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Technical University of Denmark

DTU

P^2M^2:

Physical and physiological properties of membrane-aerated and membrane-supported biofilms

Carles Pellicer i Nàcher

DTU Environment
Department of Environmental Engineering

PhD Thesis
April 2013

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The synopsis part of this thesis is available as a pdf-file for download from the DTU research database ORBIT: http://www.orbit.dtu.dk

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Preface

This thesis summarizes the results of the above-mentioned PhD project, carried out at the Department of Environmental Engineering of the Technical University of Denmark from October 2009 to February 2013. Professor Barth F. Smets supervised the project and Veolia Water supported it financially.

The present document is composed of a synopsis and four appendices. The synopsis contains a short literature review and a summary of the main contributions of this work to the current state of the art. Each paper is a publication in a scientific journal that shows the results obtained and the experimental protocols used in more detail:

I. **Pellicer-Nàcher, C.**, Franck, S., Gulay, A., Ruscalleda, M., Terada, A., and Smets, B.F. (2013). Sequentially aerated membrane biofilm reactors for autotrophic nitrogen removal: Microbial community composition and dynamics. *Submitted to Microbial Biotechnology.*

II. **Pellicer-Nàcher, C.**, Domingo-Félez, C., Mutlu, G., and Smets, B.F. (2013). Critical assessment of methods for the extraction of extracellular polymeric substances from mixed culture biomass. *Submitted to Water Research.*

III. **Pellicer-Nàcher, C.**, and Smets, B.F. (2013). Structure, composition, and strength of nitrifying membrane-aerated biofilms. *Submitted to Water Research.*

IV. **Pellicer-Nàcher, C.**, Domingo-Félez, C., Lackner, S., and Smets, B.F. (2013). Microbial activity catalyzes oxygen transfer in membrane-aerated nitritating biofilm reactors. *Submitted to the Journal of Membrane Science.*

In this online version of the thesis, the papers are not included but can be obtained from electronic article databases e.g. via www.orbit.dtu.dk or on request from: DTU Environment, Technical University of Denmark, Miljoevej, Building 113, 2800 Kgs. Lyngby, Denmark, reception@env.dtu.dk

The following authored and co-authored publications are closely related to the topic of the thesis, but not explicitly comprised here.

Papers:

Pellicer-Nàcher, C., Sun, S.-P., Lackner, S., Terada, A., Schreiber, F., Zhou, Q,, and Smets B.F. (2010). Sequential Aeration of Membrane-Aerated Biofilm Reactors for High-Rate Autotrophic Nitrogen Removal: Experimental Demonstration. *Environmental Science and Technology*. **44**: 7628–7634.

Sun S.-P., **Pellicer-Nàcher, C.**, Merkey, B., Zhou, Q., Xia, S.-Q., Yang, D.-H., Sun J.-H., and Smets, BF. (2010). Effective Biological Nitrogen Removal Treatment Processes for Domestic Wastewaters with Low C/N Ratios: A Review. *Environmental Engineering Science*. **27**:111–126.

Ni B.-J., Ruscalleda, M., **Pellicer-Nàcher, C.**, and Smets, B.F. (2011). Modeling Nitrous Oxide Production during Biological Nitrogen Removal via Nitrification and Denitrification: Extensions to the General ASM Models. *Environmental Science and Technology*. **45:** 7768–7776.

Book chapters:

Ruscalleda, M., Balaguer, M.D., Colprim, J., **Pellicer-Nàcher, C.**, Ni, B.-J., Smets, B.F., Sun, S.-P., and Wang R.-C. (2011). Biological Nitrogen Removal from Domestic Wastewater. In: Moo-Young, M., editor. Comprehensive Biotechnology 2nd Ed. Burlington: Elsevier, pp. 329–340.

Presentations at international conferences:

Pellicer-Nàcher, C., Domingo-Félez, C., and Smets, B.F. (2012). In-situ microbial activity in membrane-aerated biofilms performing autotrophic nitrogen conversion. *14th International symposium in microbial ecology*. Copenhagen, Denmark.

Pellicer-Nàcher, C., and Smets, B.F. (2012). Redox stratified biofilms to support completely autotrophic nitrogen removal: Principles and results. *IWA Conference on Autotrophic Nitrogen Removal*. Milano, Italy.

Pellicer-Nàcher, C., Roth, B., and Smets, B.F. (2011). Assessing the impact of physical and physiological factors on the oxygen mass transfer process in membrane-aerated biofilm reactors. *IWA Specialist Conference on Membrane Technology for Water & Wastewater Treatment.* Aachen, Germany.

Pellicer-Nàcher, C., Franck, S., Ruscalleda, M., Terada, A., and Smets, B.F. (2011). Who is who? Assessing the microbial diversity in wastewater treatment biofilms for completely autotrophic nitrogen removal. *IWA Biofilm Conference*. Shanghai, China.

Pellicer-Nàcher, C., Sun, S.P., Lackner, S., Terada, A., Schreiber, F., Zhou, Q,, and Smets B.F. (2010). Sequential Aeration of Membrane-Aerated Biofilm Reactors (MABRs) Yields for High-Rate Autotrophic Nitrogen Removal: Experimental Demonstration. *WEF/IWA Biofilm Reactor Technology Conference.* Portland, USA.

Pellicer-Nàcher, C., Ruscalleda, M., Terada, A., and Smets, B.F. (2010). Microbial community stratification in Membrane-Aerated Biofilm Reactors for Completely Autotrophic Nitrogen Removal. *Water Research Conference*. Lisbon, Portugal.

Presentations at national conferences:

Thygesen, I., **Pellicer-Nàcher, C.**, Jakobsen, M-H., and Smets, B.F. (2012). Chemical and physicochemical characterization of membrane-aerated bacterial biofilm for ammonium removal in wastewater. *6th Annual Water Research Meeting of the Danish Water Forum.* Copenhagen, Denmark.

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- And to Annette for always being there… for her love.

Abstract

Autotrophic nitrogen removal has become the process of choice to treat nitrogen-rich wastewaters due to its significantly lower operation costs. This technology makes use of stratified biofilm or bioaggregate structures to enrich aerobic and anaerobic ammonium oxidizing bacteria that catalyse the conversion of ammonium to nitrogen gas via nitrite in a single reactor. Recent work on membrane-aerated biofilm reactors (MABRs) has shown that this concept can be taken even further by growing these biocatalysts on aeration membranes, hereby significantly lowering aeration costs and greenhouse gas emissions without compromising performance.

Preliminary experimental work manifested the difficulty of reducing the activity of Nitrite Oxidizing Bacteria (NOB), which lowered the removal efficiency of the system. Advanced molecular biology tools were used to confirm that periodic aeration of MABRs can serve as a control strategy to outcompete NOB and stimulate the metabolism of anaerobic ammonium oxidizing bacteria. Furthermore, it could be observed that the accurate control of the oxygen load, unique for MABRs, allowed the construction of a highly stratified biofilm structure with aerobic ammonium oxidizing bacteria (AOB) growing on the membrane surface, and anaerobic ammonium oxidizing bacteria (AnAOB) distributed in a very thin stratum by the liquid phase. AOB and AnAOB communities, both dominated by fast-growing genera, were relatively more diverse than observed in other conventional biofilm reactors performing the same process.

Our results suggested that the detachment of large amounts of biofilm could seriously impair reactor performance due to the washout of AnAOB, growing in the outer anaerobic regions of the biofilm. It has been suggested that the excretion of extracellular polymeric substances (EPS) can enhance biofilm strength under certain conditions. Despite their crucial importance, there is currently no agreement within the scientific community on a protocol that optimizes EPS recovery from microbial samples without significantly compromising the viability of the embedded bacterial cells. Thus, we performed a rigorous benchmarking study on the effect of a wide range of published EPS extraction techniques on cell lysis and biopolymer extraction yields. According to our results, ultrasonic treatments could retrieve a larger amount of EPS from

most studied samples, were less biased by molecular interactions, and did not have a significant impact on cell integrity.

Further experimental work partially rejected the possibility of enhancing the strength of a model autotrophic MABR biofilm by either modifying the shear stress or the oxygen supply rates under cultivation. Overall, the biofilm layers closer to the biofilm-liquid interface displayed relatively lower cohesion forces against shear stresses, but still higher than observed in other conventional biofilms grown under similar environmental conditions. None of the biofilms tested detached completely from the substratum and proved to have very high adhesion strengths. Microscopic observations confirmed that this adhesion layer was dominated by very compact cell structures encapsulated in a dense layer of protein and carbohydrate. Observations at various scales were further used to confirm that a higher level of shear in the bulk liquid made the biofilms thinner, denser (less porous), more homogeneous, and with a higher content of volatile material than the rest of assayed conditions.

The factors impacting oxygen transfer with and without biofilm, unclear until now, were successfully identified using sophisticated microprofiling investigations under undisturbed reactor operation. It could be concluded that conventional methods to characterize oxygen transfer rates in clean water underestimated those observed when a biofilm was present considerably. Higher degrees of bacterial activity at the biofilm base catalysed oxygen transfer. This behaviour was described with the addition of two terms (depending on system characteristics and reactor loading conditions) to existing model structures.

In conclusion, we presented control strategies to engineer the microbial communities catalysing autotrophic nitrogen removal in MABRs, proposed methods to minimise the risk and effect of bacterial sloughing, and developed novel strategies to characterize, optimize, and better regulate oxygen transfer. Overall, the present work should serve to better design reactors supporting a cleaner, more robust, and cost-effective nitrogen removal. Furthermore, the large dataset of structural biofilm data reported here should facilitate the calibration of process models for the implementation of advanced process control.

Dansk Resumé

Fuldstændig autotrof nitrogenfjernelse er blevet den foretrukne metode til at behandle nitrogenrigt spildevand på mange aktiv-slamanlæg over hele verden på grund af de betydeligt lavere operationelle omkostninger. Denne teknik benytter sig af stratificerede biofilm eller bioaggregatstrukturer til at berige aerob og anaerob ammonium oxiderende bakterier, der fungerer som katalysatorer i omdannelsen af ammonium til nitrogengas via nitrit. Nye undersøgelser af beluftede membranbiofilmsreaktorer (MABRs) har vist, at dette koncept kan føres videre endnu ved at lade disse biokatalysatorer gro på beluftede membraner. Derved sænkes omkostningerne i forbindelse med beluftningen samt udledningen af drivhusgasser, betydeligt uden at gå på kompromis med ydeevnen.

Fluorescent insitu hybridization (FISH), kvantitativ PCR, samt højresolutions-, mærkningsbaserede 16S rRNA sekvensdatabaser blev anvendt til at undersøge og kvantificere den mikrobielle mangfoldighed, struktur og fysiologi i denne MABR. Vores resultater bekræftede en yderst stratificeret biofilmsstructur, hvor aerobe ammonium oxiderende bakterier (AOB) groede nær membranens overflade, og anaerobe oxiderende bakterier (AnAOB) groede i et tyndt stratum tæt på den flydende fase. AOB- og AnAOB samfundene blev fundet til at være mere mangfoldige end det før er blevet observeret i andre reaktorer, som udfører den samme proces. Disse mikrobielle grupper blev domineret af hurtigtvoksende slægter. Nitrit oxiderende bakterier kunne påvises, men på niveauer tæt på kvantificeringsgrænsen. Sekventiel beluftning var sandsynligvis grunden til, at disse bakterier blev udkonkurreret. Det var også den primære faktor bag den højere mangfoldighed i AOB- og AnAOB grupperne.

Vores resultater pegede imod, at løsrivelsen af store mængder biomasse havde en yderst negativ indvirkning på reaktorens ydeevne, grundet udvaskningen af AnAOB, der groede i de ydre anaerobe dele af biofilmen. Det er blevet foreslået, at udskillelsen af ekstracellulære polymeriske substanser (EPS) kan forstærke biofilm under visse betingelser. På trods af deres afgørende vigtighed er der endnu ikke nogen enighed blandt forskere angående hvilke protokoller, der bedst optimerer inddrivelsen af EPS i mikrobielle prøver uden at kompromittere de bakterielle celler deri. Derfor udførte vi en grundig benchmarkingundersøgelse af en lang række udgivne EPS-udtagelsesteknikker. Blandede biomassekulturprøver med forskelligartede fysiologier blev anvendt som testmateriale. Ifølge vores

resultater kunne ultrasonicationsbehandlinger indsamle den største mængde EPS fra de fleste prøver med det laveste bias og uden nogen betydelig påvirkning af celleintegriteten.

Ydermere undersøgte vi mulighederne for at styrke en model autotrof MABR biofilm ved enten at ændre det tangerende tryk (eng: shear stress) eller oxygenfortætningsraten under opdyrkningen. Observationer på forskellige skalaer afslørede, at højere niveauer af tryk i væsken resulterede i tyndere, mindre porøse, mere homogene biofilm med en større mængde volatilt materiale end fundet under andre testede forhold. Dog havde ingen af de undersøgte biofilm styrke til at hænge sammen, når trykket blev sat til imellem 0,2 og 0,9Pa. Derimod var de udviklede biofilm gode til at modstå normalt tryk, og de havde den bedste bindingsevne set hidtil. Mikroskopiske observationer bekræftede, at bindingslaget var domineret af yderst kompakte celleklyngestrukturer, omgivet af et tykt lag proteiner og kulhydrater. Samlet set forbedrede biomasseopdyrkning i MABR biofilm kulhydratindholdet i EPS betydeligt.

Faktorerne, der påvirker oxygenfortætningen med og uden biofilm, og som indtil nu ikke har været tydeligt beskrevet, blev med succes identificeret ved at anvende sofistikerede mikroprofilleringsundersøgelser i løbet af uforstyrret reaktoroperation. Det kunne konkluderes, at konventionelle metoder til at karakterisere oxygenfortætningsraten i rent vand i stor grad undervurderede de rater, der kan observeres, når en biofilm anvendes. En større bakteriel aktivitet ved biofilmens fundament syntes at fungere som en katalysator ved oxygenfortætningen. Denne opførsel blev mere præcist beskrevet ved at tilføje to termer (baseret på systemkarakteristika og reaktortilførelse) til de eksisterende modelstrukturer.

Afslutningsvis identificerede vi de mikrobielle samfund, der katalyserede autotrof nitrogenfjernelse i MABRs, foreslog metoder til at minimere bakteriel afrivning, og udviklede nyskabende strategier til at karakterisere, optimere og forbedre reguleringen af oxygenfortætningen. Dette arbejde kan hjælpe med at identificere muligheder for bedre at kunne udvikle reaktorer, membranmoduler og biofilm, som vil understøtte en ren, hårdfør og omkostningseffektiv nitrogenfjernelse. Desuden skulle den store mængde strukturerede biofilmdatasæt gennemgået her fremme justeringen af procesmodeller til at implementere fremskreden proceskontrol.

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1. Introduction and objectives

The main advantage of biofilm-based bioreactors over conventional suspended growth systems (i.e. activated sludge) is their higher ability to retain larger amounts of slow-growing bacteria, while still being able to be operated at short hydraulic retention times. For example, for high-performance nitrogen removal, dependent on slow growing organisms (aerobic and anaerobic ammonium oxidizing bacteria), biofilm-based reactors are preferred. In these biofilms, chemical gradients can be established with proper control of the oxygen flux. This stratification of redox conditions allows aerobic ammonium oxidizing bacteria (AOB) to grow in the aerobic biofilm regions, converting all the oxygen transferred and half of the ammonium loaded to nitrite, while anaerobic ammonium oxidizers (AnAOB), which populate the remaining anoxic portion of the biofilm, metabolize the remaining ammonium and the produced nitrite to nitrogen gas (Van Hulle et al., 2010).

However, to attain such redox-stratified environments, it might be more advantageous to grow biofilms directly on aeration membranes, because oxygen delivery can be controlled independently from the ammonium load and hydraulic disturbances in the bulk phase. With *in silico* analysis, it has been confirmed that (i) redox-stratified biofilms performing completely autotrophic nitrogen removal can be engineered, (ii) shown that they have a wider operational range than co-diffusion systems, and (iii) inferred the relative surface loading of oxygen and ammonium as key operational parameter driving system performance. However, several operational challenges have been identified that make full-scale implementation of membrane-aerated biofilm reactors (MABRs) difficult: detachment of large amounts of biomass (sloughing), oxygen loading control, and microbial community control (Terada et al., 2007; Lackner et al., 2008).

While the feasibility of the process has been recently reported, little is known about the biofilm that catalysed the conversion of ammonium to nitrogen gas. Nitrite oxidizing bacteria (NOB) growth has been identified as one of the critical factors affecting MABR performance during completely autotrophic nitrogen removal because their development at the biofilm base provides spatial protection, and it is difficult to be reverted (Lackner et al., 2010; Pellicer-Nàcher et al., 2010; Gilmore et al., 2013). Current characterization methods do not predict well oxygen fluxes in MABRs during reactor operation. An incorrect

estimation of the oxygen flux may lead to the accumulation of dissolved oxygen in the biofilm, inhibiting AnAOB activity, and stimulating the growth of NOB.

