show the same concentration trend. Even the Manganese and

Aluminium concentrations were rising although it is not clear from which materials these metals were released.

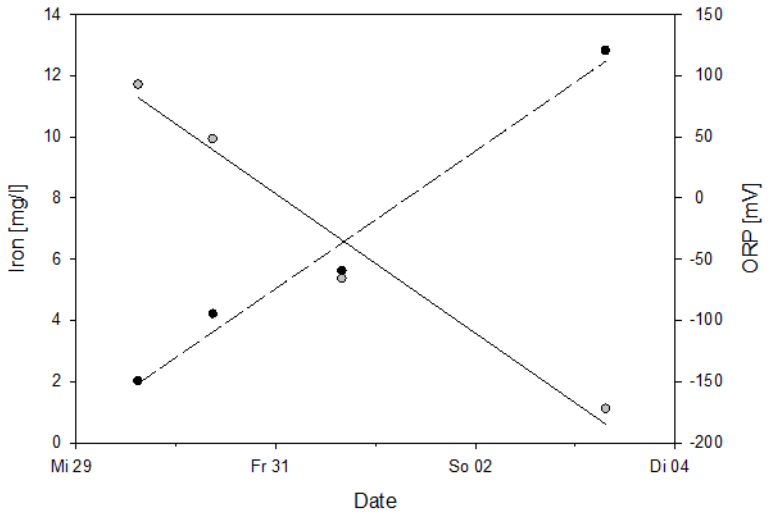

Fig. 26: Development of the Oxidation-Reduction Potential and iron concentration in the system over 5 days

Microorganisms were not detectable any more by the end of the experiment. An analysis of the situation revealed that the material of the pumps might be a major reason for corrosion. Therefore, pumps were changed for pumps made of stainless steel. Subsequently, the system was flushed and rinsed until the effluent was clear and showed the same iron concentration as the tap water used to fill the system. Thereafter, the system was refilled. However, the iron concentration increased within 4 days from 0.16 mg/l (initial value) to 0.75 mg/l. Therefore, the system was cleaned again, and copper tubes were completely removed.

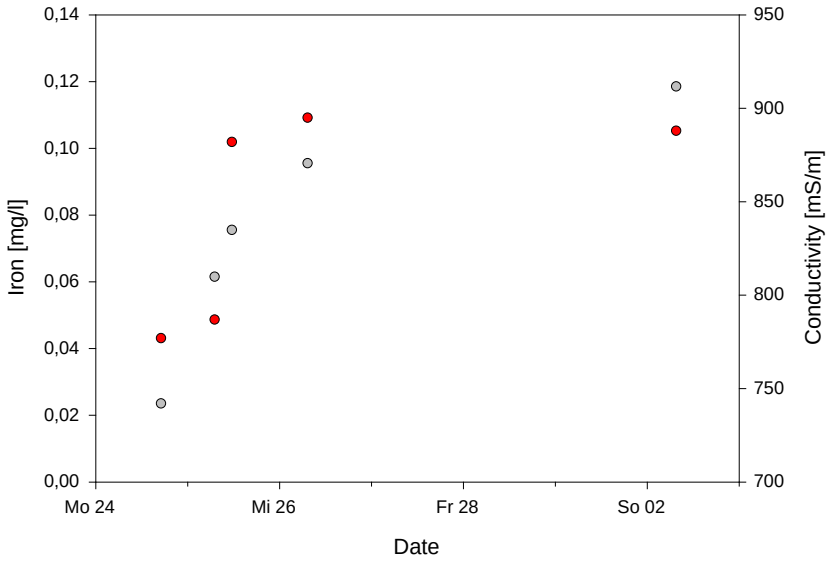

Fig. 27: Rise of iron concentration and conductivity after removal of copper tubes.

However, even after several cleaning steps and multiple exchange of the water in the system, iron concentrations increased again as depicted in Fig. 27, however, on a much lower level as in the earlier experiments.

Therefore, as iron concentrations were in the area which may be found in drinking water installations, another biofilm formation experiment was initiated. This time, additionally to the experimental conditions described before, also the zinc-plated steel tubes were removed and substituted against plastic tubes. Again, a small amount of nutrient broth (100 ml in 20 liter of filling water in the system) was added. However, due to the addition of nutrient broth the TOC increased up to 27 mg/l.

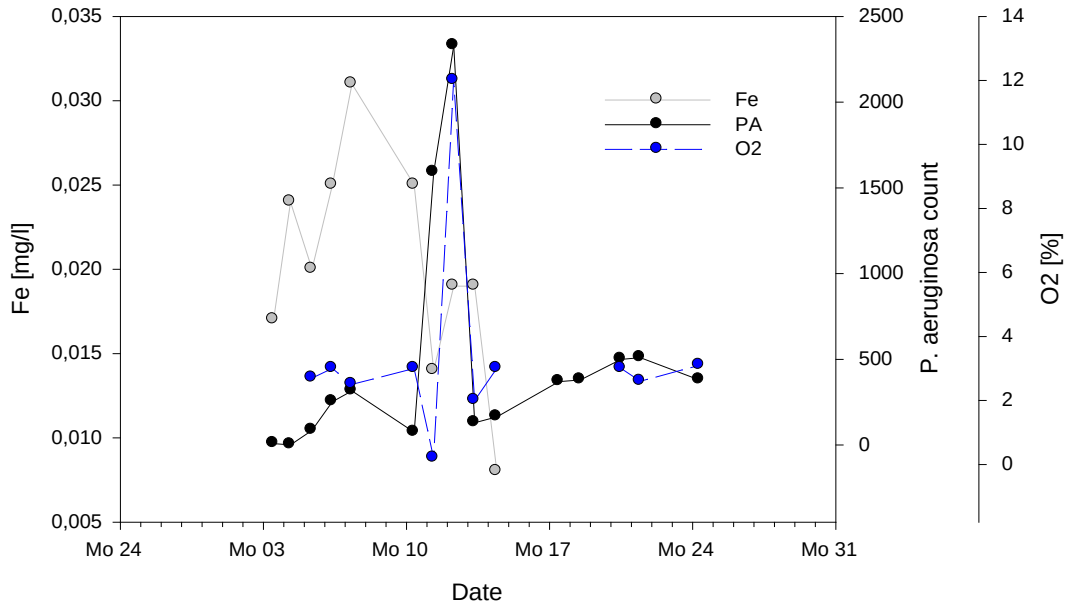

Fig. 28: Development of P. aeruginosa, iron and oxygen concentration after repeated cleaning and removal of both copper and zinc-plated steel tubes.

As shown in Fig. 28, iron concentration only rises to 0,03 mg/l (starting from 0,01 mg/l). However, there is only limited bacterial growth until the ninth day of the experiment. Then, iron concentration significantly decreases, and oxygen and colony count increases sharply due to unknown reasons. After that peak, oxygen concentration as well as colony count remains on a much lower level.

The major conclusion of this part of the work was that the oxygen in the system is consumed by oxidation processes leading to adverse growth conditions for *P. aeruginosa*. However, corrosion of metals was only limited in this experiment. Therefore, the system was improved by exchanging one of the sample tubes against another tube equipped with a gas inlet system.

3.11 Identification of P. aeruginosa in robotic pool cleaners

During the three samplings conducted in both robotic pool cleaners all green halos founded on CA plates and on TSA contact plates (Fig. 29), were analysed by MALDI-TOF MS, to confirm *P. aeruginosa* identification. All *P. aeruginosa* isolates had high-confidence identification scores (> 2.3, identification to the species level) by MALDI-TOF MS. Table 12 and Table 13, suggest the presence of P. aeruginosa in both robotic pool cleaners. *P. aeruginosa* locations varied during each sampling in both robotic pool cleaners, indicating the presence of *P. aeruginosa* in most of the different parts sampled of the cleaning devices.

It should be noted that both brands are used daily, with which the robotic pool cleaners were always wet, unable to be dry at room temperature. Furthermore, the maintenance after use carry out on each of the brands was the same, washing with water and let dry, without the use of any disinfectant.

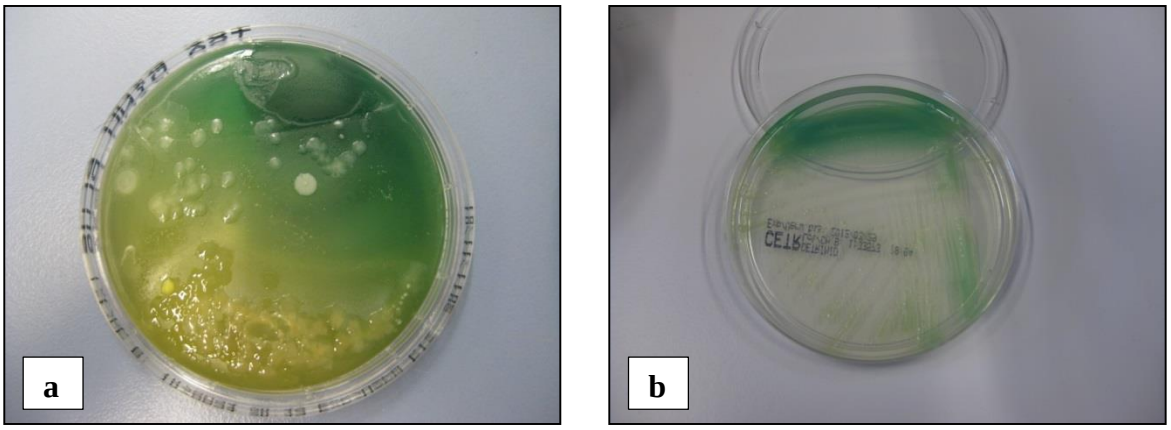

Fig. 29: *P. aeruginosa* samples founded during sampling (a) On TSA contact plate. (b) On cetrimid agar plate

Table 12: Performance of MALDI-TOF MS identification of *P. aeruginosa* samples isolated in Brand 1

Sampling date	Location in the robotic pool cleaner	MALDI-TOF score
November 2011	Filter holding	2.40
February 2012	Inside of the housing	2.46
April 2012	Roller	2.41

Table 13: Performance of MALDI-TOF MS identification of *P. aeruginosa* samples isolated in Brand 2

Sampling date	Location in the robotic pool cleaner	MALDI-TOF score
November 2011	Inside of the housing	2.50
February 2012	Inside of the housing	2.39
	Filter	2.52
	Outside of the suction pipe	2.39
April 2012	Inside of the housing	2.43

3.11.1 PFGE patterns of *P. aeruginosa* strains isolated from automatic robot cleaners

Pulsed-field gel electrophoresis of genomic DNA was carried out on *P. aeruginosa* strains to study the genetic relationships between the two samples founded in Brand 1 and Brand 2, thereby knowing if there was a common origin in the problem with *P. aeruginosa* in both cleaning devices. The interpretation of the PFGE patterns was done according to Tenover et al. (Tenover *et al.*, 1995) guidelines. When the band differences between two strains are seven or more by PFGE, as in our case, means that they have three or more independent genetic events, having the two strains different genotypes (Fig. 30). Thus, DNA sequence analysis suggested that each cleaning device was contaminated by a different strain, discarding a common strain in the contamination of both cleaning devices.

