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Characterization of Bacterial Biofilms for Wastewater Treatment

SOFIA ANDERSSON

Royal Institute of Technology
School of Biotechnology
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Royal Institute of Technology
School of Biotechnology
Division of Environmental Microbiology
AlbaNova University Center
SE-106 91 Stockholm
Sweden

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Sweden

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Cover illustration: Scanning electron microscopy (SEM) image of a dual strain biofilm formed by *B. denitrificans* and *A. calcoaceticus*.

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Abstract

Research performed at the Division of Environmental Microbiology has over the last years resulted in the isolation of possible bacterial key-organisms with efficient nutrient removal properties (*Comamonas denitrificans, Brachymonas denitrificans, Aeromonas hydrophila*). Effective use of these organisms for enhanced nutrient removal in wastewater treatment applications requires the strains to be retained, to proliferate and to maintain biological activity within the process. This can be achieved by immobilization of the organisms using an appropriate system.

Two putative immobilization systems, agar entrapment and biofilm formation, were assessed. Surface attached biofilm growth provided better results with respect to cell retention, proliferation and microbial activity than immobilization in agar beads. Thus, biofilm physiology was further characterized using simplified systems of single, dual or multi strain bacterial consortia containing the key-organisms as well as other wastewater treatment isolates. Mechanisms for initial adherence, biofilm formation over time, dynamics and characteristics of extracellular polymeric substances (EPS) and exopolysaccharides, nutrient removal activity as well as the effect of bacterial interactions were investigated. The results showed that all the assessed bacterial strains could form single strain biofilm providing that a suitable nutrient supply was given. Production of EPS was found to be critical for biofilm development and both EPS and polysaccharide residue composition varied with bacterial strain, culture conditions and biofilm age. Denitrification and phosphorus removal activity of the key-organisms was maintained in biofilm growth. Co-culturing of two or more strains resulted in both synergistic and antagonistic effects on biofilm formation as well as the microbial activity within the biofilm. Bacterial interactions also induced the synthesis of new polysaccharides which were not produced in pure strain biofilms.

The complexity of single and mixed strain biofilm development and the implications of interactions on biofilm performance were underlined in this study. The data presented can be useful for modeling of biofilm systems, serve as a tool for selection of bacterial strain combinations to use for bioaugmentation/bioremediation or provide a base for further experiment design.

Keywords: Biofilm, extracellular polymeric substances, exopolysaccharides, interspecies interactions, wastewater treatment, denitrification, phosphorus removal

Sammanfattning

Flera nyckelorganismer (*Comamonas denitrificans*, *Brachymonas denitrificans* och *Aeromonas hydrophila*), har isolerats från avloppsreningsverk med en hög och stabil kväve- och fosforreduktion. Syftet har varit att med hjälp av dessa nyckelorganismer om möjligt kunna utforma en stabil och effektiv avloppsreningsprocess. För detta krävs att bakterierna kan stanna kvar i processen med bibehållen enzymaktivitet. Dessutom krävs att bättre förstå hur bakterierna interagerar med varandra och alla andra organismer som naturligt finns i systemet. Ett sätt att få selekterade bakterier att stanna kvar i systemet är att immobilisera dessa på lämpligt sätt. Två olika system undersöktes. Dessa bestod av (i) inneslutning i en agarmatris och (ii) bildning av biofilm på ett antal olika bärarmaterial. Biofilm systemet resulterade i en högre denitrifikationsaktivitet, retention och etablering av de utvalda bakterierna jämfört med agarmatrisen. En ingående karaktärisering av biofilmfysiologi utfördes därmed med hjälp av förenklade, kontrollerade system av en, två eller fler bakteriestammar. Nyckelorganismerna samt andra avloppsresningsisolat användes.

De mekanismer som gör att bakterier fäster på ytor, tillväxt av biofilm över tid, dynamik och sammansättning av extracellulära polymerer och polysackarider, denitrifikationsaktivitet och fosforupptag samt påverkan från bakteriell växelverkan i biofilmer med fler arter undersöktes. Resultaten visade att alla de undersökta bakterierna kunde utveckla biofilm i renkultur i närvaro av en lämplig näringsämnessammansättning. Syntes av extracellulära polymerer var avgörande för biofilmutveckling. Polymererna bestod av kolhydrater, protein, fetter och nukleinsyror. Både de extracellulära polymererna och polysackaridsammansättningen varierade med odlingsförhållanden och biofilms ålder. Nyckelorganismernas förmåga att denitrifiera respektive ta upp fosfor upprätthölls i biofilm. Blandkulturer gav upphov till både synergistiska och antagonistiska effekter på biofilmtillväxten såväl som denitrifikation och fosforreduktion. Interaktioner mellan nyckelorganismerna gav dessutom upphov till syntes av helt nya polysackarider som inte tillverkades i renkulturerna.

Denna studie visar på komplexiteten i biofilmtillväxt av ren- och blandkulturer samt det betydande inflytandet av bakteriella interaktioner. De data som presenteras här kan användas som underlag för modellering av biofilmsystem eller val av bakteriesammansättning vid bioaugmentering och bioremediering.

List of publications

This thesis is based upon the following six papers, which are referred to in the text by their roman numerals (I-VI). The papers are found in the appendix.

I **Andersson S**. and Dalhammar G. (2006) Bioaugmentation for enhanced denitrification in a lab-scale treatment system. *Proceedings (peer reviewed) of The Second IASTED International Conference on advanced technology in the environmental field*, 6-8/2 2006, p. 63-67

II **Andersson S**., Kuttuva Rajarao G., Land C. J., Dalhammar G. (2008). Biofilm formation and interactions of bacterial strains found in wastewater treatment systems. *FEMS Microbiology Letters*. 283:1 p. 83

III **Andersson S.**, Nilsson M., Dalhammar G. and Kuttuva Rajarao G. (2008). Assessment of carrier materials for biofilm formation and denitrification. *Vatten* 64 p. 201–207

IV **Andersson S.**, Dalhammar G., Land C. J., Kuttuva Rajarao G. (2009) Characterization of extracellular polymeric substances from denitrifying organism *Comamonas denitrificans*. *Applied Microbiology and Biotechnology* 82:3 p. 535-543

V **Andersson S**., Dalhammar G., Land C. J. and Kuttuva Rajarao G. (2009) Biological nutrient removal by individual and mixed strain biofilms. *Submitted manuscript*

VI. **Andersson S**., Dalhammar G., Kuttuva Rajarao G. (2009) Persistence and competition of denitrifying biofilms subjected to a natural wastewater flora. *Submitted manuscript*

Contribution to papers:

I, VI	Principal author, outlined experiments, performed all experimental work
II, IV, V	Principal author, took part in outlining the experiments, performed all experimental work
III	Principal author, took part in outlining the experiments, performed minor part of the experimental work

Related papers:

Andersson S., Misganaw F, Leta S and Dalhammar G. (2006) Evaluation of nitrogen removal in a small-scale system for biological treatment of tannery wastewater. *Proceedings of the 7th Specialized Conference on Small Water and Wastewater Systems*, 7-10/3 2006

Andersson S., Norström A. (2007) Potential of hydroponics for graywater treatment, two case studies. *Proceeding of the International Conference on Sustainable Sanitation "Water and Food Security for Latin America",* 23-25/11 2007

Gunaratna K. R., Garcia B., **Andersson S**. and Dalhammar G. (2008) Screening and evaluation of natural coagulants for water treatment. *Water Science & Technology: Water Supply*, 7:5-6, p. 19–25

Contents

Abbreviations

AOB	ammonia oxidizing organism
CLSM	confocal laser scanning microscopy
CRA	congo red agar
EBPR	enhanced biological phosphorus removal
EPS	extracellular polymeric substance
FISH	fluorescent *in situ* hybridization
GC	gas chromatography
HPAEC	high performance anionic exchange chromatography
MS	mass spectrophotometry
NCBI	National Center for Biotechnology Information
NOB	nitrite oxidizing organsim
PAO	polyphosphate accumulating organism
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SEC	size exclusion chromatography
SEM	scanning electron microscopy
TCA	trichloroacetic acid

Introduction

1. Wastewater treatment

Water is essential for all known lifeforms, still, water pollution and the destruction of ecosystems continue to increase. Water contamination is now a major problem in the global context as a consequence of industrialisation, globalization, population growth, urbanisation and warfare combined with increased wealth and more extravagant lifestyles [1]. From a Swedish perspective eutrophication of lakes and the Baltic sea, caused by discharge of nutrients originating from human activities, industries and agriculture, threatence the maintenance of biodiversity and human health. Biological wastewater treatment is therefore of outmost importance for the wellbeing of our waterbodies. In Sweden there are around 500 large scale municipal wastewater treatment plants and more than 800 small scale plants. Nevertheless, the nutrient load (from activities within Sweden) on the Baltic sea has not decreased in over 30 years. In 2006 the discharge of nitrogen and phosphorus from Swedish wastewater treatment plants and industries reached 12 000 and 500 tonnes respectively [2, 3]. Although Sweden has a good existing infrastructure of well functioning treatment plants, we can do even better. This calls for a continuous development and refinement of wastewater treatment techniques as part of the effort to make the world a cleaner place.

1.1 Historical overview

During mid-19th centurey, several epidemics of waterborne diseases such as cholera and typhoid fever ravaged throughout Europe. The emerging knowledge of the role of microorganims and sanitary systems for the spreading of disease resulted in the construction of sewer systems in several large cities. In the late-19th centurey, the vast population increase in urbanised areas lead to severe pollution of rivers and lakes, creating a demand for wastewater treatment. The first treatment plants used in Europe were simple and consisted mainly of primary treatment, i.e. screens, grits, strainers and settling tanks [4]. In UK, the leading nation on watewater treatment of this time, a full-scale biological treatment plant employing biofilm technique (trickling filter) was operated as early as the 1880s [5]. Widespread large scale biological wastewater treatment, secondary treatment, was established in Europe during the first half of the 20th century, introducing the activated sludge process and modified versions of the trickling filter [4].

Around 1950, the incentive for wastewater treatment switched from disease prevention to prevention of eutrophication, as the nutrients nitrogen and phosphoros started to attract attention. Still, it was not until the 1970^{s} that tertiary treatment, nutrient removal, was generally incorporated into european treatment plants [4]. Precipitaion of phosphorus in combination with biological nitrogen removal soon became the leading technique (Figure 1). The ambition

to achieve a strictly biological treatment set up resulted in the introduction of the enhanced biological phosphorus removal process (EBPR) in the 1980s, after more than 20 years of research [6]. The increased amounts of wastewater, stricter discharge regulations and lack of space in urbanised areas in the modernized society accelerated the development of alternative methods for biological wastewater treatment. This resulted in a boost of reaserch on biofilm systems during the 80s leading to the development of innovative and flexible processes including various designs of both fixed and moving bed biofilm reactors [5].

During the last two decades, improved analytical tools have lead to the the discovery of a new type of micro-pollutants [7, 8], resulting in yet another switch of the incentive for wastewater treatment. The activated sludge wastewater treatment configurations widely used today (Figure 1) do not remove these compounds to an acceptable extent [9, 10]. Physical, chemical and biological methods for micro-pollutant removal are currently beeing evaluated and developed [11].

While parts of the world strive to upgrade existing treatment systems to handle stricter standards, more complex wastewaters and lack of space, others have only just begun. Epidemics of water borne disease, eutrophication and micro-pollutants in combination with underdeveloped infrastructure and weak economy constitute a challenge for the global community to solve. Therefore, parallel research on efficient low cost and low maintenance processes for wastewater treatment is simultaneuously carried out [12].

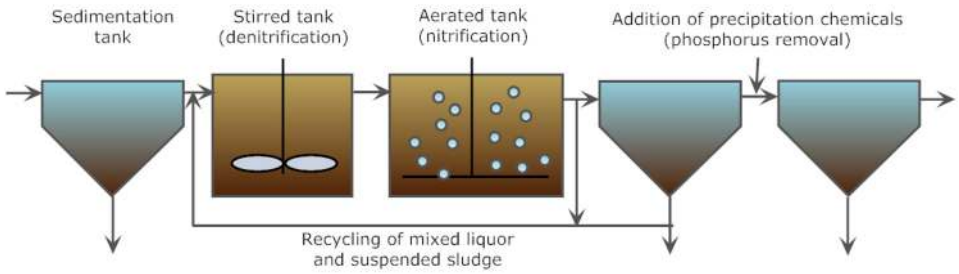

Figure 1. A common wastewater treatment set up for tertiary treatment including biological nitrogen removal in a pre-denitrification configuration and chemical phosphorus removal using post-precipitation.

1.2 Biological processes

Biological wastewater treatment is mainly carried out by prokaryotes, even if fungi, protozoa, algae and rotifers may also be represented [13]. The microorganisms remove carbon and nutrient from sewage by employing various metabolic and respiratory processes. The most frequently found prokaryotes in biological wastewater treatment systems belong to the classes *Alpha-*, *Beta-* and *Gammaproteobacteria*, *Bacteroidetes* and *Actinobacteria* [14]. Municipal wastewater is composed of organic material, i.e. proteins, carbohydrates, fats and oils; nutrients, mainly nitrogen and phosphorus; as well as trace amounts of recalcitrant organic compounds and metals [13]. Biodegradable organic material is biochemically oxidized by heterotrophic bacteria under aerobic conditions resulting in production of carbon dioxide, water, ammonia and new biomass. Under anaerobic conditions methanogenic archaea, partially oxidizes organic material to yield carbon dioxide, methane and new biomass [15].

Biological nitrogen removal is achieved by a combination of nitrification, the oxidation of ammonia to nitrate, and denitrification, the reduction of nitrate to nitrogen gas. Nitrifying bacteria are chemolithotrophs, using the inorganic nitrogen compounds as electron donors. Ammonia oxidizing bacteria (AOB), like e.g. *Nitrosomonas*, *Nitrosospira* and *Nitrosococcus*, convert ammonia to nitrite according to equation (1). Nitrite oxidizing bacteria (NOB), like e.g. *Nitrobacter*, *Nitrospira*, *Nitrococcus* and *Nitrospina* subsequently convert nitrite to nitrate consistent with the stoichiometric formula described by equation (2) [16]:

$$15\ CO_2 + 13\ NH_4^+ \rightarrow 10\ NO_2^- + 3\ C_5H_7NO_2\ 23\ H^+ + 4\ H_2O \quad (1)$$

$$5\ CO_2 + NH_4^+ + 10\ NO_2^- + 2\ H_2O \rightarrow 10\ NO_3^- + C_5H_7NO_2 + H^+ \quad (2)$$

The denitrification process reduces the nitrates to nitrogen gas, thus removing nitrogen from the water phase. In the absence of molecular oxygen denitrifying organisms can respire nitrate or nitrite through a chain of enzymatic reactions coupled to the bacterial inner membrane (Figure 2). Synthesis of the enzymes involved in denitrification is induced under anoxic conditions. In the presence of molecular oxygen the aerobic electron transport system is employed since the redox potential of oxygen is higher than for nitrate [16]. The stoichiometric formula for the overall process, here with acetate as electron donor, is presented below [17]:

$$5CH_3COOH + 8NO_3^- \rightarrow 8HCO_3^- + 2CO_2 + 6H_2O + 4N_2 \quad (3)$$

The ability to denitrify is widespread among heterotrophic bacteria and archaea making it difficult to determine which microorganisms are most important for *in situ* denitrification in wastewater treatment plants [14]. Members of the genera *Pseudomonas*, *Alcaligenes*, *Acinetobacter*, *Paracoccus*, *Methylobacterium*, *Bacillus* and *Hyphomicrobium* are commonly identified as part of the denitrifying microbial flora in wastewater treatment plants when culture dependant isolation methods are used [13, 17, 18].