Sloughing events can also seriously impair autotrophic nitrogen removal in MABRs because AnAOB biomass, potentially growing in the biofilm regions neighbouring the bulk liquid, have very low growth rates and it could take up to several months to recover the bacterial numbers prior to the disturbance. While information on biofilm strength remains dispersed for co-diffusion biofilms, essentially nothing is known about this property for autotrophic MABR biofilms.

In summary, it remains essential to develop approaches and methodologies to better estimate and control oxygen supply, to make these biofilms less prone to sloughing, and to process instabilities caused by unwanted changes in the microbial community. Hence, this study will aim to combine measurements at various scales to find methodologies to optimize the activity, structure and physical strength of MABR biofilms performing autotrophic nitrogen conversions. More in detail, the objectives can be divided in three groups:

- **Paper I:** Examine the dynamics, structure, composition, and diversity of the microbial community performing completely autotrophic nitrogen removal in MABRs.
- **Paper II+III:** Establish the different operational conditions (chemical or hydrodynamic) that can impact biofilm physical strength
- **Paper IV:** Study oxygen transfer and control in MABRs (with and without biofilms) and create model structures that allow a more comprehensive description of oxygen fluxes in these bioreactors.

2. Theoretical background

2.1. Wastewater treatment biofilms

Biofilms are as old as wastewater treatment itself. Back in 1883 J. König, one of the wastewater treatment pioneers, decided to spray wastewater regularly on a metallic mesh in order to investigate the mechanisms driving the light “self-purification” of sewage occurring in the very polluted rivers during the late ages of the industrial revolution. After some weeks he could observe a biofilm growing on that surface and could conclude that microorganisms could catalyse the process. Not long after that, the first trickling filters were in use in Berlin. They were able to remove 66% of the dissolved organic carbon and convert most of the incoming ammonium to nitrate (Wiesmann et al., 2006). Many biofilm wastewater treatment systems have been developed since then in order to accommodate a very wide range of treatment needs.

2.1.1. Why biofilms?

The success of biofilm technologies is based on their ability to decouple two very important process parameters in wastewater treatment: solids retention time and hydraulic retention time. The combination of both characteristics allows biofilm reactors to achieve high biomass concentrations (and hence high treatment efficiencies) and high biomass retention times in relatively small reactor volumes. The latter is of special importance for processes relying on slow-growing bacterial groups, such as those mediating nitrogen conversions or xenobiotic removal (Metcalf & Eddy et al., 2003).

Biofilms can be very efficient nutrient scavengers and thicknesses of only a few hundreds of micrometres allow the establishment of very steep redox gradients within their structure (Stewart and Franklin, 2008). This strong stratification supports the growth of very diverse microbial communities, which improves the reliability of the reactor against toxic shock loads and changes in wastewater composition. Moreover, the development of oxic and anoxic niches permits the development of synergies between bacterial groups that can catalyse the removal of organic carbon, nitrogen and phosphorous from wastewater.

Biofilm growth represents a significant competitive advantage for the individual microbe, which is why 99% of all bacteria on the planet are attached to surfaces. The most obvious reason to prefer this way of life is probably the increased protection against unfavourable conditions in the bulk liquid (unfavourable pH, inhibitors, predators, shear, etc., Flemming and Wingender, 2010). However, bacterial growth in very compact structures also facilitates cell-to-cell communication mechanisms (also known as quorum sensing) that allow microorganisms to give a coordinate response against environmental stresses that can compromise their survival (nutrient scarcity, antibiotics, etc., Shrout and Nerenberg, 2012). Extracellular polymeric substances (EPS), a group of biopolymers with excellent gelation properties, bind microbial cells together and are responsible for the physical structure and strength of biofilms.

2.1.2. Bacterial adhesion

The cornerstone of these biofilm technologies, moving millions of Euros all around the world, is the attachment of certain microbes with a specific function to a surface of our choice. This process, called microbial adhesion, is the result of the combination of very complex mechanisms.

The first step involves the transport of microbial cells to a surface (Flemming and Wingender, 2010). The flow of wastewaters along bioreactors (turbulent in most cases) leads to the establishment of viscous forces on top of the biofilm attachment surfaces and, subsequently, to the creation of liquid boundary layers in which mass transport is diffusion-limited. The thickness of this layer is inversely correlated to the degree of turbulence in the reactor bulk. Transport of microorganisms in the bulk liquid of bioreactors is normally mediated by a combination of convective forces and gravity. Once in the surroundings of the boundary layer, Brownian motion, diffusion or active transport (if bacterial cells are motile) assist microbial transport to the vicinity of the attachment surface. At this stage, the success of bacterial adhesion will be dependent on energy interactions arising between the attachment surface, the bacterial surface and the bulk liquid (Oliveira et al., 2003).

These energy interactions, mainly due to electrostatic forces, acid-base interactions or Liftshitz-Van der Waals forces, are not strong enough to fix bacteria irreversibly to the attachment surface. However, they allow short-range forces of higher magnitude (e.g. hydrogen bonding, dipole momentum, etc.) to

take over and anchor bacteria permanently to the substratum. Once a single microbial cell or a small cell aggregate has anchored to a surface, the gap between cell and surface can be filled with excreted EPS. In environments with high ionic strength (>0.1 M) the adhesion process is driven only by acid-base and Liftshitz-Van der Waals interactions (An and Friedman, 1998), which can be estimated from the contact angles of several liquids on the substrata and on the bacteria of study (Oliveira et al., 2003).

As a rule of thumb, some studies suggest that adhesion is enhanced by hydrophobic substrata no matter what are the properties of the bacterial surface (Van Loosdrecht and Zehnder, 1990; Annachhatre and Bhamidimarri, 1992; Bos et al., 1999). These authors propose that bacterial adhesion is mediated through highly localized hydrophobic cell-surface organelles or appendages (also known as adhesins). The resulting hydrophobic interactions between the bacterial surface and the substratum, with very favourable adhesion energies, facilitate strong attachment forces. It has also been shown that the calculation of the total surface free energy can be a good parameter to estimate the bacterial attachment rates to a certain material (Khan et al., 2010).

Modification of the chemical composition of a substratum can accommodate the wettability and surface energy of the untreated material to make bacterial attachment more energetically favourable (Ista et al., 2009). For example, surface conditioning with -NH_2 terminated polymer chains have demonstrated to enhance the biofilm-forming ability of nitrifying bacteria on surfaces as different as polyethylene, polypropylene or glass (Lackner et al., 2009; Khan et al., 2010).

Proteins and other macromolecules may adhere to the substratum surface faster than bacteria, creating a layer known as conditioning film. Even though this film tends to mask, change and equalize the physico-chemical properties of the substrata, most microbial surfaces still seem to probe interactions towards the original substratum material (Bos et al., 1999).

Bacterial EPS encapsulation and physiological state impact adhesion as well, reducing the extent of electrostatic repulsions between cells and enhancing hydrophobic interactions between the adhering cells and their supporting materials (Habash et al., 1997; Liu and Tay, 2001; Sanin et al., 2003; Walker et al., 2006; Tsuneda et al., 2003; Long et al., 2009).

It is not only the energetics of the cell membranes or the substrata that impact attachment. Surfaces with increased roughness can protect bacteria from shear

forces in the bulk liquid and facilitate bacterial adhesion (Teughels et al., 2006). Such effect can be also accomplished with the use of reinforced fibrous fabrics or plastic carriers, which offer very high surface areas and shelter from hydraulic disturbances in the bulk liquid (Tsushima et al., 2007; Terada et al., 2009).

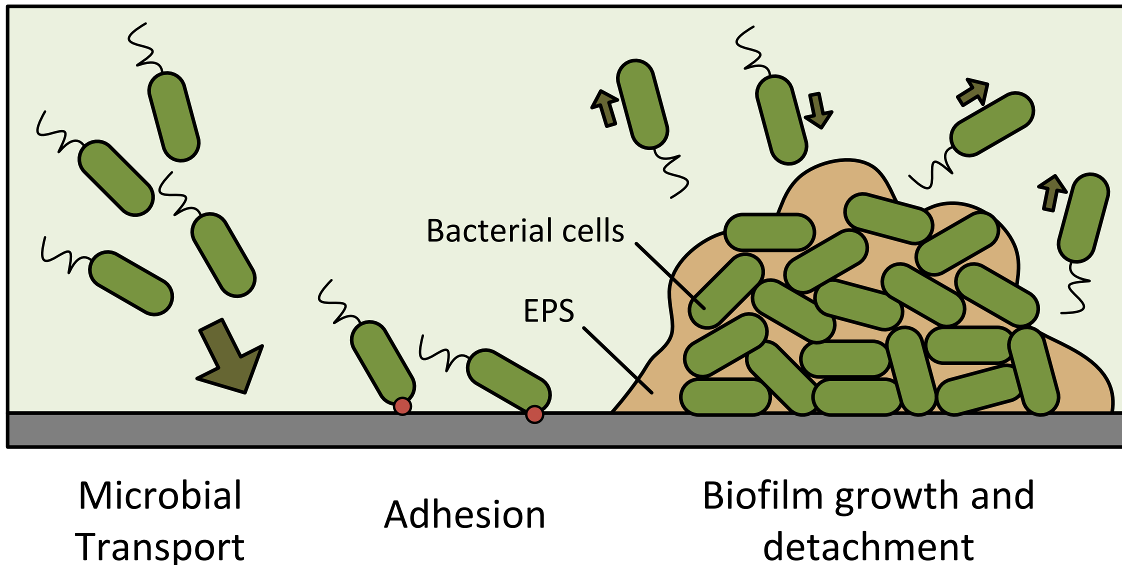


Figure 1: Dynamics of biofilm formation

2.1.3. Biofilm structure and development

After attachment, the surface colonizers grow on the substrates present in the bulk liquid, reproduce, and form biofilms. The resulting biofilm structure depends on bacterial physiology and environmental factors. Van Loosdrecht et al. (1995) suggested that biofilm structure is a balance between growth velocity and detachment forces. Therefore, conditions favouring rapid cell growth (i.e. high specific growth rates or loads) will result in the development of thick biofilms and protuberances (Kwok et al., 1998; Picioreanu et al., 2001) while slow-growing biofilms will be better shaped by shearing conditions, which will slowly erode any irregularity and lead to the development of thinner, more compact, and more regular structures. Biofilms with the similar physiological properties grown under more intense shear conditions will lead to the formation of thinner and denser biofilms (Characklis, 1981; Peyton, 1993; Derlon et al., 2008).

Partial dissolution of the EPS matrix can create an internal network of pores that can facilitate solute exchange with the bulk liquid. The upper biofilm layers, with easier access to the nutrients in the bulk liquid, grow on older biofilm strata and

assist the consolidation and compaction of the biofilm base. As a result, these biofilm layers, formed mainly by debris and inerts, display lower porosities and higher densities than the rest of the matrix (Zhang and Bishop, 1994).

Large detachment events can model biofilm structure as well. These events can occur due to (i) shearing forces higher than the biofilm cohesive force, (ii) predation by multicellular microorganisms, (iii) abrasion by solids present in the bulk liquid, or (iv) dissolution of the EPS matrix by hydrolytic or enzymatic processes.

Biofilm structure can significantly affect nutrient transport. Some authors suggest that higher biofilm roughness can assist the creation of convection currents on the biofilm surface that enhance mass transport from the bulk liquid towards the biofilm (Lewandowski, 2000). However, this effect has been neglected by modelling and experimental approaches (Picioreanu et al., 2000; Chen et al., 2005). Mass transport inside the biofilm matrix is generally governed by diffusion and the rate of chemical reaction, being the diffusion resistance highly correlated with the porosity (or density) of the biofilm. Convection through pores could also play a role on the overall transport processes, but its influence remains unclear (Rasmussen and Lewandowski, 1998). For modelling purposes all factors affecting mass transfer within the biofilm are lumped together in a single term called effective diffusivity, which is used instead of the conventional diffusion coefficient in typical mass transport equations.

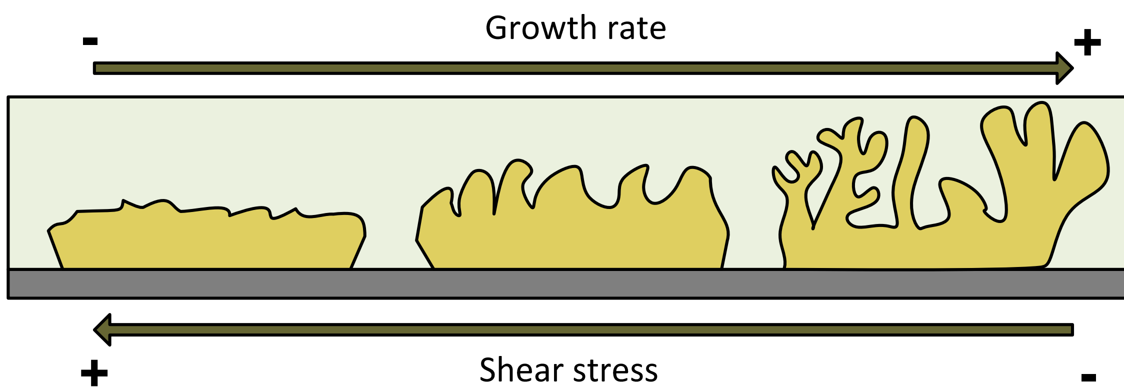


Figure 2: Typical biofilm structures as a function of shear and growth rates

2.1.4. Biofilm composition

Biofilms are formed by a variable fraction of inerts, ions and volatile material. Flemming and Wingender (2010) stated that only 10% of the volatile biofilm mass can be attributed to microbial cells (the rest is expected to be EPS), highlighting the importance of extracellular biopolymers to the structural stability and function of the biofilm matrix.

Some microorganisms are able to excrete EPS under certain environmental conditions. However, EPS can also accumulate as a result of cellular lysis, hydrolysis of macromolecules, and adsorption of organic compounds from the water matrix. Thus, EPS could also be seen as a pool of nutrients, extracellular enzymes and genetic material for the attached bacteria, which can allow the creation of unique synergic interactions (Sheng et al., 2010).

It has been proposed that EPS can be found in three layers: tightly-bound EPS or capsular EPS, found on the cell wall and bridging cells together in clusters; loosely bound EPS, gluing clusters to form microcolonies and biofilms; and soluble EPS, lightly adsorbed and involved in the formation of conditioning films (Nielsen and Jahn, 1999). The amounts and compositions of these biopolymers are variable and extremely dependent on the bacterial species involved and the existing environmental conditions (substrates, growth phase, shear, etc., Sheng et al., 2010). Nevertheless, the scientific community has agreed that EPS is mainly composed of proteins and polysaccharides, and minor amounts of DNA, lipids and humic acids (Vu et al., 2009).

Different protocols have been explored to identify and quantify EPS in wastewater biofilms. Some authors have studied the EPS distribution *in situ* via confocal laser scanning microscopy (CLSM, Adav et al., 2010), X-ray photoelectron spectroscopy (XPS, Boonaert et al., 2001), Fourier-transformed infra-red spectroscopy (FTIR, Hadjiev et al., 2007), Raman spectroscopy (Wagner et al., 2009), and nuclear magnetic resonance (NMR, Garny et al., 2010). However, these techniques require expensive equipment and the conclusions arising from their data are qualitative and potentially biased by sample preparation procedures, interferences, and the representability of the points chosen for analysis. Consequently, most EPS characterization studies are based on chemical or physical extraction methods from representative biomass samples and successive analysis of its composition.

Different amounts of accumulated EPS and different compositions have a strong influence on the behaviour of the biofilm matrix with respect to the adsorption of heavy metals (Späth et al., 1998), its biodegradability (Park and Novak, 2007), hydrophobicity (Dignac et al., 1998), or microbial cohesion (Ahimou et al., 2007).

2.1.5. Biofilm mechanics

Testing biofilm structures from a material science perspective can be a valuable tool to predict bacterial detachment and establish operation strategies to better manage biofilm thickness. This field of research is termed biofilm mechanics (Guélon et al., 2011).

Many studies have investigated the mechanical properties of biofilms by different experimental methods. However, most of them agree that biofilms are viscoelastic materials. When a certain stress (force per unit area in a certain direction) is applied to these materials, they show a time dependent strain (deformation). When the stress disappears, the material recovers its original appearance after a certain period of time (relaxation time).