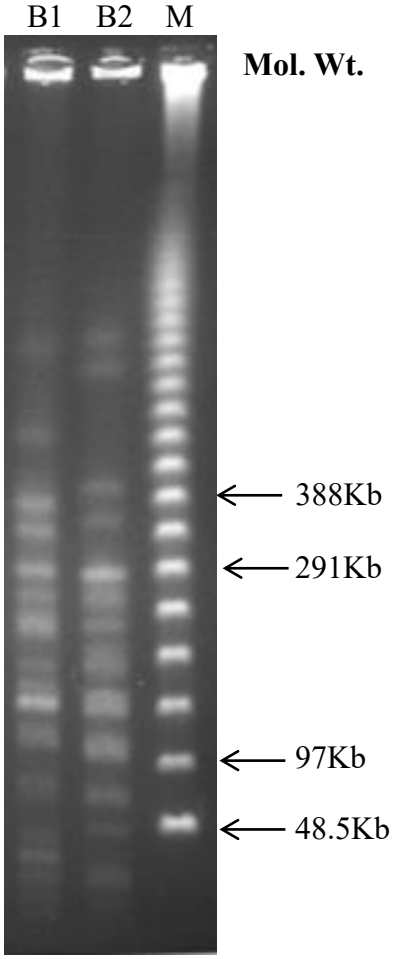

Fig. 30: PFGE analysis of *P. aeruginosa* strains isolated from robotic pool cleaners after genomic *Spe*I digests. Lanes: M (λ DNA marker) with the sizes at right in Kb, PFGE patterns B1 (*P. aeruginosa* from Brand 1), B2 (*P. aeruginosa* from Brand 2)

3.11.2 Subsequent *P. aeruginosa* findings in robotic pool cleaners

A summary of the results obtained during the sampling of 18 more robotic pool cleaners from different indoor swimming pools is presented in Table 14. From the 18 robotic pool cleaners sampled, 5 cleaning devices were the same brand as Brand 1, 4 cleaning devices were the same brand as Brand 2 and the remaining 11 cleaning devices belonged to different brands, most of which had a similar structure and mode to Brand 1 and 2. Thus, we can conclude that all cleaning devices sampled in this study had similar structure and similar mode.

After gathering the necessary information on the use and maintenance after use of the robotic pool cleaners, all pool attendants agreed that after use of the robotic pool cleaners, they were washed with water and left to dry until the next use. In the case of Brand 1 or brands similar to this one, in most cases the filter bag was washed in the washing machine at 95°C after its use. The curious thing is that nobody uses disinfectants to clean the robotic pool cleaners, arguing that they follow manufacturer´s recommendations. And the only article found related to cleaning devices (Prassler *et al.*, 2000) mentions just characteristics of the robotic pool cleaners, but until today´s date has not been found any scientific article on the need for disinfection of cleaning devices in swimming pools.

It should be noted, that we found several commonalities in all robotic pool cleaners that were contaminated with *P. aeruginosa*. The first thing in common was that at the time of sampling, all of them were still wet. And the second thing was that all robotic pool cleaners were used at least three times per week, so it was not possible that they could be totally dry. Organic contamination, constant humidity and a temperature range (25-42°C) in indoor swimming pools (Tsuji *et al.*, 1982), are the ideal conditions that take place in robotic pool cleaners to trigger bacterial build up and therefore, *P. aeruginosa* biofilm growth.

Table 14: Prevalence of *P. aeruginosa* in robotic pool cleaners

Samples	Brand	Sampling results	*P. aeruginosa* location	Times per week used	Robotic pool cleaner state
1	B3	-		1	dry
2	B1	-		3	dry
3	B4	+	FH, I, R	4	wet
4	B5	-		1	dry
5	B1	+	FB, I, R	7	wet
6	B6	-		1	dry
7	B7	+	R	3	wet
8	B1	-		3	dry
9	B8	-		1	dry
10	B2	-		1	dry
11	B2	-		3	wet
12	B1	-		3	dry
13	B9	+	FH, I	7	wet
14	B2	+	B	3	wet
15	B2	+	I, B, S	3	wet
16	B10	-		1	dry
17	B1	-		1	dry
18	B11	-		1	dry

(+ : *P. aeruginosa* finding, - : no *P. aeruginosa* finding)
(B: brush, F: filter, FH: filter holding, FB: filter bag, I: inside of the housing, R: roller, S: suction pipe)

3.12 *P. aeruginosa* biofilm in water systems of a hospital

All *P. aeruginosa* strains founded in the samples were identified by MALDI-TOF MS obtaining a score $x \geq 2.0$, being an accurate identification at the species level.

In this study were taken a total of 186 samples, of which 17 were positive for *P. aeruginosa* (9%). Following the classification of multidrug-resistant gram-negative (MRGN) *P. aeruginosa* according to the Robert Koch Institut, in Table 15 are represented the two types of multidrug-resistant for *P. aeruginosa*. The designation 3MRGN corresponds to the multidrug-resistant gram-negative *P. aeruginosa* with resistance against 3 of the 4 groups of antibiotics, and the designation 4MRGN corresponds to the MRGN *P. aeruginosa* with resistance against 4 of the 4 groups of antibiotics. *P. aeruginosa* possesses intrinsic resistance to many antibiotic classes and has the ability to develop resistance by mutations in different chromosomal loci or by horizontal acquisition of resistance genes carried on plasmids, transposons, or integrons. The frequent acquisition of antimicrobial resistance in *P. aeruginosa* limits the utility of antimicrobial susceptibility patterns as a tool in epidemiologic typing (Murray *et al.*, 2007).

The susceptibility testing results showed that only one 3MRGN *P. aeruginosa* was founded in the water sample from sink in Unit 3 (Table 16), being the remaining samples not multidrug-resistant. Unfortunately, it was not possible to find the focus of 4MRGN *P. aeruginosa*, so further studies should be conducted in the future to find the source of 4MRGN *P. aeruginosa* outbreak.

Table 15: Classification of MRGN *P. aeruginosa* on the basis of their phenotypic resistance characteristics (Institut, 2011)

Antibiotic group	Antibiotics	*Pseudomonas aeruginosa*	
		3MRGN	4MRGN
Penicillins	Amoxicilline/Clavulanate and/or Piperacilline/Tazobactam	only one	R
Cephalosporins	Ceftriaxone and/or Ceftazimide	of the four	R
Carbapenems	Imipenem and/or Meroponem	antibiotic groups	R
Fluoroquinolones	Ciprofloxacin and /or Moxifloxacin	susceptible	R

(R = resistant or intermediate susceptible)

Table 16: Results of susceptibility test performed on *P. aeruginosa* samples

Location	Sample collection	Susceptibility test of antibiotic groups				Conclusion
		PCs	Cs	CARs	FQs	
Unit 1, sink	smear	R	R	S	S	-
Unit 1, sink	water	R	S	S	S	-
Unit 1, shower	smear	R	S	S	S	-
Unit 1, toilet tank	water	R	S	S	S	-
Unit 2, sink	water	R	S	S	R	-
Unit 2, sink	smear	R	S	S	S	-
Unit 2, toilet tank	water	R	S	S	S	-
Unit 2, sink	smear	R	S	S	S	-
Unit 2, sink	water	R	S	S	S	-
Unit 2, toilet tank	smear	R	S	S	S	-
Unit 2, toilet tank	water	R	S	S	S	-
Unit 3, shower	smear	S	S	S	R	-
Unit 3, toilet tank	water	R	S	S	S	-
Unit 3, shower	smear	R	S	S	R	-
Unit 3, sink	water	R	S	R	R	3MRGN
Unit 3, shower	water	R	S	S	R	-
Unit 3, toilet tank	water	R	S	S	S	-

Abbrevations: PCs, Penicillins; Cs, Cephalosporins; CARs, Carbapenems; FQs, Fluoroquinolones
(R = resistant, S = susceptible), (-) No multidrug-resistant gram-negative *P.aeruginosa*

Furhtermore, drug resistance and tolerance greatly diminish the therapeutic potential of antibiotics against pathogens. Antibiotic tolerance by bacterial biofilms often leads to persistent infections, but its mechanisms are unclear. A study by Lin Chua et al. shows that their work provides insights on the mechanisms underlying the formation of antibiotic-tolerant populations in bacterial biofilms and indicates research avenues for designing more efficient treatments against biofilm-associated infections (Zhang *et al.*, 2015).

3.13 Results of the qualitative and quantitative suspension test for evaluation of bactericidal and/or fungicidal activity

3.13.1 Results of the qualitative suspension

Firstly, it was tested the substance 1, which would be used to clean and disinfect the DWST. As can be seen in Table 17, the substance 1 only worked for *S. aureus* and not for the rest of bacteria, where after 30 and 60 min was still bacterial growth.

Table 17: Qualitative suspension test for evaluation of bactericidal and fungicidal activity of the substance 1

Bacteria	Colony count (CFU/ml)	200g/l sub.1, Time (min)			
		5	15	30	60
Pseudomonas aeruginosa	$6.2*10^7$	+	+	+	+
Staphyloccocus aureus	$5.4*10^8$	-	-	-	-
Enterococcus hirae	$1.7*10^8$	+	+	+	+
Escherichia coli	$1.8*10^8$	+	+	+	+
Candida albicans	$6.1*10^6$	+	+	+	+

(+) growth, (-) no growth

For this reason, it was decided to use another substance in addition with substance 1. Thus, different substances were tested to find the most suitable for this purpose. A total of six substances were examined and for each substance different concentrations (from 0.1% to 16%) were tested. After many experiments, substance 2 with the concentration 1.5% was the appropriate substance for mixing with substance 1 to disinfect DWST, since after 30 min there was neither bacterial growth nor growth of *C. albicans* (Table 18).

Table 18: Qualitative suspension test for evaluation of bactericidal and fungicidal activity of the substance 1 + 1.5% substance 2

Bacteria	Colony count (CFU/ml)	200g/L sub. 1+1.5% sub.2,	Time (min)
		1	30
Pseudomonas aeruginosa	$5.1*10^8$	-	-
Staphyloccocus aureus	$5.7*10^8$	-	-
Enterococcus hirae	$1.5*10^8$	-	-
Escherichia coli	$3.8*10^8$	-	-
Candida albicans	$2.3*10^7$	+	-

(+) growth, (-) no growth

3.13.2 Results of the quantitative suspension

For the validity of the cleaning disinfectant must take place a reduction of at least 10^5 CFU/ml (for bacteria) and a reduction of at least 10^4 CFU/ml (for yeast and mould fungus) in a time not exceeding 30 min. As organic load was used 0.03 % bovine serum albumin (for low organic load) and the neutralization medium was the same than for the qualitative suspension test. 200g/l sub.1 + 1.5% sub.2 showed a bactericidal effect (Table 19), not existing more bacterial growth after 10 min of exposure with the substance. But fungicidal activity was not demonstrated for *C. albicans* (Table 20), being fungicidal reduction less than 10^4 CFU/ml. Therefore, it was not necessary to test the fungicidal activity for *A. niger*.

Table 19: Quantitative suspension test for evaluation of bactericidal activity of the 200g/l sub.1 + 1.5% sub.2

Bacteria	Colony count (CFU/ml)	200g/L sub.1+1.5% sub.2, Time (min)			
		1	2	5	10
Pseudomonas aeruginosa	$2.9*10^8$	0	0	0	0
Staphyloccocus aureus	$1.5*10^8$	$4.5*10^3$	28	4	0
Enterococcus hirae	$1.6*10^8$	$1.2*10^3$	2	0	0
Escherichia coli	$4.7*10^8$	8	4	4	0

Table 20: Quantitative suspension test for evaluation of fungicidal activity of the 200g/l sub.1 + 1.5% sub.2

Fungi	Colony count (CFU/ml)	200g/L sub.1+1.5% sub.2, Time (min)			
		5	10	15	30
Candida albicans	$1.5*10^7$	$6.5*10^4$	$1.5*10^4$	$9.6*10^3$	$6.2*10^3$

Since 200g/l sub.1 + 1.5% sub.2 did not show fungicidal activity, it was decided to increase the concentration of substance 2 to 2%, showing a bactericidal effect (Table 21) and a fungicidal activity, but only for *C. albicans* (Table 22).

