THE CO-EXISTENCE OF HETEROTROPHS AND NITRIFIERS IN NITRIFYING BIOFILM

A study on the relationship between heterotrophic and nitrifying bacteria in a marine environment.

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Abstract

As world fish stock decrease the aquaculture industry becomes more and more lucrative. Conventional aquacultures such as caged-based or flow-through systems can have a strong impact on surrounding benthic environment by inorganic nutrient loading creating high anoxic conditions. Closed recirculation aquaculture systems (RAS) has proven to be a more sustainable because a major part of the organic waste is sorted out and undergone nitrification and de-nitrification reducing the output of nitrate into the oceans. In RAS the utilization of nitrifying bacteria to reduce and control concentration of ammonium NH_4^+ and ammonia NH_3 referred to as total ammonia nitrogen TAN is critical since these are highly toxic for fish. Ammonium is secreted from the gills of the fish and from the decomposition of organic nutrients by a wide range of heterotrophic bacteria naturally present in marine and freshwater. In bio filtration of a RAS nitrifying bacteria are growing in biofilm on bio filters designed to increase the surface area making more room for the nitrifiers to create biofilm. Biofilm can be described as a three-dimensional structure consisting of different layers in which the microorganisms are embedded. Ammonium oxidation has a very low energy yield and nitrifying bacteria are known to reproduce very slowly. It is therefore a common problem in many bio filtration systems that nitrifying biofilm gets overgrown by heterotrophic bacteria which leads to a decrease in ammonium conversion. Ammonia oxidation is catalyzed by the enzyme ammonia monooxygenase (amoA) which has been used in a wide range of phylogenetic studies describing different strains of ammonia oxidizing bacteria and as a biomarker for ammonia oxidation. In this study the colonization of nitrifying biofilm of *Nitrosomonas marina* sp. 22 by the marine heterotrophic bacteria *Shewanella japonica* was examined and how different surfaces may influence the rate of colonization by *S. japonica*. The effect on the expression of amoA and conversion rate of TAN by *N. marina* sp. 22 was described by the use of quantitative real-time PCR and measurements of TAN decrease over a 12 day period. Results from qPCR and the effect on TAN conversion was unfortunately inconclusive. The rate of biofilm production by *S. Japonica* when grown on a surface coated with biofilm from *N. marina* sp. 22 and on a surface coated with a synthetic biofilm of Bovine serum albumin (BSA) showed a significant increase in exponential growth rate when compared to a uncoated surface, indicating that heterotrophic bacteria may be stimulated to colonize nitrifying biofilm and biofilm like surfaces. A comparison of a live/dead staining of a biofilm with both strains and one with only *N. marina* sp. 22 also indicated a difference.

Danish summary (Dansk sammendrag)

Som verdens fiskebestand mindskes, bliver aquakultur industrien mere og mere lukrativ. Konventionelle akvakulture så som bur-baserede eller gennemstrøms systemer kan have en kraftig indvirkning på det omkringliggende bentiske miljø ved inorganisk nærings tilførelse, som skaber kraftigt iltfattige vilkår. Lukkede recirculerings akvakultursystemer (RAS) har vist sig at være mere bæredygtigt, da størstedelen af det organiske affald er sorteret fra og er undergået nitrification og de-nitrification, hvilket reducerer afkastet af nitrat til havet. I RAS bruges nitrificerende bakterier til at reducere og kontrollere koncentrationen af ammonium (NH_4^+) og ammoniak (NH_3), også kaldet Total Ammoniak Nitrogen (TAN), da dette er kritisk eftersom det er meget giftigt for fisk. Ammonium secreres fra fisks gæller og fra forrådnet organisk materiale af en lang række naturligt tilstedeværende marine heterotrophiske bakterier. I biofiltration i RAS gror de nitrificerende bacteria på biofiltre, som er designet til at have stor overflade og derved øge arealet, hvorpå nitrifcernde bakterier kan lave biofilm. Biofilm kan beskrives som en tredimensionel struktur bestående af forskellige lag, hvori mikroorgansimer er indlejret. Ammonium oxidation har et meget lavt energi udbytte, og nitrificerende bakterier er kendt for at vokse meget langsomt. Det er derfor et meget almindeligt problem, at mange biofiltrationssystemer bliver overgroet af heterotrophe bakterier, hvilket kan lede til en mindskning i TAN omsætning. Oxidation af ammonia er katalyseret af enzymet ammonia monooxygenase (amoA), som er blevet brugt i en lang rækkephylogenetiske studier af forskellige stammer af ammonia oxiderende bakterier og som en biomarkør for ammonia oxidation. I det pågældende studie undersøges raten, hvorved biofilm fra Nirosomonas marina sp. 22 koloniseres af den marine heterotrophe bakterie *Shewanella japonica* og hvorvidt dette påvirker udtrykket af amoA og omsættelsen af TAN over en 12 dages periode. Ved brug af quantitative real-time PCR blev effecten af amoA udtrykkelsen undersøgt. Resultater fra qPCR og effekten på TAN omsætningen gav resultat. Vækst raten af biofilm production fra *S. japonica* når den gror på en overflade, som er behandlet med biofilm fra N. marina sp. 22 og en overflade behandlet med Bovine Serum Albumin (BSA) viste en significant forskel sammenlignet med en ikke behandlet overflade. Dette kan indikere at heterotrophiske bacterier bliver stimuleret af biofilm fra nitrificerende bakterier og biofilm lignende overflader. En sammenligning af en live/dead farvning af biofilm med begge stammer og en med kun *N. marina* sp. 22 indikerede også en forkel.

Preface

This thesis was written as a 60 ECTS point interdisciplinary master's thesis in molecular and environmental biology. All experiments were conducted at Roskilde University in the laboratories of Håvard Jenssen and Ole Skovgård and in the climate room of Benni Winding Hansen.

My supervisors for this project were Professor Benni Winding Hansen, Roskilde University, Department of Environmental, Social and Spatial Change (ENSPAC), and Associate Professor Håvard Jenssen, Roskilde University, Department of Nature, Systems and Models (NSM).

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Contents

1. Introduction

As the world's fish stocks are drastically decreasing and the demand for fish is constantly increasing the aquaculture industry is growing rapidly. The use of aquacultures can have a serious impact on the surrounding benthic environment by inorganic nutrient loading creating high anoxic conditions (Holmer et al., 2003). Conventional aquacultures such as caged-based or flow-through systems have no way of dealing with the bi-products that are created. Eutrophication from fish feed and feces can lead to oxygen depletion in shallow waters and result in major fish kills if wastewater is not diluted or treated (Wu, 1995, Holmer et al., 2003). This has become a major concern in many countries that wish to avoid further organic pollution. A solution to this problem is to use more advanced closed recirculation aquaculture systems (RAS) (fig1). The impact of RAS on the surrounding environment is easier controlled and has proven to be a much more sustainable solution (Cytryn et al., 2003). Besides being more eco-friendly there are many other advantages with using RAS when compared to the caged-based or flow-through systems. For example up to 90-99% of the water in RAS is re-circulated making it easier and cheaper to maintain at a fixed temperature, and food conversion efficiency is much higher creating less food waste. In countries with high restrictions on waste discharge RAS has proven to be well suited for intense fish farming because a major part of the organic waste is sorted out and undergone nitrification and de-nitrification reducing the output of nitrate into the oceans (Heinen et al., 1996, Losordo et al., 1998, Wolters et al., 2009). Having to recycle water comes with its own challenges compared to the cage-based and flow-through systems. Salinity, temperature, oxygen, pH, alkalinity and waste bi-products however are all parameters that have to be controlled in order for the system to function. Salinity is fairly simple, since the water is recycled and by minimizing evaporation it is considered constant. Temperature, pH, oxygen and waste products have to be monitored and controlled thoroughly since these factors are crucial for fish survival(Kim et al., 2000). Waste products such as dissolved ammonium are of primary concern when dealing with waste in RAS. Ammonium exists in two aqueous forms, NH_3 and NH_4^+ (eq. 1). Both are toxic for fish but the unionized form, ammonia (NH_3) gets toxic at lower concentrations. The equilibrium of the two

(1) $H_3O^+ + NH_{3(aq)} \leftrightarrow H_2O + NH_4^+{}_{(aq)}$

are affected by pH, temperature and salinity (Chen et al., 2006). In seawater equilibrium is obtained at pH 9.3 at 25 C° (Clegg and Whitfield, 1995). Lower pH moves the equilibrium to the left, and

higher pH moves the equilibrium towards the right (Chen et al., 2006). Since dissolved ammonium exists in these two forms it is referred to as Total Ammonia Nitrogen (TAN) for the entire system. Other waste products are nitrite-nitrogen (NO_2^-), nitrate-nitrogen (NO_3^-) and carbon dioxide (CO_2). They can also have an impact on fish health but are either easier managed or less toxic. TAN and CO_2 arise from protein metabolism and respiration of the fish and are secreted from the gills. It will also be formed from the bacterial breakdown of fatty-acids, proteins and carbohydrates that are not assimilated by the fish, and from fecal solids or uneaten feeds. It is therefore important to remove all waste solids as soon as they appear (Losordo et al., 1998)