Two parameters are most widely used to report mechanical failure in biofilm structures: cohesive strength and adhesive strength. While the first parameter refers to the stress leading to the detachment of parts of the biofilm, the second is the stress that leads to the complete detachment of the biofilm from the substratum. Different experimental approaches for their characterization yield very different parameter values. However, several studies conclude that biofilm cohesion is stratified, being the deeper layers more resistant against hydrodynamic stresses in the bulk liquid (Coufort et al., 2007; Mohle et al., 2007). This layer with better mechanical properties has been termed basal biofilm layer and it is able to withstand wall shear stresses as high as 12 Pa (Coufort et al., 2007). The exact composition of this layer is unknown, but CLSM observations suggest that it is very rich in EPS. Some studies also suggest that the mechanical behaviour of this layer is elastic, and hence different from the higher biofilm layers (Paul et al., 2012).

Despite the very interesting implications of these results, it remains unclear how to convey all this knowledge into real wastewater treatment systems where high substrata packing densities do not seem to be compatible with the application of homogeneous shear stresses inside the reactor. Further research is needed in

order to develop new reactor technologies that allow for a better management of solid detachment from biofilms.

2.1.6. Experimental techniques for the study of biofilms

In spite of being only a few hundreds of micrometres thick, very steep substrate gradients can develop inside biofilm structures. Such a high diversity in environmental conditions, which is difficult to estimate just from bulk measurements, complicates the analysis of the reasons leading to poor process performance in biofilm reactors. Consequently, a wide range of experimental techniques have been developed over the last 20 years for the study of the microbial ecology and composition of biofilms.

Microsensors

Microsensors are used for the undisturbed characterization of nutrient transport and nutrient concentration gradients in biofilms. These devices with tips in the micrometre range are able to generate signals (galvanometric or potentiometric) that can be correlated with the solute concentration they are sensing (Revsbech and Jørgensen, 1986). Concentration profiles with resolutions up to 10μm can be obtained when the microsensor is mounted on a micromanipulator. Even though the concentration of a wide range of solutes can be quantified, oxygen and pH microsensors are most reliable and have more often been used in literature studies. As biofilm structures can be very irregular, multiple measurements at different points are necessary in order to obtain representative concentration trends.

FTIR-ATR

In attenuated total reflectance Fourier-transformed infrared spectroscopy (FTIR-ATR) the biofilm under study is grown on top of an internal reflection element (IRE). Then, infrared radiation is focused on the inner part of this IRE and is reflected between its upper and lower surface. These reflections create an evanescent wave, which is able to penetrate a few micrometres the biofilm attachment layer. Some of the functional groups present at the biofilm base (lipid chains, amides, carboxyl groups, etc.) are able to absorb part of this IR radiation (Wolf et al., 2002), generating an IR spectra that is representative of the chemical composition of the sample. This technique has been used to estimate the evolution of the biofilm composition over time. Analyses on pure culture

biofilms have also revealed that different microbial species present different absorption spectra. However, water (80% of the biofilm) also absorbs IR radiation, masking signals from amine and lipid groups and complicating their quantification (Nivens et al., 1995).

Nuclear Magnetic Resonance

Nuclear magnetic resonance is another non-invasive imaging technique, which can track the mobility and distribution of paramagnetic ions within hydrated structures in the microscale. For example, the technique has been used to measure the distributions of water protons (^{1}H) inside and around biofilms, allowing in-situ monitoring of wall and bulk shear stresses in biofilm reactors. The spectra of biofilms incubated with ^{13}C can be used to differentiate hydrocarbons, carboxylic groups, EPS, or anomeric groups in sugars in a specific point of that sample (Garny et al., 2010; Wagner et al., 2010).

XPS

This technique is used to estimate the elemental composition of a relevant biofilm surface (cell surface, conditioning films, capsular EPS, etc.). The sample under study is freeze-dried and bombed with X-rays, which ionize the outermost atoms (first 10 nm). Each electron of a certain orbital in a certain atom has a characteristic energy, which is used to sort the generated electrons. The percentage of C, O, N or P can be then quantified by regular counting (McArthur, 2006). The use of high resolution scans can also be used to chemical environment of the ionized atoms (i.e. which other atoms are they bound to).

CLSM

Confocal laser scanning microscopy (CLSM) is a very powerful tool used for the visualization of biofilm structures. This technique requires the labelling of one or more biofilm components (nucleic acids, EPS components, structural fluorescent proteins, etc.) with fluorophores. Biofilms can be then imaged by exciting those fluorophores with lasers and collecting their fluorescence, which is magnified and filtered to account only for light coming from a single focal plane and a single fluorophore. CLSM microscopes are able to obtain images with higher resolutions, thinner optical sections and higher contrast than those obtained by conventional fluorescent microscopy (Lawrence and Neu, 1999). The biofilm structure can be inspected in a non-invasive way by taking thin optical sections

of the biofilm at different depths (Figure 3). The study of the biofilm structure by CLSM does not necessarily imply sacrificing the biofilms: the microorganisms under study can be genetically modified to produce different types of fluorescent proteins that allow *in-vivo* tracking of the evolution of the biofilm structure in special flow cells (Palmer et al., 2006).

Several software solutions can be used in order to quantify structural parameters from biofilms such as their average thickness, roughness, surface area, biovolumes, porosities, etc. Those programs rely on the conversion of the images taken by the microscope to binary images. This process, called thresholding, quantifies which is the minimum pixel intensity that can be considered to come from a biofilm. Many thresholding algorithms are available to minimize the amount of manual input that could bias the process. However, the result is not always satisfactory and semi-automatic thresholding protocols are often recommended (Yang et al., 2001).

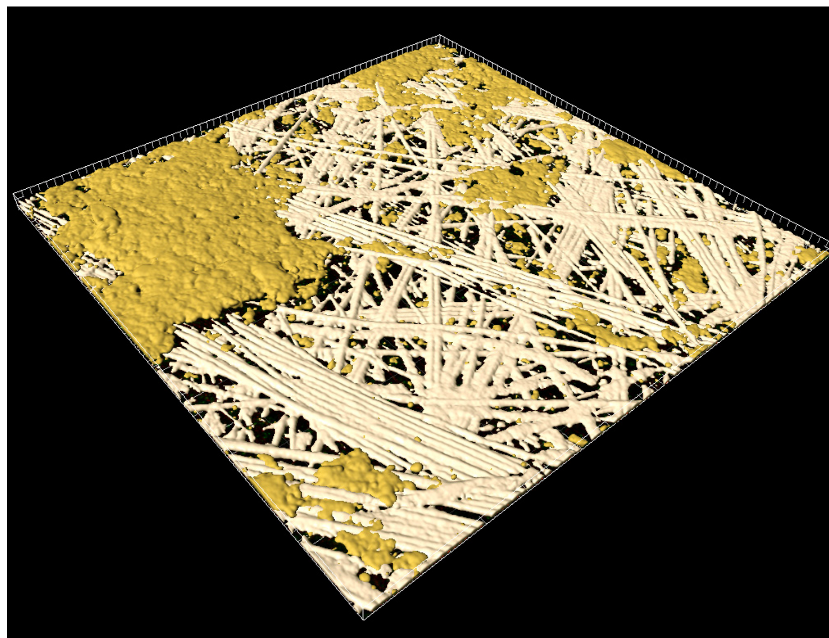


Figure 3: CLSM reconstruction of a biofilm (stained with a generic nucleic acid stain) growing on a fibrous support

Laser light can be severely scattered and attenuated within very thick or dense biofilm structures. In those conditions the laser will not be able to fully penetrate the matrix and the characterization of the whole biofilm will not be possible. The use of two-photon laser scanning microscopy can reduce the problem in some

cases. However, many authors working with these types of biofilm samples tend to embed their specimens and perform cross sections with a cryostat microtome. Shrinkage of the specimen might occur during sample preparation (Wang et al., 2009).

Raman Microscopy

In Raman spectroscopy the molecules of the biofilm specimen are irradiated with a laser of a certain wavelength. When the photons in this laser hit a functional group in a molecule, the electrons of orbitals with lower energy than the photon can be excited to a higher energy level. After a certain time, the excited electron emits the absorbed photon and returns to an orbital of a lower energetic state (equal or not to the original). That photon can be slightly scattered in comparison to the original. Raman spectroscopy measures the scattering of a certain type of emitted photons. Each molecule scatters these photons in a different way (Raman shift), and depending on the amount of molecules in the sampled spot (about 1µm) this scattering will be more or less intense. The Raman spectrophotometer can be coupled with a microscope to assist on localization of the area to sample. Furthermore, if the microscope is confocal, the operator will also have the opportunity to decide which focal plane to scan. This technique has been widely used to qualitatively measure the main functional groups of the EPS matrix without any staining. Contrarily to the other spectroscopy techniques reviewed, Raman does not require any sample preparation and is not interfered by most molecules in the EPS sample (Geesey et al., 2002; Haagensen et al., 2011).

FISH

Fluorescence *in situ* hybridization (FISH) is based on the *in situ* fluorescent labelling of 16S RNA oligonucleotide fragments (characteristic from a certain microbe or microbe family) and subsequent observation by CLSM. Therefore, this method does not only facilitate the reliable detection of species, genus, family, order and domain of bacteria forming part of a biofilm, but also their position within the biofilm structure. This duality permits the identification of synergic or competitive interactions between the different microbial communities targeted. The description many 16S RNA oligonucleotide probes are freely available online, which has favoured the popularization of this method (Wagner et al., 2003; Loy et al., 2007).

Samples from wastewater treatment environments are ideally suited for FISH, given the very high concentration of cells and their very high physiological activity. However, when working with substrate-limited biofilms, starved cells may display a reduced 16S RNA content and identification using this technique might be challenging. In those situations it might be useful to employ methods that allow the amplification of the fluorescent signal or the use of probes with two fluorophores. Contrarily, some bacteria are known to retain high 16S RNA concentrations even after long periods of inactivity and care should be taken when drafting conclusions (Daims et al., 2005).

Sometimes specific FISH probes can bind to genetic material they are not supposed to bind to. In those cases it is recommended, to add an unlabelled competitor that binds preferentially to the potentially wrong sites, making it less likely that the FISH probes will do so. In order to better discriminate specific from unspecific binding, it is also advisable to always use other probe or stain that targets all bacteria in the sample. A double positive serves as quality control check to test whether the selected probe is targeting microbes.

FISH can be used to target different microbial populations in the same sample by making use of several probes labelled with different fluorophores. In those cases it might be also interesting to perform image analysis on the obtained micrographs to determine the relative abundance, biovolumes, possible colocalization patterns, etc. of the detected microorganisms (Daims et al., 2006).

The combination of FISH with microautoradiography (MAR-FISH) can also help determining the physiology of the detected microorganisms. Here, the biofilm samples are incubated with radiolabelled substrates of interest before proceeding with the usual FISH protocol. After all the hybridizations have been completed, the sample is coated with a liquid film emulsion that reveals which sites within the sample have incorporated the radiolabelled substrate. A double positive by FISH and microautoradiography indicates that the bacteria targeted by the FISH probe have incorporated the labelled substrate into their cellular structure in that position of the biofilm. This strategy has been also explored for the identification of complex feeding interactions (also known as cross feedings) within the bacterial communities conforming the biofilm (Okabe et al., 2004).

qPCR

Quantitative polymerase chain reaction is another popular technique used in molecular biology. It is based on the amplification and quantification of the selected phylogenetic markers (16S rRNA sequences or functional genes from a specific bacterial guild or genus) with fluorescently labelled primers, that allow the replication of the DNA regions they bind to. Unlike FISH, this technique requires prior DNA extraction, which completely destructs the architecture of the specimen. However, qPCR can be performed on biofilm cryosections parallel to the attachment surface to describe the stratification of the microbial community within depth. The quantification limit of this technique is higher than the one for FISH, however, different cells can harbour different numbers of the phylogenetic markers targeted by the primers, complicating the quantification of the exact microbe numbers.

Pyrosequencing

Pyrosequencing allows the determination of the nucleotide order of up to thousands of sequences of genomic material partially amplified by PCR. The reconstructed oligonucleotide sequences are later processed by bioinformatic tools and compared to previously identified sequences in known microorganisms. An estimation of the total microbial diversity or total number of sequences of a certain type can be done with several models and estimation techniques.

2.2. Biological nitrogen removal

On the 13th October of 1908 the German scientist Fritz Haber filed the patent of a novel process that allowed the mass-scale fixation of atmospheric N_2 as ammonia in a cheap and reliable way. His discovery followed the intensification of agricultural production that, among other factors, sustained an unprecedented growth in human population (7 times more people populate the planet now than in 1900). Unfortunately, this growth has not been sustainable and has broken the natural balance in the biogeochemical cycles of many nutrients. A recent report has estimated that European population are introducing into to the environment three times more reactive nitrogen than the continent would be able to produce itself by natural means. Large amounts of this nitrogen accumulate in the planet as undesirable species that cause destruction of the ozone layer (N_2O, NO_x), global warming (N_2O), acid rain (NO_x), pollution of aquifers and water bodies (NH_4^+, NO_2^-, NO_3^-), etc.

Seen from an urban perspective, our cities, which comprise only 2% of the continental area, have the potential of dumping 15% of the total reactive nitrogen that is introduced in Europe every year to our water bodies. However, the stringent wastewater regulations and technological developments in the field of wastewater engineering allow only 0.7% of this nitrogen to end up in our lakes, seas and rivers (Sutton et al., 2011).

Three microbial groups catalyse nitrogen removal in most wastewater treatment plants: aerobic ammonium oxidizing bacteria (AOB), nitrite oxidizing bacteria (NOB) and heterotrophic bacteria (HB). The first two assist the aerobic conversion of ammonium to nitrite (AOB) and nitrate (NOB), while HB anaerobically reduces either nitrite or nitrate to innocuous nitrogen gas using different sources of organic carbon as electron donor (Figure 4). These processes occur concomitantly in wastewater treatment plants with the aerobic oxidation of biodegradable organic carbon to CO_2.

The reactor configuration and the operation regimes making these processes possible are very dependent on the wastewater composition and the space requirements. A detailed description of these configurations can be found elsewhere (Sun et al., 2010; Ruscalleda et al., 2011). In the following sections we will centre on the diversity, function and ecophysiology of the main microbial groups involved in biological nitrogen removal.

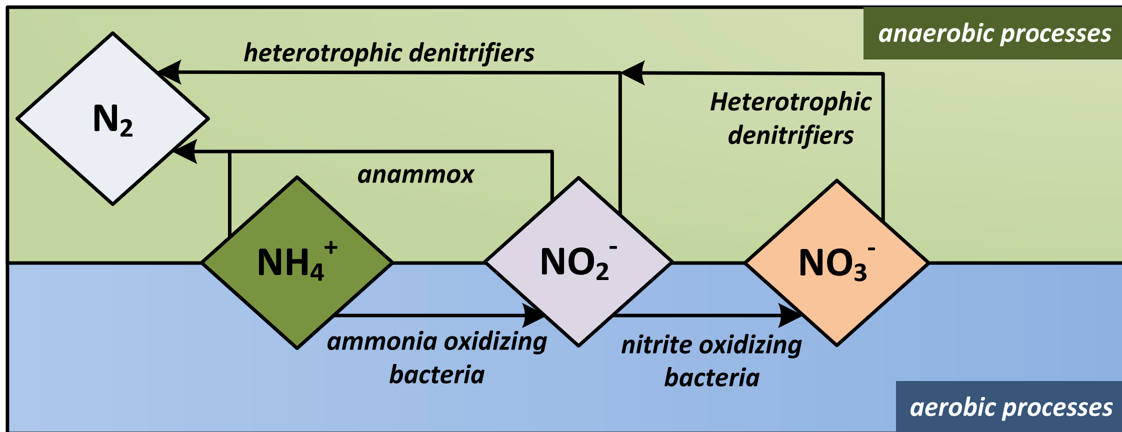
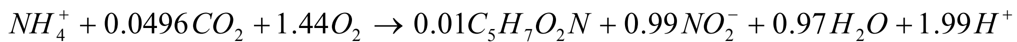


Figure 4: Main nitrogen pathways during wastewater treatment

2.2.1. Aerobic ammonia oxidizing bacteria

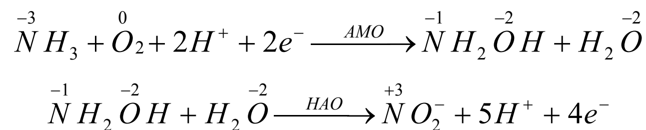
Physiology and biochemistry

The aerobic ammonia oxidizer bacterial group (AOB) comprises all bacterial species able to convert ammonium to nitrite under aerobic conditions.



$$NH_4^+ + 0.0496CO_2 + 1.44O_2 \rightarrow 0.01C_5H_7O_2N + 0.99NO_2^- + 0.97H_2O + 1.99H^+$$

They members of this group are mainly chemolithoautotrophs (i.e., able to obtain energy and carbon from inorganic sources), even though some of them are also able to metabolize organic substrates of low molecular weight under anaerobic conditions (Sayavedra-Soto and Arp, 2011). The aerobic conversion of ammonia to nitrite in the cells of these microorganisms takes place in two steps, as outlined below:



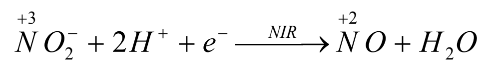
$$\overset{-3}{N}H_3 + \overset{0}{O}_2 + 2H^+ + 2e^- \xrightarrow{AMO} \overset{-1}{N}H_2\overset{-2}{O}H + H_2\overset{-2}{O}$$

$$\overset{-1}{N}H_2\overset{-2}{O}H + H_2\overset{-2}{O} \xrightarrow{HAO} \overset{+3}{N}O_2^- + 5H^+ + 4e^-$$

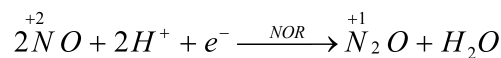
Ammonia monooxygenase (AMO) catalyses the incorporation of an oxygen atom into the ammonia molecule, leading to the formation of hydroxylamine (NH_2OH). The remaining oxygen atom is used for water synthesis (2 electrons and 2 protons are involved in the formation of the latter). The second step of the pathway, catalysed by the enzyme hydroxylamine oxidoreductase (HAO), converts hydroxylamine to nitrite and releases four electrons. Two are redirected to support further oxidation of ammonia, while the other two are utilized for CO_2 fixation and generation of proton motive forces.