Table 21: Quantitative suspension test for evaluation of bactericidal activity of the 200g/l sub.1 + 2% sub.2

Bacteria	Colony count (CFU/ml)	200g/L sub.1+2% sub.2, Time (min)			
		1	2	5	10
Pseudomonas aeruginosa	$4.7*10^8$	0	0	0	0
Staphyloccocus aureus	$1.9*10^8$	8	0	0	0
Enterococcus hirae	$3*10^8$	0	0	0	0
Escherichia coli	$3.1*10^8$	0	0	0	0

Table 22: Quantitative suspension test for evaluation of fungicidal activity of the 200g/l sub.1+2% sub.2

Fungi	Colony count (CFU/ml)	200g/L sub.1+2% sub.2, Time (min)			
		5	10	15	30
Candida albicans	$2.6*10^7$	94	0	0	0
Aspergillus niger	$2.2*10^6$	$5.6*10^5$	$6.6*10^5$	$6.2*10^5$	$4.9*10^5$

Finally, three controls were performed for the both substances to confirm that the test procedures were correct: control A (control of the experimental conditions), control B (control of the neutralization medium) and control C (process validation). To verify that controls gave good results, values of the controls A, B and C had to be higher than half the test-organism suspension for validation. Controls carried out for both substances were correct, as seen in Table 23, Table 24 and Table 25.

Table 23: Quantitative suspension test for evaluation of bactericidal activity of the 200g/l sub.1 + 1.5% sub.2 with the three controls

	Preparations for validation				Test method	
Test-organism	Test-organism Suspension for validation	Experimental conditions „A“	Non-toxicity of the neutralization meidum “B”	Inactivation by dilution neutralization method “C”	Test-organism suspension for the test	Test concentration 16.7% pH 6.7 Residence time 10 min.
Staphylococcus aureus ATCC 6538	V_c=70/84 N_v=7.7*10^3 N_{v0}=7.7* 10	V_c=46/47 A =46.5	V_c=49/52 B = 50.5	V_c=38/40 C = 39	N= 1.5*10^{10} N_0=1.5*10^8	V_c=0/0 N_a<10^3 R >1.5*10^5 %R >99.999
Pseudomonas aeruginosa ATCC 15442	V_c=59/61 N_v=6*10^3 N_{v0}=6*10	V_c=40/41 A =40.5	V_c=43/50 B =46.5	V_c=43/47 C =45	N= 2.9*10^{10} N_0=2.9*10^8	V_c=0/0 N_a<10^3 R >2.9*10^5 %R >99.999
Enterococcus hirae ATCC 10541	V_c=94/98 N_v=9.6*10^3 N_{v0}=9.6*10	V_c=78/87 A =82.5	V_c=85/89 B =87	V_c=78/88 C =83	N= 1.6*10^{10} N_0=1.6*10^8	V_c=0/0 N_a<10^3 R >1.6*10^5 %R >99.999
Escherichia coli DSM 682	V_c=90/90 N_v=9*10^3 N_{v0}=9*10	V_c=54/60 A =57	V_c=68/72 B =70	V_c=54/56 C =55	N= 4.7*10^{10} N_0=4.7*10^8	V_c=0/0 N_a<10^3 R >4.7*10^5 %R >99.999

N	Viable count (CFU) per ml test organism suspension	V_c	Viable count
N_0	Viable count (CFU) per ml test mixture at the beginning of residence time (t = 0), N_0 = N/10, product concentrate: N_0 = N/100	A	Viable count (CFU) per ml control preparation for experimental conditions
		B	Viable count (CFU) per ml control preparation for non-toxicity of the neutralization medium
N_a	Viable count (CFU) per ml test mixture at the end of residence time, before neutralization	C	Viable count (CFU) per ml control preparation for inactivation
N_v	Viable count (CFU) per ml test organism suspension for validation	R	Reduction of viable count, R = N_0/N_a
N_{v0}	Viable count (CFU) per ml in “A”, “B” and “C” at zero time (t = 0), N_{V0} = N_V/10, product concentrate: N_{V0} = N_V/100	%R	Percent reduction: %R = 100 - (N_a*100/N_0)

Table 24: Quantitative suspension test for evaluation of bactericidal activity of the 200g/l sub.1+2% sub.2 with the three controls

	Preparation for validation			Test method		
Test-organisms	Test-organism Suspension for validation	Experimental conditions „A“	Non-toxicity of the neutralization medium “B”	Inactivation by dilution neutralizytion method „C“	Test-organism suspension for the test	Test concentration 16.7% pH 6.7 Residence time 2 min.
Staphylococcus aureus ATCC 6538	V_c=70/84 N_v=7.7*10^3 N_{v0}=7.7* 10	Vc=50/51 A =50.5	Vc=50/45 B = 47.5	Vc=39/40 C = 39.5	N= 1.9*10^{10} N0=1.9*10^8	Vc=0/0 Na<10^3 R >1.9*10^5 %R >99.999
Pseudomonas aeruginosa ATCC 15442	V_c=59/61 N_v=6*10^3 N_{v0}=6*10	Vc=38/39 A =38.5	Vc=46/34 B =40	Vc=34/31 C =32.5	N= 4.7*10^{10} N0=4.7*10^8	Vc=0/0 Na<10^3 R >4.7*10^5 %R >99.999
Enterococcus hirae ATCC 10541	Vc=94/98 N_v=9.6*10^3 N_{v0}=9.6*10	Vc=88/76 A =82	Vc=92/90 B =91	Vc=91/87 C =89	N= 3.0*10^{10} N0=3.0*10^8	Vc=0/0 Na<10^3 R >3.0*10^5 %R >99.999
Escherichia coli DSM 682	V_c=90/90 N_v=9*10^3 N_{v0}=9*10	Vc=86/62 A =74	Vc=75/65 B =70	Vc=65/64 C =64.5	N= 3.1*10^{10} N0=3.1*10^8	Vc=0/0 Na<10^3 R >3.1*10^5 %R >99.999

Table 25: Quantitative suspension test for evaluation of fungicidal activity of the 200g/l sub.1+2% sub.2 with the three controls

	Preparations for validation			Test method		
Test-organisms	Test-organism Suspension for validation	Experimental conditions „A“	Non-toxicity of the neutralization medium “B”	Inactivation by dilution neutralizytion method „C“	Test-organism suspension for the test	Test concentration 16.7% pH 6.7 Residence time 10 min.
Candida albicans ATCC 10231	V_c=260/270 N_v=2.65*10^4 N_{v0}=2.65* 10^2	V_c=230/240 A =235	V_c=250/230 B = 240	V_c=210/200 C = 205	N= 2.6*10^9 N_0=2.6*10^7	V_c=0/0 N_a<10^3 R >2.6*10^4 %R >99.998

3.13.3 Cleaning of iron-soiling plate and manganese-soiling plate

Once it was confirmed the disinfectant activity, it was also tested the effectiveness of both disinfectants to remove soiled substrates with iron and manganese (Fig. 31). Both cleaning disinfectants required about 2 min to clean the manganese-soiling, whereas for iron-soiling took approximately 25 min. So both disinfectants are suitable for removing iron- and manganese-soiling.

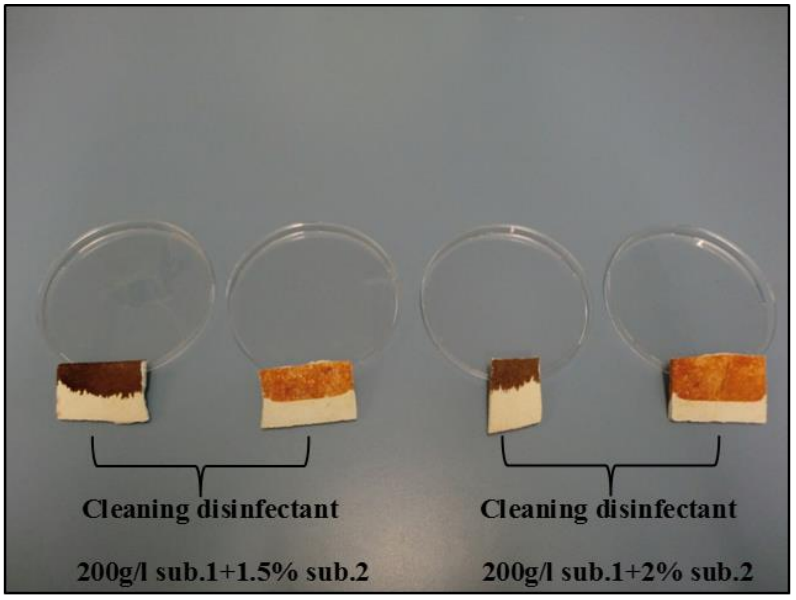

Fig. 31: Iron- and manganese-soiling plates cleaning by both cleaning disinfectants

3.13.3.1 TOC values of the cleaning disinfectants

According to the technical rule DVGW 319 (Reinigungsmittel für Trinkwasserbehälter; Einsatz, Prüfung und Beurteilung), positive control must have TOC values higher than 80 mg/l (+ 0.2 mg/l), negative control must have TOC values lower than 1mg/l (+ 0.2 mg/l) and cleaning disinfectants were accepted as harmless when the TOC values were not higher than 3mg/l (+0,6mg/l). And in this way, could be confirmed that cleaning disinfectants were harmless for their use in DWST, since TOC values for both cleaning disinfectants were lower than 3 mg/l (Table 26). A standard of 10 mg TOC was measured to check the validity of the measurement.

Table 26: TOC values of the cleaning disinfectants

	TOC (mg C/l)
Cleaning disinfectant A	1.62
Cleaning disinfectant B	2.63
Positive control	> 80
Negative control	0.78
Standard 10 mg TOC/ 10 mg IC	10.69

3.14 Physical-chemical methods of disinfection against *P. aeruginosa* biofilm on water surfaces

3.14.1 Desiccation method

Disinfection of biofilms in water containing systems may be challenging. Depending on the specific strain of *P. aeruginosa*, the structure of the biofilm may provide an effective shelter against the attack of chemical agents. However, biofilms need at least certain moisture in their environment and therefore may react sensitive to drying out.

For example, conservation by drying has been and still is a common method for food preservation. Nevertheless, reliable data on sensitivity of *P. aeruginosa* to dryness are limited. Whilst certain bacterial strains are highly sensitive to desiccation stress, other bacteria may be conserved by freeze-drying and may recover as soon as conditions of living recover.

Usually, viability of cells may be detected by cultivation and plate count methods. However, desiccation stress may lead to the formation of a special survival form of microbes known as VBNC (viable but nonculturable). Microbial cells may react to desiccation stress by loss of water and decrease of the cell size finally leading to VBNC. As VBNC bacteria are not detectable by cultivation methods, other means of detection are necessary.

In this section, desiccation of *P. aeruginosa* biofilms grown on glass slides as described before was analyzed.

Desiccation experiments were performed using a desiccator and sodium sulphate (Na_2SO_4) as desiccant. Samples were taken by scraping of the biofilm and resuspension of the sample in 3 ml of sterilized WSH.

To measure the number of bacterial cells, all samples were at first analyzed using conventional cultivation methods on Petri dishes (bacterial count), and additionally viability of cells was checked using a commercial test for ATP generation based on measurement of

luciferase. ATP activity was measured using the BacTiter-Glo assay (Promega, USA). ATP concentration in cells will rapidly decay after cell death. However, living cells in VNBC state will still show high ATP levels. Therefore, comparison of the results of the cultivation method and simultaneously measured ATP concentration will provide information of the reaction of *P. aeruginosa* to desiccation stress.

Table 27: Variation of colony count and ATP activity under mild desiccation conditions (desiccator/Na_2SO_4)

Hours	ATP activity	PA/ml
0	1,56E+05	4,00E+06
1	1,22E+05	1,15E+08
2	1,59E+05	9,50E+06
3	1,14E+05	8,13E+07
6	6,30E+04	0,00E+00
7	7,18E+04	0,00E+00
48	7,88E+04	0,00E+00

As expected and observed in Table 27, colony count drops from an initial concentration of $4*10^6$ per mL to "not detectable" within 6 hours. Although colony count of the biofilms on the glass slides shows a wide variety, there is a clear downwards tendency. After 6 hours, there are no more living cells detectable by cultivation methods and analysis performed after 7 and 48 hours of drying shows no recovery.