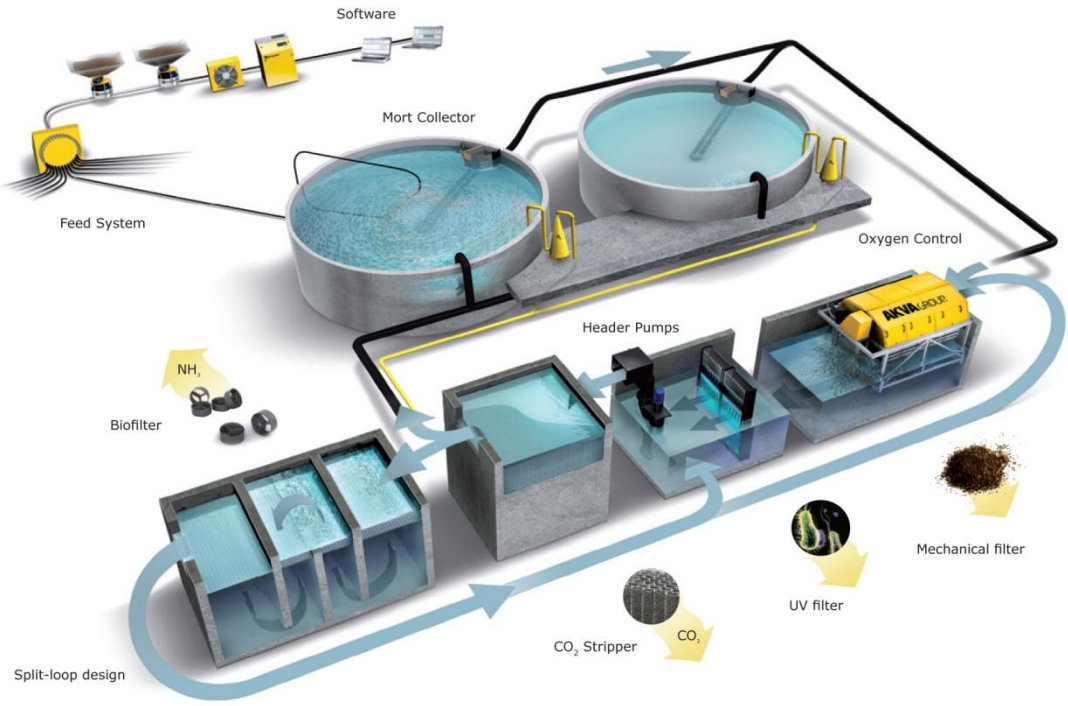


Figure 1: An illustration of a Recirculating Aqua System (Akvagroup.com). Water is passed from the fish tank to mechanical filtration which removes all particles larger than 50 microns. The water is then passed through a UV filter, which radiates the water, controlling the levels of pathogens and ecto-parasites. Hereafter the water is passed through a CO_2 stripper where gasses such as CO_2, N_2 and sulphide residues are removed. Approximately 70% will be transferred back to the fish tank. Approximately 30% of the water is then transferred to the bio filtration, where ammonia will be oxidized into nitrite and nitrogen, securing ammonia levels are below lethal levels. The water is then passed back to the UV chamber.

Even with thorough waste removal it is unavoidable that the concentration of TAN will increase in the fish tank and if left unattended it will reach toxic levels. By utilizing microorganisms that are able to perform nitrification, a two part reaction in which ammonium (NH_4^+) is oxidized to nitrate as shown in eqs. (2) and (3), lethal levels of toxic bi-products from waste can be avoided. This is referred to as bio-filtration (Kim et al., 2000).

(2) $NH_4^+ + 1.5O_2 \rightarrow 2H^+ + H_2O + NO_2^-$

(3) $NO_2^- + 1.5O_2 \rightarrow NO_3^-$

Nitrifying organisms and optimization of nitrifying properties has therefore become of great interest in the field of aqua culturing. Nitrifying bacteria form biofilm on bio filters and oxidize ammonium to nitrate. It is imperative that the conversion rate is high enough to keep TAN concentration below approximately 30µM (Grommen et al., 2005). Many different strategies for optimal bio filters have been tested. The main idea of most bio filters is to increase the surface area thereby permitting more nitrifying biofilm to be formed to increase the level of nitrification (Malone and Pfeiffer, 2006)

1.1 Structure and nature of biofilm:

Microorganisms in nature do not live as pure cultures or dispersed single cells. Many have a tendency to form polymicrobial aggregates known as biofilm. This phenomenon is very common and is done by a wide range of microorganisms. Microorganisms within the biofilm make up for less than 10% of the biofilm dry weight whereas the matrix itself consists of more than 90% (Flemming and Wingender, 2010). The biofilm matrix is formed of a conglomeration of different bi-polymers produced by the microorganisms. These are referred to as extracellular polymeric substances (EPS) (Bales et al., 2013). The biofilm matrix is a three-dimensional structure consisting of different layers in which the microorganisms are embedded. EPS are closely related to the cell since they create the immediate environment in which the microbes exists (Decho, 2000). The nature of different EPS is crucial for the structural maintenance of a multicellular biofilm, as well as the three-dimensional structure such as pores and channels (Gamini et al., 2002). Concentration, charge and sorption capacity are all properties that will determine the interaction with other substrates and other cells. The nature of the surrounding EPS also determines the morphological shape of the biofilm, whether it is rough, smooth, rigid or even sponge like. By forming a biofilm the microorganisms enter a stationary phase and are no longer motile which means the life form is completely changed from the planktonic phase. EPS enable microorganisms to create a micro-environment within the biofilm which is suitable for them. Different cells in close proximity of one another allows for cell to cell communication known as quorum sensing. This is done by the use of specialized receptors and secreted proteins. Quorum sensing even takes place between different species (Shrout and Nerenberg, 2012). By the use of quorum sensing and creation of micro-environments a high biodiversity of a long-sustained multi cellular population is promoted. Although the importance of EPS is clear the specific role of most EPS secreted is still very poorly

understood, but it is believed to have a key role in both cooperation and competition between microbial species within a biofilm (Flemming and Wingender, 2010).

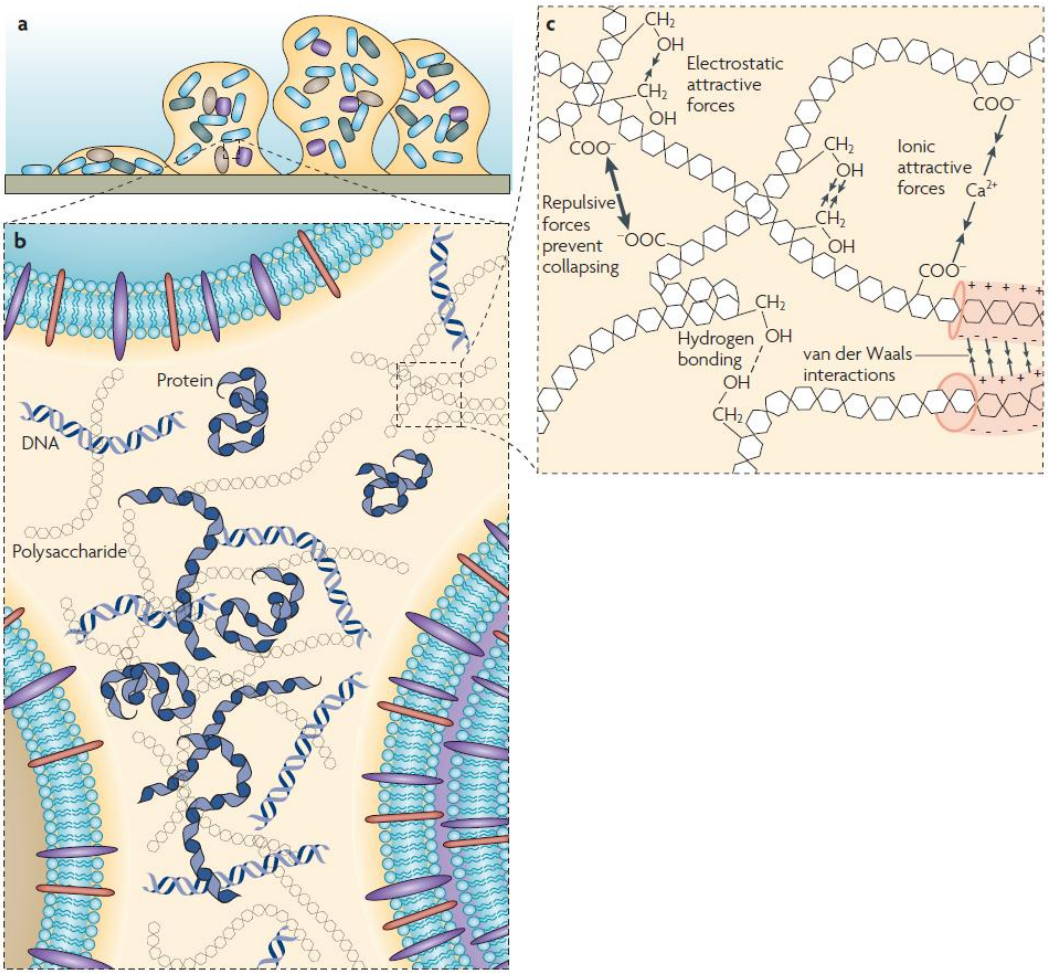


Figure 2: An illustration of Extracellular Polymeric Substances in a biofilm at different dimensions. a: The colonization of a solid surface by a bacteria. It starts with the attachment of a single cell to a solid surface. It then multiplies and starts to form the biofilm matrix, forming a micro-colony consisting of only a few cells. As the biofilm matures other cells may be recruited and a more complex biofilm matrix is formed. b: The extracellular space inside the biofilm with different matrix components. Polysaccharides, proteins and eDNA secreted by neighboring cells in a non-homogenic fashion. c: Physicochemical interactions between different biopolymers that ensures the stability of the biofilm matrix (Flemming and Wingender, 2010).