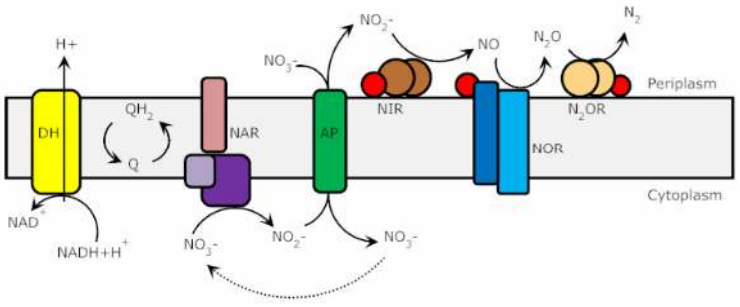

Figure 2. The enzymatic reactions involved in denitrification in bacteria. All enzymes are located within or on the surface of the inner membrane. The enzymes involved are nitrate reductase (NAR), nitrite reductase (NIR), nitric oxide reductase (NOR) and nitrous oxide reductase (N_2OR).

Biological phosphorus removal is achieved by intracellular accumulation of polyphosphates in combination with cell uptake for growth. The most efficient phosphate removal bacteria are called polyphosphate accumulating organisms (POAs). PAOs require alternating anaerobic and aerobic environments to obtain a high net uptake of phosphorus. The process is described in Figure 3. The phosphorus content in bacterial cells is usually around 1-3 % of the dry weight while the corresponding percentage for PAOs can reach 10% [13, 19]. By removing biomass after the aerobic step, the phosphorus is removed from the wastewater. Traditional isolation procedures have failed to identify bacteria possessing the characteristics ascribed to PAOs. However, cultivation-independent molecular techniques have identified a group of *Rhodocyclus*-related bacteria, named "Candidatus *Accumulibacter phosphatis*", as PAOs [20]. Some bacterial strains have been found to take up enhanced amounts of phosphorus under solely aerobic conditions. The possibility to by-pass the anaerobic step is advantageous from a process design point of view. Bacteria with enhanced aerobic phosphorus uptake ability are for example *Acinetobacter calcoaceticus*, *Acinetobacter iwoffi* and *Aeromonas hydrophila* [19, 21, 22].

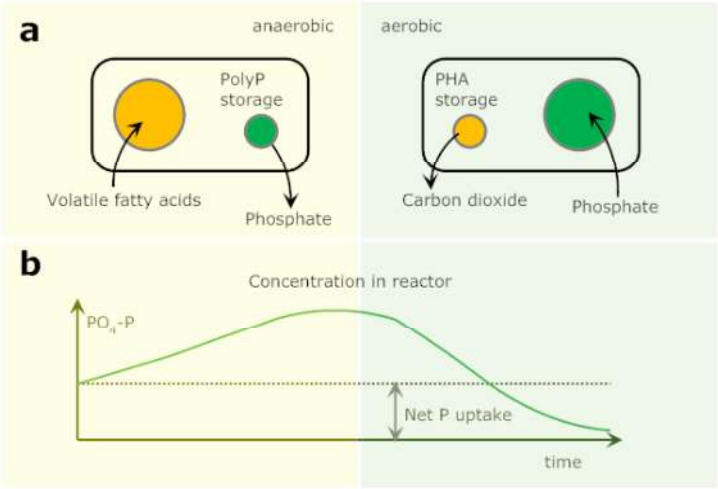

Figure 3. Schematic overview of the EBPR process. a) Under anaerobic conditions PAOs take up volatile carbohydrates for intracellular storage using energy derived from digestion of intracellular polyphosphates. Under subsequent aerobic conditions the stored carbohydrates is used as energy reserve for an enhanced uptake of phosphate which is intracellularly stored as polyphosphates. b) The concentration of phosphate in the bulk phase of a typical wastewater treatment reactor employing biological phosphate removal with alternating anaerobic and aerobic conditions as a function of time. A net PAO uptake of phosphorus leads to removal from the bulk.

2. Biofilms

The discovery of microorganisms, 1684, is usually ascribed to Antoni van Leeuwenhoek, who was the first person to publish microscopic observations of bacteria [15]. Although the most common mode of growth for microorganisms on earth is in surface associated communities [23, 24], the first reported findings of microorganisms "attached in layers" were not made until the 1940s. During the 1960s and 70s the research on "microbial slimes" accelerated but the term "biofilm" was not unanimous formulated until 1984 [25]. Various definitions of the term biofilm have been proposed over the years. According to the omniscient encyclopedia Wikipedia a biofilm is "*a structured community of microorganisms encapsulated within a self-developed polymeric matrix and adherent to a living or inert surface*" (http://en.wikipedia.org, 20090205). Dental plaque, surfaces of slippery stones and pebble in a stream, slimy coatings in showers or on boat hulls, gunge on infected wounds or the mass clogging water distribution pipes are examples of biofilms that may be encountered in ones everyday life.

Microorganisms in biofilms produce extracellular polymeric substances (EPS) that hold the cell aggregates together and form the structural biofilm matrix scaffold [26-28]. The fact that EPS is produced even under growth-limiting conditions, despite the high energy consumption it requires, emphasizes the advantages for bacterial cells to be in biofilm [29]. The biofilm matrix shelters the bacterial cells from antimicrobial agents and environmental stress by acting as a physical barrier [30].

Other ecological advantages of the biofilm lifestyle are metabolic cooperation, presence of microniches and facilitated gene transfer. Efficient metabolic cooperation or mutual dependence (syntrophism) frequently evolves within biofilms due to interspecies substrate exchange facilitated by the spatial proximity of the cells. Development of microniches with diverse oxygen and nutrient concentrations within biofilms creates favorable conditions for a great variety of species. Enhanced gene transfer rates, often detected in biofilm communities, guarantees a progressive evolution and genetic diversity increasing the competitiveness of the bacterial cells [30].

Bacterial cells adapted to a surface-associated lifestyle express phenotypic traits distinct from those expressed during planktonic growth. For example, increased tolerance to antimicrobial agents, altered metabolic or biochemical reaction rates, enhanced degradation ability of toxic chemicals and changed synthesis of biomolecules have been observed [28]. Biofilms were initially thought of as homogenous systems of cells entrapped in slime but recent research findings point in the opposite direction. Nowadays, the perception of physiologic and genetic heterogeneity in biofilms is generally accepted in the research

community [23, 31]. Natural biofilms usually harbour a multitude of microbial species forming complex differentiated populations capable of developing highly convoluted structures, often separated by a network of water channels [23, 32]. This requires a sophisticated organization which in some organisms is controlled by a cell-cell communication system, known as quorum sensing. The biofilm structure is also affected by numerous other conditions, such as surface and interface properties, nutrient availability, microbial community composition and hydrodynamics [30].

2.1 Biofilm in wastewater treatment

Wastewater treatment with biofilm systems has several advantages compared to suspended growth systems. Operational flexibility, low space requirements, reduced hydraulic retention time, resilience to changes in the environment, increased biomass residence time, high active biomass concentration, enhanced ability to degrade recalcitrant compounds as well as a slower microbial growth rate resulting in lower sludge production are some of the benefits with biofilm treatment processes [5, 33-35]. Biofilm systems also permit enhanced control of reaction rates and population dynamics [5].

Biofilm reactor configurations applied in wastewater treatment include trickling filters, high rate plastic media filters, rotating biological contactors, fluidized bed biofilm reactors, air-lift reactors, granular filters and membrane immobilized cell reactors, as can be seen in Figure 4 [5]. A general division between fixed and moving bed processes based on the state of the support material is usually done. Fixed bed systems include all systems where the biofilm is formed on static media such as rocks, plastic profiles, sponges, granular carriers or membranes [5]. The liquid flow through the static media supplies the microorganisms with nutrients and oxygen. Moving bed systems comprise all biofilm processes with continuously moving media,

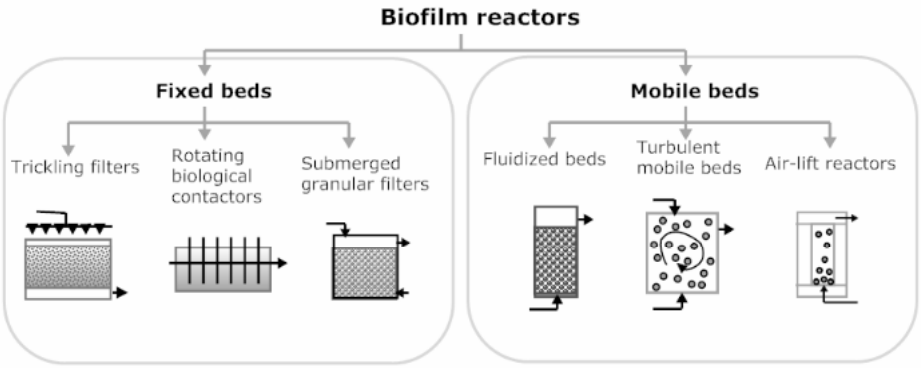

Figure 4. Overview of common configurations for biofilm wastewater treatment

maintained by high air or water velocity or mechanical stirring [36]. Biofilm carrier material (media) is selected based on size, porosity, density and resistance to erosion [37, 38]. By using a material with a large specific surface area (m^2/m^3) high biological activity can be maintained using a relatively small reactor volume. The biofilm thickness in the reactors is usually controlled by applying shear force, which is achieved by altering the stirring intensity, flow velocity or by backwashing [36].

Besides primary, secondary and tertiary wastewater treatment, biofilm systems have also been successfully used to treat industrial wastewaters. Biofilms used in wastewater treatment take advantage of a number of removal mechanisms such as biological degradation, biosorption, bioaccumulation and biomineralisation [39]. Efficient biosorption of heavy metals [40] and organic solvents [41] by biofilm matrix components have been found. Reactors using natural microbial flora or specific strains with the ability to remove e.g. chlorophenols [42-44], pyrene and phenanthrene [45], n-alkanes [46], carbon tetrachloride [47] and mixed effluent from pharmaceutical industry [48] have been described in literature.

The use of specific bacterial strains to enhance the performance of wastewater treatment is called bioaugmentation. Stephenson and Stephenson defined bioaugmentation as a process which attempts to improve treatment by increasing diversity and/or activity through direct introduction of either selected naturally occurring or genetically altered microorganisms to the system [49]. To achieve a successful bioaugmentation the survival, activity and retention of the inoculated microorganisms have to be guaranteed in the new environment [50]. Thus, biofilm-mediated bioaugmentation which offers the selected microorganisms protection against toxic compounds, protozoa grazing and washouts within the sheltered biofilm matrix, is a technique with potential use in wastewater treatment [39].

2.2 Biofilm formation and development

Biofilm formation and development is a fascinatingly intricate process, involving altered genetic genotype expression, physiology and signal molecule induced communication. Biofilms can form on all types of surfaces, biotic or abiotic, in most moist environments. Several distinct steps essential in the biofilm formation process have been identified and a simplified sketch of the most crucial ones can be seen in Figure 5. Surfaces in aquatic environments generally attain a conditioning film of adsorbed inorganic solutes and organic molecules (Figure 5-1). Bacteria move towards the surface by chemotaxis or Brownian motion, resulting in a temporary bacteria-surface association (Figure 5-2) mediated by non-specific interactive forces such as Van der Waals forces, electrostatic forces, hydrogen bonding, and Brownian motion forces [51]. At the surface, production of extracellular polymeric substances will firmly

anchor the cells to the surface. This state is commonly referred to as irreversible attachment (Figure 5-3), truly irreversible only in the absence of physical or chemical stress. Synthesis of exopolysaccharides which form complexes with the surface material and/or secretion of specific protein adhesins that mediate molecular binding are known mechanisms for irreversible attachment [52]. A large group of such proteinaceous adhesins are the β-sheet-rich, water insoluble amyloid fibrils found in 5-40% of the strains present in both freshwater and wastewater treatment biofilms [53]. During the initial attachment various short range forces are involved, including covalent, hydrogen and ionic bonding as well as hydrophobic interactions. The initially adhered cells rarely come in direct contact with the surface because of repulsive electrostatic forces, instead the secreted polymers link the cells to the surface substratum [54]. The shift from reversible to irreversible attachment is relatively rapid. Various studies report firm attachment within a few minutes or less [55]. Once anchored at the surface, cell division and recruitment of planktonic bacteria results in growth and development of the biofilm community, i.e. maturation (Figure5-4).

Surface attached bacterial cells use the nutrients in the conditioning film and the aqueous bulk to grow and produce more EPS resulting in the formation of microcolonies. Eventually the microcolonies expand to form a layer covering the surface [54]. During biofilm growth a differentiation of the gene expression pattern can be seen compared to planktonic cells. The production of surface appendages involved in bacterial motility is down-regulated due to cell immobility in the biofilm matrix while production of EPS and membrane transport proteins such as porins is up-regulated [56]. The up- and down-regulation of genes is mainly dependent on population density and is controlled by a signal molecule driven communication system known as quorum sensing [52].

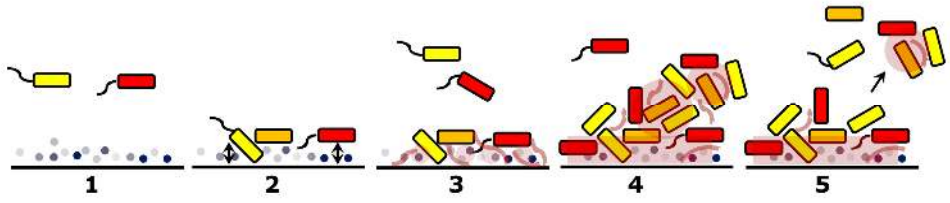

Figure 5. Schematic representation of the steps involved in biofilm formation. 1. Formation of conditioning film on the surface, 2. initial adherence of bacterial cells, 3. irreversible attachment of bacteria, 4. maturation of the biofilm, 5. detachment.

Mature bacterial biofilms are dynamic, spatially and temporally heterogeneous communities which can adopt various architectures depending on the characteristics of the surrounding environment (nutrient availability, pH, temperature, shear forces, osmolarity) as well as the composition of the microbial consortia [57]. Complex structures such as mushroom-like towers surrounded by highly permeable water channels, facilitating the transport of nutrient and oxygen to the interior of the biofilms, are commonly observed [23, 32, 57]. The biofilm development process is fairly slow, several days are often required to reach structural maturity [23]. A mature biofilm is a vibrant construction, with an advanced organisation which continuously adapts it self to the surroundings, meaning that under adverse conditions bacteria may leave their sheltered existence within the biofilm community in the search for a new, more favourable habitat to settle down in. This step is known as detachment (Figure 5-5).

The biological, chemical, and physical factors that drive detachment are complex. Degradation of the extracellular polymeric substances, absence of sufficient nutrients or oxygen, quorum sensing, hydraulic shear and normal forces, sloughing and erosion are all factors believed to influence biofilm detachment [58]. Active detachment involves an up-regulation of genes encoding carbohydrate degrading enzymes resulting in weakened cohesive forces within the biofilm and subsequent detachment of single cells or biofilm units. Simultaneously the expression of porin proteins is down-regulated and the operon encoding flagella proteins is up-regulated, preparing the cells for a planktonic lifestyle [23, 56].

2.3 Extracellular polymeric substances

The production of an extracellular matrix is a prerequisite for biofilms formation [26, 27, 32]. The biofilm matrix generally consist of up to 97% water, 2-5% microbial cells, 3-6% EPS and ions [24]. The EPS, in turn, is normally composed of 40-95% polysaccharides, 1-60% proteins, 1-10% nucleic acids and 1-40% lipids [59]. The composition of the EPS varies with the composition of the microbial consortia and the environmental conditions [32]. In addition to structural, protective and biosorptive properties, discussed in previous sections, EPS can serve as substrate for cell growth under conditions of starvation [60, 61]. A compilation of common EPS components and their role in biofilms is shown in Table 1. The distribution of EPS in a biofilm varies both temporally and spatially. In general, more EPS in relation to cells is found in older and thicker biofilms [62]. Thin biofilms are composed of less EPS compared to cells and the EPS is often rich in proteins [63]. The highest cell densities in biofilms are found in the top layer, decreasing with depth while the EPS is more abundant in the biofilm interior

[64]. The EPS produced by most bacteria in biofilms also differs in composition from the EPS produced by the same bacteria in planktonic culture [65].