On the other hand, ATP activity is detectable in all samples, even after 48 hours of desiccation. Samples taken after 6, 7, and 48 hours still show activity, although no more organisms are cultivatable.

3.14.2 Measurement of catalytic activity of TiO_2-coated microscope glass slides

3. 14.2.1 Degradation of methylene blue

When the photoactive system, the TiO_2-coated microscope glass slides were illuminated by UV-A light, it was observed discoloration of the dye molecule methylene blue (MB). It has been proposed that the oxidation of surface adsorbed water and the reduction of O_2 by the illuminate semiconductor lead to the formation of hydroxyl and hydroperoxy radicals, respectively (Martin *et al.*, 1995). These radicals are mobile along the surface of the

semiconductor and potentially able to oxidize organic compounds such as MB at or near to the surface (Tschirch *et al.*, 2008). Therefore, those samples where MB had a lower absorbance at 660 nm, it meant they were more active than those samples where MB had a higher absorbance at 660 nm.

In samples made by reactive pulse magnetron sputtering we observed in Fig. 32, that when the samples were made by magnetic assembly up (MO), they appeared to be more active than by assembly down (MU). Furthermore, it was noticed that with increasing the thicknesses of the TiO2 layer, the catalytic activity was higher.

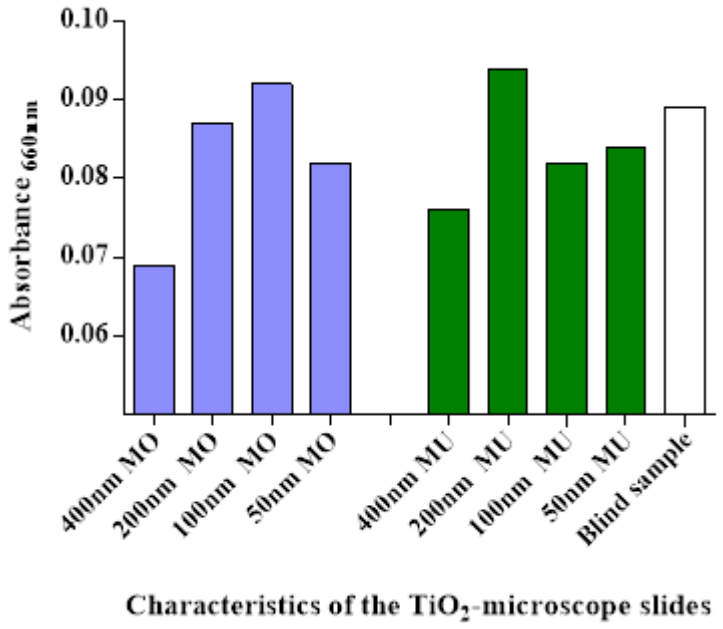

Fig. 32: TiO2-coated microscope glass slides made by reactive pulse magnetron sputtering

In samples made by electron beam evaporation we observed in Fig. 33 that when the samples were made by evaporation material Ti_3O_5, they were more active than by evaporation material TiO_2, being also more active when the thickness layer of the materials was thicker.

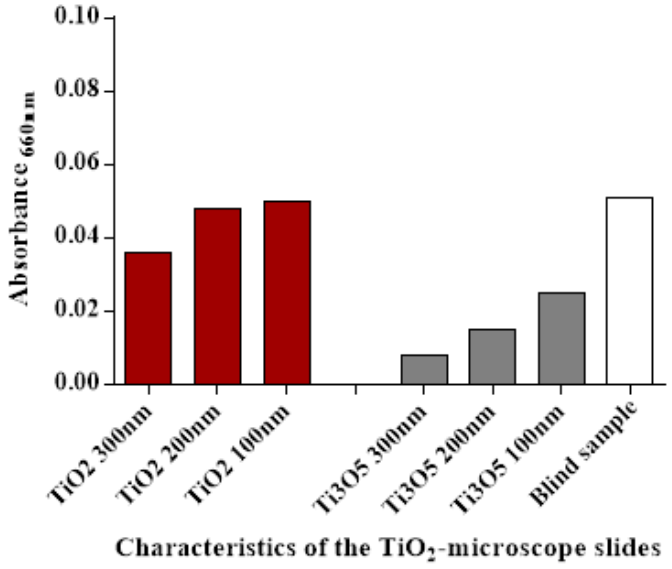

Fig. 33: TiO_2-coated microscope glass slides made by electron beam evaporation

3.14.2.2 Total mineralization method based on the formation of CO_2

This process consists of a total mineralization of glucose, where the photocatalytic activity of the samples is reflected in the carbon conversion of glucose to CO_2. Hence, those samples where the CO_2 concentrations were higher, it meant they were more active than samples where the CO_2 concentrations were lower. In samples made by reactive pulse magnetron sputtering we observed in

Fig. 34 that with increasing the thicknesses of the TiO_2 layer, the catalytic activity was higher. The sample made by magnetic assembly up (MO) with a layer thicknesses of 400 nm showed to be the most active.

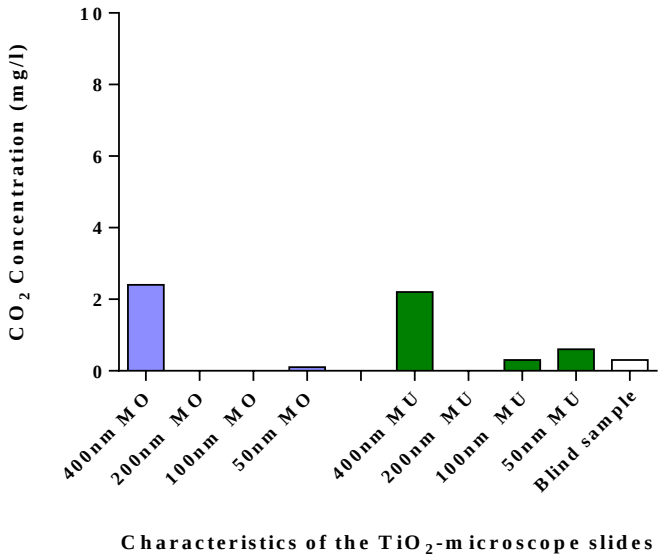

Fig. 34: TiO_2-coated microscope glass slides made by reactive pulse magnetron sputtering

In samples made by electron beam evaporation we observed in Fig. 35 that when samples were made by evaporation material Ti_3O_5, the photocatalytic activity was higher than by evaporation material TiO_2, being also more active when the thickness layer of the materials was thicker.

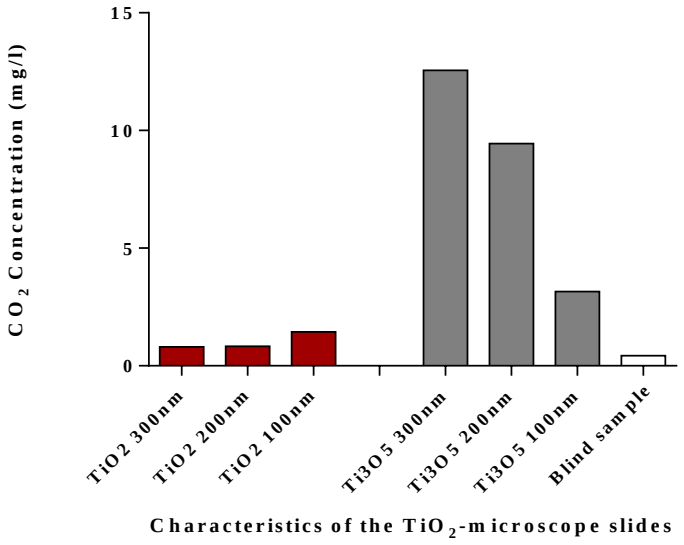

Fig. 35: TiO_2-coated microscope glass slides made by electron beam evaporation

Correlation between both methods was calculated, noting that values of the correlation coefficients for both processes were optimal, with an r2 = 0.75 (Fig. 36) by reactive pulse magnetron sputtering and with an r2 = 0.81 (Fig. 37) by electron beam evaporation. Thus, both methods are shown to be suitable for measuring the catalytic activity of TiO2-coated microscope glass slides.

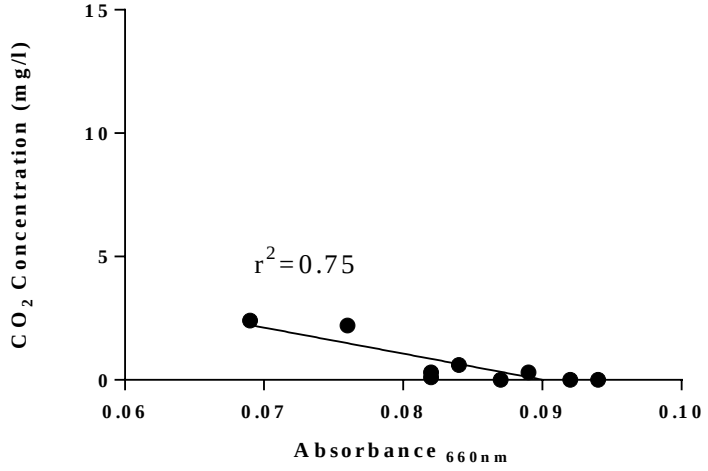

Fig. 36: Correlation between MB method and total mineralization method in reactive pulse magnetron sputtering process

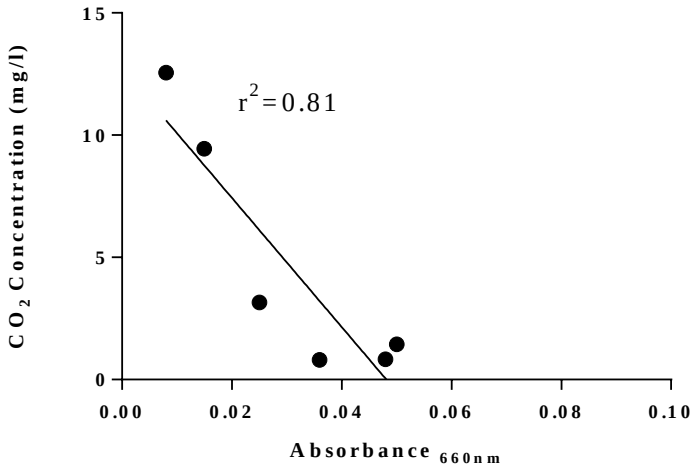

Fig. 37: Correlation between MB method and total mineralization in electron beam evaporation process

3.14.3 Biofilm production on the different TiO_2-coated microscope glass slides

3.14.3.1 Reactive pulse magnetron sputtering process

The conclusion we can draw from these results is that the sample, which was made by magnetic assembly up (MO) and has a layer thicknesses of 400 nm, has less bacterial growth than the other samples (Fig. 38), decreasing biofilm growth by 2 log CFU/ml (compared with blind sample). Furthermore, the bacterial viability of biofilm was lower in this sample than in the others (Fig. 39).