The biofilm presents many advantages for the biofilm biota. Many extracellular enzymes function as an external digestive system where external bi-polymers are broken down to low-mass molecules which can then be taken up and utilized as carbon or as an energy source. Also cell components from lysed cells are trapped in the matrix making neighboring cells able to recycle important components such as nucleotides, lipids and even whole genes. Utilization of genes from lysed cells creates an available gen-pool for horizontal gene transfer within the biofilm (Sutherland, 2001).

The biofilm also works as a shield protecting against many physical and chemical threats such as desiccation, oxidizing, metallic cat ion, UV-radiation and even protozoan grazers (Elasri and Miller, 1999). In any natural environment changes are constant among available oxygen, organic/ inorganic nutrients, temperature or pH. The microbial community within a biofilm is therefore under constant selective pressure, and constant adaption to ecological changes and competition with rivaling cells makes the biofilm community very dynamic (Sutherland, 2001).

A major part of the EPS matrix consists of extracellular polysaccharides. These are in general very long molecules, both linear and branched, and have been demonstrated to be ubiquitous in the biofilm matrix. Many have been observed being attached to cell surfaces forming complex networks (Chen et al., 2013). Extracellular polysaccharides have shown to be very diverse even between different strains of the same species. In *P. aeruginosa* 3 distinct extracellular polysaccharides was described; alginate, psl, and pel. Different strains of *P. aeruginosa* was able to form functioning biofilm with only one of the three (Ghafoor et al., 2011). All were very important for architectural stability of the biofilm. Cells that are not able to form extracellular polysaccharides will not be able to form a biofilm. Some can still attach to surfaces and form micro colonies, but will never mature into a biofilm (Danese et al., 2000). It has been observed though, that in multispecies biofilms, these extracellular polysaccharide mutants are still able to colonize and integrate into a biofilm, as long as another species will form the matrix. That is why the amount of different extracellular polysaccharides does not necessarily show how many species a specific biofilm contains, since they do not contribute equally to the structure of a biofilm (Sutherland, 2001).

Extracellular proteins, primarily enzymes, are another important aspect when discussing biofilm. Proteins are so abundant that they in some cases can exceed the total mass of extracellular polysaccharide. Many extracellular enzymes are used in the degradation of different biopolymers such as proteins, polysaccharides, nucleic acids, lipids, inorganic matter and organic matter trapped in the biofilm. In some cases even chitin and cellulose. Some enzymes are also involved in degrading structural EPS (Zhang and Bishop, 2003). In the case of environmental changes, nutrient starvation or availability of a new nutrient, biofilm may be needed in order to adapt to the new environment. Enzymes degrading the structural EPS are then released by cells in proximity of the changes. This causes dispersion of sessile cells allowing new biofilm to be formed. Since most naturally occurring biofilms are multispecies, many different EPS are formed depending on species

and possible mutations. For each new EPS, a new enzyme with matching binding site is required. A universal enzyme that degrades all EPS is yet to be found (Wingender et al., 2001).

It is very costly for the cells to produce enzymes. It is therefore important for the cells to make sure that they gain something in return. An efficient way of retaining enzymes in the matrix is to have them form weak bonds with the structural extracellular polysaccharides thereby decreasing the distance they diffuse from the cell that secreted them. This ensures that the reactions catalyzed by the enzyme happens in close proximity of the cell that in the first place secreted them, making sure it benefits from the work performed (Mayer et al., 1999, Skillman et al., 1999).

All proteins in the biofilm matrix are not enzymes. Structural proteins such as lectins are important part of stabilizing the structural extracellular polysaccharides. Studies on *Psuedomonas aerguginsa* further testament to this. Mutants with inhibition of a lectin called LecB (Johansson et al., 2008) or LecA (Diggle et al., 2006) both showed dispersion of biofilm. Also when LecA was overproduced so was the biofilm when compared to the wild-type (Diggle et al., 2006). However when the two different strains with different mutations where introduced into the same biofilm, the lectins were able to assemble outside the cells, thereby creating a functional biofilm, showing how different cells may support each other (Johansson et al., 2008).

Ekstracellular DNA (eDNA) has been found to be important for the structural integrity of the matrix for some species of bacteria. However the importance differs between different species. For some it is vital, where in others it is less significant. eDNA have different structural roles when secreted from different species. When eDNA was first discovered many believed it to be leftovers from lysed cells. This however proved not to be true, since there is a fundamental difference in the composition of nucleotids compared to genomic DNA (Frolund et al., 1996, Kaplan et al., 2011)

1.2 The Nitrifying Community:

The nitrifying bacteria are chemo litho autotrophic, and consist of two groups; the ammonia oxidizing bacteria (AOB) and the nitrite oxidizing bacteria (NOB). With the exception of two species, all known AOBs belong to the monophyletic group within the *β*-subclass of *Proteobacteria* (Head et al., 1993, Aakra et al., 2001), where NOB belong to four groups within the α-subclass, γ-subclass and δ-subclass of *Proteobacteria* (Grommen et al., 2005).

As mentioned earlier, in aquatic media ammonia consists of two forms. Unionized NH_3 and ionized NH_4^+. (Chen et al., 2006). The conversion of ammonia to nitrite and nitrite to nitrate is unique for the nitrifying microorganisms.

A simplified reaction is depicted in eq. (5) and (6) and as illustrated in figure 3.

(5) $NH_4^+ + 1{,}5O_2 \rightarrow 2H^+ + H_2O + NO_2^-$ (AOB)

(6) $NO_2^- + 1{,}5O_2 \rightarrow NO_3^-$ (NOB)

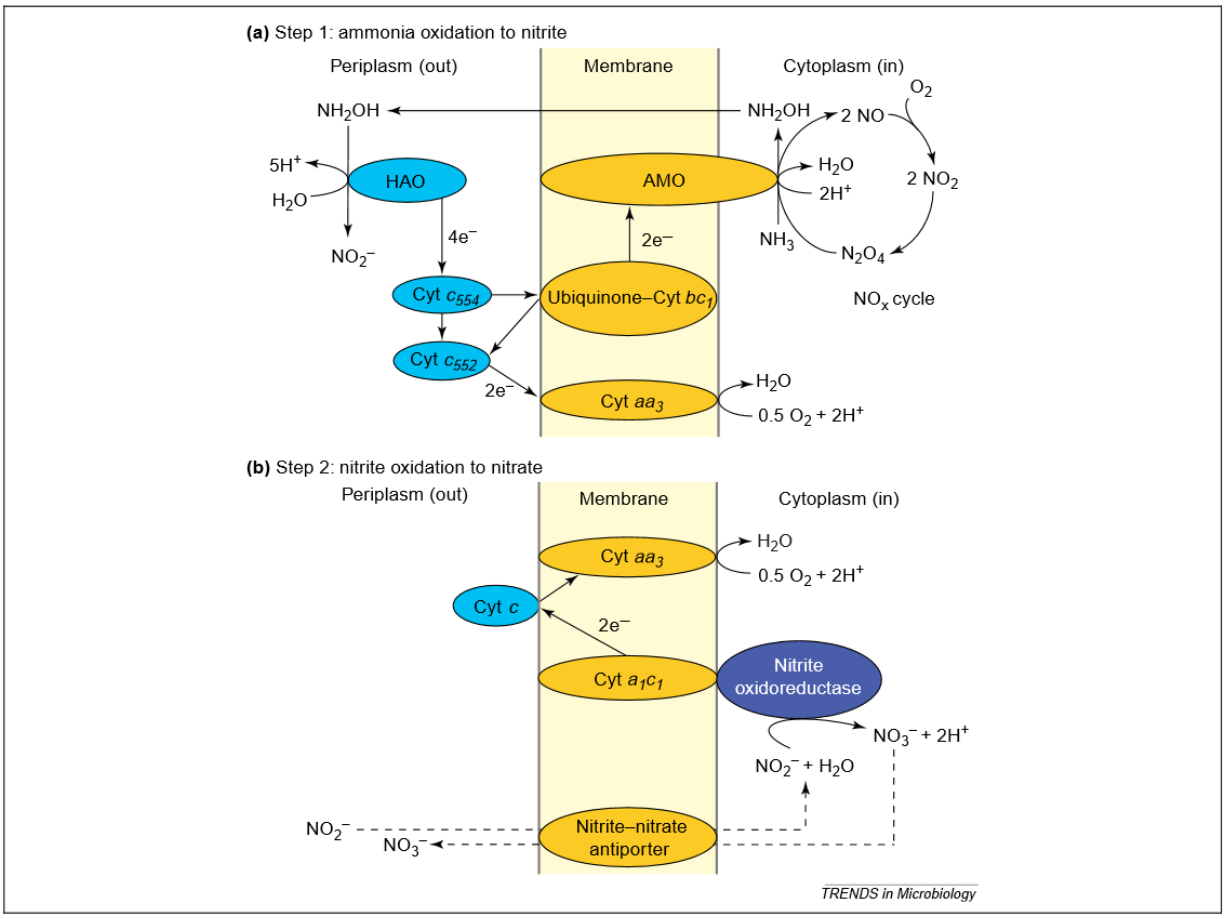


Figure 3: A simplified illustration of the two steps in nitrification from a model AOB (N*itrosomonas)* and NOB (*Nitrobactor*. (Costa et al., 2006). It shows how the NO_x cycle is catalyzed by the membrane bound enzyme ammonia monooxygenase (AMO).