The overall energy yield of these biochemical reactions is very low (-275 kJ/mol), which explains their very low growth rates (2 1/day) and yields (0.15 g-COD/g-N). They have been found to grow at temperatures from 5-35ºC, even though their optimum temperature has been reported between 25-35ºC. AOB have preference towards slightly basic pH media, being their optimum between 7.9 and 8.2. Ammonia oxidation produces protons as well. Therefore, alkalinity should be controlled in order to avoid acidic conditions that could inhibit the process. Free ammonia, free nitrous acid, heavy metals, methane, carbon monoxide or ethylene are known inhibitors of ammonia oxidizing bacteria. Even though AOB cannot grow directly from hydroxylamine, their lag phase can be reduced with spikes of this intermediate in their growing medium (Wiesmann, 1994; Ahn, 2006; Paredes et al., 2007).

When oxygen concentrations are low, some AOB are able to obtain energy from the reduction of nitrite, a process catalysed by two additional enzymes: nitrite reductase (NIR) and nitric oxide reductase (NOR).



$$\overset{+3}{N}O_2^- + 2H^+ + e^- \xrightarrow{NIR} \overset{+2}{N}O + H_2O$$



$$2\overset{+2}{N}O + 2H^+ + e^- \xrightarrow{NOR} \overset{+1}{N}_2O + H_2O$$

The two electrons required for both reduction processes come from the oxidation of hydroxylamine (Ni et al., 2011). Hydroxylamine, nitric oxide (NO) and nitrous oxide (N_2O) transients are often observed in AOB enrichments when they are periodically aerated (Noophan et al., 2004; Kostera et al., 2008). The emissions of N_2O, a very powerful greenhouse effect gas, can be very important in autotrophic processes. Abundant research is currently being done on the mechanisms to control and minimize these emissions (Kampschreur et al., 2009).

Phylogeny and adaptation strategies

All the members of the AOB group belong to the *Proteobacteria* phylum. While the majority of species belong to the *Betaproteobacteria* class (*Nitrosomonas*, *Nitrosospira, Nitrosovibrio,* and *Nitrosolobus*), a few AOB, mostly found in marine environments, are classified as *Gammaproteobacteria* (*Nitrosococcus and Nitrosococcus oceani*). All members of this bacterial group share 16S rRNA fragments and the *amoA* gene (gene that directs the synthesis of the subunit A of the AMO enzyme), which facilitates their quantification by molecular techniques (Norton, 2011). *nirK* is the only gene known to encode NIR synthesis in AOB.

However, many other HB are known to contain this same gene, invalidating it as marker of autotrophic N_2O production in wastewater systems (Braker et al., 1998; Casciotti and Ward, 2001).

Nitrosomonas and *Nitrosospira* are known to dominate different types of wastewater treatment environments. Seen from an ecology perspective, *Nitrosomonas* spp. are often considered as *r*-strategists that display higher growth rates than their competitors in niches with higher ammonia availability. On the other hand, *Nitrosospira* spp. are regarded as *K*-strategists, growing faster than its competitors when oxygen is available at lower concentrations (Figure 5).

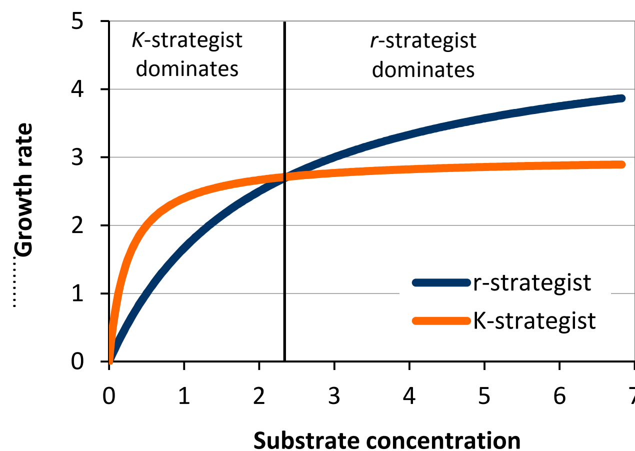


Figure 5: Specific growth rate of *K*- and *r*-strategists

Different *Nitrosomonas* sub-lineages are also known to dominate environments with different ammonium concentrations. While *Nitrosomonas oligotropha* are only found in systems with low to moderate ammonium concentration, *Nitrosomonas europaea*, *Nitrosomonas communis*, or *Nitrosomonas eutropha* adapt better to a wider range of ammonia concentrations (Norton, 2011). The higher resistance to salt of *Nitrosomonas* spp. and *Nitrosococcus* spp., make them the prevalent AOBs in systems with a higher salt content. The ability to denitrify nitrite using hydrogen gas or small organic compounds also represents a competitive advantage for *N. europaea* or *N. eutropha* living in mixotrophic and microaerophilic environments (Stein, 2011).

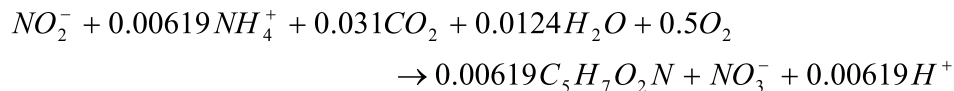
A wider microbial diversity, even inside the same functional group, is always preferable in wastewater treatment bioreactors. Enriching different species with the ability to remove ammonia under different environmental conditions ensures that the process will still be performed even after one of those species is affected

by a certain disturbance (shock load, predation, infection, etc.). Some authors have named this concept functional redundancy. Wastewater bioreactors operated at moderate ammonium concentrations (20-60mg-N/L) have proved to foster a more diverse AOB community. The same observation has been reported in reactors that are operated in batches, where ammonium concentration decreases with time, potentially allowing several AOB species to perform the oxidation of ammonia at different times within the reaction cycle. Ammonium stratification within biofilms is also known to allow the enrichment of different AOBs (Daims and Wagner, 2010; Okabe and Kamagata, 2010).

2.2.2. Nitrite oxidizing bacteria

Physiology and biochemistry

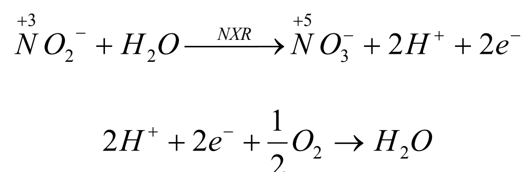
Nitrite oxidizing bacteria (NOB) is the functional group that contains all the bacteria with the ability to gain energy from the oxidation of nitrite to nitrate. This equation is often used to describe their substrate needs



$$NO_2^- + 0.00619NH_4^+ + 0.031CO_2 + 0.0124H_2O + 0.5O_2 \rightarrow 0.00619C_5H_7O_2N + NO_3^- + 0.00619H^+$$

Like AOB, they are considered to be aerobes and chemolithoautotrophs, although some of its members can assimilate small organic molecules and even derive energy from phototrophic growth or anoxic denitrification with nitrate. This relatively higher diversity in physiology is mirrored by a higher diversity in their phylogeny as well. Unlike AOB, this functional group contains bacteria from three phyla: *Proteobacteria*, *Nitrospirae* and *Chloroflexi* (Daims et al., 2011; Starkenburg et al., 2011; Sorokin et al., 2012).

The difficulty to obtain pure cultures of single NOB species has significantly complicated the study of their physiology and their metabolic pathways. Pure cultures of the *Alphaproteobacterium Nitrobacter* have been available for more than 20 years, making it, by far, the most studied NOB. This bacterial specie is able to derive energy from the combination of the following chemical reactions



$$\overset{+3}{N}O_2^- + H_2O \xrightarrow{NXR} \overset{+5}{N}O_3^- + 2H^+ + 2e^-$$

$$2H^+ + 2e^- + \frac{1}{2}O_2 \rightarrow H_2O$$

The nitrite oxidoreductase enzyme (NXR) catalyses the first step of their metabolic pathway. The two electrons and the two protons derived are used for the synthesis of water, which liberates the energy used for biosynthesis. This reaction yields even less energy than ammonia oxidation by AOB (about -75 kJ/mol), which results in even lower growth rates and cell yields than reported for ammonia oxidizers (1.45 1/day, and 0.045 g-COD/g-N). Temperatures ranging between 25 and 35ºC, and pH from 7.2 to 7.6 seem to be optimal for their reproduction. *Nitrobacter* spp. can be inhibited by free ammonia, free nitrous acid, nitric oxide, and several heavy metals (Wiesmann, 1994; Paredes et al., 2007). On the other hand, their growth rate can be slightly enhanced by mixotrophic growth with small organic carbon molecules (Starkenburg et al., 2011). Some sub-lineages of the *Nitrobacter* genus are known to synthetize NIR and NOR, enzymes required to catalyse the conversion of nitrite to N_2O under microaerophilic conditions. However, the environmental impact of this second process does not seem to be as important reported for AOB (Kampschreur et al., 2009; Starkenburg et al., 2011).

Phylogeny and adaptation strategies

Five genera from five different classes are reported as being the main NOBs in nature: *Nitrobacter* (*Alphaproteobacteria*), *Nitrotoga* (*Betaproteobacteria*), *Nitrococcus* (*Gammaproteobacteria*), *Nitrospina* (*Deltaproteobacteria*) and *Nitrospira* (known to be the only phyla within its class). The organism *Nitrolancetus hollandicus* (from the bacterial class *Chloroflexi*) has been recently added to this list, although their environmental significance is still an open question (Daims et al., 2011; Sorokin et al., 2012). This higher diversity in their phylogenetic traits makes their quantification more difficult. Only *Nitrobacter* has been accurately quantified by qPCR using primers targeting the *nxrA* gene (Poly et al., 2008; Wertz et al., 2008). Individual 16S rRNA oligonucleotide probes have been designed to detect each genus and their sub-lineages. However, each of these probes require different hybridization conditions, complicating significantly their simultaneous application (Loy et al., 2007).

Nitrobacter and *Nitrospira* spp. are the most common NOBs in wastewater treatment systems. *Nitrospira*-like spp., considered *K*-strategists, have often been found in activated sludge systems, where the concentrations of nitrite and oxygen are kept low. Contrarily, *Nitrobacter spp.*, defined as *r*-strategists, are normally detected in reactors operated at higher loads and concentrations (i.e. industrial

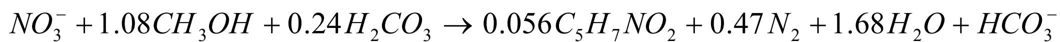
wastewaters, treatment of centrate, etc.). Both NOBs could be simultaneously enriched in sequencing batch reactors, where high and low nitrite concentrations are possible during the reaction phase. Both genera can also tolerate high salinities (Starkenburg et al., 2011).

Preliminary reports on the physiology of the *Nitrospira* genus have revealed a high diversity in the adaptation strategies of its sub-lineages. Sub-lineages I and IV could fix both CO_2 and pyruvate under aerobic conditions, which could be a competitive advantage in systems with limited alkalinity. *Ca.* Nitrospira defluvii contains chlorite (ClO_2^-) dismutase, an enzyme that can assist the detoxification of chlorite, giving them the ability to persist in systems disinfected with this chemical. The novel *Nitrospira* sub-lineage VI can adapt to environments with high temperatures and acidic conditions (Daims et al., 2011). Sub-lineages I and II have been shown to coexist in nitrifying flocs, with the first showing preference towards floc regions with a higher nitrite availability.

2.2.3. Denitrifying bacteria

Physiology and biochemistry

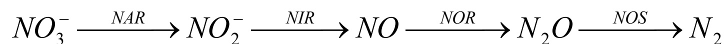
Even though some nitrifying organisms can denitrify, the term denitrifying bacteria is normally applied to heterotrophic bacteria able to degrade biodegradable carbon to CO_2 using nitrite or nitrate as final electron acceptors:



$$NO_3^- + 1.08CH_3OH + 0.24H_2CO_3 \rightarrow 0.056C_5H_7NO_2 + 0.47N_2 + 1.68H_2O + HCO_3^-$$

$$NO_2^- + 0.67CH_3OH + 0.53H_2CO_3 \rightarrow 0.04C_5H_7NO_2 + 0.48N_2 + 1.23H_2O + HCO_3^-$$

The conversion to nitrogen gas takes place in four reduction steps:



$$NO_3^- \xrightarrow{NAR} NO_2^- \xrightarrow{NIR} NO \xrightarrow{NOR} N_2O \xrightarrow{NOS} N_2$$

This process is carried out by a vast group of microorganisms with very distinct physiologies from very different phyla that contain the right enzymes: nitrate reductases (NAR), nitrite reductases (NIR), nitric oxide reductases (NOR), and/or nitric oxide reductases (NOS).

Each individual step can occur at microaerophilic or anoxic conditions, depending on the main phylogenetic affiliations of the microorganisms involved (different microorganisms can have a different set of enzymes inhibited at different oxygen concentrations). Although the process takes place under

anaerobic conditions, the energetics of denitrification are still far more favourable than for nitrification (heterotrophs grow about 2-3 times faster and at 4 times higher yields). Denitrification proceeds at optimal rates at pH between 7.2 and 7.6, and temperatures between 25 and 35ºC. The composition of the microbial community, the presence of inhibitors (NO, salinity, heavy metals, etc.), pH, the biodegradability of the organic substrates or temperature can also have an effect on the process rate and on what is the end product of the process. Heterotrophic denitrification has net generation of alkalinity, a factor that is often considered when designing nitrogen removal processes (the flowchart in most reactor systems alternate between nitrifying and denitrifying conditions in order to, among other things, maintain the pH in the system).

The COD requirements to achieve complete denitrification depend on the amounts of nitrate (3.7 g-COD/g-N) or nitrite (2.3 g-COD/g-N) to denitrify. The C/N ratio in wastewater does not always allow for the complete removal of nitrogen with the organic carbon available in solution and organic compounds of low molecular weight (e.g. methanol or acetate) have to be added to achieve full denitrification (Sun et al., 2010).

Phylogeny and adaptation strategies

Very distantly related microbes feature the same denitrifying genes, indicating that horizontal gene transfer has been a common process during all the evolutionary history of prokaryotes. The vast diversity within this group makes the phylogenetic mapping of all denitrifiers via FISH a very resource intensive process with a large number of 16S rRNA oligonucleotide probes involved. Therefore, qPCR targeting *nirK* or *nirS* genes has been the most widely used approach to quantify bacterial numbers within this microbial guild (Daims and Wagner, 2010).

Culture-independent techniques like MAR-FISH have allowed the characterization of the niches of some relevant denitrifiers from wastewater treatment plants. Thomsen et al (2007) observed that *Curvibacter*-like microorganisms only used amino acids during denitrifying conditions; *Azoarcus* could assimilate amino acids, acetate and pyruvate while *Thauera* proved to be the most versatile denitrifier among the tested communities, uptaking amino acids, ethanol, propionate, and pyruvate. Hagman et al. (2008) partially confirmed this hypothesis and observed that *Azoarcus* spp. could utilize both acetate and methanol.

Acetate and methanol, often added to denitrifying reactors to improve the efficiencies of the denitrification process, have been also suggested to select for different denitrifying communities. While acetate enriched *Comamonas*, *Acidovorax*, and *Thauera*, methanol selected for *Methylophylus*, *Methylobacilus* and *Hyphomicrobium* (Osaka et al., 2006).