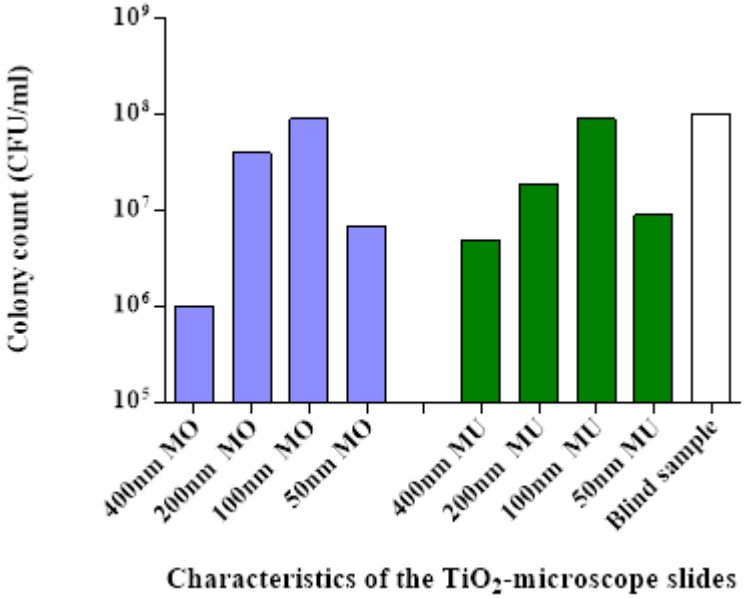

Fig. 38: Quantification of biofilm growing on TiO2-coated microscope glass slides

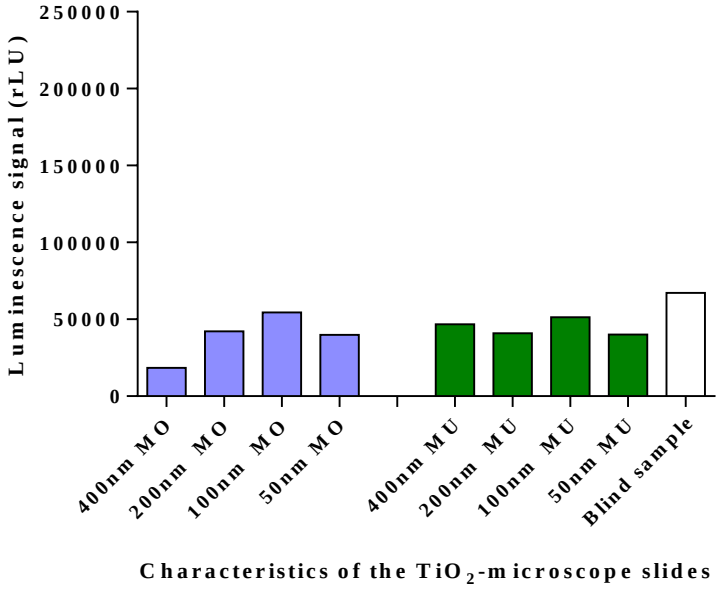

Fig. 39: Bacterial viability of biofilm growing on TiO_2-coated microscope glass slides

3.14.3.2 Electron beam evaporation process

The finding in these results was that the sample, which was made by evaporation material Ti_3O_5 and has a layer thickness of 400 nm, has less bacterial growth than the other samples (Fig. 40), decreasing biofilm growth by 0.5 log CFU/ml (compared with blind sample). Besides, the bacterial viability of biofilm was lower in this sample than in the others (Fig. 41).

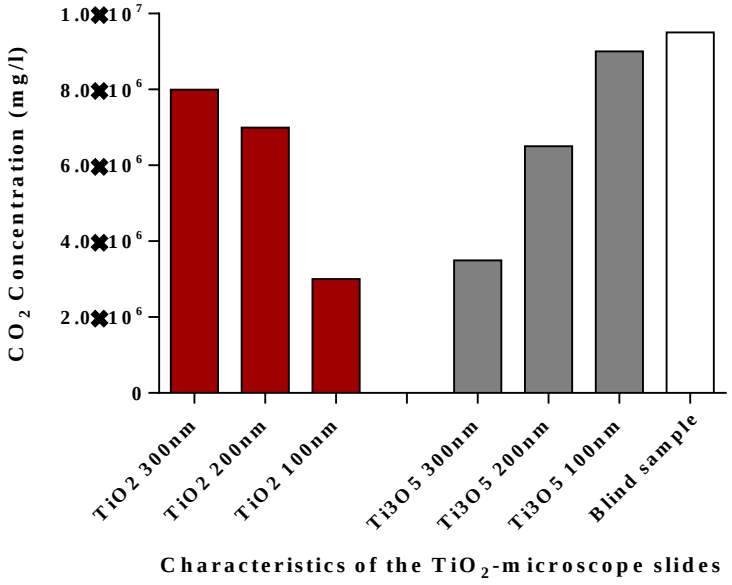

Fig. 40: Quantification of biofilm growing on TiO_2-coated microscope glass slides

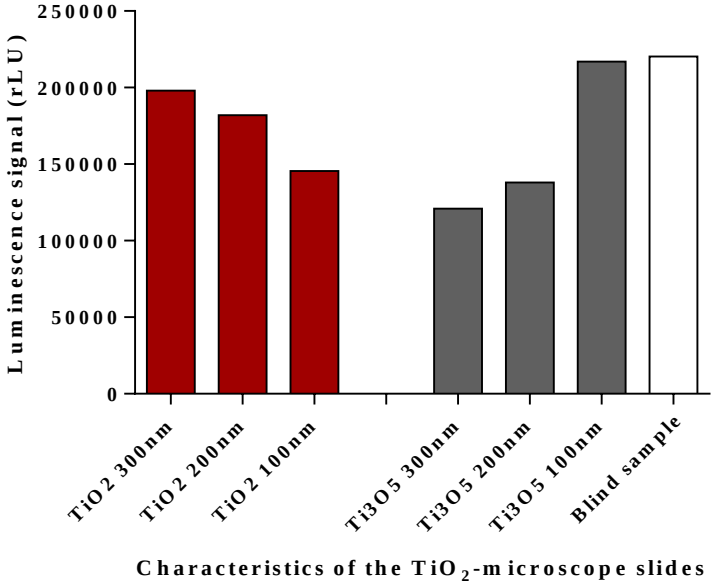

Fig. 41: Bacterial viability of biofilm growing on TiO2-coated microscope glass slides

Some published works on the photocatalytic activity of TiO_2 against *P. aeruginosa* biofilm showed that neither the photocatalytic treatment with TiO_2 film nor that with TiO_2 nanopowder had any effect on *P. aeruginosa* biofilms at all the interfaces investigated (Polo *et al.*, 2011), whereas our results showed that there is a reduction in the formation of biofilm. Our experiments showed that biofilm seems to be influenced by TiO_2 film thickness and by TiO_2 sputtering methods. TiO_2 coatings may provide another tool for the prevention of biofilm growth in water systems, so further works must be done on the subject in order to have a better understanding of the impact of photocatalytic treatment with TiO_2 film on biofilms.

3.15 Results of clogging analysis

3.15.1 Well screen observations

In Fig. 42 a sequence of pictures of four different pieces taken at different times after the beginning of the experiment is shown. It can be seen that the pieces were gradually covered by orange-brown fine viscous sediments, with the consequent clogging of the piece holes. In the column experiment, after 80 days of continuous injection without any backwash, the grid openings were still open but some of them have reduced their open area.

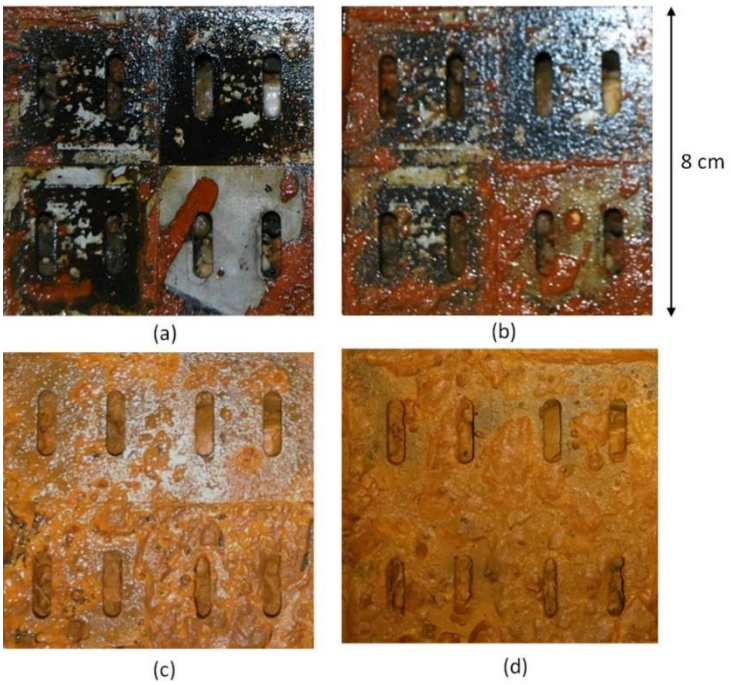

Fig. 42: Evolution of the column well screen clogging after 1(a), 20(b), 29(c) and 73(d) days

3.15.2 Biofilm growth evolution determination

Biofilm formation is a complex process depending on the microbial species, its communication and motility skills, the specialties of the surface where the biofilm will be attached, and, of course, the quality as well as chemical and physical properties of the surrounding aquatic environment. On a surface such as metal, biofilms allow for a variety of microorganisms with differing redox potential requirements to reside in close proximity and metal (Else *et al.*, 2003). In this way, biocorrosion can occur during biofilm formation, as was in the case of metallic pieces of the well screen. Biofilms are composed primarily of microbial cells and EPS. EPS may account for 50% to 90% of the total organic carbon of biofilms (Flemming, 2000) and can be considered the primary matrix material of the biofilm. For this reason was carried out a study of the concentration of EPS to be able to analyze the phases of biofilm formation on the well screen. Fig. 43 depicts EPS content related to the five stages of biofilm formation:

1. Attachment: during the first ten days EPS concentrations of the order of 1µg glucose-eq/cm2 were observed, corresponding to the time at which planktonic bacteria adhere to the well screen surface. Previous researchs shows thatsingle bacteria will reach the surface through the liquid phase usually by swimming. Single bacteria will attach to surfaces using flagella and other surface appendages (Kearns, 2010). This attachment is generally reversible and it is largely mediated by van der Waals forces. However, early stages of

biofilm development will depend on the specific strain. In nature biofilms, other eukaryotic organisms interact with the biofilm, forming part of it, such as fungi, algae, yeasts, protozoa and other microorganisms.

2. Adhesion: the following twenty days was observed as the concentration of EPS increased progressively, passing from 1 to 30µg glucose-eq/cm2 in about 20 days. As observed by other researchers (Monroe, 2007), during the second stage the bacteria will slowly but tightly adhere to the surface via pili, proteins, polysaccharides and fimbriae. Filamentous fungi will carry out deposition of spores or other propagules such as hyphal fragments or sporangia. Diatoms will attach to the substratum by the production of mucilage, which will encapsulate the cells.

3. Proliferation: from day 30 to day 50 it was observed a concentration of EPS around 45µg glucose-eq/cm2, corresponding with a subsequent step characterized mainly by the proliferation and production of extracellular polymeric substances (EPS). During this stage, cells lose their flagella-driven motility and the whole system turns to be immobilized. EPS are not unique to bacteria; some of the most abundant EPS producers are microalgae (in particular, diatoms). Fungi (yeasts and molds) also produce EPS (Flemming and Wingender, 2010).

4. Biofilm maturation: this phase is the most elongated in time, approximately 100 days, and the maximum EPS formation occurs during the maturation phase, reaching a maximum value of 60 µg glucose-eq/cm2. During this step microorganisms continue to proliferate and will excrete larger amounts of hydrated EPS consisting of polysaccharides, proteins, nucleic acids and lipids, providing stability to the biofilm as a whole and additional shelter to individual microorganisms (Conrad, 2012).

5. Release or detachment: during this last stage the EPS concentration decreased progressively, going from 60 to 12µg glucose-eq/cm2 in about 60 days. This phase corresponds to the moment when motile cells may disperse from the film; by diverse mechanisms. Cells from the biofilm will attach at other places and will promulgate the spreading of the film (Imam *et al.*, 2011). Dispersal of fungi involves spore dispersal or release of biofilm fragments.