The energy yield from nitrification is fairly low compared to oxidation of sugars that happens in most heterotrophic bacteria. This makes nitrifying bacteria grow very slowly and harder to isolate, but also quite a hassle to work with.

The nitrifying community on bio filters is mainly categorized by AOB and NOB, all though in recent years, ammonia oxidizing archaea (AOA) have also been reported to have a significant role in the nitrifying community (Bollmann et al., 2011). Whether it is AOB or AOA that dominate the biofilters in RAS seems to differentiate depending on where different studies are done (Foesel et al., 2008, Sakami et al., 2012, Bollmann and Laanbroek, 2002). Many cases do point out that it is often within the *Nitrosomonas* and *Nitrospira* linage that dominate the bacterial ammonia oxidizers in marine RAS. In nature the *Nitrospira* linage has proven to be most common in marine system(Ward, 2005) where *Nitrosomonas* dominate most estuaries and are often associated with higher ammonia concentrations (Bollmann and Laanbroek, 2002). The present study will only focus on AOBs.

The phylogenetic relationship between ammonia oxidizing bacteria (*Nitrosospira* lineage and *Nitrosomonas* lineage), has been extensively studied, by the use of 16S rRNA PCR (Koops et al., 1991, Head et al., 1993) and by amoA microarray (Purkhold et al., 2003, Abell et al., 2012). The 16S rRNA is highly conserved in most lineages, since mutations in the rRNA will induce changes in all proteins synthesized. This also means that horizontal gene transfer is unlikely to happen. Because of rRNA evolutionary stable nature different species can be catalogued through this. But since rRNA is not an enzyme, it does not show the impact of the environment on the microorganisms and does not show selective pressure (Ward, 2005). Synthesis of active enzymes, like ammonia monooxygenase enzyme (amoA), generally has a higher rate of mutations and isoforms are more common. amoA is more likely to depict in which environment the bacteria thrives and thereby diversity within different lineages (O'Mullan and Ward, 2005). It has also been shown that amoA mRNA may function as a biomarker of nitrification in wastewater management (Aoi et al., 2004).

Since AOBs are very slow growing, fast growing heterotrophic bacteria will quickly outcompete them, in means of space and available oxygen, if available organic nutrients are present. Heterotrophic bacteria will colonize existing biofilm, gradually taking over, thereby strangling the original nitrifying biofilm. This is believed to have a negative impact on the bio filters nitrifying capability (Michaud et al., 2006).

1.3 Heterotrophic bacteria in recirculation aqua system:

The main focus on heterotrophic bacteria in recirculation aquaculture systems has only been on pathogenic bacteria since these affects the direct health of the fish. The non pathogenic community

has therefore never been studied in full. The heterotrophic bacteria can consist of more than 80% of the total bacterial population on bio filters (Hovanec and DeLong, 1996). Heterotrophic bacteria can however have an indirect negative effect on the fish population. As mentioned earlier undigested food pellets as well as fecal matter will be converted to ammonium. A constant problem in most RAS is heterotrophic growth on the nitrifying biofilm, which leads to a decrease in ammonia oxidation. UV radiation of water entering the bio filtration chambers decreases heterotrophic growth but despite these efforts heterotrophic growth is still observed in bio filtration (Elasri and Miller, 1999). When heterotrophic bacteria invade existing nitrifying biofilm, competition for dissolved oxygen will intensify. The slow growing nitrifies will most likely be outcompeted by faster growing heterotrophs. Access to the bulk water will also be limited, decreasing the access to dissolved ammonia. A RAS with completely overgrown bio filters will be in serious threat of reaching toxic levels of TAN (Michaud et al., 2006).

1.4 Objective:

RAS depends on bacteria naturally present in the water to form nitrifying biofilm. Two biofilm are never the same and the phylogenic relationship in a biofilm is often very different. This is partly due to the dynamic nature of a biofilm. Very little is known on the actual development of a biofilm, and how competing bacteria may influence the development and maintenance in a biofilm. Some strains such as *Nitrosomonas* and *Nitrobactor* have been suggested to be the dominant nitrifiers in RAS bio-filtration, but this again varies between studies. Observations from many different RAS describe that bio filters will eventually become polluted and colonized by other heterotrophic bacteria, even though the water is passed through UV-radiation. A detailed study on how the development of a nitrifying biofilm is influenced by the heterotrophs is lacking. In this study the focus will be on the development of a nitrifying biofilm and how heterotrophic interaction might disrupt nitrification, and outcompete the nitrifiers in a simulated environment. The following questions will be sought to be answered:

- How will heterotrophic bacteria influence the development of nitrifying biofilm?

- How will co-existence with heterotrophic bacteria influence the rate of nitrification and the expression of the amoA gene in a nitrifying biofilm?

- Will pre-existing nitrifying biofilm increase the rate by which heterotrophic bacteria will form biofilm?

2 Materials and Methods

The inhibition of nitrification in RAS can be a serious problem. Observations suggest this is because of heterotrophic bacteria growing on the bio filters. In order to examine this problem an experiment to determine how nitrifying biofilm is affected by the presence of biofilm producing heterotrophic bacteria. *Nitrosomonas marina* sp. 22 was used as a model nitrifying bacteria and *Shewanella japonica* was used as a model heterotrophic bacteria. The difference in ammonium oxidation will be measured as well as the expression of the amoA gene by the use of qPCR and if a pre-formed biofilm will affect biofilm production of a heterotrophic bacterium. This chapter will describe how different experimental procedures were designed and executed.

2.1 Strains, enrichment and storage:

2.1.1 Bacterial strains:

Bacterial strains were chosen in order to model the nitrifying community and the heterotrophic community observed in RAS. *Nitrosomonas marina sp.* 22 (*N. marina sp. 22*) was chosen as a model ammonia oxidizing bacteria due to its frequent association with marine biofilm. The strain was kindly supplied by Professor A. Pommerening-Röser from University of Hamburg, faculty of biology. *Shewanella japonica* (*S. japonica*) was used as a model aerobic heterotrophic bacterium due to its biofilm forming capability. *S. japonica* was kindly supplied by PhD-student Agnete Krabbe Katholm from Roskilde University, Department of Environmental, Social and Spatial Change (ENSPAC).

2.1.2 Storage and enrichment of strains:

S. japonica and *N. marina sp. 22* had to be stored in different ways. *N. marina* sp 22 was not suited for freeze storage as with most bacteria and had to be stored as a continuous batch culture. *S. japonica* was able to be stored as a freeze culture and inoculated when it was to be used.

Medium for enrichment of marine litoautotrophic ammonia-oxidizing bacteria was mixed as described by Koops et al. (2006).

Growth media for litoautotrophic ammonia oxidizers was made by mixing artificial seawater with different minerals in order to obtain optimal growth conditions for marine nitrifiers (table 1).

Table 1: Ingredients list for marine ammonium media. All media was adjusted to pH 8 with NaOH before it was auto-claved. Phenol red acts as an indicator if ammonium oxidation by turning the media yellow when below pH 7. The mixed and ready to use media was stored at 5˚ C.

Seawater 40 ‰	1000 ml
$(NH_4)_2SO_4$	1320 mg/l
$MgSO_4 \cdot 7H_2O$	200 mg/l
$CaCl_2 \cdot 2H_2O$	20 mg/l
K_2HPO_4	114 mg/l
Chelated iron (13% Geigy Chemical)	1 mg/l
$Na_2MoO_4 \cdot 2H_2O$	1 µg/l
$MnCL_2 \cdot 4H_2O$	2 µg/l
$COCl_2 \cdot 4H_2O$	2 µg/l
$CuSO_4 \cdot 5H_2O$	20 µg/l
$ZnSO_4 \cdot 7H_2O$	100 µg/l
Phenol red (0,05%)	1 ml/l

After mixing all the ingredients the pH was adjusted to pH 8 with NaOH. Phenol red was added to indicate growth. When ammonium is oxidized to nitrite the pH value will decrease due to the acidic effect of nitrite. Phenol red turns the media yellow when pH drops below pH 7 thereby indicating ammonium oxidation. All media was auto-claved and stored at 5˚ C.

Batch cultures were made up of 8 Erlenmeyer flasks with 200 ml of ammonium media at 28˚ C. Every 14th day each batch culture was resuspended in 200 ml fresh media. For each flask resuspension was done by centrifugation of 4x50 ml batch culture at 4700 g for 15 minutes. The pellets were then transferred to new Erlenmeyer flasks with 200 ml of fresh ammonium media.