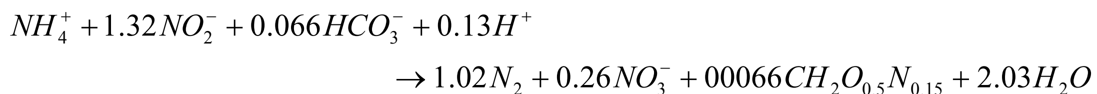
Interactions with nitrifying bacteria

Many heterotrophic denitrifiers are facultative, meaning that they can also grow using oxygen, instead of nitrite or nitrate, as final electron acceptor. Depending on the environmental conditions, heterotrophs can grow about 3-4 times faster than nitrifiers when oxygen is present (Henze et al., 2000). Therefore, if organic carbon is not limiting, heterotrophs can easily uptake most of the oxygen available, outcompetinh nitrifiers from the reactor. Consequently, wastewater treatment systems featuring nitrification and denitrification are often designed to allow the creation of ecological niches where organic carbon is limiting for heterotrophs, but oxygen is still present so that nitrifiers can use it to nitrify ammonia.

2.2.4. Anaerobic ammonium oxidizers

Physiology and biochemistry

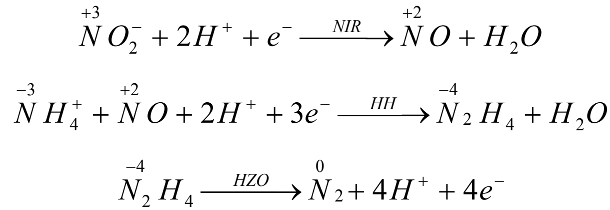
Anaerobic ammonium oxidizers (AnAOB), also known as anammox, are a bacterial guild that has the ability to obtain energy from the oxidation of ammonium to dinitrogen gas using nitrite as electron acceptor.



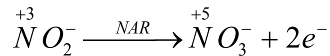
$$NH_4^+ + 1.32NO_2^- + 0.066HCO_3^- + 0.13H^+ \rightarrow 1.02N_2 + 0.26NO_3^- + 00066CH_2O_{0.5}N_{0.15} + 2.03H_2O$$

Like nitrifying bacteria, these microorganisms are chemolithoautotrophs, although some members of the group can also fix carbon from organic molecules of small molecular weight. They are all obligate anaerobes.

The enrichment of the *Ca.* Kuenenia stuttgartiensis opened the door to decipher the metabolism of these microorganisms, and three steps have been postulated to allow the oxidation of ammonium with nitrite:



At the first step, the reduction of nitrite is assisted by the nitrite oxidoreductase enzyme (NIR) to form nitric oxide. Then, hydrazine hydrolase (HH) facilitates the combination of ammonium with the formed nitric oxide (NO) to form hydrazine (N_2H_4), one of the most powerful reductants in nature. Finally, hydrazine oxidation is catalysed by hydrazine dehydrogenase (HZO) to derive 4 electrons that are used to further sustain the first two steps of the pathway. The oxidation of nitrite to nitrate (catalysed by the nitrate reductase) derives enough electrons to sustain carbon fixation and cell growth.



Although the presented pathway is very energetically favourable (-357 kJ/mol), the growth rate of this microorganism is extremely low (doubling time ranges from 3.6-17 days, Terada et al., 2011). Some researchers believe that this observation could find an explanation in the compartmentalization of the AnAOB cell (Jetten et al., 2009, Figure 5). Cell yields, on the other hand, are similar to the ones reported for AOB. Nitrite, oxygen, phosphate or methanol at high concentrations inhibit their growth (Jetten et al., 1998; Strous et al., 1999; Isaka et al., 2007). Hydroxylamine and nitric oxide have been reported to reactivate the metabolism of AnAOB after long times of biomass inactivation (Kartal et al., 2010; Zekker et al., 2012). Temperatures between, 37-40ºC and pH at 8 are optimal for their growth (Egli et al., 2001). Anammox consumes protons, hence care should be taken to ensure that the pH stays inside the range that allows bacterial growth.

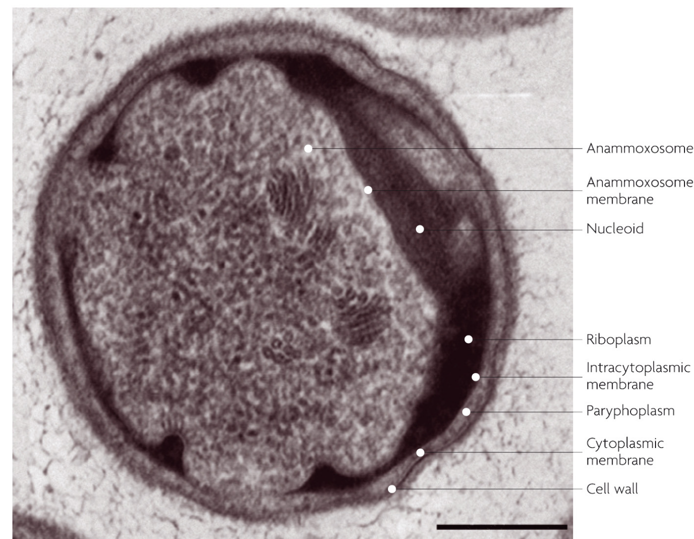


Figure 6: Compartmentalization of the AnAOB cell as presented by Kuenen (2008). The anammoxome is largest organelle in the cell. All genomic material distributes around it.

Phylogeny and adaptation strategies

AnAOB were discovered about 20 years ago. Since then, 5 genera have been detected in nature, all of them belonging to the *Planctomycetes* phylum: *Ca.* Brocadia, *Ca*. Kuenenia, *Ca*. Scalindua, *Ca*. Anammoxiglobus, and *Ca*. Jettenia, all very phylogenetically similar. Consequently, they are all very easily detected with 16S rRNA oligonucleotide probes or primers. AnAOB quantification is also possible with primers targeting the *hzoq* gene, which encodes the synthesis of the HZO enzyme.

Ca. Brocadia and *Ca.* Kuenenia have been commonly reported as the main AnAOB genera in wastewater treatment systems. It has been observed that *Ca.* Brocadia behave as *r*-strategists, dominating systems operated at higher ammonium loads and concentrations, while *Ca.* Kuenenia, with a higher affinity for nitrite at low concentrations, are regarded as *K*-strategists (Van der Star et al., 2008).

Salinity is also a selection factor for different types of AnAOB microorganisms, being *Ca.* Scalindua the AnAOB specie found in high salinity environments. Similarly, the presence of small organic compounds favours the enrichment of *Ca.* Brocadia (with acetate), *Ca.* Anammoxiglobus propionicus (propionate), and *Ca.* Scalindua (acetate, formate and propionate, Terada et al., 2011).

AnAOB in wastewater treatment

The advantages of denitrifying ammonium via the anammox pathway are notable: only half of the ammonia has to be converted to nitrite (lowering the aeration costs), the process still occurs even when there is not organic carbon available (reducing operation costs), and cell yields are 4 times lower (reducing biosolids treatment costs). These figures result even more attractive knowing that aeration, sludge treatment, and energy costs related to the recirculation of wastewater streams to remove nitrogen represent more than 50% of the total cost of a typical wastewater treatment plant (De Gussem et al., 2011).

A lot of effort has been put into the industrial implementation of this process during the last few years (Terada et al., 2011). The slow growth rate of AnAOB has favoured the selection of reactor configurations that allow for very high biomass retention times (mainly biofilm and granular systems). Two very important factors required for the development of AnAOB in wastewater treatment bioreactors are (i) the accumulation of ammonium and nitrite at concentrations below their inhibiting threshold, and (ii) the presence of low concentrations of dissolved organic carbon. While the first is just a physiological condition necessary for growth, the latter is a requirement to avoid the development of heterotrophic denitrifiers that could outcompete AnAOB for nitrite (heterotrophs can grow up to 75 times faster that AnAOB). Temperatures up to 40ºC can be beneficial to sustain the process at higher rates, but not strictly necessary.

Two approaches have traditionally been followed to supply nitrite to AnAOB biomass. The first industrial implementation of the process used a two-tank reactor system where AnAOB (enriched only in the second reactor) catalysed the conversion of approximately equimolar mixtures of ammonium and nitrite (effluent of the first reactor) to nitrogen gas. Nitrite accumulation was achieved in the first reactor (a conventional CSTR) after operation at temperatures that allowed AOB to grow faster than NOB (>28ºC) and subsequent selection of hydraulic residence times that retained AOB and washed NOB out.

Another possibility is the use of 1-tank reactor systems, where both AOB and AnAOB are enriched within the oxygen-stratified structure of granules or biofilms. AOB, present in the aerobic zones of the biological matrix, convert part of the ammonium loaded to nitrite with oxygen while, at the same time, AnAOB microcolonies in the anoxic regions complete the transformation of the generated

nitrite and the remaining ammonium to nitrogen gas. NOB are subjected to very strong selective pressures in these systems. On the one hand, AOB, known to have better affinities for oxygen at small concentrations, consume most of the oxygen present. On the other hand, AnAOB, more efficient nitrite scavengers, deprive them at the same time of electron donor. Some authors suggest that the elevated pH values at which this process is normally run could increase the concentrations of free ammonia at levels that are inhibitory for NOB, but not for AOB. However, it has been shown that NOB populations can still adapt and thrive in these environments.

Single tank systems require less space than 2-tank reactor systems, are less likely to be affected by nitrite toxic loads and display lower nitrous oxide emissions. However, fluctuating ammonium loads can also lead to the accumulation of oxygen in single tank systems, inhibiting AnAOB growth and favouring NOB outgrowth. Appropriate control of the oxygen load is hence essential in these systems. The lower investment costs are moving the industrial implementation of the anammox process towards one-reactor systems, and several plants are now operative worldwide (Szatkowska et al., 2007; Wett, 2007; Joss et al., 2011). Specific removal rates within the range 0.7-1.9 g-N/m^2/day have been reported.

2.3. Membrane-aerated biofilm reactors

A very attractive aspect of membranes is their potential to assist bubbleless gas transfer to liquid streams in system volumes that are much smaller than those required in conventional absorption towers. (Klaassen and Jansen, 2001). This high-rate gas transfer concept was tested 30 years ago to supply gaseous electron donors (H_2) or acceptors (O_2) to the liquid phase of wastewater bioreactors. However, once the membrane modules got in contact with microbes, these irreversibly attached to the membrane and formed biofilms (Wilderer et al., 1985). This observation was probably the start of the end for bubbleless gas aerators in wastewater treatment, but it was also the beginning for a new bioreactor technology that since then has filled thousands of pages of scientific literature and that has inspired 10 patents: the membrane biofilm reactor.

2.3.1. Concept

Membrane-aerated biofilm reactors (MABRs) are a relatively novel biotechnology based on the use of bubbleless gas-transfer membranes as support material for biofilm growth. The microorganisms attached at the membrane-biofilm interface consume oxygen as it diffuses from the lumen, without any additional mass transfer resistance than the one offered by the membrane material. The rest of solutes supporting growth penetrate into the biofilm after diffusing through the liquid boundary layer developing on the biofilm surface. These biofilms are often referred to as counter-diffusion biofilms, since substrates diffuse into their structure from opposite sides (Syron & Casey, 2008).

The MABR is a technology that retains all the benefits of conventional biofilm growth (solids retention, redox stratification, diversity, etc.), but with the additional advantage of a very efficient and flexible gas delivery. Theoretically, for a set of bacterial kinetics, biofilm transport properties and pollutant load, the oxygen penetration into the biofilm can be conveniently adjusted increasing or decreasing the partial pressure of oxygen in the membrane lumen (Casey, Glennon, et al., 2000). Such high flexibility in the application of the oxygen load supports the spatial-temporal creation of anaerobic niches within the biofilm. This zonation is known to favour the development of bacterial communities that catalyse the removal of organic carbon, nitrogen, phosphorous or several xenobiotics (Martin and Nerenberg, 2012).

Unlike conventional biofilm systems, the adjustment of the oxygen load does not modify the hydrodynamic conditions in the bulk liquid, and can be completely decoupled from the load of pollutants. Furthermore, since aeration is bubbleless, stripping of unwanted gases (volatiles, greenhouse gas effect gases, etc.) and foaming are avoided. Recent studies have shown that MABRs can display very competitive oxygen transfer rates even under low pressurization conditions and constant airflows through the membrane lumen. Such an aeration mode lowers considerably the aeration costs since the air compressor only has to supply enough hydraulic head to overcome the head losses occurring in the gas lumen, which are much lower than in other conventional bubble-aeration systems. Oxygen can be used instead of air when the oxygen content in air is not enough to sustain the needs of a certain microbial process (Semmens, 2005).

The efficient control of the oxygen load can also stimulate a higher degree of microbial activity at the biofilm base. Thus, nutrient limitations at the biofilm base, known to trigger biofilm detachment events in conventional biofilms, can be avoided. Even more, since the biofilm in the biofilm-liquid interface is often anoxic, the detached solids could have the potential of being partially digested, which could reduce their retention time in sludge digesters.

2.3.2. Biofilm properties in MABRs

We have previously reviewed that the rate of microbial growth and biofilm composition can impact significantly the mechanical behaviour and performance of conventional biofilm systems. Since both factors are affected by oxygen availability, it could be hypothesized that the counter diffusion gradients observed in MABRs can lead to the creation of biofilms with very different properties from the ones observed in conventional biofilms. Unfortunately, very few studies have focused on the physicochemical characterization of these biofilms.

Experimental studies in MABRs with heterotrophic biomass have revealed that low shear conditions can result in a high stratification of the biofilm density with depth (Cole et al., 2004). Contrarily, high shears are known to equalize the density distribution within the biofilm thickness, stimulate convective transport within pores and cavities of the biofilm (Picard et al., 2012), increase detachment rates during biofilm formation, and yield thinner and denser structures (Casey, et al., 2000).

Ahimou et al. (2007) reported that the biofilm cohesion of counter-diffusion biofilms increased with depth. The authors concluded that the higher oxygen availability at the biofilm base, which concomitantly stimulated the excretion of carbohydrates, was the responsible for the observed stratification in the mechanical properties.

2.3.3. Factors to consider during MABR design and operation

Oxygen loading

Two operational regimes can be used to feed air to MABRs. In the dead-end mode, air flows into the membrane lumen from one end while the other end remains closed, hence allowing mass transfer efficiencies up to 100%. However, gasses dissolved in the bulk liquid, or even water vapour can back diffuse, accumulate and even condense in the downstream lengths of the gas compartment. This phenomenon leads to the establishment of oxygen gradients along the lumen that can significantly impact biofilm development and compromise the overall oxygen transfer ability of the system (Ahmed et al., 2004).

In flow-through mode, the airflow fed is continuously vented, lowering the transfer efficiencies, but equalizing oxygen concentrations along the length of the membrane. At the same time, unwanted gases dissolved in the bulk liquid can back-diffuse and eventually be transported to the atmosphere, which may represent a significant drawback for this feeding regime.

The oxygen transfer rate (OTR, g-O_2/m^2/day) in MABRs has been traditionally estimated from the overall oxygen mass transfer coefficient (K_{OL}, m/day). This parameter allows the calculation of the overall oxygen flux transferred from the gas phase (with an oxygen concentration $S_{O2,g}$, g/m^3) to the bulk liquid (with a dissolved oxygen concentration $S_{O2,BL}$, g/m^3) with the following expression

$$OTR = K_{OL} \cdot \left(\frac{S_{O2,g}}{H} - S_{O2,BL} \right)$$

The driving force of the process is the oxygen gradient between the equilibrium concentration of oxygen in water given an oxygen concentration in the gas phase ($S_{O2,g}/H$, H being the Henry coefficient describing the equilibrium gas-liquid) and (ii) $S_{O2,BL}$.

A gas valve installed in the outlet of the gas compartment operated in flow-through mode can be then used in order to increase the oxygen partial pressure (correlated to the oxygen concentration in the lumen) and consequently the OTRs. Oxygen transfer efficiencies up to 60% have been reported for a hollow fibre system operated in flow-though mode at 120 kPa and flow rates as low as 500mL/min (Soreanu et al., 2010). Even higher oxygen transfer rates can be obtained by using oxygen instead of air. However, the cost of using pure oxygen is only justified for systems treating high loads of pollutant. Some authors have also reported a reduction in biofilm activity when using pure oxygen in MABRs (Osa et al., 1997).

Membrane selection

The ideal MABR membrane should combine excellent oxygen transport properties (e.g. high oxygen diffusivity and low thickness), with high durability, high mechanical strength, and surface characteristics that facilitate biofilm attachment (rough and with high free energies). Hollow fibre geometries (with diameter in the order of 100-500μm) are favoured in industrial applications since they permit a higher membrane surface are to be packed in relatively small reactor volumes. Flat sheets can be more convenient in laboratory operations where easy inspection is required.