The protein fraction of EPS is generally quite large but yet, very little is known about its role in biofilms. For example, it is not clear if the proteins function as structural components or if they mainly have other functions, independent of the mechanical integrity [66]. For some bacterial species, proteins are shown to have an important function in the initial adherence to a surface. Adhesins, cell surface associated proteins like pili, flagella, curli and amyloid fibres are believed to be important factors for biofilm formation [27, 53, 67] as well as a homologous group of large proteins, referred to as biofilm-associated proteins, found in e.g. *Staphylococcus*, *Enterococcus* and *Salmonella* [68]. In EPS produced by a *Pseudomonas putida* strain, only one type of extracellular protein, a flagellin, was found [69] indicating presence of flagella. Apart from adhesins, extracellular enzymes are often detected within the biofilm matrix. The presence of mainly proteases, but also glycosidases, retained in the EPS is suggested to be involved in the community metabolism [70].

Nucleic acids detected in extracted EPS were at first believed to originate from intracellular contamination during the extraction procedure or the presence of dead cells in the matrix. However, Whitchurch and colleagues [71] showed that extracellular DNA is required for initial establishment of biofilms by *Pseudomonas aeruginosa* and later on Böckelmann and colleagues [72, 73] demonstrated the structurally importance of DNA in biofilms.

Table 1. EPS functionality. Extracted from [27]

Effect of EPS component	Nature of EPS component	Role in biofilm
Constructive	Neutral polysaccharides Amyloids	Structural component Structural component
Sorptive	Charged or hydrophobic polysaccharides	Ion exchange, sorption
Active	Extracellular enzymes	Polymer degradation
Surface active	Amphiphlic Membrane vesicles	Interface interactions Export from cell, sorption
Informative	Lectins Nucleic acids	Specificity, recognition Genetic information, structure
Redox active	Bacterial refractory polymers	Electron donor/acceptor
Nutritive	Various polymers	Source of C, N, P

Filamentous networks of extracellular DNA, possibly protected from enzymatic digestion by methylation, was shown to stabilize the biofilm architecture.
The extracellular DNA had a different sequence than the genomic DNA implying active production and transport [72]. The mechanism for the structural function of DNA is proposed to involve cross-bridging [74].

The lipid fraction of the EPS is probably the least investigated one and originates from three sources: (i) direct sorption from the wastewater or culture medium, (ii) cell lysis, and (iii) microbial metabolism. Studies on EPS from activated sludge granules reveal the presence of glycolipids, phospholipids, neutral lipids and lipopolysaccharides [75]. The lipid EPS is most likely not structurally important [76] but may, however, play an important role in the hydrophobic properties of EPS [75].

2.3.1 Exopolysaccharides

The carbohydrate fraction of EPS mainly consists of polysaccharides. This fraction have been extensively studied since several commercial applications of bacterial exopolysaccharides have been found, such as gelling agents, flocculants, foam stabilizers, hydrating agents and biosurfactants [59]. In biofilms, exopolysaccharides are postulated to be responsible for the structural stability and architecture [77]. The β-linked polysaccharides are thought to form the backbone of a network where other EPS components can bind [76]. The exopolysaccharides are essentially very long with a molecular weight of 500-2000 kDa and they often associate to form even bigger molecules. Both filamentous networks and gel-like structures have been reported depending on the exopolysaccharide composition [77]. Bacterial polysaccharides can be divided into capsular or released. Capsular polysaccharides are tightly associated with the cell surface and may even be covalently bound while the released polysaccharides are not associated to the cell after secretion [78].

Biosynthesis of exopolysaccharides is generally performed at the cell membrane, although exceptions where the synthesis is extracellular are known [79]. Precursors for exopolysaccharide synthesis, nucleoside *di*phosphate mono-sugars (UDP-sugars), are manufactured in the cytoplasm. At the periplasmic membrane different glycosyl transferases assembles the precursors to repeating units. Another group of enzymes located outside the cell membrane polymerizes the macromolecules forming extruding polysaccharides [78, 80].

The carbohydrates found in bacterial exopolysaccharides are extremely diverse. Few of the exopolysaccharides are homo-polymers, e.g. cellulose, curdlan, dextran and sialic acid, but the vast majority are hetero-polymers composed of 2-4 types of mono-sugars in di- to octasaccharide repeat units, like alginate, emulsan, gellan and xanthan to mention a few [77, 80]. The polysaccharide chains can be linear or branched. To further complicate the situation, it is common that a strain can produce more than one type of exopolysaccharide [66, 77]. Bacterial polysaccharides are made up of a variety of mono-sugar derivates. Among the more common ones are D-glucose, D-galactose, D-mannose, L-fucose, L-rhamnose, L-arabinose, N-acetyl-D-glucose amine and N-acetyl-D-galactose amine as well as the uronic acids D-glucuronic acid, D-galacturonic acid, D-manuronic acid and L-guluronic acid. Other sugar monomers less frequently occurring are D-ribose, D-xylose, 3-keto-deoxy-D-*manno*octulosonic acid and several hexoseamineuronic acids [60, 80-82]. The composition and conformation of sugar monomers has a huge impact on the properties of the polysaccharides and thereby also the biofilm matrix properties. For example, a high uronic acid fraction conveys polyanionic polymers which readily interact with cations, stabilizing the polysaccharide conformation [80]. High arabinose content in *Azospirillum brasiliense* polysaccharides have been found to induce cell aggregation [60, 83]. Linear, neutral, water insoluble 1,3-β-D-glucan polysaccharide forms gels while a similar but branched polysaccharide with β-D-glucosyl side-chains forms highly viscous aqueous solutions.

The physical properties of polysaccharides are dependant of the arrangement of mono-sugars and the polysaccharide chain association [78]. The polysaccharide synthesis of individual bacterial species is generally independent of the carbon source available. However, strains capable of synthesizing more than one polysaccharide may produce different products depending on the carbon substrate present. One example is *Pseudomonas syringae* that produce levan when the substrate is sucrose and alginate when the substrate is glucose [80]. The amount of produced exopolysaccharide is also dependant on the carbon substrate. The availability of nutrients such as nitrogen or phosphorus in relation to carbon can determine if the cell uses its energy for cell division or exopolysaccharide production. In general, low concentrations of nitrogen, phosphorus or other substrates required for cell division and high concentration of carbon substrate promote production of exopolysaccharides [80, 84].

2.4 Activity

The biofilm (B) activity, or the reaction rate, is directly proportional to the biochemical substrate (S) conversion rate (kgS m^{-3}B h^{-1}) of the microorganisms in the biofilm if there are no substrate transport limitations in the film [85]. Transport of substrate into biofilms is the result of diffusion in the denser aggregates and potentially convective transport within pores and water channels. In many biofilm systems, diffusion has been shown to dominate mass transport [86]. If the biofilm is under diffusion control, the reaction rate is additionally dependent on the specific diffusion constant (m^2 s^{-1}) and the bulk substrate concentration (kgS m^{-3}). Diffusion limited reactions are generally of ½ order meaning that a four times higher substrate concentration results in a doubled reaction rate [87]. In diffusion controlled biofilms substrate and metabolite gradients will arise within the film (Figure 6). This means that cells in the interior of the biofilm may not contribute to the biochemical substrate conversion.

The diffusion constant is specific for each substrate, depending on size, hydrophobicity and electrical charges, but it also depends on biofilm properties such as density, porosity, cell surface charges and hydrophobicity of the matrix components [86, 88]. A higher reaction rate is usually obtained in thin and dense biofilms due to high amounts of active cells in relation to EPS [63, 84].

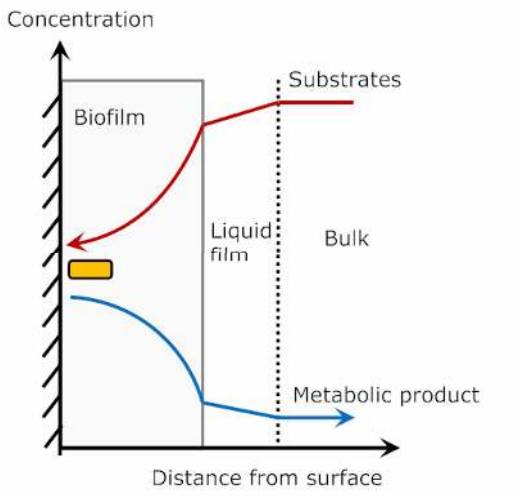

Figure 6. The transport limitations in a diffusion controlled biofilm leads to concentration gradients of both substrates and metabolic products within the biofilm, thus affecting the biofilm activity.

In biofilms without substrate limitations high biofilm densities are usually obtained [84] while diffusion limited biofilms show a decreasing density with increasing biofilm thickness [85]. The heterogeneity of most biofilms conveys variations in the diffusion constants in different regions of the biofilm, however, most models use empiric average values for the diffusion constants.

The biochemical substrate conversion rate for denitrification is proportional to the number of active denitrifying bacteria per biofilm volume and the accessibility of electron donor (organic carbon) and electron acceptor (nitrate/nitrite). The anoxic conditions required for the denitrification process can either be obtained in the aquatic bulk phase or within zones of the biofilms. For efficient denitrification it is also important that organic carbon is not the limiting substrate. A C:N ratio above 3.4 in the culture medium ensures that nitrate, and not organic carbon, is the limiting substrate [87].

Factors influencing enhanced aerobic phosphorus uptake are e.g. phosphate and molecular oxygen concentration and diffusion. Phosphorus uptake relies on intracellular storage and in order to decrease the phosphorus concentration in the system a controlled biomass removal is essential. This can be achieved by temporally applying shear forces, causing biofilm sloughing [89]. Nutrient removal activity in a biofilm wastewater treatment process involve mechanisms for substrate elimination other than biochemical conversion, like adsorption or external degradation by extracellular enzymes [16].

2.5 Interactions

The complex web of interactions within biofilm consortia is the key to understand biological community structure, composition and function [90]. Inter- and intraspecies interactions mast likely influence all the above discussed aspects of biofilms; the formation, structure, EPS and polysaccharide production and composition as well as the biofilm activity [90, 91]. Biofilms are heterogeneous systems hosting different microenvironments with bacterial cells immobilised in relatively fixed positions. In such an environment microbial interactions are unavoidable. Compared to suspended systems where the behaviour of planktonic bacteria in mixed cultures often can be predicted based on the performance of each respective single strain, biofilm systems are much more complex. Studies have shown that two strains can coexist in biofilms even though one strain consistently outcompeted the other in planktonic culture due to production of inhibiting compounds [92] or superior growth rate [93, 94].

Interactions which are beneficial to a population are called synergistic while those with a negative impact on the population are called antagonistic [95]. Synergism in biofilms include reciprocal protection from environmental stress [96-98], enhanced degradation of organic compounds [99, 100] or increased biofilm formation [94, 96]. A protective mechanism observed in dual-strain biofilms subjected to toxic organic compounds is the adoption of a spatial arrangement where a sensitive strain is surrounded by cells of a tolerant strain [97, 101, 102]. Other mechanisms known to offer increased protection in biofilms due to interactions are horizontal gene transfer of antibiotic resistance genes [103] and enzyme complementation [104]. Enhanced degradation of organic compounds is often the result of cooperative metabolism [105, 106] or by the establishment of oxygen gradients allowing both anaerobic and aerobic species to coexist [30]. Increased biofilm formation can be the result of enhanced coaggregation, i.e. specific protein-saccharide mediated interactions [107], facilitated initial surface adherence [108] or rheological interactions between EPS, altering the matrix physical property [109]. Antagonism may be caused by competition for space and substrates or by production of inhibiting substances. Inhibiting substances include extracellular antibacterial protein [110], proteinaceous toxins known as bacteriocins [92] or metabolites causing lowered pH [96]. Negative interactions might lead to suppression or outcompeting of one or more species [91] or in deficient biofilm formation [95].

A phenomenon which cannot be overlooked when discussing interactions in biofilms is cell-cell signalling. The signals often referred to as autoinducers allow organisms to behave in a co-ordinated manner including regulation of biofilm formation, development and bacteriocin production [111, 112]. Interspecies signalling is mediate by the same molecules as in intraspecies signalling. Moreover some strains which do not synthesise autoinducer molecules themselves can respond to foreign molecules and adapt their behaviour accordingly [112]. The importance of autoinducers for coordinated behaviour, microbial interactions, maintenance and function of microbial community structures is not clear. Although single species biofilms have been extensively studied, the knowledge of mixed species biofilms and their interactions is very limited [93, 113] and the underlying mechanisms are diverse and not well characterised [103].

2.6 Biofilms and research

"*In comparison to what is known about the cells themselves, very little is known about the biofilm matrix*"

Philip S. Stewart [66]

Research on biofilm formation, matrix composition, interspecies interactions and biofilm activity as well as the interrelation between these issues has proved difficult to perform on natural biofilms. The difficulty to isolate individual events and specific interactions as well as the lack of reproducibility [114] of complex natural systems have lead to development of simplified laboratory systems comprised of one or a few bacterial strains kept in controlled environments [94, 115, 116]. The use of such systems provides the possibility to investigate specific characteristics and functions under reproducible conditions [32]. Mechanisms for quorum sensing [117], resistance to antibiotics and toxic compounds [96-98], synergistic degradation of recalcitrant organic compounds [100], surface adherence [118-121], biofilm specific genetic expression patterns [122] and production, composition and function of EPS [123] are just a small selection of biofilm related properties which have been illuminated using simplified biofilm systems. Although very useful, one should bear in mind when working with simplified systems that results obtained may or may not be applicable to natural biofilm systems.

Biofilm research on a molecular-microbiological level have to date mainly been performed on clinically relevant bacteria [124, 125], strains involved in food spoilage [126, 127] or strains with potential use in fine chemical production [128, 129]. Despite wastewater treatment plant being the most widespread bioreactors in the world, little is known about the biofilm characteristics of the participating microorganisms. Understanding the underlying mechanisms of coexistence and competition of the organisms involved is therefore essential in order to further the development of biofilm system design. Knowledge of micro scale function and structure of the biological components in a biofilm can help to adjust specific biofilm wastewater treatment processes to a high efficiency [64].

Experimental techniques

3. Methodology

In recent years, experimental practice used to study biofilm has advanced greatly. A wide range of techniques; microscopic, microbiological, molecular-biological, chemical and physical, are nowadays available for the exploration of different aspects of biofilm morphology, physiology and genetics [130]. This chapter aims to summarize the techniques used in the current investigation.

3.1 Growth

The fundamental base for all biofilm studies is the use of appropriate cultivation techniques. Whether the aim is to study the time-course of biofilm formation, interspecies interactions, the matrix composition or the genetic expression of biofilm microbes, the first step is always to culture the selected microorganisms on a surface substrate. The two main categories of biofilm growth systems are batch and continuous flow systems [130]. Batch systems are generally simpler and easier to operate while continuous flow systems provide hydrodynamic conditions similar to natural systems. Two of the most commonly used techniques for laboratory studies of biofilm formation and development are the flow-cell and the microtiter plate.

The microtiter plate is a batch system that allows a high-throughput screening of biofilm formation over time by different species, mutant strains or growth factors [130]. The wells in the microtiter plate (polystyrene, 96-well) are inoculated and incubated aerobically or anaerobically for a selected time interval, allowing biofilm to be formed on the inner surface of the wells. For studies of mature biofilms requiring extended growth time the medium has to be regularly replaced. After rinsing the wells the attached biofilm can be analyzed in different ways. The most widely used method for quantification of biofilm growth is crystal violet staining [131]. Crystal violet is a basic dye which binds to negatively charged molecules, including cell surfaces and EPS [132]. By staining with crystal violet, rinsing and subsequently dissolving the bound dye in ethanol, the biofilm can be semi-quantitatively measured using a spectrophotometer. A good correlation between crystal violet readings and viable counts confirm the reliability of the method [133]. Qualitative analyses of biofilms cultured in microtiter plates can also be performed. By selecting a plate with thin, flat and clear bottom the formed biofilms can be visualized microscopically by light, phase contrast, EPI-fluorescent or confocal microscopy.