Samples of *S. japonica* were plated on Difco™ Marine Broth 2216 agar plates and incubated at room temperature (20-24 ˚ C) overnight. A colony from the agar-plate was then inoculated in 5 ml marine broth media in a shaking bath at 28˚ C overnight. 500 µl overnight culture and 500 µl 50% glycerol solution was transferred to a cryotube and mixed by vortexing. The cryotube was transferred to a -80˚ C freezer for storage.

2.2 Doubling time of *S. japonica.*

A sample of the freeze culture was platted on a Difco™ Marine Broth 2216 agar plate and incubated overnight at room temperature. A colony from the agar plate was selected and inoculated in 5 ml marine broth in a shacking bath at 28˚ C overnight. The overnight culture was then diluted

1/50 and 1 ml of the dilution was transferred to 100 ml marine broth in an Erlenmeyer flask in a shacking bath at 28˚ C. Samples were taken out and measured at OD_{600} every half hour for a total of 7 hours.

The doubling time was calculated for an exponential function as $T_2 = \frac{Log(2)}{Log(a)}$ estimated from the mean values.

2.3 Effect of existing biofilm on colonization by *S. japonica*

To answer how preformed biofilm might affect the colonization by heterotrophic bacteria an experiment designed to investigate how different coated surfaces might influence the exponential growth rate of a biofilm. A coating of living biofilm from *N. marina* sp. 22 would represent active nitrifying biofilm. To test if it might be the structural matrix of a film that makes it easier for planktonic bacteria to colonize, bovine serum albumin (BSA) and gelatin was chosen to mimic the rugged surface of a biofilm. The experiment would be made by three different setups. In setup 1 the wells were coated with a biofilm from *N. marina sp. 22*. In setup 2 and 3 synthetic biofilm was used made from BSA or gelatin. Each setups would be made up of an optically clear flat-bottom 96-well plate (fig 4) divided into a positive or negative group with 4 replicates in each. The positive groups would be coated with a biofilm and the negative groups would not be coated.

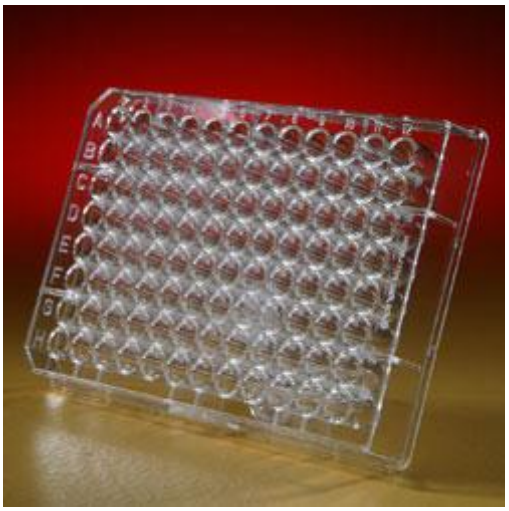


Figure 4: Picture of an optically clear flat-bottom 96-well plate used in the experiments.

Rows A,B,C and D in the optically clear flat-bottom 96-well plate were the negative groups and was not coated with biofilm in any of the setups. Rows E, F, G and H were the positive groups and were coated with different biofilm depending on the setup (fig. 5, fig. 6, fig. 7). Setup 1 contained

pre-formed biofilm from *N. marina* sp. 22. Cells were harvested from 50 ml batch culture. The bacteria were transferred to 4.5 ml of ammonium media. A volume of 90 µl sterile ammonium media was transferred to all wells in row A, B, C and D. A volume of 90 µl of the suspended bacteria was transferred to each well in row E, F, G and H. The plate was incubated at 28˚ C for 7 days (fig 5).

	1	2	3	4	5	6	7	8	9	10	11	12
A	÷	÷	÷	÷	÷	÷	÷	÷	÷	÷	÷	÷
B	÷	÷	÷	÷	÷	÷	÷	÷	÷	÷	÷	÷
C	÷	÷	÷	÷	÷	÷	÷	÷	÷	÷	÷	÷
D	÷	÷	÷	÷	÷	÷	÷	÷	÷	÷	÷	÷
E	Nm22	Nm22	Nm22	Nm22	Nm22	Nm22	Nm22	Nm22	Nm22	Nm22	Nm22	Nm22
F	Nm22	Nm22	Nm22	Nm22	Nm22	Nm22	Nm22	Nm22	Nm22	Nm22	Nm22	Nm22
G	Nm22	Nm22	Nm22	Nm22	Nm22	Nm22	Nm22	Nm22	Nm22	Nm22	Nm22	Nm22
H	Nm22	Nm22	Nm22	Nm22	Nm22	Nm22	Nm22	Nm22	Nm22	Nm22	Nm22	Nm22

Figure 5: An illustration the optically clear flat-bottom 96-well plate used in setup 1. The ÷ indicates wells where no biofilm was present at the start of the experiment. Nm22 indicates preformed-biofilm formed by N. marina sp. 22 incubated for 7 days at 28˚ C.

In setup 2. and 3. the wells were coated with a synthetic film mimicking the structure of a biofilm. This was done by coating the wells with 90 µl of a 1% solution of either BSA (fig. 6) or gelatin (fig. 7). Each plate was incubated at room temperature for 2 hours. As with setup 1 only row E,F,G and H were coated with a film of either BSA or gelatin (fig. 6, fig. 7).

	1	2	3	4	5	6	7	8	9	10	11	12
A	÷	÷	÷	÷	÷	÷	÷	÷	÷	÷	÷	÷
B	÷	÷	÷	÷	÷	÷	÷	÷	÷	÷	÷	÷
C	÷	÷	÷	÷	÷	÷	÷	÷	÷	÷	÷	÷
D	÷	÷	÷	÷	÷	÷	÷	÷	÷	÷	÷	÷
E	BSA	BSA	BSA	BSA	BSA	BSA	BSA	BSA	BSA	BSA	BSA	BSA
F	BSA	BSA	BSA	BSA	BSA	BSA	BSA	BSA	BSA	BSA	BSA	BSA
G	BSA	BSA	BSA	BSA	BSA	BSA	BSA	BSA	BSA	BSA	BSA	BSA
H	BSA	BSA	BSA	BSA	BSA	BSA	BSA	BSA	BSA	BSA	BSA	BSA

Figure 6: An illustration of an optically clear flat-bottom 96-well plate used in setup 2. The ÷ indicates wells where no biofilm was present at the start of the experiment. BSA indicates wells coated with a synthetic film formed by a 2 hour incubation of a 1% bovine albumin serum solution.

	1	2	3	4	5	6	7	8	9	10	11	12
A	÷	÷	÷	÷	÷	÷	÷	÷	÷	÷	÷	÷
B	÷	÷	÷	÷	÷	÷	÷	÷	÷	÷	÷	÷
C	÷	÷	÷	÷	÷	÷	÷	÷	÷	÷	÷	÷
D	÷	÷	÷	÷	÷	÷	÷	÷	÷	÷	÷	÷
E	Gelatin	Gelatin	Gelatin	Gelatin	Gelatin	Gelatin	Gelatin	Gelatin	Gelatin	Gelatin	Gelatin	Gelatin
F	Gelatin	Gelatin	Gelatin	Gelatin	Gelatin	Gelatin	Gelatin	Gelatin	Gelatin	Gelatin	Gelatin	Gelatin
G	Gelatin	Gelatin	Gelatin	Gelatin	Gelatin	Gelatin	Gelatin	Gelatin	Gelatin	Gelatin	Gelatin	Gelatin
H	Gelatin	Gelatin	Gelatin	Gelatin	Gelatin	Gelatin	Gelatin	Gelatin	Gelatin	Gelatin	Gelatin	Gelatin

Figure 7: An illustration of an optically clear flat-bottom 96-well plate used in setup 3. The ÷ indicates wells where no biofilm was present at the start of the experiment. Gelatin indicates wells coated with a synthetic film formed by a 2 hour incubation of a 1% gelatin solution.

For each of the setup an overnight culture of *S. japonica* was diluted 1/100 in Difco™ Marine Broth 2216 media and 90 µl of the solution was added to column 2-12. Sterile Difco™ Marine Broth 2216 media was added to column 1 which acted as a negative control. The negative controls indicate where no biofilm is present and would measure the absorbance if the plastic. Sj indicate where only *S. japonica* is present. Mix indicates where the preformed-biofilm from *N. marina sp* 22, BSA or gelatin was present together with *S. japonica* (fig 8).