Microporous membranes (pore size: 0.03 to 10μm) have been widely used as biofilm substrata in MABRs. Since they are manufactured with hydrophobic materials, their pores remain dry when the membranes are wetted and oxygen transfer into the liquid phase proceeds by fast molecular diffusion through the gas-filled pores (Henry constant describes the equilibrium in the gas-liquid interface). Operation at pressures higher than a few kPa can result in air leaks at the liquid-biofilm interface that could lead to severe biofilm sloughing. Moreover, accumulated EPS can hydrophilize the membrane surface, making it water-permeable and leading to the formation of a water film on the membrane lumen, which deteriorates significantly the oxygen transfer rates and eventual reactor drainage.

Dense films of oxygen-permeable materials such as PDMS and other rubbers have been also assayed as biofilm substrata in MABRs. Mass transfer through these polymers takes place via absorption of the gas on the membrane, molecular diffusion and desorption at the gas-liquid interface (both absorption and desorption steps have their own equilibrium constant). The diffusion rates

attainable here are significantly lower than those observed in microporous membranes and the thicknesses of the material are substantially higher. However, faster oxygen transfer rates can still be achieved by operating the system at higher oxygen pressures (in the order of hundreds of kPa). Many of these materials are flexible. Therefore, changes in the lumen pressure could result in expansion or retraction of the membrane and eventual breakage of the biofilm. The low surface roughness of these films can be increased by abrading their surface with sandpaper (Suzuki et al., 2000).

Composite membranes are normally preferred since they embrace the advantages of both microporous and dense membranes: they can be operated at high pressures and deliver outstanding oxygen transfer rates without compromising the integrity of the biofilm. Their architecture normally consists on a thin film of dense polymeric material supported by a microporous structure.

Membrane modules

In industrial applications, hollow fibre membranes are normally potted into pressure headers in order keep the membranes shape and to simplify air supply and collection to and from the fibres. The structure formed by the headers and the membranes is called module. Modules should be designed to maximize the membrane area of the reactor (32-813m^2/m^3 have been reported for pilot scale studies), keep solute transfer into the biofilm high (minimizing the liquid boundary layer thickness), and allow energy efficient flow management of liquids and gases (Martin and Nerenberg, 2012). Spacing between the membranes should be also sufficient to prevent membrane clumping by biofilm growth. Shell and tube systems where hollow fibres are installed inside a tube and the water flows in longitudinal direction is one of the preferred configurations. The structure formed by the membranes and headers can even allow rotation in order to enhance the mixing conditions in the liquid phase or apply additional shear on the biofilms to prevent excessive biofilm growth.

Another configuration allowing high membrane areas are cross-flow systems. Here, hollow fibres are stitched together into a fabric or net, which is then potted into structures called cassettes. Several of these cassettes are installed along prismatic reactors with water flowing perpendicularly to the hollow fibres. The flow pattern here has a higher advective component, which maximizes mass transfer into the biofilm, but can be more expensive to maintain (Motlagh et al., 2008).

Figure 7: Pressure header of a hollow fibre MABR module

Thickness control

Managing the biofilm thickness can improve the removal rates in MABRs featuring heterotrophic processes (Casey et al., 1999). The most common method to assist biofilm detachment is the enhancement of the bulk turbulence with increases of the water flow, intermittent gas sparging, or mechanical mixing. Unfortunately, the detachment mechanisms are poorly understood and many times biofilm control strategies often result in massive sloughing events that compromise reactor performance. Innovative approaches to control biofilm thickness include the use of surfactants, sodium chloride or ultrasounds to destabilize the EPS components of the biological matrix and hence the biofilm structure (Freitas Dos Santos and Livingston, 1995; Celmer et al., 2008; Hwang et al., 2009b). However, these methods can result too aggressive and reduce the viability of the remaining biofilm. The reactor should be prepared for an adequate management of the detached solids (settling and withdrawal).

While there is an obvious need for biofilm thickness control in MABR studies involving heterotrophic bacteria, excellent process rates and thinner biofilm structures have been reported for a wide range of MABR systems performing autotrophic nitrogen conversions (Brindle et al., 1998; Motlagh et al., 2008; Terada et al., 2009; Pellicer-Nàcher et al., 2010). Insignificant impact of mass transfer limitations and very low production rates of suspended solids were observed in these studies, which highlights the better fit of the MABR technology to host processes with slower bacterial growth.

Hydraulic conditions

Lab scale studies tend to use completely mixed conditions in order to facilitate mass balance analyses, control strategies and reactor modelling. However, the industrial implementation of the process, like with other biofilm systems, seems to benefit from plug-flow operation. Here, several completely mixed reactors are connected in series and treat wastewater in stages. The precise adjustment of the oxygen oxygen dosing in each stage makes MABR operation very robust and stable when treating shock loads (Stricker et al., 2011).

2.3.4. Modelling MABRs

Mathematical models are essential tools in process engineering. They are routinely used to test hypotheses, train personnel in the control and behaviour of a certain process, design reactors and predict their performance, and to apply automated process control. In the field of biofilm technology reliable mechanistic models are of special interest since they allow the systematic description (in time and space) of simultaneous interactions between substrates and bacteria that otherwise would be very costly to verify experimentally.

During the last few decades a large number of models have been created to describe biofilms mathematically. Engineers can often use one-dimensional models in order to describe reactor performance and dynamics well. On the other hand, researchers, more interested in finding a mechanistic explanation to macroscale observations, lean towards the development of multidimensional models that describe the composition, cohesion, and the mass transfer phenomena within the biofilm with more detail. Both models are based on the same fundamental equations, but with a different level of simplification.

Membrane aerated biofilm reactors have also been part of this development. Casey et al. (1999) created the first pseudo steady state model inspired by those used to predict mass transfer limitations in porous catalytic media. Despite being able to describe their experimental observations well, the model assumed a high degree of simplification, which does not allow the description of the complex interactions existing in systems where different microbial pathways exist.

Model structure for MABR multipopulation models

After Casey et al. (1999), several attempts have been made in order to describe the competitive and synergic interactions in MABRs with more than one microbial group. Published one-dimensional multipopulation modelling studies make use of a compartmenftalized structure in which each compartment of the MABR (lumen, biofilm or bulk liquid) is connected with its neighbouring counterpart through diffusive links (Figure 8).

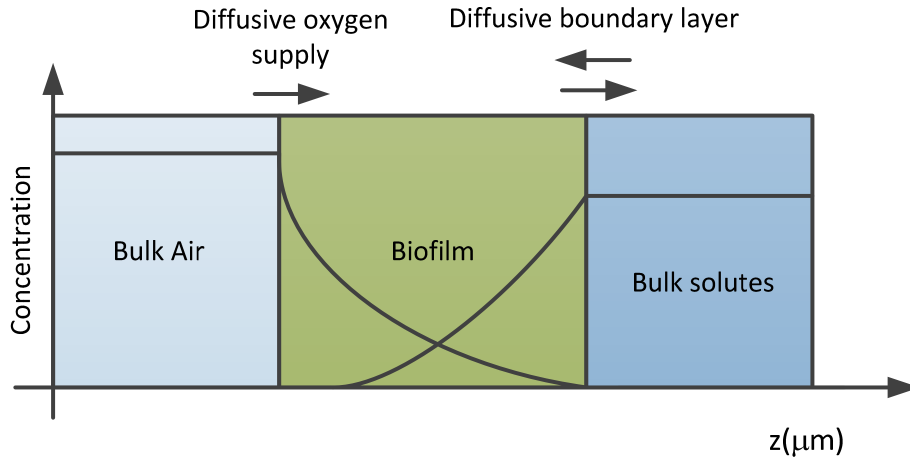


Figure 8: Conceptualization of substrate fluxes into MABR biofilms

Both gas and liquid compartments are considered completely mixed. One-dimensional models assume that diffusion only takes place in the direction of biofilm growth and that chemical reaction is only relevant inside the biofilm matrix. The equation describing substrate diffusion, reaction and growth is the well-known diffusion-reaction equation, which assumes that the concentration of a certain solute *i* at a certain time in a point of the biofilm *z* is the result of two processes: diffusion and reaction.

$$\frac{\delta C_i(z)}{\delta t} = \frac{\partial}{\partial z}\left(D_{i,bf}(z) \cdot \frac{\partial C_i(z)}{\partial z}\right) \pm R_{C,i}(z)$$

$$\frac{\delta X_b(z)}{\delta t} = \pm R_{X,b}(z)$$

Given a point z in the biofilm, $C_i(z)$ and $X_b(z)$ are the concentration profiles of substrate *i* and biomass *b*, respectively (g/m^3), $D_{i,bf}(z)$ are the effective diffusion coefficients of the substrate *i* within the biofilm depth (m^2/day) and $R_{s,i}(z)$ and $R_{X.i}(z)$ are the net production (or consumption) rate of solutes *i* and biomass *b*

within the biofilm depth (g/m^3/day). The substrate diffusivity of each compound *i* is normally assumed to vary with the biofilm porosity or density (ρ(z))

$$D_{i,bf}(z) = \varepsilon(z)\cdot D_{i,w} \qquad\qquad D_{i,bf}(z) = D_{i,w}\cdot\left(1-\frac{0.43\cdot\rho(z)^{0.92}}{11.19+0.27\cdot\rho(z)^{0.92}}\right)$$

being $D_{i,w}$ the biofilm diffusivity of the substrate *i* in water, and ε(z) the biofilm porosity at a depth *z*. The net growth of biomass is normally expressed as

$$R_{X,b}(z) = X_i(z)\cdot\mu_{\max,b}\cdot\frac{C_i(z)}{C_i(z)+K_{b,i}}\cdot\frac{C_j(z)}{C_j(z)+K_{b,j}} - b_i\cdot X_i(z)$$

Where the first term defines the specific growth rate of biomass *b* on two substrates (*i* and *j*) at a depth *z* and the second is the decay rate of that same biomass at that position. In more detail, μ_{maxb} stands for the maximum bacterial growth rate (1/day), $K_{b,i}$ (g/m^3) is the affinity constant of the biomass *i* for the substrate *i*, $K_{b,j}$ (g/m^3) is the affinity constant of the biomass *i* for the substrate *j*, and b_i is the decay rate of *i*. The consumption rate of substrate i is related to biomass growth rate of *b* through the yield coefficient $Y_{B,i}$

$$R_{C,i}(z) = \frac{1}{Y_{B,i}}\cdot X_i(z)\cdot\mu_{\max,b}\cdot\frac{C_i(z)}{C_i(z)+K_{b,i}}\cdot\frac{C_j(z)}{C_j(z)+K_{b,j}}$$

The biofilm growth velocity (u_{bf}, m/day) can be defined as a function of the net rate of biofilm volume production for n biomass components (first term in the equation below), and the detachment rate (u_{det}, g/day)

$$u_{bf} = \frac{1}{A}\cdot\left(\int_{z=0}^{z=Lf}\frac{1}{1-\varepsilon(z)}\cdot\sum_{b=1}^{b=n}\frac{R_{X,b}(z)}{\rho(z)}\cdot dz - \frac{u_{\det}}{\rho(Lf)}\right)$$

Where A is the area of biofilm parallel to the substratum, and ρ is the biofilm density (g/m^3). The biofilm thickness can be calculated as

$$u_{bf} = \frac{dLf}{dt}$$

The problem then gets reduced to a system of partial differential equations. In order to solve it will be necessary to assume an initial condition for all variables and two boundary conditions: one for z=0 (substratum), and another for z=Lf (biofilm thickness). For z=0 it can be assumed that none of the substrates or biomass components can diffuse through the substratum

$$\left.\frac{dC_i}{dz}\right|_{z=0} = 0 \qquad \left.\frac{dX_b}{dz}\right|_{z=0} = 0$$

but oxygen, which diffuses from the membrane

$$D_{O2,w} \cdot \varepsilon(z=0) \cdot \left.\frac{dC_{O2}}{dz}\right|_{z=0} = k_M \cdot \left(C_{O2,g} - C_{O2}(z=0) \cdot H\right)$$

Where $C_{O2,g}$ is the oxygen concentration in the gas phase, $C_{O2}(z=0)$ is the oxygen concentration at the biofilm base, H is the Henry coefficient and k_M is the mass transfer coefficient of oxygen through the membrane (m/day).

The boundary condition at the biofilm surface is based on the assumption that the flux of substrate *i* diffusing at the biofilm surface is the flux transported through the liquid boundary layer (diffusion-limited):

$$D_{i,w} \cdot \varepsilon(z=Lf) \cdot \left.\frac{dC_i}{dz}\right|_{z=LF} = \frac{D_{i,w}}{L_{BL}} \cdot \left(C_i(z=Lf) - C_{i,bulk}\right)$$

For particulates, it is assumed that the detached solids diffuse out to the bulk liquid

$$D_{b,w} \cdot \left.\frac{dX_b}{dz}\right|_{z=LF} = -\frac{u_{\det}}{A}$$

Results from current modelling efforts

Shanahan and Semmens (2004) presented the first multipopulation model to describe nitrification and denitrification in MABRs following the above concept. Their model was unable to describe neither the substrate dynamics in the liquid bulk nor the biofilm stratification observed with FISH by other researchers (Hibiya et al., 2004). The inclusion of density stratification in biofilm thickness did not improve their results (Shanahan, 2005). Further modelling attempts revealed that the formulation used to describe oxygen transport mechanisms in these models, does not describe well the oxygen dynamics observed during reactor operation with biomas (Lackner et al., 2010). Individual-based models (two-dimensional) have also been used to study the stratification of the microbial communities performing nitrification-denitrification and EPS production in MABRs. The results obtained described well the oxygen and microbial

stratification observed in the modelled system, but only during a single steady state.

The compilation of datasets describing fundamental physical parameters such as u_{det}, $\rho(z)$, or $\varepsilon(z)$ under well defined operational conditions, and model calibration with experimental data ($C_i(z)$, $X_b(z)$, etc., never undertaken in MABR modelling studies), should broaden our understanding on the processes influencing the structure and activity of counter-diffusion biofilms and lead to the creation of more accurate process models.

3. Physical and physiological properties of membrane-aerated biofilms for ammonium removal

3.1. MABRs for ammonium removal

3.1.1. Nitrification

Full nitrification of inorganic solutions containing ammonium and bicarbonate has been widely reported in MABRs. Most of the studies centred on process performances and did not provide any further detail on the structural and physical characteristics of the developed biofilms.

Ammonium conversion rates ranging from 0.14 to 26.5g-N/m^2/day were achieved in different setups using pure oxygen (Brindle et al., 1998; Hsieh et al., 2002; Hwang et al., 2009a) or air (Terada et al., 2006, 2009, 2010; Wang et al., 2011) as feeding gas (pure oxygen did not always yield higher removal rates). Higher biofilm coverages (Shanahan et al., 2005; Motlagh et al., 2008; Terada et al., 2009), advective flow (Motlagh et al., 2008) and increasing ammonium loading rates (Wang et al., 2011, Hwang et al., 2009a) seemed to affect the process performance to a higher or lower extent. Overall, biofilm thicknesses ranging from 200 to 600μm were observed over long operation periods (hundreds of days). Some studies using dead end configurations described clumping of membrane fibres at areas closer to the inlet (closer to each other and with a higher oxygen content). Sloughing events were reported only in studies with a very uneven hydrodynamic regime over the membrane (Shanahan, 2005).

Hsieh et al. (2002) coated their membranes with a polyvinyl alcohol gel that contained biomass from a nitrifying enrichment. The film was able to withstand shear stresses well, but it impaired the oxygen transfer rates significantly, even when pure oxygen was used.

Schramm et al. (2000) described the microbial ecology of nitrifying MABRs for the first time. He observed that *Nitrosomonas* spp (*r*-strategist AOB) and *Nitrobacter* spp (*r*-strategist NOB) dominated at the biofilm base (where oxygen was at its highest), while *Nitrospira* spp (*K*-strategist NOB) and *Nitrosospira* spp (*K*-strategist AOB) populated microerophillic biofilm areas. AOBs were higher in numbers overall, which was in agreement with their higher yield and growth

rate. The oxygen supplied by the membrane penetrated only the deepest 200µm of the biofilm.

3.1.2. Nitritation

Nitrite accumulation can bring substantial energy savings to the treatment of nitrogen rich wastewaters when combined with anammox or heterotrophic denitrification. Downing and Nerenberg (2008a) suggested that the dissolved oxygen concentration at the biofilm base is a key parameter to attain partial nitritation in MABRs. Concentrations at the membrane-biofilm interface lower than 2mg/L selected for *Nitrospira* spp, NOBs with lower growth rates that can be easily outcompeted by AOB. Contrarily, dissolved oxygen concentrations higher than 2mg/L enhanced ammonia conversion fluxes, but stimulated the growth of *Nitrobacter*, which could compete better with AOBs for oxygen.