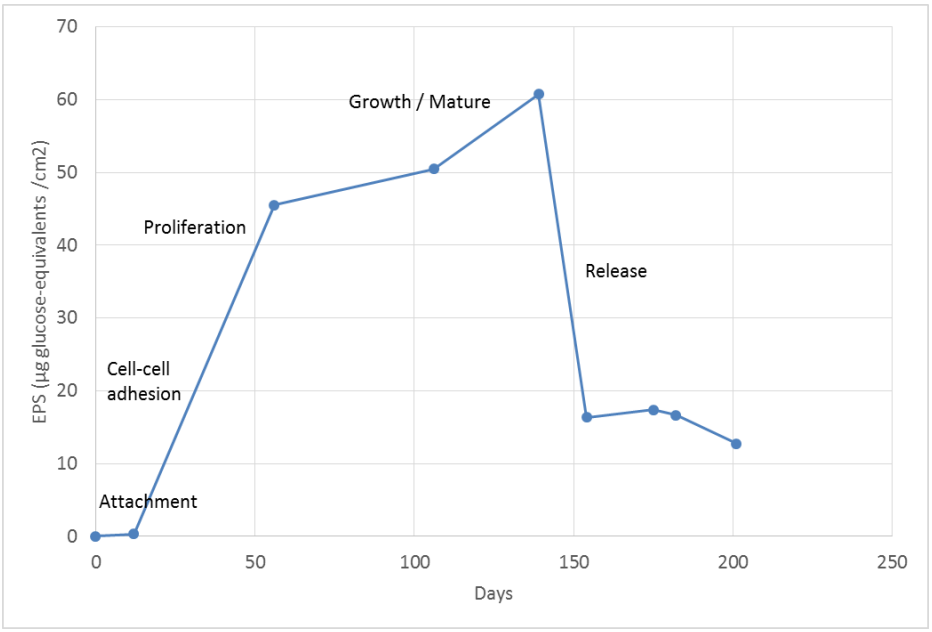

Fig. 43: Biofilm content evolution by the EPS analysis. Data plotted in dots and lines corresponds to the EPS quantified in the column experiment. Numbers from (1) to (5) and draws are extracted from (Monroe, 2007) to better illustrate biofilm evolution

3.15.3 SEM observations and EDX microanalysis

The solid-liquid interface between the surface (well screen) and aqueous medium (SFSW) provides an ideal environment for the attachment and growth of microorganisms. Fig. 44a corresponds to the most frequent image observed during SEM observations, which shows a highly developed biofilm with a dense matrix of EPS. EDX revealed an elemental composition of these irregular pellet formations of carbon, oxygen and hydrogen, corresponding to organic substances but also revealed the presence of iron, calcium, magnesium, potassium, silica and aluminium corresponding the main fraction of inorganic material.

SEM examination also revealed a complex morphological diversity composed of diverse microorganisms such as bacteria, algae and fungi, and their exopolymers, diatom skeletons, detritus and other biomatarial build-up (Fig. 44a-b). Organic filaments closely resembling fungal hyphae were observed through the whole piece surface, together with branching filamentous structure of fungi, and a sporangium, enclosure in which spores (Fig. 44c). Furthermore, siliceous diatom skeletons of diverse shapes and sizes were occasionally observed across the examined piece surface (Fig. 44d).

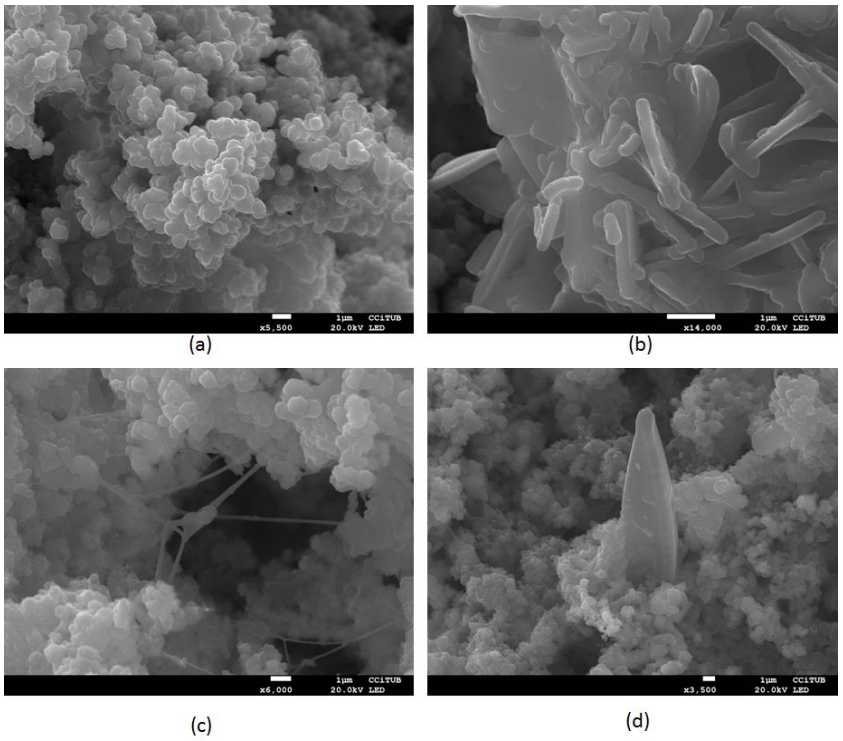

Fig. 44: SEM images of the biofilm. (a) Bacteria covered by the biofilm matrix. SEM image at 17,000x magnification (b) Bacilli present on the surface (14,000x magnification) (c) Filamentous fungi present in the biofilm structure at 6,000x magnification (d) Front-side view of a diatom (3,500x magnification**)**

4. DISCUSSION

This research should identify current problems that exist with *P. aeruginosa* biofilm in different water systems, thereby to understand the need of a proper hygiene and disinfection. *P. aeruginosa* has become a major cause of nosocomial infections worldwide (about 10% of all such infections in most European Union hospitals) and a serious threat to Public Health (de Bentzmann and Plesiat, 2011). When swimming pools are not well treated and disinfected, *P. aeruginosa* can also cause important infections in swimmers such as otitis externa, dermatitis… One of the main objectives of this study is the implementation of methods for the identification, characterization and disinfection of *P. aeruginosa* in water systems.

4.1 Method for quantification of *P. aeruginosa* biofilm under laboratory conditions

Biofilm often exhibits resistance to typical cleaning, disinfection and antibiotic regimens, presenting new challenges for preventive measures in both hospital and manufacturing environments. Therefore, the study of biofilm formation is an important and struggle to minimize infections. Investigations of biofilms have caught the interest of many different groups of microbiologists, most likely because the significance of sessile life of bacteria has penetrated to several areas of microbiology, but also because the relevant technologies (microscopy combined with molecular techniques and tools) now permit very detailed studies of even the most complex communities (Heydorn *et al.*, 2000).

Although previous reports have discussed the measurement of biofilm through the TOC, the method that we used here has never been described before. TOC content gives an idea about the proportion of organic matter in a sample and can be interpreted as biological material. The main purpose of our research was to present and evaluate a method for quantitative *P. aeruginosa* biofilm analysis under laboratory conditions, with focus on mass density measurements. The idea is to use this method to quantify biofilm on actual surfaces where biofilm formation can occurs, such as swimming pools surfaces, pipes… Delahaye conducted a similar research in three water treatment plants (WTP) from Paris, where biofilm incubators were installed at the outlet of the WTPs to allow the biofilm formation and quantification in terms of fixed TOC (Delahaye *et al.*, 2006). Furthermore, results of biofilm measurements by TOC analyser were compared with observations of biofilm under CLSM. Thanks to observations by CLSM, we could observe a correlation between different stages of biofilm formation and TOC measurements in each of stages. In the initial stage

with an irreversible attachment, TOC concentration starts to grow. Then comes the stage of biofilm maturation, in which TOC concentration starts up and finally the dispersion stage, where TOC concentration remains constant.

In relation to research on biomolecules responsible for *P. aeruginosa* biofilm formation, we reached the following conclusions. According to our results, *P. aeruginosa* showed that when it was treated with $NaIO_4$ the OD_{570nm} was lower than when it was treated with proteinase K, but occurred during both treatments biofilm formation. Results indicate that polysaccharides may play a more important role than the proteins in the biofilm formation. A recent research has shown that the Psl polysaccharide of *P. aeruginosa* forms a matrix, which facilitates surface adherence and maintains biofilm architecture during a biofilm developmental cycle. Overall, data indicates that Psl is a key scaffolding component of the *P. aeruginosa* biofilm matrix, a property that likely plays a critical role in *P. aeruginosa* persistence. A better understanding of the biofilm matrix formation and ultra-structure may open up avenues for therapeutics of biofilm-related complications in medical, industrial, and environmental settings (Ma *et al.*, 2009). *P. aeruginosa* has become the model organism for proteobacterial biofilms, and much work has been done in order to identify consensus in biofilm formation and to formulate a general biofilm model (Stoodley *et al.*, 2002).

4.2 Identification of *P. aeruginosa* in different water systems

4.2.1 Biofilm samples collected in drinking water storage tanks

Virtually every water distribution system is prone to the formation of biofilms, regardless of the purity of the water, type of pipe material, or the presence of a disinfectant. Growth of bacteria on surfaces can occur in the distribution system or in household plumbing. It is reasonably well documented that the suspended bacterial counts observed in distribution systems are the result of biofilm cell detachment rather than growth of organisms in the water (Van Der Wende and Characklis, 1990). Produce and maintain high quality drinking water is a major challenge. The quality of water has to be controlled and monitored by drinking water suppliers during all stages of the treatment process from the water sources to the end of distribution systems. The water quality in storage tanks depends on their maintenance. Studies on water quality in distribution networks, as well as in drinking water storage tanks have been conducted in different countries around the world. A research about drinking water quality was performed in Czech Republic from 2006 to 2008. The results of the contamination degree discovered in the course of the project solution will serve as basic

data for a scale that should evaluate the degree of water tank pollution as well as for resulting corrective measures or optimisation of water tank cleaning (Ambrozova *et al.*, 2009). In Venezuela was carried out a study about bacteriological contamination of the drinking water distribution systems as well. The conclusion and the recommendation was that a maintenance program covering the complete system, including the storage tanks, must be established as a strategy for controlling bacteriological contamination. It was also necessary to control corrosion, level of nutrients and to perform adequate disinfection procedures; these measures must be applied by constantly trained personnel (De Sousa *et al.*, 2008). In Saudi Arabia was carried out a molecular identification and biofilm-forming ability of culturable aquatic bacteria in microbial biofilms formed in drinking water distribution networks. They concluded that most of isolated bacteria had ability to form biofilm at suboptimum temperature of growth. These results may provide basic information on formation of microbial biofilms and overcome the problem of deteriorating of water quality in the drinking-water distribution networks (Elhariry *et al.*, 2012). All studies come to the conclusion that the existence of bacterial contamination in water distributions networks and storage tanks is a current problem that we must solve in order to provide a good quality of drinking water. So it is necessary a properly maintenance and an adequate disinfecting of all surfaces.

The larger question is not whether biofilms or bacterial accumulations are present, but whether bacteria are associated with diseases. Biofilms in drinking water distribution systems are primarily composed of organisms typically found in the environment, and as such, are likely to be of limited health concern. Most of bacteria isolated in the four DWST during our research do not suppose a health risk to people, but the problem comes when these people are immunocompromised. Then these bacteria can become a risk to human health. *Brevibacillus parabrevis* is a Gram-positive aerobic spore-forming bacillus commonly found in soil, air, water and decaying matter. It is rarely associated with infectious diseases (Pearce, 2005). *Micrococcus luteus* can be found in many places such as the human skin, water, dust and soil. It is generally thought of as harmless bacterium, but there have been rare cases of infections in people with compromised immune systems (Madigan and Martinko, 2005). *Kocuria rhizophila* can be isolated from a wide variety of natural sources including mammalian skin, soil, rhizosphere, fermented foods, clinical specimens, fresh water and marine sediments. *Bacillus subtilis* is commonly found in soil, more evidence suggests that *B. subtilis* is a normal gut commensal in humans and is only known to cause disease in severely immunocompromised patients (Oggioni *et al.*, 1998).