3.2 Visualization

Microscopes constitute the most basic tool in microbiology. The combination of microscopy with various labeling techniques and digital imaging acquisition and analysis is extensively used for the study of biofilms [28].

3.2.1 Labeling

Depending on which characteristics of the biofilm you wish to study, a battery of labeling methods is available. One useful method for localizing species diversity and quantifying cell numbers in biofilms is fluorescent *in situ* hybridization (FISH). Fluorescently labeled oligonucleotide probes (15-25 nucleotides) are hybridized to the small ribosome subunit (16S rRNA) in bacteria. The small ribosome subunit is made up of 1542 nucleotides, containing highly preserved regions as well as highly variable regions, enabling the design of probes for different levels of specificity such as domain specific or species specific [28, 134]. Ribosomes are present in vast numbers in active prokaryotes, up to 20,000 copies per cell [135], resulting in a strong signal from the hybridized probes. The steps involved in the FISH procedure are shown in Figure 7. Before hybridization can take place, the cells should preferably be fixed in order to maintain their morphology throughout the procedure [134]. Hybridization has to be performed in the presence of salts, formamide, sodium dodecyl sulfate (SDS) and elevated

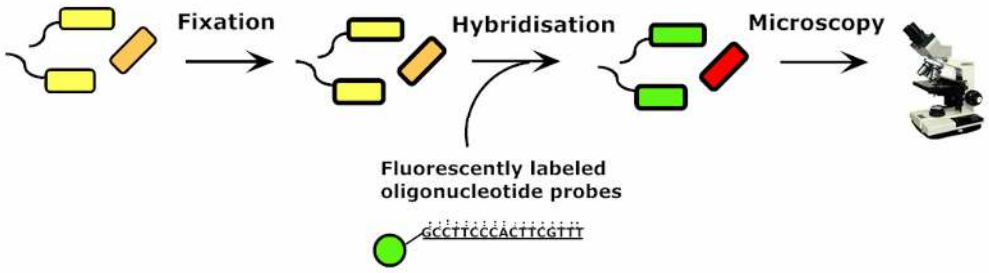

Figure 7. The steps involved in the FISH procedure

temperatures. Exact conditions for each probe have to be individually optimized to maintain high stringency. Salts reduce the repulsion between the negatively charged phosphate groups in the nucleic acids. Formamide in combination with moderately elevated temperatures disrupt the hydrogen bonds in the double helix, destabilizing the 16S rRNA molecule and SDS straightens the nucleic acid strand increasing the accessibility to hybridization [134]. The hybridized sample can subsequently be analyzed under an EPI-fluorescent or confocal

microscope. FISH is commonly used for studies of microbial ecology or bacterial interactions in biofilms [14].

Not only cells can be labeled using molecular techniques. The distribution of EPS in the biofilm matrix is commonly visualized by the use of the dye calcofluor white which stain β-D-glucopyranose polysaccharides.

3.2.2. Microscopy

The microscopic techniques available today are manifold including optical and electron based systems. Light or phase contrast microscopy can only be used on detached samples or biofilms grown on glass slides since the light beam has to pass the specimen. More appropriate techniques for the study of biofilms grown on non-transparent surfaces include EPI-fluorescent microscopy, confocal laser scanning microscopy (CLSM) and scanning electron microscopy (SEM). CLSM allows the study of live, fully hydrated biofilms as well as fluorescently labeled samples. The possibility to obtain high resolution in-focus images of thick specimens by optical sectioning can be used to create computer reconstructions of three-dimensional topologically complex objects [130]. The combination of FISH and CLSM is a perfect tool for biofilm studies. EPI-fluorescent microscopes cannot examine the depth of biofilms like CLSM and are thus suitable for examination of thin or detached biofilms.

SEM is an adequate method to visualise biofilm surface structures at high-resolution by the acquisition of three dimensional images of the surface, revealing details about 1 to 5 nm in size. Sample preparation by fixation, dehydration and coating is required. Fixation conveys conserved biofilm morphology and structure, dehydration is essential since the specimen chamber is at vacuum and coating is necessary to create an electrically conducting surface.

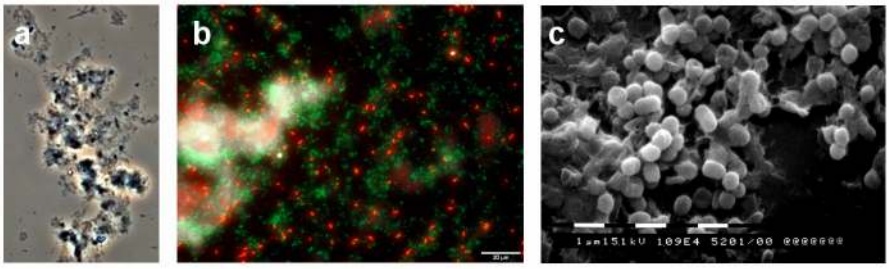

Figure 8. Images of mixed strain biofilms using three different microscopic techniques. a) Phase contrast micrograph of detached 4d *C. denitrificans* 110 biofilm, b) FISH/EPI-fluorescent micrograph of a detached natural biofilm bioaugmented with *C. denitrificans* 110 (oligonucleotide probe, EUB (green) targets all bacteria, and DEN1423 (red) targets *Comamonas* sp.) c) SEM image of 3d biofilm of *B. denitrificans* B79 and *A. calcoaceticus*. (Pictures by Sofia Andersson and Kaj Kauko).

Biofilm samples which are not properly prepared can cause structural misconception since EPS have a tendency to dry up and be visualised as fibrous threads [31]. Sample images of biofilms produced by phase contrast microscopy, EPI-fluorescent microscopy in combination with FISH and SEM are shown in Figure 8.

3.3 Activity and Removal rates

The simplest and most widely used method to assess biofilm activity is to measure the substrate removal rate. Colorimetric analyses provide the bulk concentration of organic carbon and/or nutrients at different time points, enabling calculations of the removal rate per bulk volume, biofilm mass or biofilm support area. This method evaluates the whole system and not the local activity within regions of a biofilm. Since the method does not distinguish between microbial conversion/degradation and other mechanisms such as adsorption or precipitation, the term substrate removal is used.

3.4 EPS characterization

The importance of EPS for biofilm formation, function and integrity combined with findings of commercially important exopolysaccharides have intensified the ambition to characterize the EPS produced by various organisms. Characterization often includes EPS extraction followed by purification and fraction separation before the analysis.

3.4.1 Extraction and purification

Extraction methods can be boldly grouped into physical and chemical ones. Physical methods include centrifugation, stirring, sonication, heating and cation exchange resin while chemical methods comprise the use of e.g. aldehydes, sodium hydroxide (NaOH) and ethylenediaminetetraacetic acid (EDTA) [136-138]. The choice of extraction method affects the quality of the product and must therefore be done with awareness. An optimal extraction method should give a high yield of native EPS without disrupting the cells. In general, chemical methods result in higher yields than physical methods, however, drawbacks such as reactions with the EPS or contamination of the product must be taken into account [136].

One extraction method which demonstrates good EPS yield, small interference with the biopolymers and low contamination with intracellular material is the formaldehyde-NaOH method [138]. Detached biofilms (e.g. by scraping or sonication) suspended in isotonic NaCl solution are incubated with formaldehyde, which fixates the cells and prevents them from lysis, and NaOH, which increases EPS solubility. Centrifugation is then used to separate the cells from the dissolved EPS.

The extracted crude EPS contains a mixture of polysaccharides, protein, nucleic acids, lipids and salt. A schematic overview of a common purification and fractionation procedure is shown in Figure 9. First, salts, trace amounts of culture medium that might have been trapped within the biofilm and partially degraded biopolymers are removed by dialysis. The next step is to separate the proteins and nucleic acids from the solution. Precipitation with trichloroacetic acid (TCA) removes proteins and nucleic acids larger than 20 nucleotides long without affecting the polysaccharides in the solution [139]. Subsequent precipitation with ethanol renders a purified polysaccharide fraction.

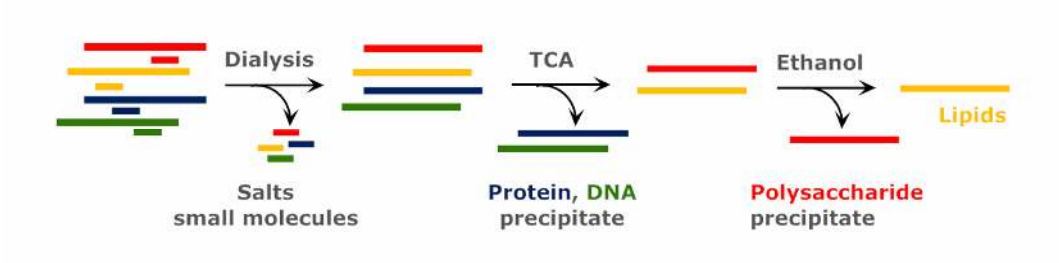

Figure 9. Purification scheme for EPS components

3.4.2 Polysaccharides

The most well characterized fraction of bacterial EPS is the polysaccharide fraction. The simplest way to estimate the amount of polysaccharides in EPS is to determine the overall carbohydrate content by colorimetric analysis using e.g. the phenol-sulfuric acid method [140]. The size distribution of the polysaccharides can be analyzed with size exclusion chromatography (SEC). Results from SEC can sometimes reveal if more than one polysaccharide type is present. In order to find out the composition of the mono-sugar molecules constituting the polysaccharides, hydrolysis of the glycosidic bonds with acid at elevated temperatures is performed. Identification and quantification of the mono-sugars is done using high-performance anion exchange chromatography (HPAEC) or, after reduction and acetylation, gas chromatography coupled to a mass spectrophotometer (GC-MS). These analyses provide the molar ratio between the sugar residues [80]. GC-MS can also be used for sugar linkage analysis if the polysaccharide hydroxyl groups are methylated prior to hydrolysis [141]. The knowledge of the mono-sugar molar rates and relative abundance of different glycosidic bonds can sometimes be sufficient to infer the actual polysaccharide structure. However, bacterial exopolysaccharides are often highly heterogeneous and a complete determination of the structure is not always possible (Göran Widmalm, personal communication).

3.4.3 Proteins, Lipids and Nucleic acids

The other EPS fractions, proteins, lipids and nucleic acids have not received as much attention as the polysaccharides. Colorimetric and spectrophotometric methods are widely used to determine the content of each fraction respectively in crude EPS [142, 143]. More detailed analysis of the protein fraction have been done with SDS-polyacrylamide gel electrophoresis (SDS-PAGE) which separates the proteins based on molecular weight [144]. This provides fingerprint from each sample and is suitable for comparative studies [145]. Further characterization of the proteins can be made by MS based partial sequencing of tryptic peptides from cut protein bands [146, 147]. Detection of proteinaqueous amyloid adhesins can be readily done by growth on Congo red agar (CRA). The congo red dye binds to the characteristic β-sheets in the protein, coloring the colonies red [148].

The overall lipid content is commonly analyzed using calorimetric methods [143]. Analysis of nucleic acid can be done with the colorimetric diphenylamine method [149] or by UV-spectrophotometric measurement at 260nm, the absorption maximum of the nitrogenous bases. By reading the absorbance at 230 (absorbance maximum for phenols and sugars), 260 and 280nm (absorbance maximum for proteins) and calculate the ratios, the purity of the samples can be estimated [150]. By the use of a nanodrop instrument a sample volume as small as 1-2μL is enough for a quantitative analysis. Another both semi-quantitative and qualitative method for nucleic acid analysis is agarose gel electrophoresis which separates nucleic acid fragments with respect to electrical charge density, i.e. size [151, 152].

Present Investigation

4. Objective

A number of efficient nutrient removing bacteria (information below) have been isolated from different wastewater treatment environments as parts of previous research efforts at the Division of Environmental Microbiology. The ambition to use these putative key-organisms for incorporation in new treatment systems or in existing malfunctioning plants was the driving force motivating the work within the scope of this thesis. By immobilizing selected bacteria an enhanced spatial control can be obtained as well as a good retention of the cells within a system. Thus, the work presented here had the overall objective to find and characterize an appropriate immobilization method for increased retention with maintained biological activity of selected bacterial strains for wastewater treatment. Specific goals included (i) selection of a suitable immobilization system for the key-organisms described below, (ii) characterization of the mechanisms mediating immobilization (iii) study of the influence of shifting environmental conditions on the stability of the immobilization technique and (iv) nutrient removal activity of the immobilized strains.

With these goals in mind, investigations of two putative immobilization systems, agar embedding and biofilm growth, were assessed (paper I and III). The preferred system, biofilm growth, was subsequently characterized, using up to thirteen different bacterial strains, with respect to surface attachment properties (paper II), nutrient dependence of biofilm formation (paper II, IV), dynamics of EPS and polysaccharide composition (paper IV and V), influence of interspecies interactions on biofilm formation and EPS composition (paper II, V and VI), biological activity of the key-organisms in pure and mixed strain biofilm (paper V and VI) and persistence of selected strains in biofilm subjected to a competitive environment (VI). Figure 10 provides an overview image of the present investigation.

Putative key-organisms isolated at the division of Environmental microbiology

Comamonas denitrificans:

Efficient denitrifying strain isolated from Gustavsberg wastewater treatment plant, Sweden. Can rapidly switch from aerobic respiration to denitrification without lag-phase and have been found in sludge from various sites. [153, 154]

Brachymonas denitrificans:

Denitrifying strain isolated from Ethio-tannery wastewater treatment plant, Ethiopia. Insensitive to presence of the toxic compound chromium. [155]

Aeromonas hydrophila:

Strain with enhanced aerobic phosphorus uptake (bypassing the anaerobic phase). Suggested to be commonly present in biofilm wastewater treatment systems. [22, 156]

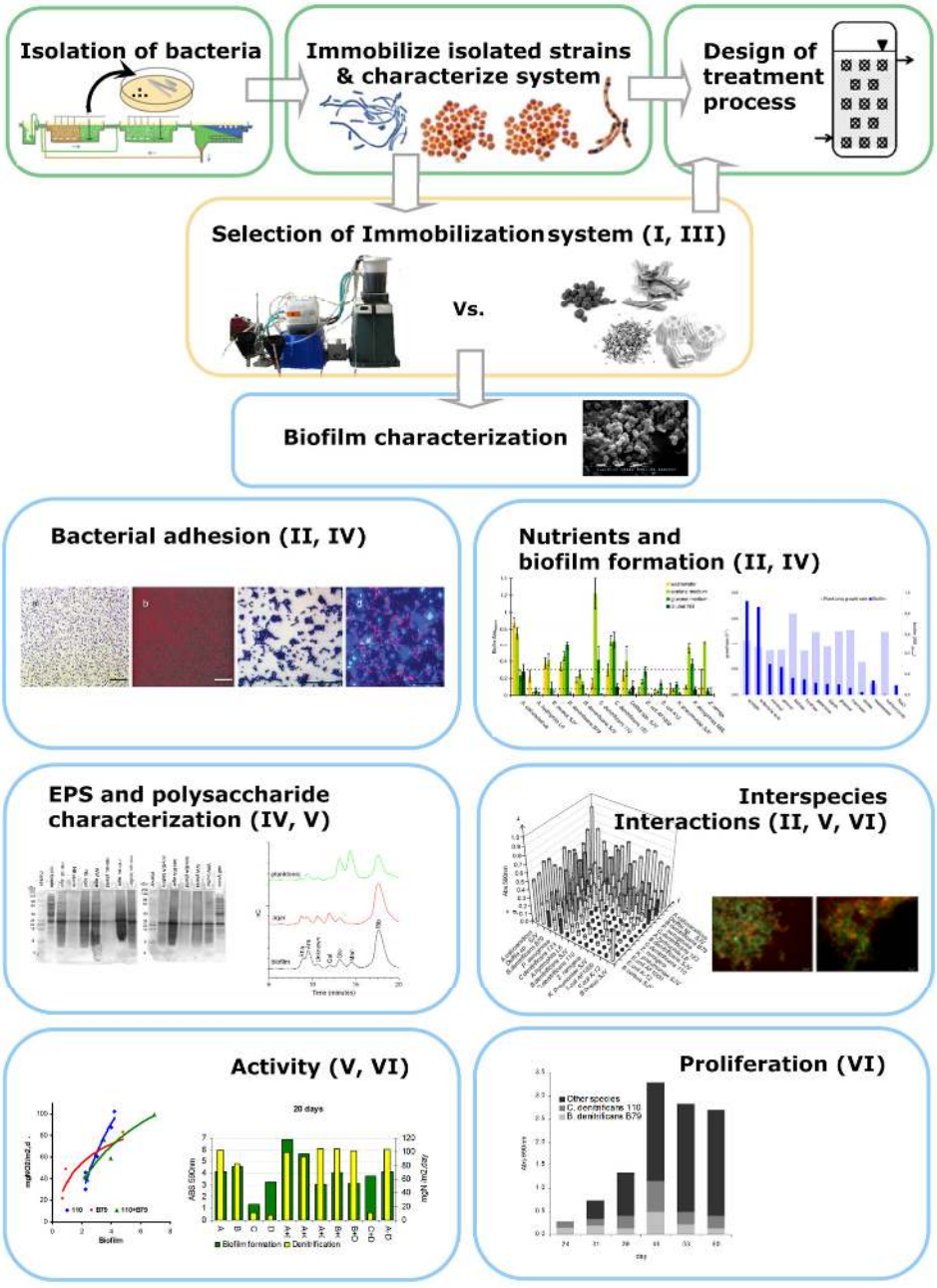

Figure 10. Summary of the research activities performed in the present investigation. The roman numerals refer to the respective papers found in the appendix.