Lackner and Terada (2010) demonstrated with experimental and modelling studies that reduced low oxygen concentration at the membrane-biofilm interface cannot alone explain NOB outcompetition and suggested that the composition of the microbial community also plays an important role in the success of nitritation in MABRs. The authors reported that biofilms presenting higher ammonia removal rates and lower nitrite oxidizing rates had high cell numbers of *Nitrosomonas* spp. and lower densities of *Nitrobacter* spp., while biofilms with poorer nitritating abilities featured higher numbers of *Nitrosospira* spp. and *Nitrobacter* spp..

Wang et al. (2009) showed with his sensitivity analysis of a multipopulation biofilm model that the value of the membrane mass transfer coefficient has a significant impact on the ability of a system to accumulate nitrite, and suggested that membrane selection could be a crucial step in designing nitritating MABRs. He also reported that operation at high pH values in the bulk liquid could slightly increase nitrite production.

3.1.3. Completely autotrophic nitrogen removal (nitritation-anammox)

Partial nitritation and anammox can also be coupled in a membrane-bound biofilm to achieve a more energy-efficient wastewater treatment. Terada et al. (2007) first postulated the feasibility of the process in a modelling study. Their simulations revealed that AOB would populate the biofilm base, creating a niche for the enrichment of AnAOB at the outer edge of the biofilm (Figure 9). The process allowed for higher nitrogen removal efficiencies than its co-diffusion equivalent at a wider range of operating conditions. The relative surface loading of oxygen and ammonium was the key operational parameter driving system performance.

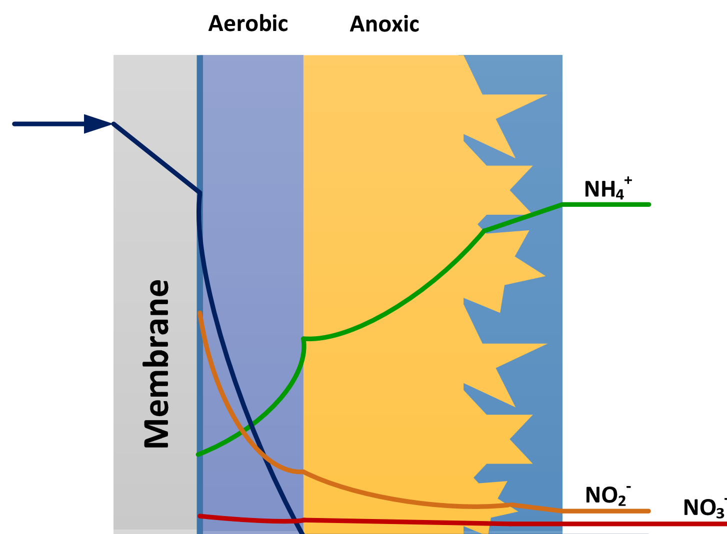


Figure 9: Expected oxygen, ammonium and nitrite microprofiles during autotrophic nitrogen removal in MABRs

Lackner et al. (2008) later added heterotrophs to the model structure and showed that the process was only feasible for COD/N loading ratios below 2. Simulated sloughing events removing up to 90% of the biofilm impacted reactor performance severely, which took more than 6 months to recover.

The first experimental MABR study that successfully coupled nitritation and anammox used fibrous supports on top of carbon aeration membranes in order to stimulate biofilm formation (Gong et al., 2007). The authors first inoculated the reactor with an AOB enrichment. After a brief period of nitrate production by NOB, nitrite started to accumulate by reducing the lumen air pressure in a step-wise pattern. After 42 days of operation both nitrite and ammonium accumulated at ratios suitable for AnAOB. AnAOB were then inoculated, immediately

attached to the supports, and 40 days later the reactor achieved a maximum nitrogen removal efficiency of about 80% (0.7 g-N/m^2/day).

Pellicer-Nàcher et al. (2010) and Gilmore et al. (2013) demonstrated that the process was also feasible without any structural support. In none of the studies neither the inoculation with AOB-enriched and AnAOB enriched biomass, nor pH control, nor oxygen control at the biofilm base, nor operation at high temperatures helped outcompeting NOB in early stages of the study. The exact causes explaining the decrease in NOB activity and the onset of AnAOB were undetermined in both studies. Once a stable AnAOB population established in the reactors, the process performance could be increased by tuning the relative surface loading of oxygen and ammonium. Removal rates up to 5.5g-N/m^2/day were reported.

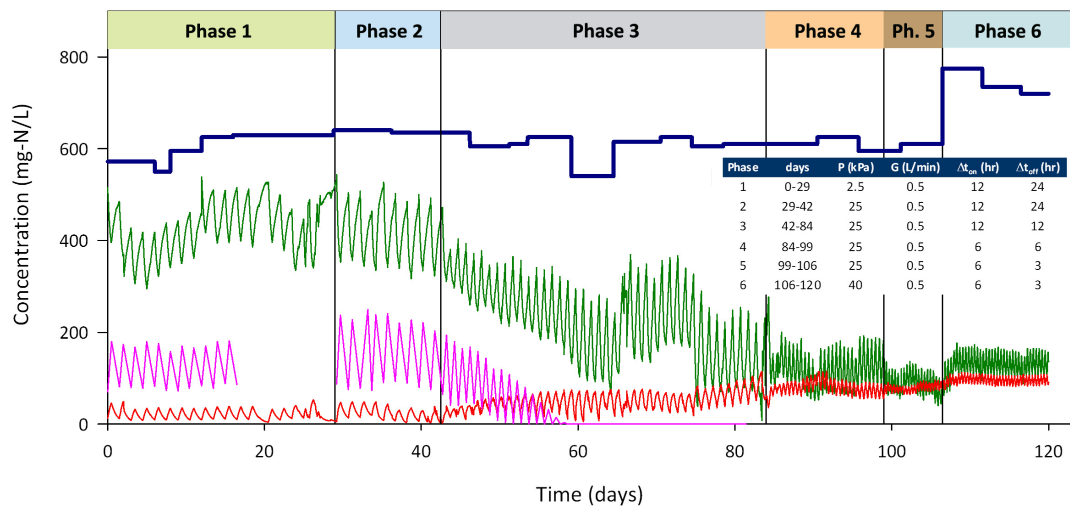
3.2. Diversity and structure of microbial communities performing autotrophic nitrogen removal in MABRs (Paper I)

Wastewater treatment bioreactors have been traditionally considered black-box systems for which a defined set of operating conditions is known to yield a desired reactor performance. While that approach has proven successful for many years in systems with fast-growing bacteria (e.g. heterotrophic bacteria in activated sludge reactor), it has encountered many problems in systems with slower microbial dynamics (e.g. nitrifying basins), where disturbances within the microbial community supporting the process could impair reactor performance for long periods of time. As a reaction to this issue, there has been a growing interest in incorporating molecular biology tools into the study of the microbiology of wastewater treatment.

In Paper I, Pellicer et al. applied these methodologies to study the microbial community performing autotrophic nitrogen removal in a MABR (Pellicer-Nàcher et al., 2010). The main purpose was to explore (i) the effect of operation strategies on the microbial dynamics, (ii) the possibility to control the stratification and diversity of the microbial populations involved, and (iii) the abundances required from the main bacterial guilds in order to make the process possible.

The system was first inoculated with nitrifying biomass enriched from a wastewater treatment plant. In spite of being operated under oxygen limitation, and at the oxygen to ammonium fluxes suggested by Terada et al. (2007), the reactor was never able to perform partial nitritation in the first month of operation and most of the converted ammonium accumulated as nitrate in the bulk liquid. An AnAOB enrichment performing stable anaerobic ammonium oxidation was then inoculated in order to impose higher selective pressures to the NOB community (AnAOB have higher affinities for nitrite). Neither this operation strategy nor the later operation at increased temperatures, pH control at values that stimulated high concentrations of free ammonia, or reduction of the lumen pressures to lower oxygen concentrations at the membrane-biofilm interface were successful at increasing the reactor performance.

Several batch tests after 390 days of operation demonstrated that nitrite accumulation was still feasible. Therefore, the authors decided to operate the reactor as a continuous batch where ammonium was continuously supplied and the air supply was turned on and off periodically (Figure 10). Nitrite immediately accumulated in the bulk after the onset of this control strategy, and the first signs of AnAOB activity could be detected only 40 days after. AOB and AnAOB catalysed the conversion of all the ammonium loaded to nitrogen gas 16 months after AnAOB were first inoculated. The reactor was further operated until day 630, attaining ammonium surficial rates up to 5.5 g-N/m^2/day and emitting 100 times less nitrous oxide than other co-diffusion nitritation-anammox systems.



Phase	days	P (kPa)	G (L/min)	Δt_{on} (hr)	Δt_{off} (hr)
1	0-29	2.5	0.5	12	24
2	29-42	25	0.5	12	24
3	42-84	25	0.5	12	12
4	84-99	25	0.5	6	6
5	99-106	25	0.5	6	3
6	106-120	40	0.5	6	3

Figure 10: Evolution of bulk concentrations of ammonium (green), nitrite (magenta) and nitrate (red) after the onset of the sequential aeration strategy (day 0). Influent ammonium concentration represented in blue. Further information in *Pellicer et al., 2010.*

Biofilm samples were collected directly from the membrane with a Pasteur pipette at selected time points during reactor operation. After DNA extraction, those samples were analysed by qPCR with primers for the detection of AOB (16S primer), *Nitrobacter* (16S primer), AnAOB (16S primer), denitrifiers (*nirK* and *nirS* primers), and all bacteria (16S primer). The time series describing the evolution in abundance of the amplified primers clearly reflected the performance trends in the system after the onset of sequential aeration: *Nitrobacter* abundances decreased an order of magnitude and AnAOB increased about the same amount. Interestingly, the numbers of the *nirK* gene (gene encoding nitrite reductase) decreased also significantly, which we suggested to be related to the decrease in nitrous oxide emissions after the enrichment of AnAOB in the system.

Biofilm samples were randomly collected after the reactor was sacrificed. FISH analyses on biofilm microsections perpendicular to the membrane surface verified that the imposed oxygen limitation resulted in the creation of well-defined niches for AOB (at the membrane-biofilm interface) and AnAOB (at the biofilm-liquid interface). Both niches were separated by a wide transition region (up to 350µm) that could potentially support heterotrophic growth (Figure 11). The use of probes with a higher phylogenetic resolution revealed that *Nitrosomonas* spp and *Ca.* Brocadia spp (*r*-strategists) were most abundant within the biofilm. *Nitrosomonas oligotropha* and *Ca.* Kuenenia stuttgartiensis (*K*-strategists) were also identified, but at significantly lower numbers, which was not usual in high-rate systems like this. We suggested that the dynamics in the nitrogen concentrations caused by the sequential aeration could have facilitated the survival of these *K*-strategist species in the system. Pyrosequencing of DNA sampled on the last day of operation confirmed these results and suggested that heterotrophic denitrifiers were an integral part of the microbial community.

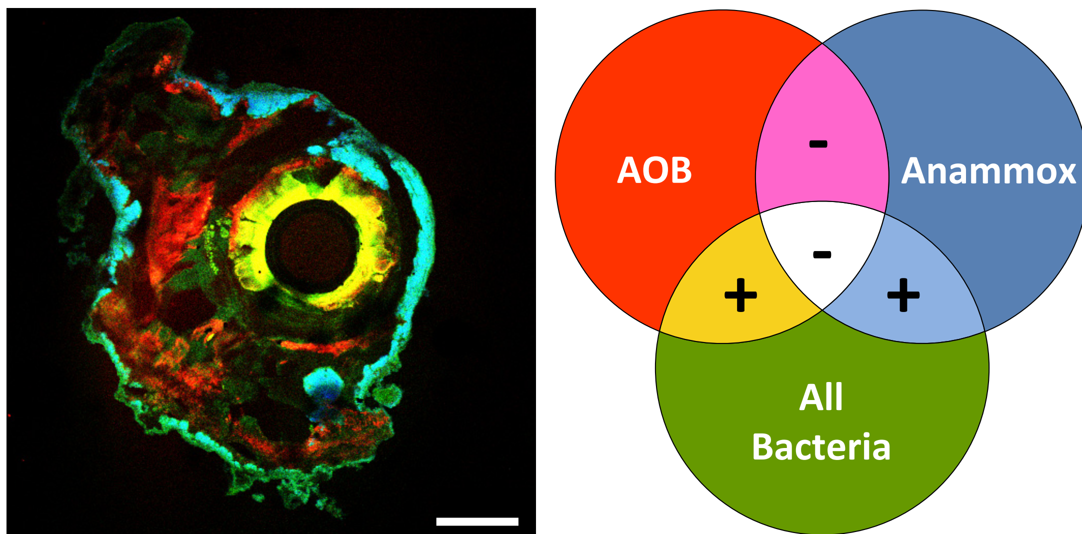


Figure 11: Distribution of the AOB and anammox (AnAOB) microbial guilds within the biofilm structure. Scale bar: 250 µm.

Only NOB of the genus *Nitrospira* could be identified in sparse clusters at microaerophilic biofilm regions, and as individual cells close to the membrane surface. The oxygen concentrations at the biofilm-membrane interface were as high as 8mg/L, which suggests that NOB control is also feasible at high oxygen loads with sequential aeration.

Image analyses on the micrographs obtained with FISH showed that AOB were the most abundant guild within the biofilm (54%), followed by AnAOB (38%) and NOB (11%). These results, however, did not compare well with the results obtained by qPCR and pyrosequencing, which clearly underestimated the bacterial numbers of the species present in the aerobic zones of the biofilm. Such observation suggests that the sampling strategy used (manual detachment from the membrane) was not efficient at recovering solids from the biofilm base.

3.3. Structure, composition and strength of autotrophic MABRs (Paper II&III)

As outlined in the previous section, all the active AnAOB community was distributed along a very thin stratum in biofilm regions very close to the membrane-liquid interface. Given the slow growth rate of AnAOB biomass (doubling times in the order of days), such a location can compromise process performance if the outer biofilm layers are sloughed away. Therefore, study of biofilm cohesion is also necessary in order to assess how robust autotrophic nitrogen removal can be in MABRs.

Recent work by Ahimou et al. (2007) and Möhle et al. (2007) have demonstrated that the cohesion forces within biofilms are stratified with depth, meaning that the biofilm base resisted bulk forces better than its surface. This observation was very correlated with the total EPS content and the carbohydrate concentration (both highest at the biofilm base). Thus, the study of EPS composition and accumulation should shed some light on the cohesion strength of MABR biofilms.

3.3.1. Benchmarking study of EPS extraction methods

The ideal EPS extraction protocol extracts as much biopolymer as possible with minimum cell lysis. Many published protocols have been inspired along this concept in the last few years. However, when the same extraction method is applied to the same biomass in different studies very different conclusions are often obtained, which significantly complicated the selection of a protocol for this work.

The second part of this study (Paper II) was then directed towards the critical assessment of methods for the extraction of EPS from mixed culture biomass in order to create an extraction protocol for our MABR biofilms. Nitrifying biomass, nitritation-anammox granules, and activated sludge were used as test materials. Proteins, carbohydrates, DNA, and humic acids were quantified by well-established analytical methods.

The selected biomass samples were first homogenized by light ultrasonic treatment and incubated with a formamide solution for 1 hour to enhance the extraction efficiency of later extraction steps. The extract obtained was collected and named loosely bound EPS. The tightly bound EPS was then extracted by (i)

acid extraction, (ii) basic extraction, (iii) extraction with surfactant, (iv) extraction with a cation exchange resin (CER) and (v) extraction by ultrasounds.

In parallel, we implemented and calibrated, for the first time, a quantitative method to assess cell lysis based on the cell viability of EPS-extracted pellets. Our results showed that current methods used to qualitatively estimate cell lysis, clearly underestimate cell death. More interestingly, we could also demonstrate that cell lysis does not necessarily correlate with the EPS extraction yield, as commonly reported in other studies.

Ultrasonic extraction of bacterial EPS was faster to perform, had high extraction yields, induced relatively low cell lysis, and was less biased by molecular interactions than the rest of extraction methods. Therefore it was selected for later analyses.

Both types of autotrophic biomass accumulated very similar amounts of EPS with practically the same composition (80% protein, 9% of carbohydrates and proteins and 2% of DNA, Figure 12). Both biomass types also had similar physiologies and had AOB as the main population. Thus, we decided to operate nitrification MABR biofilms in later stages of the study and use them as model autotrophic biofilm for the determination of cohesion and strength.