	1	2	3	4	5	6	7	8	9	10	11	12
A	Negative control	Sj	Sj	Sj	Sj	Sj	Sj	Sj	Sj	Sj	Sj	Sj
B	Negative control	Sj	Sj	Sj	Sj	Sj	Sj	Sj	Sj	Sj	Sj	Sj
C	Negative control	Sj	Sj	Sj	Sj	Sj	Sj	Sj	Sj	Sj	Sj	Sj
D	Negative control	Sj	Sj	Sj	Sj	Sj	Sj	Sj	Sj	Sj	Sj	Sj
E	Preformed biofilm	**Mix**	**Mix**	**Mix**	**Mix**	**Mix**	**Mix**	**Mix**	**Mix**	**Mix**	**Mix**	**Mix**
F	Preformed biofilm	**Mix**	**Mix**	**Mix**	**Mix**	**Mix**	**Mix**	**Mix**	**Mix**	**Mix**	**Mix**	**Mix**
G	Preformed biofilm	**Mix**	**Mix**	**Mix**	**Mix**	**Mix**	**Mix**	**Mix**	**Mix**	**Mix**	**Mix**	**Mix**
H	Preformed biofilm	**Mix**	**Mix**	**Mix**	**Mix**	**Mix**	**Mix**	**Mix**	**Mix**	**Mix**	**Mix**	**Mix**

Figure 8: An illustration of how all of the 3 setups were performed. A volume of 90 µl 1/100 dilution of a *S. japonica* overnight culture was added to each well in column 2-12. Sj indicate where *S. japonica* was incubated without any preformed-biofilm. Mix indicate where *S. japonica* was incubated with preformed-biofilm of either *N. marina sp* 22, BSA or gelatin. *S. japonica* was not added to any wells in column 1. Column 1 would act as a negative control.

After one hour incubation at 28˚ C the medium was removed from column 2 and all wells were colored with 100 µl 0.1% crystal violet for ten minutes. All wells in column 2 were gently washed 5 times with milli-Q water to ensure only crystal violet bound to biofilm was left. This procedure was repeated for all columns with a 30 minutes interval. At the very last column 1 was emptied and colored as described for the other columns. After all wells had been colored, 200 µl of 96% ethanol was added to all wells and incubated for 10 minutes. Then 125 µl was added from each well to a corresponding well in a new optically clear flat-bottom 96-well plate. Readings were performed by

a Bio-Tek Synergy HT at OD_{600}. The exponential growth expressed as $f(t) = f(t_0)\cdot(1+r)^t$, where r is the exponential growth rate, would be used as a rate of biofilm production. The negative controls and preformed biofilm indicate the background absorbance and would be constant throughout the experiment. Only the growth of *S. japonica* was of interest and the background absorbance would interfere with this. In order to avoid this the OD_{600} value of each negative control in column 1 was subtracted from all wells in its corresponding row thereby only the growth of *S. japonica* would be expressed.

2.4 Nitrification development

In order to describe how nitrification is developed as seen in many RAS an experiment with the purpose to describe ammonium oxidation of AOB was designed. This would be done by comparing the rate of ammonium depletion and the increase of nitrite. Also by introducing heterotrophic bacteria to the system the expected inhibition of nitrification was to be measured as well.

N. marina sp. 22 was used as a model AOB and *S. japonica* was used as a model heterotrophic bacteria.

2.4.1 Recirculating flow system setup

Eight glass tanks with 25 L of 0.2 µm filtered autoclaved seawater were setup in random order in a 17˚ C walk in climate room. All tanks were supplied with filtered air from sandstone bubbling. One hundred floating bio filter tablets of the brand RK BioElements were autoclaved and added to each tank increasing the surface area with 0,2941 m^2. The start concentration was set to be 2.0 mg/L TAN by adding 0.14873g NH_4Cl (eq. 7)

(eq. 7) $$n = \frac{m}{M}$$

$$\frac{5 \cdot 10^{-3} g}{18 g/mol} = 2{,}78 \cdot 10^{-4} mol$$

$$2{,}78 \cdot 10^{-3} mol \cdot 53{,}5 g/mol = 0{,}14873 g$$

Eight x 50 ml of *N. marina* sp. 22 batch culture were centrifuged at 4700g for 20 min, resuspended in 10 ml filtered seawater and added to each of the tanks.

Four samples of 5ml were taken out from each tank at day 1, 3, 5, 7, 12, 15, 18, 22, 25 and 28. All samples were stored at -20˚ C until.

Before the samples were measured they were defrosted at 22˚ C for 2 hours. Samples were measured for NH_4^+, NO_2 with a Spectroquant® NOVA 60 (Merck Millipore). Measurements of pH were done with a Metronohm pH 827 pH Lab pH meter. Calculation for TAN were done using the Vant Hoff equation to find the equilibrium constant K_{eq} for NH_3/NH_4^+ at 22˚ C .

(8) $\text{Log}\left(\frac{k_1}{k_2}\right) = \frac{-\Delta H^\theta}{R}\left(\frac{1}{T_2} - \frac{1}{T_1}\right) <=> k_1 = e^{\frac{-\Delta H^\theta}{R}\left(\frac{1}{T_2} - \frac{1}{T_1}\right)} \cdot k_2$

With K_{eq} for 22˚ C $[NH_3]$ was isolated from eq. (9)

$[H^+] = 10^{-pH}$

(9) $K_{eq} = \frac{[NH_3][H^+]}{[NH_4^+]}$

to get $[NH_3]$ in mg L^{-1} following calculation was made

(10) $[\text{NH}_3] \cdot \text{M} \cdot 1000 = \text{NH}_3 \text{ mg L}^{-1}$.

The calculated values of NH_3 were added to values of NH_4^+ in order to get TAN

The experiment was repeated 3 times but because of incoherent results the experiment was terminated before any heterotrophic bacteria were introduced.

The experiment was optimized so that it could be easier controlled. Each experimental replica would consist of three Erlenmeyer flasks that would be connected with silicon tubes then pumped through the system disconnected and pumped back into each flask (fig. 9). This would create the necessary flow conditions needed by AOB.

The experiment would be made with two different systems; one containing only *N. marina* sp. 22 and one with both *N. marina* sp. 22 and *S. japonica*. By comparing the expression of the amoA gene by qPCR the ammonium oxidation would be described. For each experiment three replicates were done. The rate of ammonium oxidation and the expression of rRNA and amoA would then be compared.

From observations on the batch cultures it had been observed that *N. marina* sp. 22 primarily existed in biofilm formed on the bottom of the flasks and in very small concentrations. Samples were very difficult to obtain without harvesting cells from the entire flasks. The experimental setup

had to consist of flasks that could be removed completely for each sample taken. To do this the experiment had to contain a flask for each planned sample that would then be connected. This ensured that an entire flask could be taken out as a sample without altering the total concentration of bacteria or TAN in the system.

For each experiment three Erlenmeyer flasks containing 150 ml of minimum marine media with the carbon to nitrogen ratio of 1 (table 2) were pumped by a peristaltic pump at a rate of 0,1 ml/min. The tubes were then connected and run through a bubble trap and then on through a flow channel chamber. The tube would then separate into 4 tubes and transported back into the 4 Erlenmeyer flasks (fig 9).

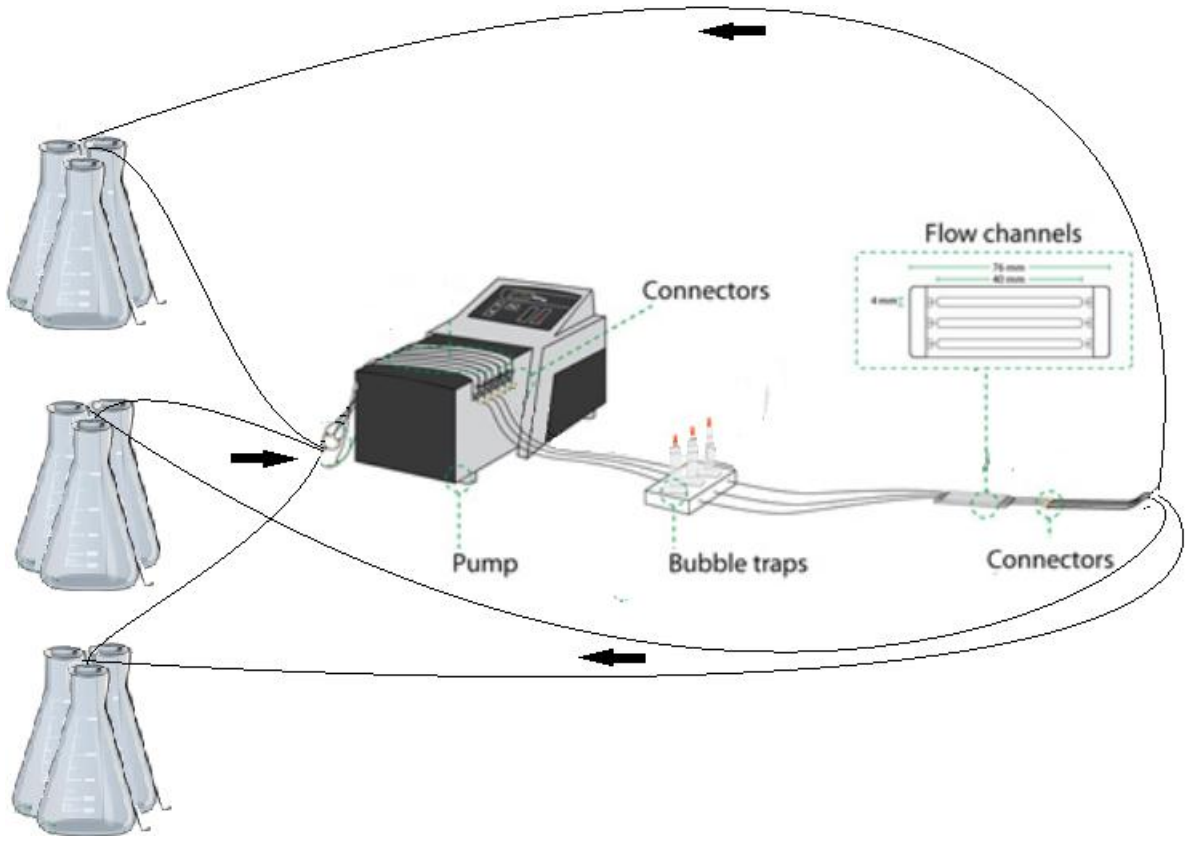


Figure 9: An illustration of one setup. The 4 Erlenmeyer flask are connected and the liquid passed through the peristaltic pump onto the bubble traps. Here potential bubbles will be trapped, and no bubble will pass on to the flow channels. In the flow channels biofilm can be viewed in a microscope. The tubes are then led back and split into 4 tubes which are then led back into the Erlenmeyer flasks.