5. Immobilization system

When using specific organisms in wastewater treatment it is important to prevent them from being washed out [39]. By immobilizing the cells a better spatial control and retention of the cells can be obtained. In the present investigation two immobilization systems were evaluated, incorporation into agar beads (paper I) and growth as biofilm on support material (paper III). The denitrifying strain *C. denitrificans* was used to investigate both systems and in addition, *B. denitrificans*, another denitrifying strain, was used to evaluate biofilm growth. In both studies denitrification activity measurement was used to monitor the reactor performance and FISH combined with EPI-fluorescent microscopy was used to monitor the bacterial population.

5.1 Agar entrapment (I)

The use of polymeric matrixes for immobilization of bacterial cells has been previously studied using e.g. alginate [157, 158] and chitosan gel [159]. Agar is a naturally derived, low cost gelling agent with good diffusion properties which is not generally hydrolyzed by bacterial exoenzymes [160]. These qualities make agar an attractive option for matrix immobilization. Hence, agar beads containing *C. denitrificans* cells were prepared. Preliminary studies showed that the cells remained viable inside the beads (24h) although the specific denitrification rate (mgN $cell^{-1}$ h^{-1}) in nutrient broth only reached 22% of the rate obtained for planktonic cells (unpublished results). The addition of cells immobilized in agar beads to a wastewater treatment reactor was thus not expected to immediately enhance the denitrification activity, the gain would instead lie in a high retention of *C. denitrificans* in the system. The agar beads were physically retained in the reactor and a slow breakdown of the beads was anticipated to convey a continuous release of cells and a subsequent establishment of *C. denitrificans*.

Two identical laboratory scale pre-denitrification reactor systems were set up (Figure 11). One system was continuously run as a reference while the other was subjected to bioaugmentation. Two types of inoculum were used, planktonic *C. denitrificans* (2.2×10^{11} cells) and agar (1%) bead embedded *C. denitrificans* (1.0×10^{11} cells, $0.5cm^3$). After the addition of inoculum to the anoxic tank the flow was stopped for two hours to allow the bacteria to acclimatize and interact with the sludge flocs. The addition of planktonic cells resulted in a rapid increase in denitrification activity with a corresponding increase of *C. denitrificans* cells in the sludge (Figure 2a, Table 2 in paper I). However, after only four days a complete washout of *C. denitrificans* cells was observed. Addition of agar embedded *C. denitrificans* did not result in enhancement of the denitrification activity or establishment of *C. denitrificans* in the system

(Figure 2b, Table 2 in paper I). Few cells were seen in the sludge 4-6 days after inoculation but thereafter no *C. denitrificans* cells were detected in the sludge or the outlet during the remaining experimental time (15 days). A physical examination of the agar beads two weeks after inoculation showed no signs of bead disruption suggesting that few bacterial cells had been released to the surrounding.

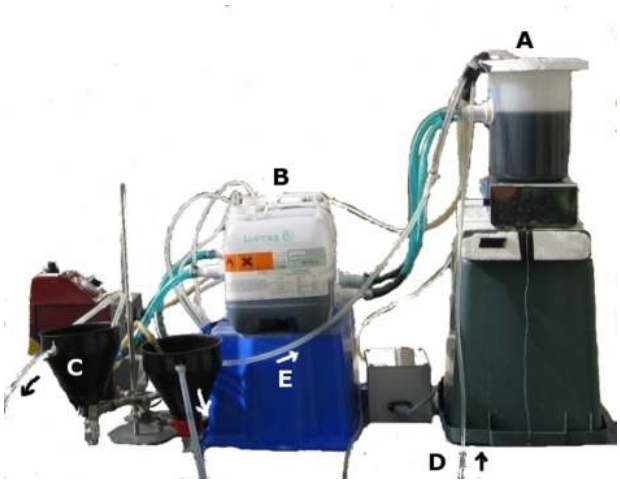

Figure 11. The two parallel lab-scale set ups used in paper I, consisting of 1.5L stirred anoxic tanks (A) followed by 3L aerated tanks (B) and 0.7L sedimentation funnels (C). The inflow (D) was 0.3L h^{-1} domestic wastewater, corresponding to 2.8g COD L^{-1} d^{-1} and the return flow from the sedimentation tanks to the anoxic tanks (E) was 200% of the feed.

5.1.1 Conclusions

The non-immobilized cells were rapidly washed out of the system, emphasizing the need for immobilization. The use of agar beads as an immobilization method to increase the retention of specific strains in a reactor was not successful. *C. denitrificans* cells were not released from the beads and no establishment of *C. denitrificans* in the reactor was seen. No further investigations were conducted on agar bead systems.

5.2 Biofilm on carrier material (III)

Biofilms on carrier material have been used in wastewater treatment since the operation of the first trickling filter in the 1880s [5]. However, it was not until the 1980s that refinement and development of moving bed biofilm systems lead to a breakthrough for biological biofilm wastewater treatment processes [161]. In biofilms, the bacterial cells are immobilized in a

matrix composed of self excreted biopolymers which prevents washout. For a successful bioaugmentation using biofilm systems, the biofilm formation ability and sessile proliferation of the bacterial strains is essential as is the selection of a suitable carrier material.

Thus, the ability of two denitrifying organisms, *C. denitrificans* and *B. denitrificans*, to form biofilm on 20 different low cost carriers (Table 1 paper III) was investigated. The biofilm formation was indirectly measured by monitoring the denitrification rate of the carriers under the assumption that a relatively linear correlation between biofilm and activity could be applied for the set up used here. The two organisms were independently able to colonize ten of the assessed materials to various degrees. Rubber and limestone materials did not support biofilm formation while other natural products like wood, lava stone (pumice) and cotton as well as processed materials like expanded clay (LECA), glass fibers and synthetic organic fibers supported biofilm growth. The plastic materials assessed gave diverse results implying that the material as such was subordinated the structure and surface characteristics. The most prominent materials, pumice, LECA, wood chips and the commercial plastic carrier Kaldnes K1 were used in a temporally extended experiment (5 weeks) in order to observe the biofilm proliferation. *C. denitrificans* colonized the materials relatively rapidly and the amount of biofilm attached to pumice, LECA and wood showed an increasing trend over time (Figure 3a in paper III) while *B. denitrificans* took longer time to colonize the materials and then maintained a constant amount of biofilm (Figure 3b in paper III). The biofilm formation on Kaldnes K1 carriers showed great fluctuations from measurement to measurement for both strains. This might be due to an uneven colonization of the individual carriers caused by the combination of a smooth surface, retarding initial adherence, and a large protected surface area, facilitating biofilm growth. Due to the different properties of the assessed materials, commonly used methods for measuring biofilm amount like crystal violet staining or detachment followed by viable count, was not applicable. The use of denitrification activity as an indirect measure of biofilm formation by denitrifying strains gave reliable results which were in parity with microscopic observations. In thicker biofilms, however, substrate diffusion limitations will convey a non-linear relationship between biofilm amount and denitrification rate, making the method inadequate.

5.2.1 Conclusions

Biofilm formation on carrier surfaces showed high potential. Both of the investigated strains could colonize the surface of several materials with various properties. Pure strain biofilm populations were established and remained stable during the experiment (5 weeks). In addition, the denitrification activity of the strains was not diminished in the biofilm. Due to the promising qualities of biofilm systems, further studies with the aim to characterize important biofilm properties were performed.

6. Biofilm characterization

Biofilm systems showed promising qualities as a technique for bacterial immobilization. Thus, a characterization of several biofilm properties of selected key-organisms and strains commonly found in wastewater treatment systems was carried out with the aim to increase the understanding of biofilm performance. Although biofilms are the most common mode of existence for microorganisms in natural environments [23, 24] very little is known about the biofilm physiology and behavior [162]. For example, it is not known if all bacterial strains have the ability to form biofilm [128] or which cell properties are most important for surface attachment [52]. This chapter describes several studies characterizing various biofilm properties such as surface adhesion, biofilm formation ability, EPS and polysaccharide composition, bacterial interactions, biological activity and proliferation of wastewater treatment isolates. This type of knowledge may be critical for development of more efficient biofilm wastewater treatment processes and continuous research in the area should be encouraged.

6.1 Adhesion properties (II, IV)

The attachment mechanism and biofilm formation ability of 13 bacterial strains commonly found in wastewater treatment systems (Table 2) cultured in sterile wastewater was investigated (paper II). Initial adherence (within 30 minutes) to a polystyrene surface, bacterial cell surface hydrophobicity, amyloid adhesin synthesis, EPS production as well as biofilm formation and corresponding planktonic growth after 24h was analyzed for all strains. Initial adherence and subsequent attachment of cells to a surface is an important step for the development of a strong biofilm since it constitutes the link that anchors the entire biofilm to the surface. Cell surface hydrophobicity and presence of amyloid adhesins have been suggested to be important factors for initial adherence and subsequent biofilm development [52, 163].

The results on initial adherence, cell surface hydrophobicity, amyloid synthesis and EPS production for the 13 strains are compiled in Table 2. No clear trend in how the hydrophobicity, amyloid fibers and EPS affected initial adherence and biofilm formation could be seen. For example, the strain *B. denitrificans* B79 which was shown to produce amyloid fibers and display a hydrophobic cell surface showed a high initial adherence, as expected. *Bacillus cereus* SJV on the other hand, produced amyloid adhesins but had a completely hydrophilic cell surface and showed deficient initial adherence properties, suggesting little influence of the amyloid adhesins on initial adherence. Further, *C. denitrificans* 110 which did not produce amyloid fibers and had a hydrophilic cell surface still adhered to the polystyrene surface within

30 minutes to a greater extent than all the other strains. Apparently *C. denitrificans* 110 adhered to the polystyrene surface by some other mechanism.

The biofilm formation and corresponding planktonic growth after 24h is shown in Figure 12a and b. Interestingly, the biofilm formation after 24h growth did not correlate to the initial adherence, seeing that adherence deficient strains like *B. cereus* SJV did form strong biofilms after 24h while *P. aeruginosa*, a strain with substantial initial adherence formed only week biofilm after 24h. Instead the biofilm formation seemed to be generally dependant on EPS production. The EPS producing strains all formed biofilms in sterile wastewater while the EPS deficient strains did not. However, two clear discrepancies were seen in the two hydrophobic strains *A. calcoaceticus* and *A. hydrophila* L6. These strains were both EPS deficient but formed biofilm anyway after 24h implying that attachment could be mediated by hydrophobic interactions. Microscopic observations showed that the initial adherence pattern of an organism was maintained in the biofilm (Figure 2 in paper II).

Table 2. The adhesion properties of the 13 strains.

Species	**Initial adherence(%)[A]**	**Hydro-phobicity(%)[B]**	**CRA-method[C]**	**FISH-Cfw[D] (cells/EPS)**
Acinetobacter calcoaceticus	7.6	79	pink	+ + / -
Aeromonas hydrophila L6	1.2	30	white	+ / -
Bacillus cereus SJV	0.1	0	red	+ / + +
Brachymonas denitrificans B79	13.5	76	red	+ + / +
Brachymonas denitrificans SJV	1.0	9	white	+ / +
Comamonas denitrificans 110	15.1	0	pink	+ / + +
Comamonas denitrificans 123	1.7	3	pink	+ + / + +
Delftia sp. SJV	5.4	5	white	+ + / +
Escherichia coli AF1000	1.6	8	white	- / -
Escherichia coli K-12	2.3	6	white	- / -
Klebsiella pneumoniae SJV	1.0	14	white	- / -
Pseudomonas aeruginosa	9.0	8	pink	+ / +
Zoogloea ramigera	0.7	10	pink	+ / +

[A]Number of cells adhering to polystyrene within 30min out of the total number of added cells [164].

[B]Percentage of cells in the xylene (hydrophobic) phase after extraction (water-xylene method) [165]

[C]Color of colonies grown on CRA; red – amyloid producing organisms, white – non-producing organisms, pink - can not be classified [29].

[D]Optical estimation of the relative amount of cells (FISH) and EPS (calcofluor white, Cfw) using CLSM. (-) absence of cells/EPS, (+) moderate and (+ +) substantial presence of cells/EPS [164].