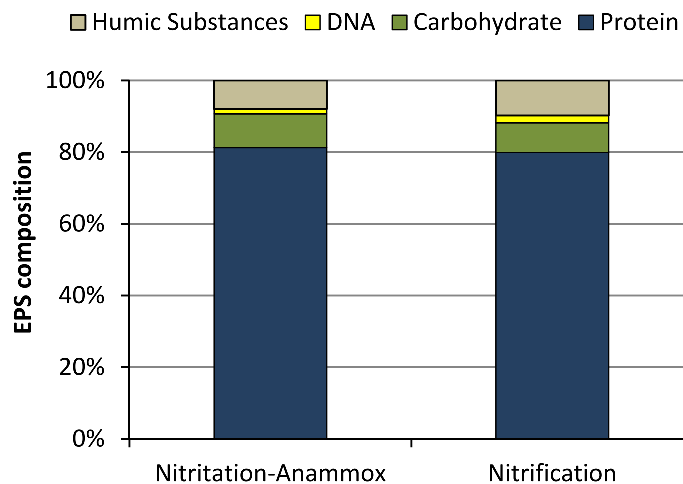


Figure 12: EPS composition of nitritation-anammox granules and activated sludge flocs

3.3.2. Structure, composition and strength of autotrophic membrane-aerated biofilm reactors

In a later step, and with a more robust protocol to assist EPS extraction, we operated flat sheet MABR flow cells performing full nitrification to identify the operation conditions that can lead to the creation of optimal biofilm structures for high-rate nutrient removal and enhanced cohesive properties (Paper III).

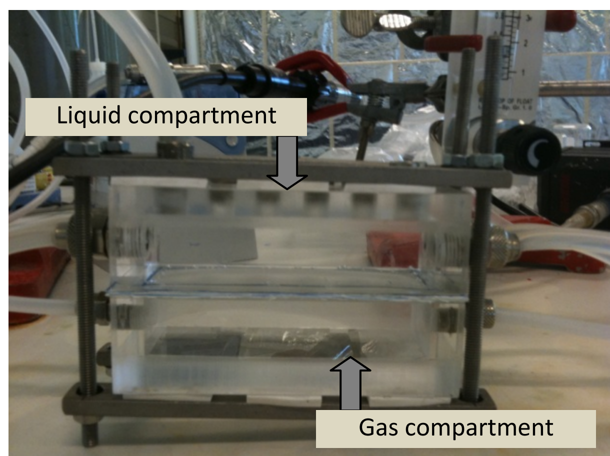


Figure 13: Flow cell used for biofilm growth in Paper III

Operation of oxygen-limited biofilms at higher hydrodynamic shears yielded thinner and denser biofilms, with a higher content of volatile material, and a lower amount of EPS than the biofilm grown under reference conditions. Biofilms that were fully penetrated by oxygen were thinner, denser, and accumulated slightly more EPS, however, they were significantly less homogeneous than the biofilms operated under oxygen limitation. Despite the different trends in total EPS accumulation, the biopolymer composition was similar for all the growth conditions tested. Unexpectedly, the EPS composition of the inoculum changed dramatically after biofilm growth in MABRs, and became dominated by carbohydrates, known to form more cohesive biofilm structures.

The composition and structure of the formed biofilms was described by fluorescent staining of cells and EPS components in biofilm cryosections perpendicular to the biofilm surface. Two distinct layers could be described in most samples: (i) a dense cluster of cells, lipids and extracellular DNA encapsulated by a layer of carbohydrate and protein at the biofilm base (potentially aerobic), and (ii) a less defined structure of EPS and cells sustained

by a network of filamentous microorganisms just on top of (i), in potentially anaerobic areas.

Literature-validated methodologies were used to determine the cohesive properties of the cultivated biofilms against different shear and tensile stresses (Figure 14). All the studied MABR biofilms were anisotropic, with a higher tendency to sloughing when subjected to higher shears. At shear stresses above 0.2 Pa (20-100 times higher than during cultivation) the biofilm fraction with a lower cohesive strength was detached, leaving a basal layer of similar protein content irrespectively of the applied growth conditions. This layer (with a higher EPS content) could be associated to the layer (i) described above and had higher cohesive forces than the biofilm regions closer to the bulk liquid. Tensile stresses up to 50Pa did not produce any significant biomass detachment from the membrane, which highlights that the biofilm behaves differently against different types of stress.

Comparison with conventional biofilms described in the literature showed that autotrophic MABR biofilms grown under similar hydrodynamic conditions accumulated more volatiles, more carbohydrates and were more cohesive.

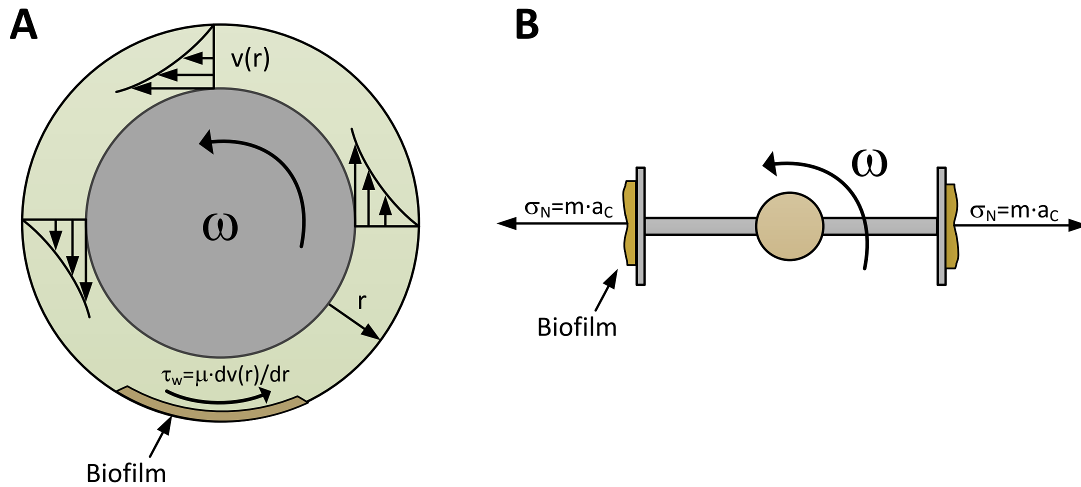


Figure 14: Experimental setups used to estimate biofilm cohesion

A (Coufort et al., 2007): The biofilm sample is placed in the inner part of a cylinder that contains a rotating drum. When the drum rotates at a certain speed ω, shear stresses (proportional to the velocity gradient inside the cell) develop on top of the sample.
B (Ohashi et al., 1994): The biofilm specimen is glued onto a rotating table turning at a velocity ω. The biofilm experiences tensile stresses proportional to its wet mass and the centripetal acceleration experienced

3.4. Oxygen use and characterization in MABRs (Paper IV)

As previously outlined, the greatest potential of MABRs lies in their ability to supply oxygen at efficiencies and operation costs very difficult to attain by conventional aeration systems. Surprisingly, the oxygen transfer mechanisms in these reactors have never been completely understood.

The oxygen transfer characteristics of an MABR are often estimated with re-oxygenation curves in clean water. Even though this method can be used to obtain a rough estimation of the mass transfer ability of a membrane, prior work has shown that it cannot predict gas transfer once a biofilm has developed on its surface, which complicates significantly reactor design and control (Brindle et al., 1998; Shanahan and Semmens, 2006; Pellicer-Nàcher et al., 2010). Several attempts have been done, to make oxygen transfer in MABRs more comprehensive (Casey et al., 1999; Gilmore et al., 2009; Lackner et al., 2010), but none of them have been able to describe the OTR dynamics seen during reactor operation well.

Even though part of the nitritation-anammox process takes place anaerobically, oxygen mass transfer plays a very important role during autotrophic nitrogen removal. Oxygen is the electron acceptor used by AOB, hence the OTR delivered will influence the rate of nitrite accumulation. Moreover, if oxygen is supplied at high rates, the NOB present at the biofilm base might be stimulated to consume nitrite that AnAOB could uptake to convert ammonium to nitrogen gas. Also, if oxygen penetrates the biofilm completely, AnAOB can be inhibited and further enhance NOB, given the lack of competitors for NO_2^-.

Thus, the correct understanding of the factors impacting oxygen mass transfer during autotrophic nitrogen removal should allow the implementation of control strategies that optimize oxygen delivery, while still preventing NOB growth. In order to study the process, we constructed a flow cell system that permitted undisturbed microelectrode inspection over the two hollow fibres installed (PDMS dense membranes, Figure 15). The reactor was kept well mixed by an aquarium pump and the membranes were pressurized with air.

During the first stage of the study, the reactor was operated without biofilm in order to investigate how oxygen mass transfer proceeds in clean water experiments. The OTR and K_{OL} obtained from re-aeration curves were within the

range of values reported for similar systems. Microelectrode profiles during clean water aeration revealed a 135 μm-thick liquid boundary layer developed over the membrane, and allowed the calculation of its oxygen mass transfer coefficient (k_L). Calculations using typical mass transfer formulations revealed that this layer was responsible for two thirds of the total resistance to oxygen transfer, a number which none of empirical correlations tested could predict. The membrane mass transfer coefficient (k_M) calculated from the microelectrode analysis was within 1% of the theoretical value for dense membranes.

The reactor was later inoculated with nitrifying biomass and aerated in cycles of 12 hours (6 hours with air on, 6 hours with air off). The oxygen and the ammonium loads were adjusted to achieve partial nitritation. Once microbial activity stabilized, the ammonium surface load was modified while keeping the oxygen pressure constant. Three ammonium surface loads were applied: 5, 13, and 31 g-N/m^2/day. The actual oxygen being transferred was estimated from the bulk concentrations of ammonium nitrate and nitrite, and stoichiometric coefficients for nitritation and nitratation.

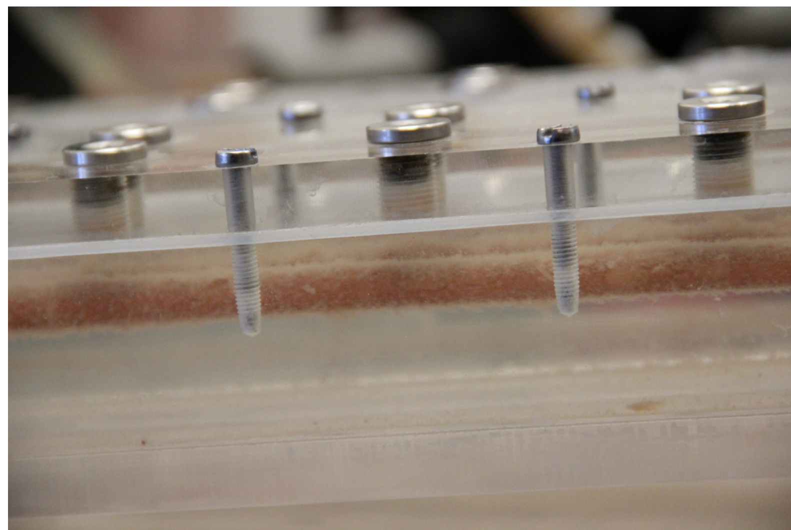


Figure 15: Nitritating biofilm and microelectrode sampling points in the designed flow cell designed in Paper IV

In spite of keeping the pressure in the lumen constant, we could observe a marked increase in the surficial nitritation rates at increased ammonium loading rates. The highest nitritation rate measured was sustained by OTRs six times

higher than predicted in clean water tests. Ammonium and oxygen microsensors were used to confirm that the higher availability of ammonia (and not ammonium) at the biofilm base was the factor enhancing AOB activity and oxygen use in the system.

All described findings were used to update existing oxygen transfer models. The oxygen transfer rates observed during the experiments described here could be successfully explained with two parameters, which depended on the relative loading of ammonium and oxygen and system-specific characteristics.

4. Conclusions and future perspectives

MABRs can significantly lower the economic and environmental impact of the nitritation-anammox process when treating nitrogen-rich wastewater streams. However, preliminary reactor experiences and several modelling studies highlighted three constraints that could hurdle the development of the technology in pilot or full scale: the lack of strategies to control the microbial community, the risk of bacterial sloughing, and the poor knowledge on the processes influencing oxygen transport in the system. During this thesis we applied methodologies at different scales that revealed how to assist the optimization of activity, structure and physical strength of MABR biofilms performing autotrophic nitrogen conversions.

The study of the microbial dynamics in nitritation-anammox MABRs clearly confirmed that the imposition of the sequential aeration strategy was key to control the NOB community, even when growing in fully aerobic areas. Nitrite and ammonium accumulation were the key drivers in increasing AnAOB numbers and enhancing process performance. Even though the AOB and AnAOB guilds were clearly dominated by fast growers, there were indicators that suggested that the sequential aeration regime may have facilitated the conservation of a few *K*-strategists. We also obtained visual confirmation that the imposed oxygen limitation was successful in controlling the stratification of the AOB and AnAOB groups. AOB and very sparse slow-growing NOBs mainly populated the biofilm base. AnAOB could only be detected in relatively thin strata close to the biofilm-liquid interface.

The delicate position of the AnAOB group within the biofilm raised the need to study cohesion in autotrophic MABRs. While the biofilms were able to withstand tensile stresses up to 50 Pa, shear stresses of only 0.2-0.9 Pa triggered detachment events of up to 40% of the total biofilm mass. The biofilm structure was clearly divided in two regions with different cohesive properties: (i) a very dense and shear-resistant cluster of cells, lipids and extracellular DNA encapsulated by a layer of carbohydrate and protein at the biofilm base; (ii) a less cohesive conglomerate of EPS components and cells linked together by a network of filamentous microorganisms (in potentially anaerobic biofilm regions). Operation at high higher shears made the layer (ii) more compact, and with a higher content of volatile solids.

Finally, we could quantitatively demonstrate that oxygen transfer experiments in clean water cannot be used to describe oxygen transfer in systems with biofilms because (i) the overall mass transfer coefficient calculated accounts for the resistance of a liquid boundary layer which is not present when a biofilm grows on the membrane, and (ii) oxygen transfer can be stimulated by microbial activity at the biofilm base. OTRs up to six times higher than the one obtained in clean water experiments were obtained under MABR operation at very high ammonium to oxygen loads.

Overall, these results have increased the knowledge on key operational factors that can assist various process optimizations in development of the MABR technology to treat nitrogen streams in a more efficient way.

Implications of the results in future modelling studies

We created novel model structures to describe oxygen transfer in a wide range of operation conditions. The extensive collection of experiments presented here also produced an important dataset of fundamental biofilm data. This new knowledge could support the development a new generation of nitritation-anammox MABR models that described detachment, porosity, distribution of the microbial communities, and abundances in a more comprehensive way. Ammonium speciation should be incorporated to describe ammonia uptake by AOB.

Implications of the results in future experimental studies

The next generation of nitritation-anammox MABR experiments should take advantage of the operation strategies described here in order to control microbial communities from earlier stages of the study. AnAOB inoculation should be postponed until nitrite accumulates in the system, and the length of the inoculation process should allow these slow growers to attach to the biofilm.

Operation conditions should be studied to favour lower separation distances between the AOB and AnAOB guilds, and eventually “bury” the AnAOB community in the biofilm for increased shear protection. Membrane modules must be designed to minimize the effect of high shear stresses on the biofilm surface (e.g. impact of solids and bubbles) and guarantee a homogeneous hydrodynamic distribution of the flow.

New experiments should also explore the benefits (both in economy and microbial diversity) of staged reactor configurations in which oxygen transfer is optimized by operating each stage of the setup at high ammonium to oxygen ratios.

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6. Papers

I. **Pellicer-Nàcher, C.**, Franck, S., Gulay, A., Ruscalleda, M., Terada, A., and Smets, B.F. (2013). Sequentially aerated membrane biofilm reactors for autotrophic nitrogen removal: Microbial community composition and dynamics. *Submitted to Microbial Biotechnology.*

II. **Pellicer-Nàcher, C.**, Domingo-Félez, C., Mutlu, G., and Smets, B.F. (2013). Critical assessment of methods for the extraction of extracellular polymeric substances from mixed culture biomass. *Submitted to Water Research.*

III. **Pellicer-Nàcher, C.**, and Smets, B.F. (2013). Structure, composition, and strength of nitrifying membrane-aerated biofilms. *Submitted to Water Research.*

IV. **Pellicer-Nàcher, C.**, Domingo-Félez, C., Lackner, S., and Smets, B.F. (2013). Microbial activity catalyzes oxygen transfer in membrane-aerated nitritating biofilm reactors. *Submitted to the Journal of Membrane Science.*

In this online version of the thesis, the papers are not included but can be obtained from electronic article databases e.g. via www.orbit.dtu.dk or on request from:

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The Department of Environmental Engineering (DTU Environment) conducts science-based engineering research within four sections:
Water Resources Engineering, Urban Water Engineering,
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The department dates back to 1865, when Ludvig August Colding, the founder of the department, gave the first lecture on sanitary engineering as response to the cholera epidemics in Copenhagen in the late 1800s.

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