Samples were harvested by scraping the bottoms with a plastic scraper. The 150 ml was then divided into three 50 ml tubes. Bacterial samples were harvested by centrifuge at 4700g for 20 min.

Measurements of NH_4^+ and NO_2 were done on the supernatants using a Spectroquant® NOVA 60 (Merck Millipore). pH measurements were done on a Metronohm pH 827 ph Lab pH meter.

Table 2: Marine minimum media used for nitrogen inhibition experiment. Ratio of $(NH_4)SO_4$ and $C_{12}H_{22}O_{11}$ was modified from (Koops et al., 2006)in order to get the ratio Carbon/Nitrogen=1. Calculation of C/N=1 was done as described in (Zhu and Chen, 2001)

Seawater 40 ‰	1000 (ml)
NH_4Cl	6 (µg/l)
$C_{12}H_{22}O_{11}$	4 (µg/l)
$MgSO_4 \cdot 7H_2O$	200 (mg/l)
$CaCl_2 \cdot 2H_2O$	20 (mg/l)
K_2HPO_4	114 (mg/l)
Chelated iron (13% Geigy Chemical)	1 (mg/l)
$Na_2MoO_4 \cdot 2H_2O$	1 (µg/l)
$MnCL_2 \cdot 4H_2O$	2 (µg/l)
$COCl_2 \cdot 4H_2O$	2 (µg/l)
$CuSO_4 \cdot 5H_2O$	20 (µg/l)
$ZnSO_4 \cdot 7H_2O$	100 (µg/l)
Phenol red (0,5%)	1 (ml/l)

2.4.2 RNA isolation

RNA is very sensitive and unstable compared to DNA. Samples are easily contaminated if not treated correctly. Because of this all equipment used for RNA isolation was treated with Sigma RNaseZAP and the isolation was done under a fume hood

Because of the relatively low amount of *N. marina sp*. 22 cells harvested from each sample. This protocol was adapted from the original TRI reagent protocol from Sigma Aldrich to insure a higher output of RNA. This was done by increasing time of centrifugation and by modifying the wash with 75% ethanol.

50 ml samples were centrifuged for 20 minutes at 4700 rpm. Each pellet was collected and resuspended in a 1.5 ml sterile Eppendorf tube with 500 µl TRI reagent and vortexed for 15 seconds. The homogenate of each sample was centrifuged at 12.000g for 10 min at 4˚ C. The supernatant from each sample was transferred to new sterile Eppendorf tubes and left at room temperature for 10 min. 200 µl of chloroform was added to the Eppendorf tubes and they were each vortexed for 15 seconds and incubated for 15 min at room temperature. Each tube was then centrifuged at 12.000g for 25 min at 4˚ C. The samples would then separate into three phases. The bottom phase called the organic phase containing lipids and proteins, the interphase containing

DNA and the aqueous phase containing RNA. The aqueous phase was transferred to new sterile Eppendorf tubes and 500 µl isopropanol was added to each. The samples were incubated for 10 min at room temperature and centrifuged at 12.000g for 20 min at 4˚ C. The supernatant was removed from each sample and the pellets were washed by storing them in 500 µl 75% ethanol at -20˚ C overnight. The samples were then vortexed and centrifuged at 7.500 g for 5 min at 4˚ C. The supernatant was removed from each sample and the eppendorf tubes were air dried for 10 min in a fume hood without drying them completely since this would decrease solubility. The pellets were then resuspended in 30 µl RNAase free H_2O. 1 µl of each sample was measured for concentration of RNA on a NanoDrop ND-1000 at 260 nm. Ratio of 260/280 nm did not exceed 1,7. This insured that there was no DNA present.

2.4.3 cDNA synthesis

For cDNA synthesis First strand cDNA synthesis kit from Sigma-Aldrich was used.

All samples and other components were stored on ice at all times.

The following description is for one sample

0,5-1 µg of RNA (volume depends on the concentration of RNA for the specific sample) was added to RNAase free H_2O, and 1 µl of random hexamer primer to a total volume of 11 µl.

4 µl 5x Reaction Buffer, 1 µl RiboLock RNase Inhibitor (20 u/µl), 2 µl 10mM dNTP Mix, 2 µl M-MuLV Reverse Transcriptase (20 u/µl) are mixed and added to the RNA. To a total volume of 20 µl

The sample was then placed in a T3000 Thermocycler with the following program

25˚ C	5 min	Initiation of primer
42˚ C	60 min	cDNA synthesis
70˚ C	5 min	Termination of reaction
4˚ C	∞ min	Storage

The finished cDNA was stored at -20˚ C

2.4.4 quantitative real time PCR (qPCR)

To describe in what magnitude different genes are expressed specific primers designed from the target gene are run in PCR. The general idea of how qPCR works is by having a fluorescent dye included in the PCR reaction. The PCR product formed from each cycle of the reaction is then able to be measured. As the reaction proceeds through each cycle, more and more product is formed making the fluorescent signal stronger. The fluorescent dye SYBR green binds to the minor groove of double stranded DNA and fluoresces 1000 times brighter when bound than when unbound. As the PCR generates more double stranded product the SYBR Green signal increases.

The primers used in the present study would comprise of a primer targeting the cDNA from 16S rRNA of *S. japonica*, a primer targeting the cDNA from 16S rRNA of *N. marina* sp. 22 and a primer targeting the amoA cDNA of *N. marina* sp. 22. qPCR run on 16S rRNA would give a reading of how strong a presence *N. marina* sp. 22 or *S. japonica* has. The primer targeting the amoA gen was to determine how intense ammonia nitrifying was happening in *N. marina* sp. 22.

Primers:

Target gene	primer melting point		primers
N. marina sp. 22 amoA	60.25° C 59.90° C	fw rv	5′- TGCTGTTGACGGGTAACTGG -3′ 5′- TTTCTGCGAGATACGGCCTC -3′
N. marina sp. 22 16-S rDNA	60.03° C 59.20° C	fw rv	5′- AGCAGGGGATCGTAAGACCT -3′ 5′- TTTCTGCGAGATACGGCCTC -3′
S. japonica 16-S rDNA	60.53° C 60.18° C	fw rv	5′- CTTACCAAGGCGACGATCCC -3′ 5′- CGTACGCTTTACGCCCAGTA -3′

Primers were designed using the primer design tool, Primer-Blast from NCBI.com

Each primer was optimized in order to find the optimal temperature for primer melting points.

A standard curve for each primer was made with the dilution factors described in table 2. Duplicate analysis was done for each sample.

Table 3: Dilution factors of cDNA used for the qPCR standard curves.

Nm 22 rDNA	1/1	1/5	1/10	1/15	1/20	1/25	0(Control)
Nm 22 amoA	1/1	1/5	1/10	1/15	1/20	1/25	0(Control)
Sj rRNA	1/10	1/15	1/20	1/25	1/30	1/35	0(Control)

A mastermix was made by mixing 5 µl SYBR-green/ROX qPCR Master Mix (2X), 1 µl Forward primer, 1 µl Reverse primer, 2 µl nuclease-free H_2O for each sample.

Each sample was added 9 µl and 1 µl cDNA template. The controls were added 1 µl DNase free H_2O. Total volume for each sample was 10 µl. All samples were transferred to a 20 µl lightcycler capillary. Each capillary was placed in a 4˚ C aluminum lightcycler centrifuge adapter and centrifuged for 5 sec at 1000g before being placed in a Roche Lightcycler 2.0 for qPCR.