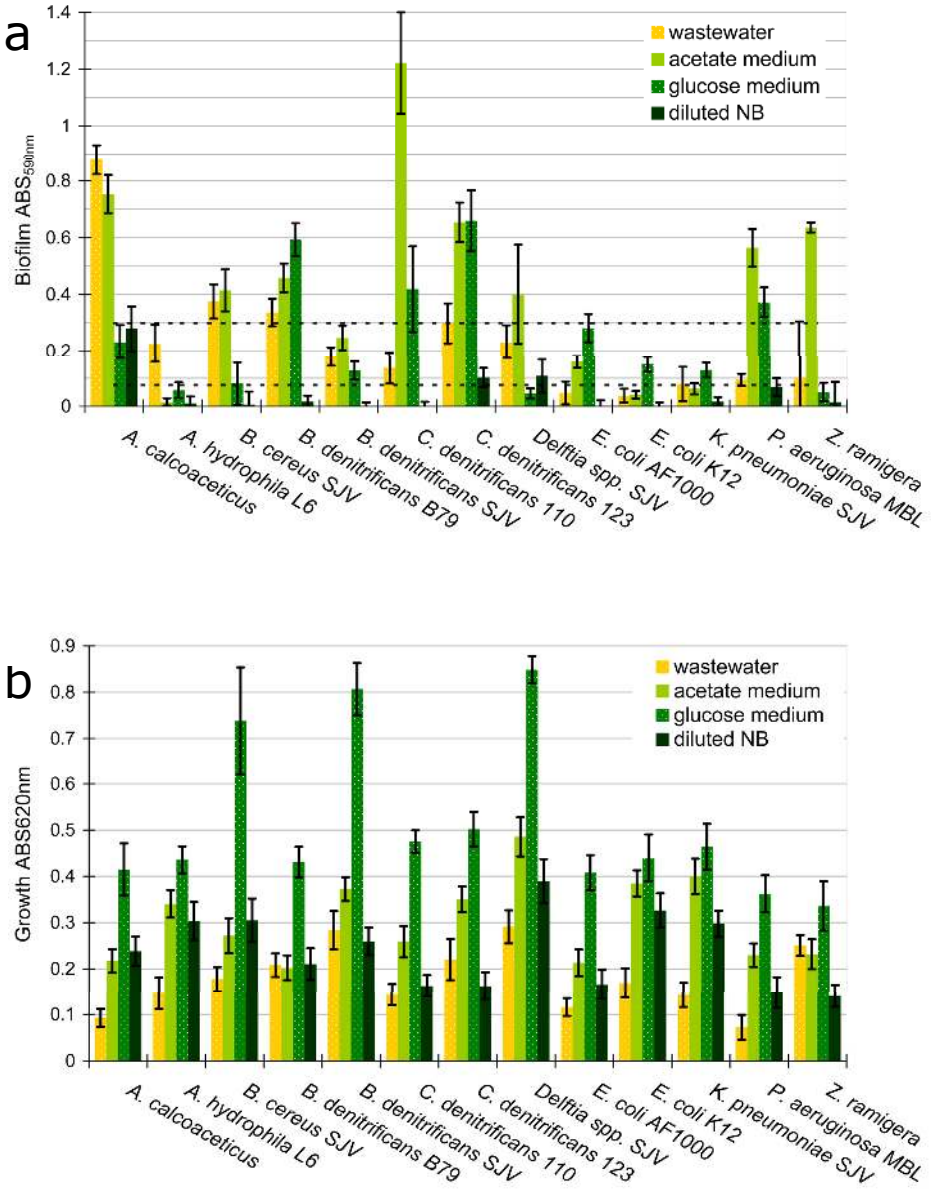

Figure 12. The biofilm formation (a) and planktonic growth (b) of the thirteen organisms described in Table 2, in sterile wastewater, minimal medium supplemented with acetate or glucose and ten times diluted nutrient broth (NB). Bars below the lower dotted line in (a) are defined as non-biofilm formers ($ABS \leq ABS_{control}$), bars above the upper dotted line are defined as strong biofilm formers ($ABS \geq 4 \times ABS_{control}$) [166].

The discovery of the involvement of extracellular DNA in biofilm formation and stability has received considerable attention in the biofilm research community in recent years [71, 72, 167]. Therefore, the ability of strain *C. denitrificans* 110 to form biofilm in the presence of DNase I was investigated (paper IV). The planktonic cell growth was not affected by the presence of the enzyme, however, the amount of biofilm formed after 24h in the presence of DNase was only 5% compared to the reference without DNase I (Figure 1b in paper IV). This implies that secretion of extracellular DNA is a very important factor for biofilm formation by strain *C. denitrificans* 110.

6.1.1. Conclusions

Altogether, the results emphasize the importance of EPS for structural development of biofilms since the EPS deficient strains either failed to form detectable biofilm within 24h or formed monolayer films unlikely to develop three dimensional structures. The mechanism responsible for formation of EPS deficient monolayer biofilm by *A. calcoaceticus* and *A. hydrophila* L6 appeared to be hydrophobic interactions. The results further show that the initial adherence of cells to a surface can be mediated by several different parameters including hydrophobic interactions, amyloid adhesin or extracellular DNA. The biofilm formation after 24h was not influenced by the extent of the initial adherence, however, the spatial distribution of cells during the initial adherence was conserved in the biofilms.

6.2 Nutrients and biofilm formation (II, IV)

The influence of the culture medium composition, carbon source and planktonic growth rate on biofilm formation was investigated in two separate studies using a micro titer plate assay. The thirteen bacterial strains defined in Table 2 were allowed to form biofilm for 24 and 48h in four different culture media; sterile wastewater, minimal medium supplemented with acetate or glucose and diluted nutrient broth (paper II). In addition, the influence of different carbon sources on biofilm formation by *C. denitrificans* 110 was studied using minimal medium supplemented with eleven different carbon sources as well as three reference media; sterile wastewater, nutrient broth and isotonic NaCl-solution (paper IV).

Figure 12a and b shows the biofilm formation and planktonic growth of the 13 bacterial strains in the four culture media after 24h. No significant differences were seen between the cultures grown for 24 and 48h, hence, only the results obtained after 24h growth are discussed here. The biofilm formation differed considerably depending on bacterial strain as well as culture medium. The strongest biofilm of all was formed by *C. denitrificans* 110 in minimal medium supplemented with acetate. However, the same organism did not form biofilm at all in diluted nutrient broth. In general, the acetate medium promoted strong biofilm

formation while the diluted nutrient broth resulted in poor biofilm formation. The results were thus in agreement with our expectations since the minimal medium was prepared with high carbon to nitrogen ratio and should promote biofilm growth while nutrient broth is rich in both nitrogen and phosphorus thus supporting cell growth. Wastewater, which is composed of both easily accessible nutrients and more complex molecules, resulted in biofilm formation by all bacteria except the two *E. coli* strains. Figure 12b shows that planktonic growth was best in minimal medium supplemented with glucose and poorest in wastewater. Figure 12a and b as well as statistical analyses using paired students t-test showed that no correlation between biofilm formation and planktonic growth could be found.

The influence of carbon sources on biofilm formation was investigated using strain *C. denitrificans* 110 and the biofilm promoting minimal medium. The results, displayed in Figure 13, show that the carbon source influences the biofilm formation and, to a lesser extent, the planktonic growth. Most biofilm was formed in the presence of acetate and isobutyric acid, two small molecules with two and four carbons respectively. Phenol, which is toxic to many bacteria, resulted in both planktonic and biofilm growth. The five 6-carbon sugars as well as the di- and polysaccharide resulted in considerably less biofilm formation. The reference, cells suspended in NaCl, showed no planktonic growth but biofilm was still formed, probably as a result of adherence of the inoculated cells to the surface.

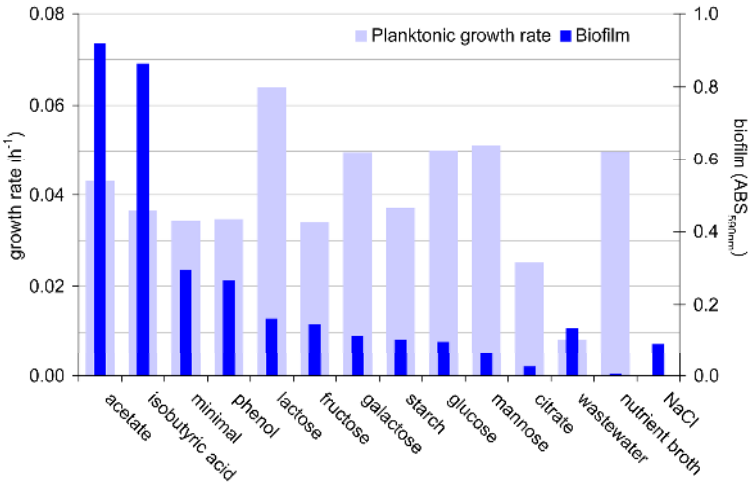

Figure 13. The biofilm formation and planktonic growth rate during 24h of *C. denitrificans* 110 in minimal medium supplied with eleven different carbon sources as well as wastewater, nutrient broth and isotonic NaCl solution.

6.2.1 Conclusions

In summary, the studies showed that all the thirteen strains assessed could form single strain biofilm if an appropriate culture medium was supplied although the amount of formed biofilm varied from strain to strain. The composition of the culture medium as well as the choice of carbon source is highly decisive for biofilm formation. The planktonic growth rate was not reflected in the biofilm formation rate, implying a switch in bacterial physiological traits when changing from planktonic to biofilm lifestyle.

6.3 EPS characterization (IV, V)

The production of EPS is essential for biofilm formation [59] and most organisms are known to produce EPS with an individual composition as well as polysaccharides with distinctive structures and composition [80]. The composition of EPS and polysaccharides produced by *C. denitrificans* 110 under different growth conditions was characterized in detail (paper IV). In addition, a less extensive characterization of the biofilm EPS and polysaccharides produced by *B. denitrificans* B79, *A. hydrophila* L6 and *A. calcoaceticus* was also performed (paper V).

C. denitrificans 110 was chosen as a model organism for the detailed EPS characterization since it possesses several interesting features such as good biofilm formation in various culture media [164], good denitrification properties [153] and abundant occurrence in wastewater treatment sludge samples from different sites not only in Sweden (Gunnel Dalhammar, personal communication). Moreover, the EPS produced by *C. denitrificans* 110 have not been previously characterized. The focus of the study was to investigate how the EPS composition varied with culture medium composition, growth mode (biofilm, agar and planktonic cultures) and time (paper IV). The extracellular polymers from cultures grown in different culture media and growth modes were extracted, purified and characterized with respect to carbohydrate, protein, lipid and nucleic acid content. The protein fraction of the EPS was characterized by SDS-PAGE separation. The carbohydrate fraction, which is normally made up exclusively of polysaccharides [77], was characterized regarding size, mono sugar composition and main sugar linkages. In addition, EPS of *C. denitrificans* 110 grown as biofilm in minimal acetate medium for different time periods spanning from 12h to 8 days were analyzed to see if the EPS composition changed with time. These samples were extracted in two steps (ultracentrifugation followed by chemical extraction) to obtain a separation of the released and cell associated EPS. For the other three strains, *B. denitrificans* B79, *A. hydrophila* L6 and *A. calcoaceticus,* EPS from biofilms grown in minimal medium supplemented with acetate were extracted, purified and characterized (EPS and polysaccharide mono sugar composition) (paper V).

We found that *C. denitrificans* 110 EPS extracted from biofilm and agar plate cultures had a protein to carbohydrate ratio above one while the ratio was below one in the planktonically grown samples (Figure 14). Unlike *C. denitrificans* 110 biofilm EPS, strains *B. denitrificans* B79, *A. calcoaceticus* and *A. hydrophila* L6 all produced EPS with a protein to carbohydrate ratio below one and a lipid fraction which made up more than 9% (*w*/*w*) of the EPS. For the *B. denitrificans* B79 sample, lipid was even the major EPS component. *A. calcoaceticus* and *A. hydrophila* L6 produced considerably smaller amounts of EPS compared to the other two strains. *C. denitrificans* 110 EPS composition was generally influenced to a lesser extent by the culture medium than the growth mode.

The mono sugar composition of the hydrolyzed polysaccharides are shown in Figure 15. *C. denitrificans* 110, A. *calcoaceticus* and *A. hydrophila* L6 exopolysaccharides were all made up of seven sugars; ribose, mannose, glucose, galactose, rhamnose, arabinose and acetyl glucosamine. Ribose was generally the main fraction in these polysaccharides which is a quite rare finding, only few studies have reported ribose to be present in more than trace amounts [168]. To make sure that the ribose was not derived from digested nucleic acid, several control experiments were made. DNA and RNA removal from the crude EPS by specifically binding matrixes or enzyme digestion followed by dialysis and subsequent HPLC analysis resulted in

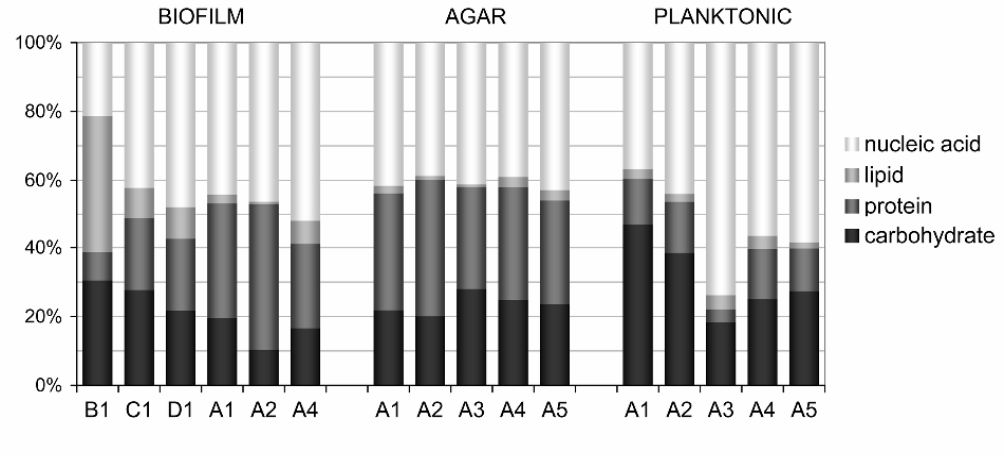

Figure 14. The composition of EPS produced by *C. denitrificans* 110 (**A**), *B. denitrificans* B79 (**B**), *A. calcoaceticus* (**C**) and *A. hydrophila* L6 (**D**) in minimal medium supplemented with acetate (**1**), isobutyric acid (**2**) or citrate (**3**) as well as sterile wastewater (**4**) and nutrient broth (**5**). EPS produced by cells grown in biofilm (7 days), on agar plates (48h) and planktonically (24h) were examined.

chromatograms identical to the samples without specific removal of potential nucleic acids. In addition, HPLC analysis of digested RNA and DNA revealed a characteristic nucleic acid peak which was not present in the polysaccharide samples. These results indicate that the ribose detected in the purified carbohydrate fraction of the EPS was indeed derived from the exopolysaccharides. *B. denitrificans* B79 polysaccharides lacked the sugar monomers ribose and acetyl glucosamine.

Similar to the EPS composition results, the growth mode had a greater impact on the sugar monomer composition and polysaccharide size than the culture medium (except sterile wastewater). Biofilm polysaccharides had a molecular weight of 500 kDa, agar plate colony grown polysaccharides weighted 300 kDa and the polymers produced in planktonic culture only reached 100 kDa.

The profile of the extracellular proteins produced by *C. denitrificans* 110 on SDS-PAGE showed few bands and a smeared look for all samples (Figure 3b in paper IV). Planktonically grown cells produced fewer protein fractions than biofilm or agar grown cells. All samples, including the reference cell lysate, displayed a strong band with a size corresponding to a molecular weight of 36 kDa. An attempt to characterize this protein by MS based partial sequencing of tryptic peptides according to standard proteomic protocol was made. However, the obtained amino acid sequences did not generate any reliable matches in the NCBI protein database, possibly due to impurities in the protein bands cut from the gel.

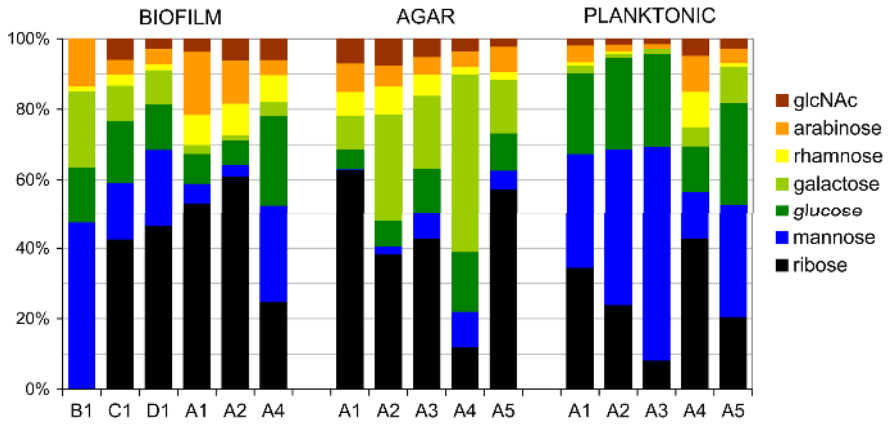

Figure 15. The mono sugar composition of exopolysaccharides produced by *C. denitrificans* 110 (**A**), *B. denitrificans* B79 (**B**), *A. calcoaceticus* (**C**) and *A. hydrophila* L6 (**D**) in minimal medium supplemented with acetate (**1**), isobutyric acid (**2**) or citrate (**3**) as well as sterile wastewater (**4**) and nutrient broth (**5**). Exopolysaccharides synthesized by cells grown in biofilm (7 days), on agar plates (48h) and planktonically (24h) were examined. glcNAc = acetyl glucosamine

The temporal study of *C. denitrificans* 110 exopolysaccharides produced during biofilm growth in minimal acetate medium showed a compositional change over time (Figure 4b in paper IV) with a decreased ribose and an increased glucose concentration. By using the combined EPS extraction method (physical and chemical) we could conclude that *C. denitrificans* 110 most likely produce at least two different exopolysaccharides, one cells associated fraction and another which is released to the surrounding.