Staphylococcus epidermidis is part of human skin flora (commensal) and it is not usually pathogenic, patients with compromised immune systems are often at risk for developing an infection (Ziebuhr *et al.*, 2006). *Pseudomonas mendocina* is an environmental bacterium that can cause opportunistic nosocomial infections, such as infective endocarditis (Aragone *et al.*, 1992) although cases are very rare. *Pseudomonas oleovorans* has been isolated in water-oil emulsions. *Staphylococcus vitulinus* was originally isolated from food (beef, chicken, lamb, and other meats) and animals (mammals including horse, vole, and whale). *Staphylococcus warneri* has been found as part of the skin flora on humans and animals. *S. warneri* rarely causes disease, but may occasionally cause infection in patients whose immune system is compromised (Incani *et al.*, 2010). Aeromonas species cause opportunistic systemic disease in immunocompromised patients, diarrheal disease in otherwise healthy individuals, and wound infections. *Pseudomonas koreensis* is a rod bacterium isolated from farming soil in Korea (Kwon *et al.*, 2003). *Staphylococcus aureus* is frequently found in the human respiratory tract and on the skin. Although *S. aureus* is not always pathogenic, it is a common pathogen in skin, soft-tissue, catheter-related, bone, joint, pulmonary, and central nervous system infections (Bamberger and Boyd, 2005). *Acinetobacter sp.* is widely distributed in nature and part of the normal mammalian flora, but can cause severe primary infections in compromised hosts. *Pseudomonas stutzeri* is a soil bacterium first isolated from human spinal fluid and it is also an opportunistic pathogen in clinical settings, although infections are rare (Noble and Overman, 1994).

Throughout history there were many examples of infectious disease outbreaks whose origin was in the drinking water distributions. The study of the water network in London during the epidemic of 1854 led to John Snow to propose that cholera was transmitted by faecal-contaminated drinking water (Ward, 2008). In 1993 took place an outbreak of *Cryptosporidium* in Wisconsin, which was associated with drinking water contamination (Eisenberg *et al.*, 2005). The Walkerton Tragedy, in Canada in 2000 took place a waterborne outbreak, which resulted from entry of *E. coli* O157:H7 and *Campylobacter spp.* from neighbouring farms into the town water supply (Clark *et al.*, 2003).

It is important to note that materials of drinking water distributions (pipes, storage tanks…) have a considerably influence of fixed biomass. Type of material has a strong influence on bacterial regrowth in distribution systems. In our research it has become clear that the material where lower *P. aeruginosa* accumulation is polyethylene. These results could help drinking water producers to choose suitable materials for the walls in drinking water storage

tanks, which minimize the possibility of biofilm development, thereby improving the quality of distributed drinking water.

4.2.2 *P. aeruginosa* biofilm in swimming pools

Water and water security issues are emerging as one of the key global challenges for the 21st century, where the goal is to ensure safe water supplies that are pathogen and chemical free. While this is mostly viewed from the drinking water perspective, it also applies to recreational waters. This is particularly true given the significant role that recreational waters (e.g. pools, spa, and reservoirs) receive over 1 billion visits per year (Rice *et al.*, 2012). As already mentioned in the introduction, a large number of microorganisms can cause serious problems in the pool water. Among these microorganisms *P. aeruginosa* is one of the most frequently isolated opportunistic pathogens in pools and hot tubs, which causes a variety of illnesses when is associated with recreational swimming, but the most common manifestation is swimmer's ear and a variety of skin infections. It has also been associated with a range of other infections, including eye, urinary and respiratory tract infections (Mena and Gerba, 2009b). Another feature of *P. aeruginosa* which increases problems in swimming pools is their ability to form biofilms. The biofilm issue is important to understand since it impacts on sampling methods, where biofilm would be responsible for shedding planktonic bacteria into the water column, and can be present anywhere in the water system of the pool, including pool and pipe surfaces, fomites and filters (Rice *et al.*, 2012). During the last decades, some researchers focused upon the *P. aeruginosa* biofilms presence in swimming pools, being shown its accumulation in filters (Uhl and Hartmann, 2005), on shower floors (Leoni *et al.*, 1999), on pool carpets (Hopkins *et al.*, 1981) and even on inflatables (Tate *et al.*, 2003).

The findings of this study indicate that *P. aeruginosa* contamination is common in swimming pools, even where chlorine concentrations are well above recommended levels. Recommendations for adequate chlorine levels in Germany according to standard DIN 19643 are 0.3 to 0.6 mg/l for indoor swimming pools, measured as free chlorine. Results of our research confirm the importance of not only adequate disinfectant levels, but also constant monitoring and adjustment to ensure that hygienic measures are always maintained correctly.

Results of our research showed that it is possible the formation of *P. aeruginosa* biofilm in robotic pool cleaners that were always moist by frequent use. Cleaning devices from indoor swimming pools can fulfil the conditions required for the development of *P. aeruginosa* biofilm: poor hygiene, constant humidity and 29±1°C. It is necessary to focus on cleaning

and maintenance of robotic pool cleaners in indoor swimming pools to prevent *P. aeruginosa* biofilm growth.

4.2.3 *P. aeruginosa* biofilm in water systems of a hospital

P. aeruginosa can cause a wide range of infections, and is a leading cause of illness in immunocompromised individuals. In particular, it can be a serious pathogen in hospitals. It can cause endocarditis, osteomyelitis, pneumonia, urinary tract infections, gastrointestinal infections, meningitis, and is a leading cause of septicaemia. *P. aeruginosa* is also a major pathogen in burn and cystic fibrosis (CF) patients and causes a high mortality rate in both populations. Tap water appears to be a significant route of transmission in hospitals, from colonization of plumbing fixtures. It is still not clear if the colonization results from the water in the distribution system, or personnel use within the hospital. Infections and colonization can be significantly reduced by placement of filters on the water taps (Mena and Gerba, 2009b).

A serious problem is the emergence of MDR *P. aeruginosa* strains in hospitals (Fujino *et al.*, 2005; Sekiguchi *et al.*, 2007). Multidrug resistance in *P. aeruginosa* is steadily increasing also worldwide. Although definitions of multidrug resistance are variable, they often involve resistance to fluoroquinolones, expanded-spectrum cephalosporins, carbapenems, and aminoglycosides (Nordmann *et al.*, 2007). The biology of *Pseudomonas* means that it is liable to colonize any moist environment and waste outlets in sinks and showers have been implicated in previous hospital outbreaks, though recent concern in the UK at least is focused more on the risk from hospital tap water. The factors which reduce the chance of these organisms spreading back to clinical areas include regular flushing of sinks/toilets/sluices, cleaning of the accessible parts of outlets to reduce scale and biofilm, and a free-flowing system that can rapidly carry away waste water. Poorly designed sinks, with water flowing directly into the plughole, will lead to splashback from the U-bend, and have been previously implicated in MDR *P. aeruginosa* outbreaks. Toilet bowls with rims and dual flushing outlets are harder to clean, and thus pose a greater infection risk. Shower trays with inadequate drainage (which is more likely if the outlets are not cleaned regularly, or if the showers deliver too much water) will lead to pooling in the shower tray: this is likely to be a mixture of tap and stagnant shower-trap water (Breathnach *et al.*, 2012).

We reported an outbreak of 4MRGN *P. aeruginosa* infection occurring over three years involving 10 immunocompromised patients in three intensive-care units of a clinical hospital in south-west Germany. Several potential sources for MDR may exist in a hospital setting, including colonized patients, staff or the environment (Mudau *et al.*, 2013). The aim

of the outbreak investigation was to identify the source of the outbreak and associated risk factors in order to halt the spread of MDR *P. aeruginosa*. During the detailed examination of sites coming into consideration for the contamination, water used for personal hygiene was thought as a possible source of the outbreak. Of 186 water samples collected in sinks, shower and tank toilets of the three intensive-care units, *P. aeruginosa* was isolated in 17 samples. After identification of bacterial isolates with VITEK 2, in none of the samples was identified the 4MRGN *P. aeruginosa*. Instead a 3MRGN was identified in one of the sinks of one intensive-care unit and no multiresistant *P. aeruginosa* were identified in some sinks, showers and toilets tanks in the three intensive-care units. We concluded from our results that we have described an outbreak of 4MRGN, but we were unable to identify the source of the organism. It is necessary to take into account the abundance *P. aeruginosa* found in the water distribution systems, in both water samples and smear samples. So this suggests that biofilm formation exists in many parts of the water system. Besides the need for further research into the origin of 4MRGN *P. aeruginosa* outbreak, it is vital to perform good hygienic control of water facilities in hospitals. It also highlights the need to create capacity for surveillance and investigation of nosocomial outbreaks.

4.3 Disinfection methods

Regarding cleaning and disinfection of DWST, potable water storage tanks which are not properly maintained can be a potential source of contamination. The disinfection of drinking water using chemicals such as chlorine has successfully protected public health against waterborne diseases. However, chemical disinfection produces potentially toxic by-products whose presence is undesirable. The use of chemical disinfection can also causes problems with taste and odour leading to customer complaints and growth in sales of bottled water and water filters (Jackson *et al.*, 1999). As with any other materials or treatment chemicals, there is a need to ensure that no new contaminants of concern will be added to the final water as a consequence of the process. For this reason, it was developed a disinfectant for cleaning and disinfecting DWST according to European Norms DIN EN 1276:1997 and DIN EN 1650:2008, which are based on evaluation of bacterial and fungicidal activity of chemical disinfectants and antiseptics used in food, industrial, domestic and institutional areas.

Regarding photocatalytic oxidation with titanium dioxide, here are our conclusions.

On the one hand, degradation of MB in aqueous solutions has been re-examined for other researchers as a method to characterize the photocatalytic activity of transparent TiO_2

coatings, but fixing some important points (Tschirch *et al.*, 2008), that we also take into account during the experiments:

- MB is easily adsorbed at the TiO_2, but also at other surfaces in contact with the aqueous MB solutions.
- Aqueous solutions are decolorized with wavelengths below 350 nm and above 480 nm. In order to avoid any type of photolysis reaction of MB the wavelength for the excitation of the photocatalyst has to lie in the range λ = 350-480 nm.
- A standard protocol has to fix the initial MB concentration and the light intensities.
- In order to characterize the activity of photocatalytic coatings, reaction rates and photonic efficiencies should be calculated by using data obtained at the beginning of the degradation reaction.

On the other hand, for the total mineralization method based on the formation of CO_2, Wolfrum (Wolfrum *et al.*, 2002) used glucose as a test of the methodology since it could be quantitatively deposited on the TiO_2 film and would not evaporate during the test. The aim of this research was to demonstrate the total oxidation and the ability to account for the carbon content and to obtain a quantitative measure of the time scale for the oxidation of biological materials on a TiO_2 surface.

It was performed a correlation of the results obtained with the two different methods for the measurement of photocatalytic activity and we came to the conclusion that with both methods we gained similar results, good correlation coefficients were obtained.

Difference between initial and final concentrations of the bacteria at the end of the experiments is found to be significant. Growth and death of bacteria are noticed with respect to sputtering methods and to TiO_2 film thickness. For the two sputtering methods used, the best results for the measurement of photcatalytic activity of different slides and for the reduction of biofilm growth were obtained when the layer thickness were thicker. Electron beam evaporation method with Ti_3O_5 as evaporation material showed better results than reactive pulse magnetron sputtering process. Our results showed that the photocatalytic action that takes places on the biofilm depends on film thickness of coated surfaces and on sputtering methods.