A substantially larger amount of cell associated polysaccharides were produced (20:1 *w:w*) although a complete separation of the polysaccharide types was most likely not achieved. Linkage analysis of the two polysaccharide fraction showed presence of terminal (t-hexopyranose), linear (3-Rib*f*, 3-Glc*p*, 2-Hex*p*, 3-Man*p* and 4-Glc*p*) and branched (2,3-Hex*p*, 2,6-Hex*p*) derivatives (Table 1 in paper IV). The released polysaccharide was found to be branched and consist of an equimolar mixture of glucose and mannose. The cell associated polysaccharide was highly branched and was built up by ribose, mannose, glucose, arabinose, acetyl glucosamine and galactose sugar monomers. The large number of sugar monomers found in the cell associated polysaccharide fraction implies that it may comprise more than one polysaccharide type.

6.3.1 Conclusions

The study of EPS, extracellular protein and exopolysaccharide from samples produced by *C. denitrificans* 110 showed that the growth mode, i.e. growth in biofilm, on agar plate or planktonically, had a greater impact on the composition and size than the culture medium. *C. denitrificans* 110 was shown to synthesize at least two different branched polysaccharides as well as one major protein component with a size of 36 kDa. *A. calcoaceticus* and *A. hydrophila* L6 produced EPS and polysaccharides with a composition rather similar to *C. denitrificans* 110 while *B. denitrificans* B79 produced polymers with diverging composition, including a large lipid fraction and lack of ribose and acetyl glucosamine monomers in the exopolysaccharides.

6.4 Interactions (II, V)

The influence of bacterial interactions is believed to be greater in biofilms than in planktonic cultures due to the proximity and immobility of the cells in the matrix combined with the vast number of different microenvironments that exist in a biofilm [28, 39]. Interactions have been suggested to convey large impact on biofilm development [169] as well as biofilm matrix composition. Therefore, different combinations of strains were co-cultured to investigate the influence of interactions on biofilm formation (paper II) and EPS and polysaccharide composition (V).

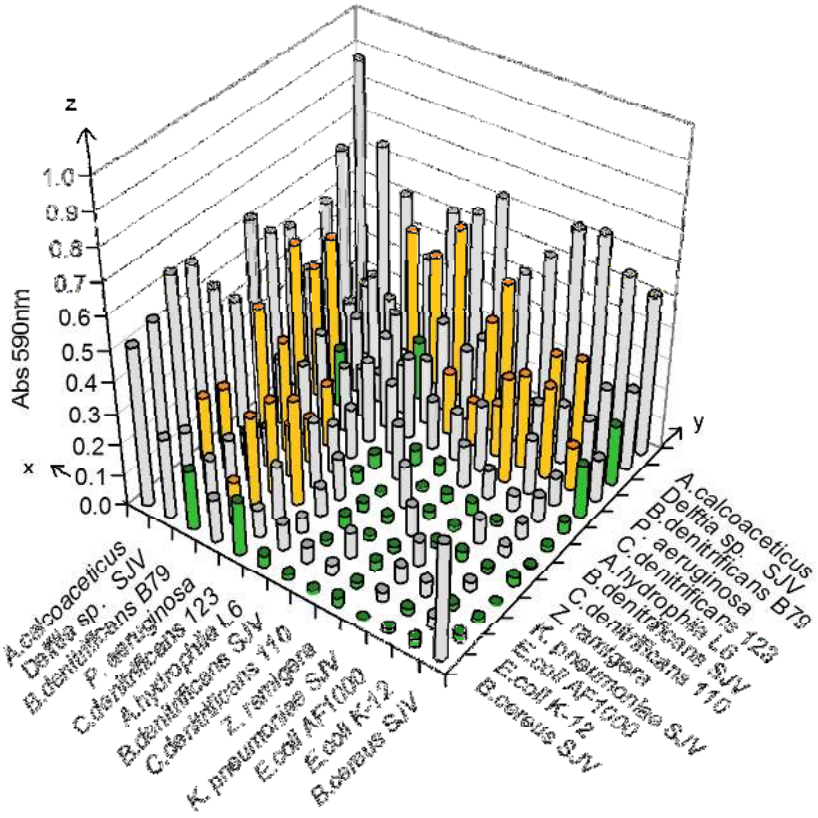

Figure 16. Dual strain biofilms formed by the thirteen strains (Table 2) in sterile wastewater (24h). The bars marked with orange color shows the strain combinations resulting in synergistic effects on biofilm formation while the green bars represent strain combinations with antagonistic effects.

Dual cultures of the thirteen strains listed in Table 2 were allowed to form biofilm on polystyrene microtiter plate wells in sterile wastewater for 24h (paper II). The results, which are displayed in Figure 16, showed that five strains, *A. calcoaceticus*, *Delftia* sp. SJV, *B. denitrificans* B79, *P. aeruginosa* and *C. denitrificans* 123, formed clearly detectable biofilm in dual culture with any of the other twelve strains. Synergistic effects on biofilm formation, i.e. when the dual strain biofilm value exceeded both single strain biofilm values [95], were observed in 14 of the 78 dual strain biofilm combinations while antagonistic effects, i.e. when the dual strain biofilm value is lower than both single strain biofilm values [95], were seen in 22 of the combinations (Figure 16). Dual strain biofilms involving either one of the strains *Delftia* sp SJV or *P. aeruginosa* most frequently resulted in synergism. *B. cereus* SJV on the other hand was the strain most commonly involved in antagonistic interactions.

To investigate if similar interactions took place also in multi strain biofilms, a set of experiments on mixed cultures were performed (paper II). The results are shown in Figure 17. The mixture of all thirteen strains defined in Table 2 formed the strongest biofilm (A). Analysis of the microbial population using FISH showed that *P. aeruginosa*, and the two *Comamonas* strains occurred in the highest relative numbers while the strongest co-adhering strain, *A. calcoaceticus*, was only detected in small numbers.

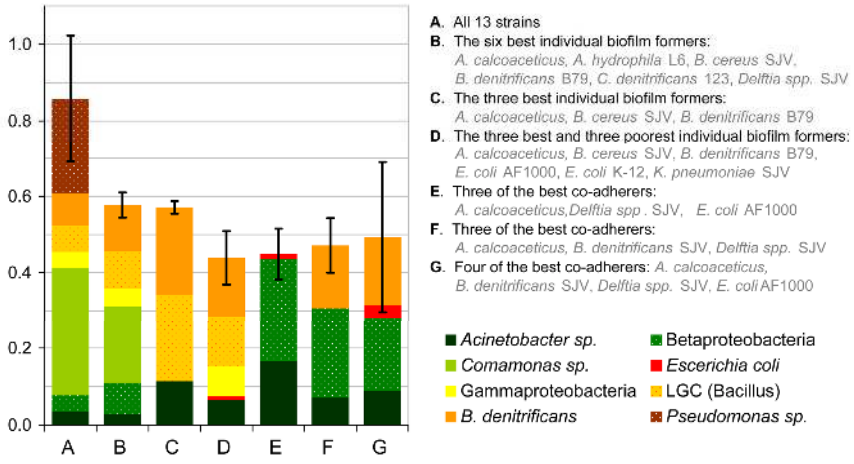

Figure 17. Multi strain biofilms. The height of the bars represents the biofilm formation and the relative presence of different strains in the biofilms, determined with FISH, is represented by the colored fractions of each bar.

The mixture of the six individually strongest biofilm formers (B) resulted in an equally strong biofilm as the mixture of the three individually strongest biofilm formers (C). However, if the three individually strongest and the three individually weakest biofilm formers were mixed (D), a significantly poorer biofilm formation was observed. Interestingly, the second best individual biofilm former, *B. cereus* SJV, was also found to be involved in the most number of antagonistic interactions. When a mixture of the three or four best co-adhering strains (E, F, G) was allowed to form biofilms together, the biofilm formation was poorer than in the respective dual strain biofilms (Figure 16).

Seeing that the biofilm formation was strongly affected by bacterial interactions in dual or multi strain cultures, the impact of interactions on the EPS production was consequently investigated (paper V). The model organism *C. denitrificans* 110 as well as the strains *B. denitrificans* B79, *A. hydrophila* L6 and *A. calcoaceticus* were assessed. Dual and quadruple strain biofilms were grown in minimal acetate medium for 20 days before the EPS was extracted and purified. Both EPS (carbohydrate, protein, lipid, nucleic acid) and polysaccharide residue composition were analyzed. In addition, the relative abundance of each strain in the extracted biofilms was examined microscopically. In the dual strain cultures, *C. denitrificans* 110 and *B. denitrificans* B79 were the dominating strains while *A. hydrophila* L6 was the least competitive strain. In the quadruple strain biofilm all strains were present in rather equal amounts, implying that the less competitive strains were favored by the multi species interactions.

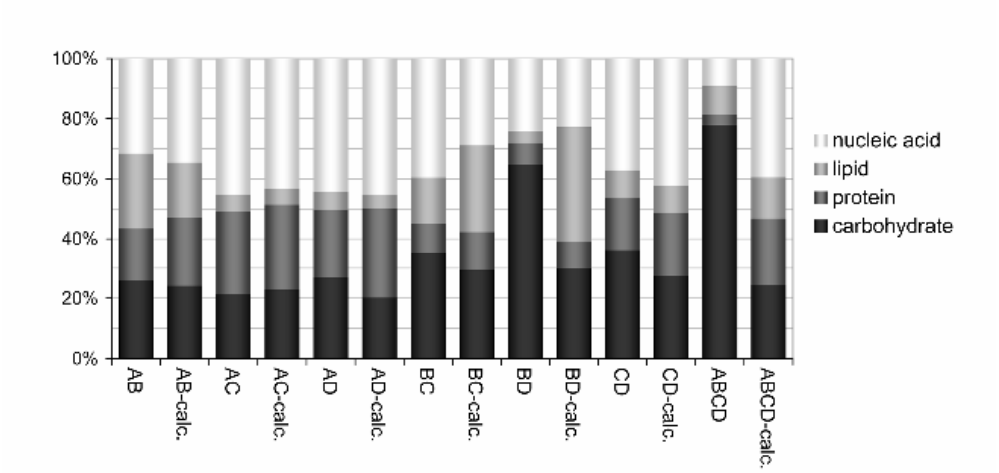

Figure 18. The EPS composition of dual and quadruple strain biofilms formed by *C. denitrificans* 110 (A), *B. denitrificans* B79 (B), *A. calcoaceticus* (C) and *A. hydrophila* L6 (D). The calculated weighted average value of each dual strain EPS is additionally shown (labeled with –calc.)

The EPS composition of the dual and quadruple strain mixtures together with a weighted average value based on the EPS composition of each single strain (from Figure 14) are shown in Figure 18. Assuming that no interactions takes place in the biofilms, the composition of the EPS produced in dual strain culture would correspond to a weighted average of the two individual EPS compositions. A similar compilation of the exopolysaccharide sugar residue compositions together with the calculated weighted average values is shown in Figure 19. The dual strain mixtures with *C. denitrificans* 110 had an EPS composition which corresponded well to the calculated value, implying few interactions. The EPS produced in all the other mixed strain biofilms had a composition which differed from the calculated values, including a higher carbohydrate fraction. The samples deviating most from the calculated values were the dual strain culture with *B. denitrificans* B79 and *A. hydrophila* L6 and the quadruple strain culture.

The polysaccharide residue composition (Figure 19) generally deviated more from the calculated values than the EPS composition. A disproportional polysaccharide production by one of the strains or imbalance caused by continuous degradation of one type of polysaccharide by the other bacteria could be reasons for this. In the quadruple strain biofilm as well as all dual strain biofilms which did not contain *B. denitrificans* B79, presence of uronic acids were detected in the hydrolyzed polysaccharides although none of the single strains produced exopolysaccharides with uronic acids (Figure 15).

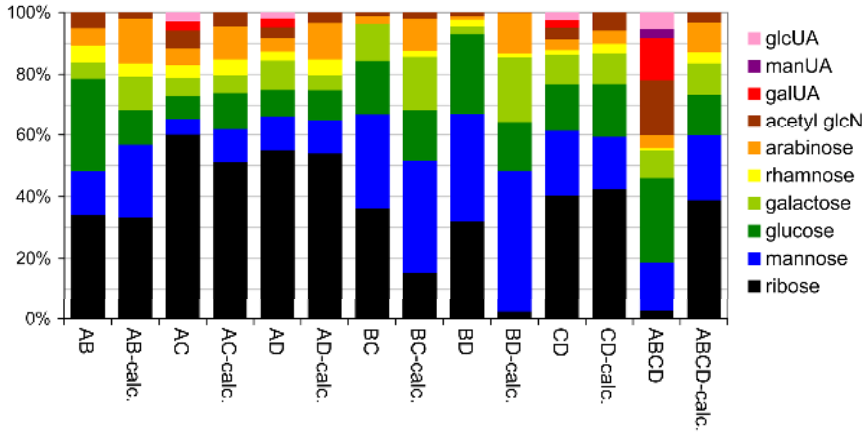

Figure 19. The mono sugar composition of the exopolysaccharides produced by dual and quadruple strain cultures *C. denitrificans* 110 (A), *B. denitrificans* B79 (B), *A. calcoaceticus* (C) and *A. hydrophila* L6 (D) grown in biofilm. The calculated weighted average value of each dual strain EPS is additionally shown (labeled with –calc.).

These results suggest that new types of polysaccharides were synthesized, induced by interspecies interactions. The quadruple strain biofilm contained a polysaccharide residue composition which deviated most from the calculated value, probably since a higher number of involved strains enhance the possibilities for interactions which can subsequently affect the matrix assembly.

6.4.1 Conclusions

Interspecies interactions were shown to have a great affect on biofilm formation, both synergism and antagonism was observed. *A. calcoaceticus* was shown to be a good co-adherer, forming strong dual strain biofilms with all twelve bacteria assessed in the study, without being the dominant strain with respect to relative cell numbers, thus *A. calcoaceticus* might be used as a bridging organism for strains with poor biofilm formation abilities. The strongest observed biofilm was formed when all 13 strains from Table 2 were grown together. Bacterial interactions were also shown to influence the EPS composition and, to an even greater extent, the exopolysaccharide residue composition. The polysaccharide residue composition from dual and mixed strain samples was unpredictable, partly due to interaction induced synthesis of polymers with new sugar monomers. The mechanisms behind the altered biopolymer synthesis could however not be concluded based on the results presented here.

6.5 Microbial activity (V, VI)

The microbial activity within biofilms relies not only on the respiratory or metabolic substrate conversion rate of the individual organisms, but also on the transport of substrates into and within the biofilm, a process which is commonly limited by diffusion [16, 86]. The key-organisms, isolated by my former colleagues, were selected based on their functional qualities. *C. denitrificans* 110 and *B. denitrificans* B79 are, as their names imply, denitrifying organisms while *A. hydrophila* L6 have an unusually high capacity to store polyphosphate intracellularly under aerobic conditions. *A. calcoaceticus,* another organism with enhanced aerobic phosphorus removal capacity, was also further investigated since it displayed interesting qualities in the interaction studies described previously. The nutrient removal activity of these strains in planktonic culture is well documented [19, 22, 153, 155], however, their nutrient removal performances in biofilm have not been previously studied. Moreover, in the previous chapter the influence of bacterial interactions on biofilm formation and matrix composition was shown to be important. Interactions are also very likely to affect the nutrient removal activity in biofilms since the substrate diffusion rate depends on the matrix composition. Therefore, the nutrient removal activity within single and mixed strain biofilms were studied using a microtiter plate assay (paper V) as well as a batch system with Kaldnes K1 carriers (paper VI).

Single, dual and quadruple strain biofilms, formed by the strains described above, were grown in microtiter plates for 20 days. The biofilms were continuously analyzed with respect to biofilm formation, bacterial diversity as well as denitrification and phosphorus removal activity (paper V). The results can be seen in Figure 20a, b and c. The general trend was an increased biofilm formation and denitrification activity over time, except for the measurement on day six when a decrease in biofilm formation was seen in all cultures (Figure 20a) as well as a drastic decline in denitrification activity in all cultures with *C. denitrificans* 110 (Figure 20b). The denitrification activity was generally well correlated with the biofilm amount in all cultures with denitrifying organisms present. Since denitrification is a respiratory process the overall nitrogen removal depend on the accessibility of the electron donor (organic carbon) and acceptor (nitrite) and the vitality of the bacteria. Thus, as long as there are no diffusion limitations within the matrix, more biofilm means a higher number of vital cells and thereby a higher denitrification activity. Interactions with synergistic effects on the denitrification performance were mainly seen in the dual strain biofilm with *B. denitrificans* B79 and *A. hydrophila* L6. Drawing parallels to the previous chapter, the compositional differences between pure strain *B. denitrificans* B79 EPS and dual strain *B. denitrificans* B79 and *A. hydrophila* L6 EPS is most likely the cause of the observed synergism. The large lipid fraction in *B. denitrificans* B79 EPS was expected to retard the diffusion of water soluble molecules into the biofilm. The low lipid fraction in the dual strain culture thus allowed for a faster diffusion and thereby also an enhance denitrification rate.

The phosphorus removal activity fluctuated from day to day but was generally highest on day one followed by day 20 (Figure 20c). Opposite to the denitrification activity, an increased phosphorus removal activity was seen on day six. The phosphorus removal is based on bacterial uptake of phosphate for subsequent incorporation in new cell mass during growth or intracellular storage of polyphosphates. The removal activity is thus growth dependant. The growth rate of cells in mature biofilms is normally lower than in planktonic culture but initial biofilm formation requires rapid cell growth in order to spread out and colonize the surface, resulting in high phosphorus removal activity on day 1. The decline in biofilm formation on day six was beneficial for the phosphorus removal since an increase in the bacterial growth rate was required to restore the biofilm. From Figure 20c the phosphorus removal may appear to be more efficient in the denitrifying strains than in the strains with enhanced aerobic phosphorus removal ability. The poor biofilm formation, which can be seen in Figure 20a, causes a low overall phosphorus removal activity by strain *A. calcoaceticus* and *A. hydrophila* L6. By comparing phosphorus removal activity per biofilm unit it can be seen that these strains perform better than the denitrifying strains. Phosphorus removal synergism was seen in the dual strain biofilms with *C. denitrificans* 110 together with either *A. calcoaceticus* or *A. hydrophila* L6.

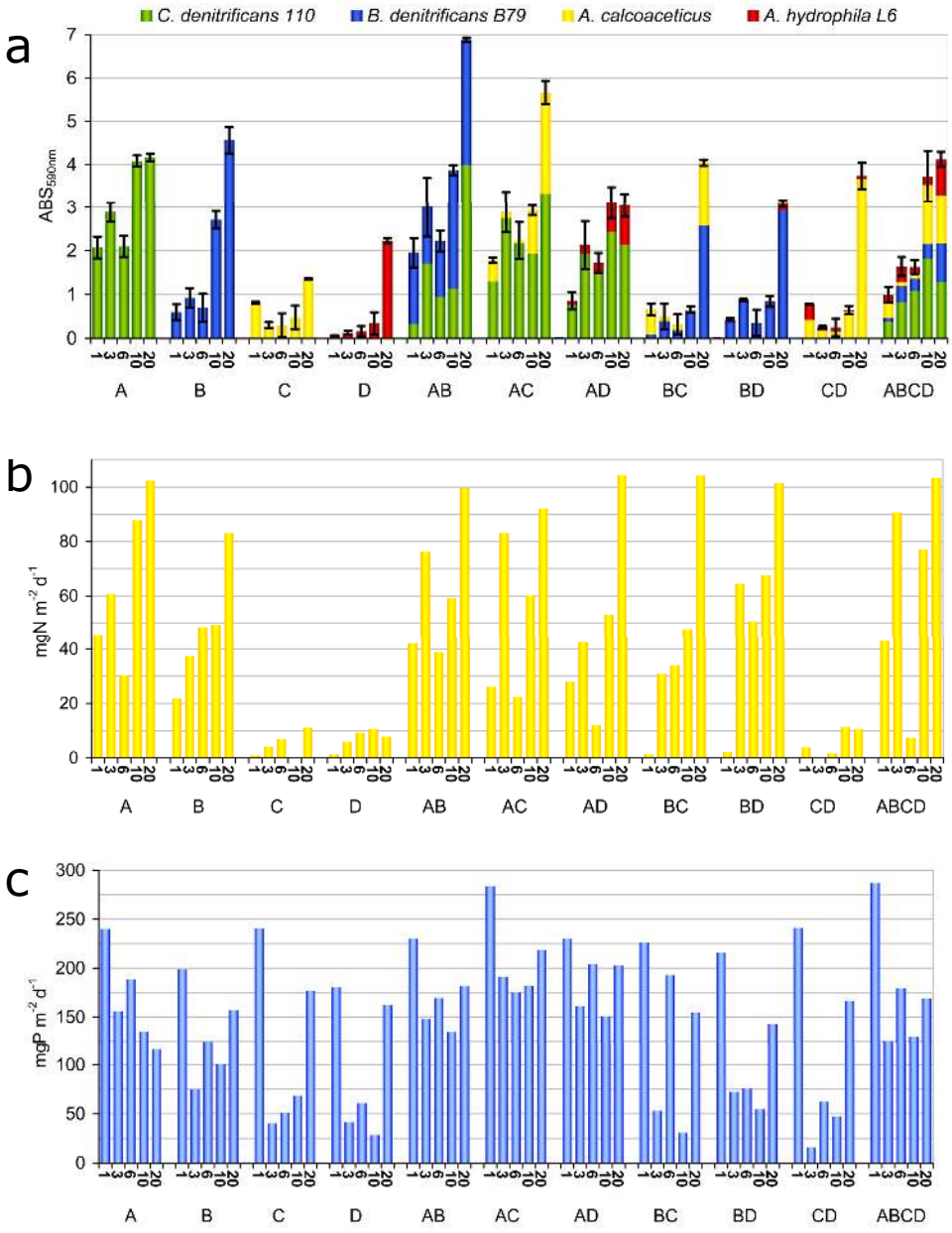

Figure 20. The biofilm formation (a), denitrification activity (b) and phosphorus removal activity (c) of dual and quadruple strain cultures of *C. denitrificans* 110 (A), *B. denitrificans* B79 (B), *A. calcoaceticus* (C) and *A. hydrophila* L6 (D) after 1, 3, 6, 10 and 20 days.

The denitrification activity was also studied in a batch system with Kaldnes K1 carriers. The two denitrifying strains and the putative biofilm bridging organism, *A. calcoaceticus*, were cultured as single, dual and triple strain biofilm in sterile wastewater during four weeks. The denitrification activity, bacterial diversity, and biofilm formation, were analyzed once a week. *C. denitrificans* 110 single strain biofilm showed the highest denitrification activity followed by *B. denitrificans* B79 in single strain culture or together with *C. denitrificans* 110. The non-denitrifying strain *A. calcoaceticus* did not contribute to higher biofilm formation, conversely, its presence seemed to disadvantage the denitrification activity (Figure 1 in paper VI). For all biofilms formed by at least one denitrifying strain a logarithmic relationship between the activity and biofilm formation was seen (Figure 21). Diffusion limitations affecting the denitrification activity in the biofilms appeared after two to three weeks of growth. The dual and triple strain biofilms were subjected to diffusion limitations in thinner biofilms than the pure strain *C. denitrificans* 110 and *B. denitrificans* B79 biofilms.

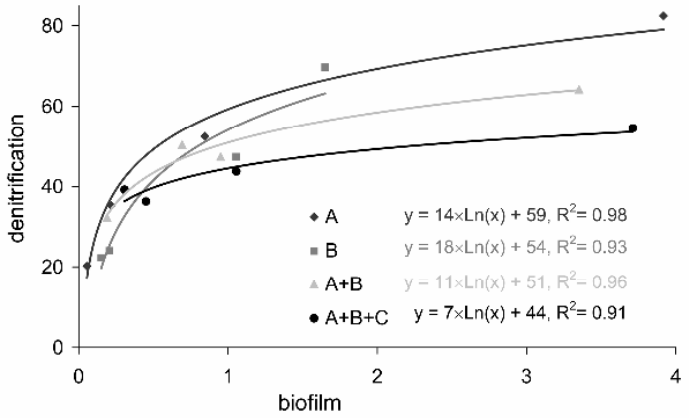

Figure 21. The denitrification activity as a function of biofilm formation. The denitrification rate is given as mg N $L_{(K1)}^{-1}$, d^{-1} and the amount of biofilm is given as ABS_{590nm}. R^2 is the correlation coefficient.

6.5.1 Conclusion

The key-organisms showed a maintained nutrient removal activity also when grown as biofilm. Both denitrification and phosphorus removal was affected by interspecies interactions, creating denitrifying synergism in biofilms with *B. denitrificans* B79 and *A. hydrophila* L6. The compositional changes in EPS within the mixed strain biofilms, compared to the pure strain biofilms, were critical for denitrification activity. The denitrification rate in biofilms demonstrated a logarithmic relationship with the biofilm formation, showing that diffusion limitations will occur in thicker biofilms.

6.6 Proliferation (VI)

The survival of *C. denitrificans* 110 and *B. denitrificans* B79 cells in a biofilm subjected to competition with a natural bacterial flora was investigated in paper VI. A dual strain biofilm was allowed to form on Kaldnes K1 carriers for two weeks in sterile wastewater. Half of the carriers were then subjected to raw wastewater with an average bacterial concentration of 2×10^5 CFU mL^{-1} for six weeks while the other half continued to be fed with sterile wastewater. The biofilm formation, denitrification activity and the microbial diversity in the biofilm was analyzed on a weekly basis. By allowing the two denitrifiers to establish a biofilm in a dual-strain system before subjecting them to a competitive environment the retention and proliferation was expected to be enhanced. The initial biofilm consisted of approximately 50% *C. denitrificans* 110 and 50% *B. denitrificans* B79 cells. After one week in the competitive environment around half of the cells incorporated into the biofilm were other types of bacteria (Figure 22). The relative abundance of *C. denitrificans* 110 and *B. denitrificans* B79 cells then continued to decrease in an exponential way until four weeks had passed and the selected strains made up just below 20% of the microbial population. From week four to six this relative abundance was kept regardless of the increase in total biofilm (Figure 2 in paper VI) The denitrification activity of the biofilms fluctuated somewhat but a clear trend of increasing activity could be seen over time.

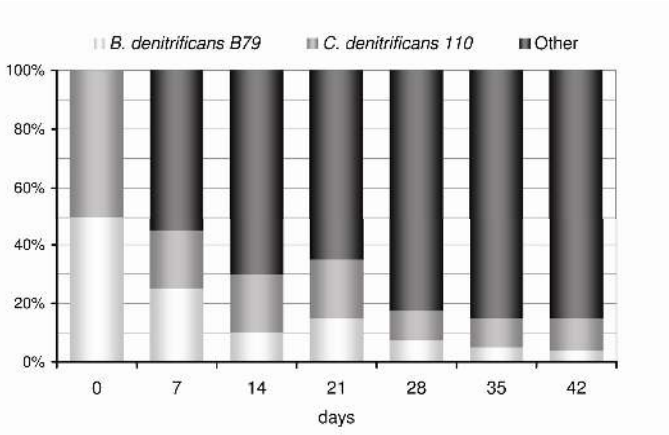

Figure 22. The proliferation of *C. denitrificans* 110 and *B. denitrificans* B79 in competition with a natural wastewater flora. The relative amount of each strain is represented by the different colors of the bars.

6.6.1 Conclusions

These results show that a stable biofilm community of selected organisms may be established in a bioreactor also when operating in the presence of a natural bacterial flora. *C. denitrificans* was found to be more competitive than *B. denitrificans* under the applied conditions.

7. Summary

The overall objective of this work was to find and characterize an appropriate immobilization method with maintained biological activity for increased retention of selected wastewater treatment key-organisms. Growth within a self produced matrix of EPS, i.e. a biofilm, showed great potential, conveying retention of the strains in the system as well as maintained denitrification and phosphorus removal activity. Consequently, a comprehensive characterization of biofilm properties and characteristics of the key-organisms as well as a number of other strains commonly found in wastewater treatment systems was made. An increased knowledge of the mechanisms controlling biofilm formation, development and function might provide improved possibilities to enhance control and performance of biofilm wastewater treatment processes.

The initial adherence of cells to a surface was found to not rely on one single factor, but rather to involve several factors including cell surface hydrophobicity and amyloid adhesin synthesis. Subsequent development of three dimensional biofilm required production of EPS. The biofilm development was greatly affected by the culture nutrient supply while the planktonic growth rate did not at all influence the biofilm formation.

The physiological properties of a biofilm are not only ascribed to its resident cells, but also the composition of the matrix housing them. The EPS contained carbohydrates, protein, nucleic acids and lipids with characteristics that varied from strain to strain but also within strains depending on growth mode and to some extent nutrient supply. The carbohydrate fraction of *C. denitrificans* 110 EPS was composed of at least two different branched polysaccharides of which one, or possibly several, was cell associated and another was released to the surrounding. The polysaccharides were made up of seven different mono sugars, in a ratio which varied with growth mode, nutrient composition and biofilm age.

A striking observation made during these studies was the increased influence of bacterial interactions in biofilm compared to planktonic cultures. Interactions were found to have a significant influence on both biofilm formation and EPS composition, causing both synergistic and antagonistic effects. The fact that new monomers, not detected in pure strain biofilm samples, were incorporated in the exopolysaccharides extracted from some of the mixed cultures indicates a much larger effect of interactions than previously anticipated. Most biofilm models today do not take this type of findings into account. By further investigation and mapping of the effects of bacterial interactions in biofilm, improved models can be developed and used in bioprocess design for various purposes, including wastewater treatment.

Bacterial By combining strains in dual or mixed cultures, interactions on biofilm formation were seen. Moreover, both the EPS and polysaccharide residue composition in

The denitrification activity of biofilm grown *C. denitrificans* 110 and, to a slightly lesser extent, *B. denitrificans* B79 was consistently high. However, substrate diffusion limited the denitrification activity in thicker biofilms. *A. calcoaceticus* and *A. hydrophila* L6 formed little biofilm, nevertheless, their phosphorus removal activity per biofilm unit exceeded the other strains. Co-culturing of the phosphorus removal organisms with *C. denitrificans* 110 greatly enhanced the over all removal activity by these strains by providing enhanced biofilms formation. By exposing existing biofilms to a competitive situation by means of a raw wastewater we showed that selected strains can survive, compete and proliferate in competitive environment.

The results presented in this thesis highlight the complexity of bacterial biofilms. By the characterization of biofilm properties of a few wastewater treatment key-organisms using simplified systems with a single, dual or multi strain consortia, we could generate relevant data which may be useful for future process development and experimental design. Bearing in mind that the knowledge of biofilm physiology, including matrix dynamics and bacterial interactions, is still very limited, the study is also significant for the general understanding of biofilm microbiology. The data on biofilm formation properties of the key-organisms together with their interactive patterns can serve as a tool for selection of bacterial strain combinations to be used in bioaugmentation or bioremediation.

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