The deactivation mechanism in the TiO_2 photocatalytic process against bacteria can be explained as follows. A first oxidative damage takes place on the cell wall when the TiO_2 photocatalytic nanoparticles make contact with intact cells. Although cell wall is damaged, the cells are still alive. The next oxidative damage takes place on the underlying cytoplasmic membrane after the eradication of cell wall protection. Photocatalytic action gradually

increases the cell permeability and subsequently allows the free efflux of intracellular contents to cause the cell death. Free TiO_2 nanoparticles may also contact membrane-damaged cells and then directly attack the intracellular components (Huang *et al.*, 2000). The self-defence and auto repair mechanisms for protecting the bacteria are more effective during the initial stage, since the active species generated begin to attack the membrane. At this stage, the active species are not sufficient to cause serious damage to the bacterial outer membrane. Later, a considerable enhancement in inactivation rate is achieved by massive generation of OH^- that rapidly overcomes the self-protection mechanisms of the bacteria and as a result the concentration of bacteria decreases with increasing time. In the next stage, inactivation of bacteria becomes slow. This is because metabolites released from the killed bacteria during the photocatalytic process form a screen to protect the remaining active bacteria (Vijay *et al.*, 2013)

The present results also showed that irradiation by UVA of TiO_2 surfaces are not enough to destroy bacteria effectively. Recent researches has shown that UV disinfection induces a VBNC state in *E. coli* and *P.* aeruginosa, so these results systematically revealed the potential health risks of UV disinfection and strongly suggest a combined disinfection strategy (Zhang *et al.*, 2015).

But TiO_2 coatings may provide another tool for the prevention of biofilm growth in water systems, so further works must be done on the subject in order to have a better understanding of the impact of photocatalytic treatment with TiO_2 film on biofilms. This research can bring great benefits in the disinfection of water surfaces. The first reason relies on fact that irradiation of coated surfaces can diminishes the biofilm growth on these surfaces. The second reason is built on the low costs and easiness in the application of the methodology proposed.

4.4 Clogging analysis

Bioclogging formation characterization by SEM photography and elemental components determination identified presence of carbon, oxygen and hydrogen corresponding to organic fraction and iron, calcium, magnesium, potassium, silica and aluminium corresponding to the main fraction of inorganic material. The pictures have shown some isolated bacillus and hifas, while most of the ubiquitous material observed with the microscope corresponds to biological mass aggregates, presumably EPS.

5. SUMMARY

The aim of this thesis was to detect and characterize *P. aeruginosa* biofilm formation in different water systems (bathroom fittings in a hospital, swimming pools and DWST), as well as methods of disinfection to prevent the presence of bacteria in DWST and biofilm formation in water systems. *P. aeruginosa* is an opportunistic pathogen that causes severe infections especially in immuno-compromised and hospitalized patients. It also causes other diseases associated with swimming pools, such as otitis externa, skin infection and other infections, including eye, urinary and respiratory tract infections.

Thanks to the research conducted during these four years, were identified some of the current problems existing with *P. aeruginosa* in water systems. Firstly, searching for the source of infection of some patients with a 4MRGN *P. aeruginosa* in several intensive care units of a hospital was found the presence of *P. aeruginosa* biofilm in bathroom fittings (sinks, showers and toilet tanks) and it was also identified the presence of a 3MRGN in one of the sinks. Secondly, trying to solve an ongoing problem of *P. aeruginosa* contamination in two public indoor swimming pools, after examining possible items as a source of the contamination, we found the presence of *P. aeruginosa* biofilm in cleaning devices, in robotic pool cleaners. This investigation was extended to other cleaning devices from other public indoor pools and results showed that those devices, which were used quite often (every two or three days), showed contamination with *P. aeruginosa*. Lastly, the research conducted in DWST did not show presence of *P. aeruginosa* on their surfaces, but it was identified biofilm formation of other bacterial species. Although these bacteria are not a risk to healthy people, the problems may occur when these bacteria come into contact with immunocompromised individuals.

Furthermore, it was developed a methodology for the quantification of *P. aeruginosa* biofilm under laboratory conditions by the TOC analyser. This methodology could be applied in the future to quantify biofilm formation on water surfaces.

For all these reasons listed above, it is required a good maintenance and hygiene of all water systems. Proper hygiene is ensured by appropriate disinfection methods. During this work, it was developed a disinfectant according to European Norm DIN EN 1276:1997 (for bacteria) and to European Norm DIN EN 1650:2008 (for fungi and yeasts), which in turn serve for cleaning and disinfection of DWST. Parallel it was investigated *P. aeruginosa* biofilm disinfection by photocatalytic oxidation in water systems in general. Results of our experiments showed a significant reduction of biofilm formation when *P. aeruginosa* grows on TiO_2 surfaces irradiated by UVA, but nevertheless still occurs biofilm formation. TiO_2

coatings may provide another tool for the prevention of biofilm growth in water systems, but it is important to continue research along these lines in order to improve these methods of disinfection for water systems.

ZUSAMMENFASSUNG

Das Ziel dieser Dissertation war es zum einen, die Biofilmbildung von P. aeruginosa in verschiedenen wasserführenden Systemen (z.B. Installationen in einem Krankenhaus, Schwimmbädern und Trinkwasserspeichern) zu detektieren und zu charakterisieren und zum anderen Desinfektionsmethoden zu untersuchen um Bakterien und Biofilme in Trinkwasserspeichern zu verhindern. P. aeruginosa ist ein opportunistischer Krankheitserreger, der insbesondere bei immunkompromittierten und hospitalisierten Patienten zu schweren Infektionen führen kann. Der Erreger kann daneben Infektionen verursachen, die in einem Zusammenhang mit Schwimmbädern stehen wie zum Beispiel Otitis externa, Haut- und weitere Infektionen wie Augen-, Harnwegs- und Atemwegsinfektionen.

Dank der Forschungsarbeiten, die vier Jahre in Anspruch genommen haben, konnten einige der derzeit bestehenden Probleme im Zusammenhang mit P. aeruginosa in wasserführenden Systemen identifiziert werden. Zunächst konnten bei der Suche nach Ursachen für Infektionen von Krankenhauspatienten in mehreren Intensivstationen durch 4-MRGN-P. aeruginosa die Trinkwasserinstallationen als durch P. aeruginosa kontaminiert identifiziert werden (Abflüsse, Duschen und Spülkästen), in einem der Abflüsse konnte ein 3-MRGN Pseudomonas aeruginosa identifiziert werden.

Daneben wurde ein hartnäckiges Problem in zwei Schwimmbädern gelöst, die durch Pseudomonas aeruginosa wiederholt kontaminiert waren. Nachdem mehrere in Frage kommende Gerätschaften untersucht wurden, konnten Pseudomonas aeruginosa Biofilme in Geräten festgestellt werden, die zur automatischen Beckenreinigung verwendet werden. Diese Untersuchungen wurden auf weitere Geräte aus anderen Öffentlichen Schwimmbädern ausgedehnt und die Ergebnisse zeigen, dass diese Geräte, die relativ häufig verwendet werden (jeden zweiten bis dritten Tag), häufig mit P. aeruginosa kontaminiert waren.

Durch die Untersuchungen, die direkt in Trinkwasserspeichern (Hochbehältern) durchgeführt wurden konnte in keinem Fall P. aeruginosa direkt auf den Oberflächen nachgewiesen werden, hier wurden jedoch Biofilme anderer Bakterienspecies identifiziert.

Obwohl diese Bakterien für gesunde Menschen kein Risiko darstellen, können Probleme entstehen, wenn immunkompromittierte Personen davon betroffen sind.

Im weiteren Verlauf der Arbeit wurde eine Methode entwickelt, mit der Biofilme unter Laborbedingungen durch Messung des TOC auf Oberflächen charakterisiert werden können. Diese Methode eignet sich besonders für Messungen auf wasserbenetzten Oberflächen.

Aus den oben genannten Gründen ist für alle Wassersysteme eine gute Wartung und Hygiene erforderlich. Gute Hygiene kann durch geeignete Desinfektionsmethoden gewährleistet werden. Im Rahmen dieser Arbeit wurde ein Desinfektionsmittel entwickelt das mit der Europäischen Norm DIN EN 1276:1997 (für Bakterien) und der DIN EN 1650:2008 (für Pilze und Hefen) konform ist, und das für die Reinigung und Desinfektion von Trinkwasserspeichern verwendet werden kann.

Parallel hierzu wurde das Verhalten von P. aeruginosa Biofilmen bei der Desinfektion durch photokatalytische Oxidaten in wasserführenden Systemen untersucht. Die Ergebnisse der durchgeführten Experimente zeigen eine signifikante Reduktion der Biofilmbildung wenn P. aeruginosa auf Titandioxid-beschichteten Oberflächen wächst, die mit UVA bestrahlt werden, aber dennoch kommt es zu einem gewissen reduziertem Wachstum. Beschichtungen auf Titandioxidbasis können eine weitere Möglichkeit zur Vorsorge gegen Biofilmwachstum in Wassersystemen darstellen, weitere Untersuchungen sind jedoch nötig um diese Desinfektionsmethoden für wasserführende Systeme zu verbessern.

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Publications

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Evaluation of Clogging during Sand-Filtered Surface Water Injection for Aquifer Storage and Recovery (ASR): Pilot Experiment in the Llobregat Delta (Barcelona, Spain)". Water, Volume 9, Issue 4 (April 2017).

Professional research presentations

11/2010 Poster presentation: Identification and disinfection of biofilm forming microorganism in drinking water storage tanks.
International Conference on Antimicrobial Research. Valladolid, Spain.

06/2012 Oral presentation: Photocatalytic oxidation with titanium dioxide for *Pseudomonas aeruginosa* biofilm disinfection in water systems
Workshop: Applications of Nanotechnology in Modern Energy concepts. Dresden, Germany.

06/2012 Participant in the Forum für Wasserhygiene und Energieeffizienz.
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04/2013 Poster presentation: TOC analysis of planktonic bacteria and biofilms
5th International Conference Swimming Pool and SPA. Rome, Italy

04/2013 Oral presentation: Cleaning devices may be sources for *Pseudomonas aeruginosa* contaminations in swimming pools
5th International Conference Swimming Pool and SPA. Rome, Italy

11/2016 Oral presentation: Biofilm analysis in distribution pipes of reclaimed water. in the Final Workshop of the LIFE aWARE project. Barcelona, Spain

06/2017 Oral presentation: Biofilm analysis in distribution pipes of reclaimed water
7th Conference 'Environment and society: guidelines for environmental management'. Barcelona, Spain

07/2017 Poster presentation: Analysis of MALDI-TOF MS profiles of isolates recovered from different drinking water sources
FEMS Microbiology Congress 2017. Valencia, Spain

10/2017 Poster presentation: Implementation of newly developed pathogen detection methods in a large water system
BioMicroWorld 2017 Conference. Madrid, Spain

11/2017 Participation in the Workshop - Managing Emerging Microbial Threats – Drinking Water Supplies
Aquavalens project (funded by EU FP7 programme)

Patent

During this investigation, different substances were tested for cleaning and disinfecting drinking waters storage tanks. One substance was finally sent to the patent office in Munich. (Substances present in detergents were kept anonymous for professional reasons of the CARELA Group).

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9. EIDESSTATTLICHE VERSICHERUNG

1. Bei der eingereichten Dissertation zu dem Thema: *"Analysis and characterization of planctonic and biofilm-forming Pseudomonas aeruginosa in water systems"* handelt es sich um meine eigenständig erbrachte Leistung.

2. Ich habe nur die angegebenen Quellen und Hilfsmittel benutzt und mich keiner unzulässigen Hilfe Dritter bedient. Insbesondere habe ich wörtlich oder sinngemäß aus anderen Werken übernommene Inhalte als solche kenntlich gemacht.

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Ort und Datum

Valencia, 14. Juni 2018

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