The Roche Lightcycler 2.0 was programmed to use the following programs:

Denaturation/Activation	**95˚**	**10 min**
Amplification 45 cycles PCR	**95˚**	**15 sec**
	64˚	**20 sec**
	72˚	**20 sec**
Melting curves	**95˚**	**15 sec**
	60˚	**20 sec**
	95˚	**0 sec**
Cooling/End	**40˚**	∞

2.5 Live/dead staining

In order to visualize how biofilm from *N. marina sp 22* is affected by *S. japonica* a live/dead staining was done on biofilm formed in batch cultures containing *N. marina sp 22* and on biofilm from a batch culture containing *N. marina* and *S. japonica*. The LIVE/DEAD® BacLight ™ Bacterial Viability Kit, L70 2, was applied to both samples to visualize how *S. japonica* may have strangled N. marina sp 22. The kit contains two dyes. SYTO®9 green-fluorescent nucleic acid strain and the red -flourescent nucleic acid strain, propidium iodide. They differ in both their spectral characteristics and in their ability to penetrate the membranes of healthy cells. SYTO®9 is able to penetrate the membrane of living bacteria and bacteria with damaged membranes. Propidium iodide is only able to penetrate damaged membranes. In healthy cells only SYTO®9 will be present giving the healthy cells a green flourescnence. In cells with damaged membranes both SYTO®9 and propidium iodide will be present. When both dyes are present red fluorescence from propium iodide overpowers the green fluorescence from SYTO®9 giving damaged cells a red glow. This means that

when the dyes are used in conjunction healthy living cells will grow green and damaged seemingly dead cells will glow red.

2 x 200 ml of *N. marina* sp. 22 batch culture was centrifuged at 4700g for 15 min. The pellets were resuspended in 200 ml fresh ammonium media. One was inoculated with a 1/100 diluted *S. japonica* overnight culture at 28˚ C for 24 hours. Both were then centrifuged at 4700g for 15 min and the pellets were transferred to 100 µl 0,9% ice cold NaCl to remove interfering media components. 1 µl of staining solution was added to both samples and incubated for 15 min in the dark. Both samples were then loaded onto agarose slips on a microscope slide. Microscope slides were prepared with 1% agarose solution. The staining solution was prepared by adding 5 µl of 3.34 mM SYTO®9 dye in DMSO (Compound A) and 5 µl of 20 mM prodpidium iodide in DMSO (Compound B) to 20 µl sterile water. Samples were inspected using a Leica DM5000B microscope with a mercury lamp. A filter cube with excitation filters 436, 535 and 630 nm was used to be able to detect healthy and damaged bacteria.

3 Results

3.1 Nitrification development

The ammonium oxidation was monitored by measuring the NH_4^+ and NO_2 values for 28 days in order to find the rate by which ammonium would be converted to NO_2. As NH_4^+ was converted NO_2 levels should increase in a similar fashion. In order to describe ammonium oxidation total ammonia nitrogen (TAN) had to be calculated. The mean values ± SD of NH_4^+, TAN, NO_2 and pH are shown in table 4.

Table 4: Data from the Nitrification development experiment. Values for NH_4^+, TAN, NO_2 and pH for the entire experiment (0-28 days) are shown as mean values ± SD. TAN has been calculated from measurements of NH_4^+, temperature and pH.

Time [days]	Mean NH_4^+ [mg L^{-1} ± SD] $t_{0\text{-}28\ d}$	Mean TAN [mg L^{-1} ± SD] $t_{0\text{-}28\ d}$	Mean NO_2 [mg L^{-1} ± SD] $t_{0\text{-}28\ d}$	Mean pH [pH ± SD] $t_{0\text{-}28\ d}$
0	1.9875 ± 0.1458	2.1730 ± 0.1592	0.0088 ± 0.0035	8.02 ±0.07
3	1.6400 ± 0.2922	1.7725 ± 0.3193	0.0175 ± 0.0084	7.95 ±0.09
5	1.3688 ± 0.4987	1.4873 ± 0.5405	0.0300 ±0.0141	7.99 ±0.05
7	1.0575 ± 0.4089	1.1326 ± 0.4338	0.0313 ±0.0125	7.91 ±0.10
12	0.8887 ± 0.3334	0.9644 ± 0.3625	0.0338 ±0.0151	7.98 ±0.06
15	0.8525 ± 0.3448	0.9205 ± 0.3691	0.0350 ±0.0160	7.96 ±0.05
18	0.4625 ± 0.2386	0.5169 ± 0.2404	0.0350 ±0.0160	7.91 ±0.08
22	0.3775 ± 0.2522	0.4052 ± 0.2681	0.0350 ±0.0160	7.93 ±0.07
25	0.4750 ± 0.2659	0.5098 ± 0.2829	0.0350 ±0.0160	7.92 ±0.08
28	0.0863 ± 0.0741	0.0921 ± 0.0792	0.0350 ±0.0160	7.90 ±0.07

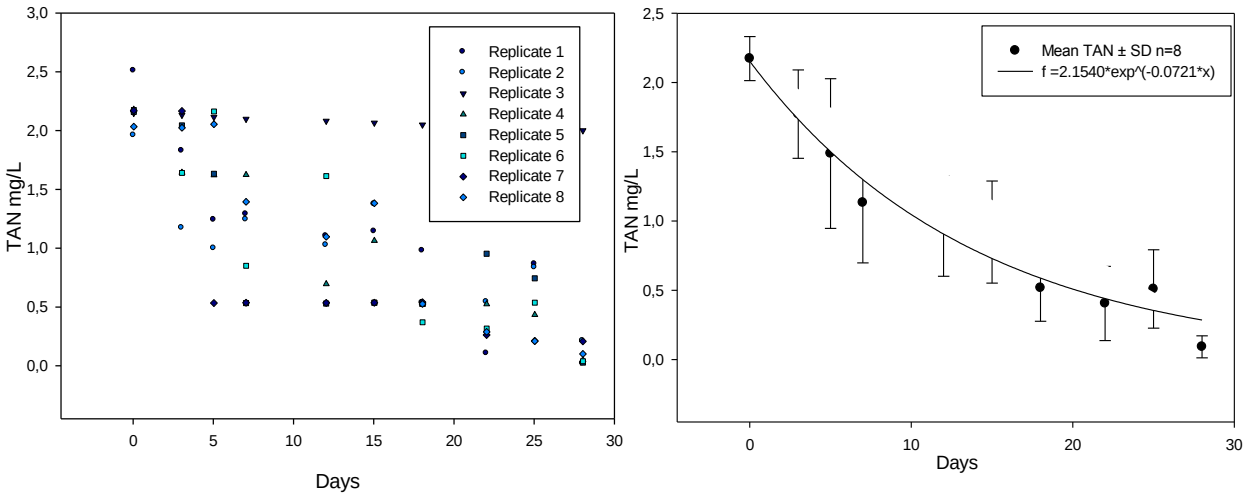


Figure 10: To the left all the calculated TAN values are plotted as a scatter plot for all 8 replicates. To the right the calculated mean value of TAN ± SD (n=8) is plotted

The calculated values of TAN are plotted as a scatter plot and as mean values ± SD (fig. 10). The standard deviations are very large on most samples making it hard to make a definitive description of TAN decay. The plotted mean values did however show an overall trend of exponential decay.

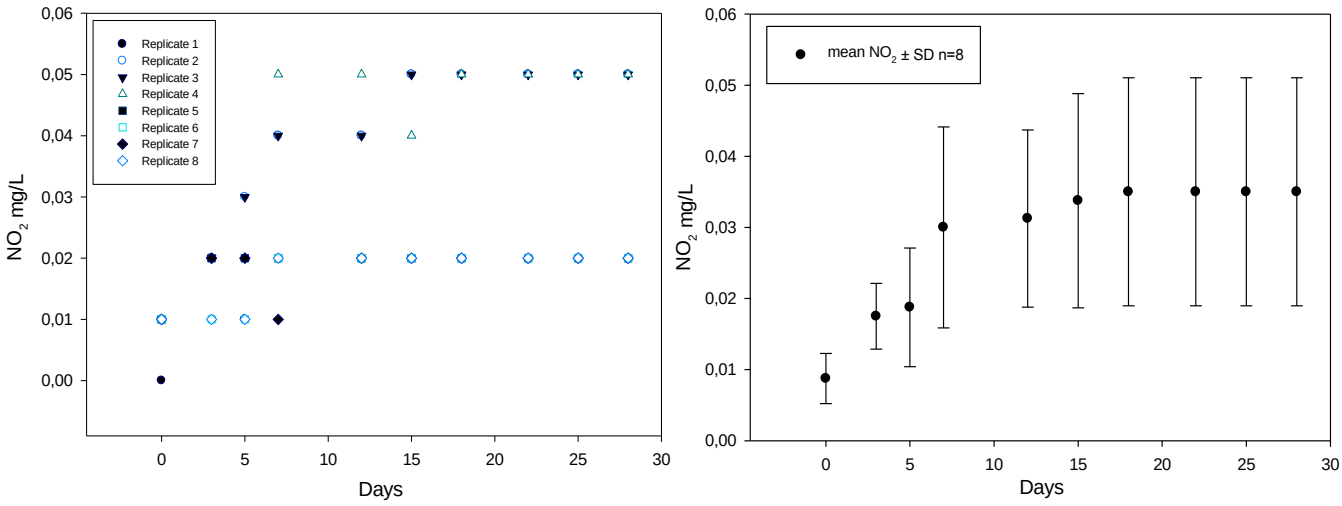


Figure 11: To the left are all measured values for NO_2 plotted as a scatter plot for all 8 replicates. To the right are the mean values ± SD has been plotted.NO_2 was expected to increase proportional with the decrease of TAN but only a small rise was observed se figure 11. The standard deviations of NO_2 are very large and the increase in concentration very small. This might indicate the NO_2 is converted to something else directly after being produced.

3.2 Estimation of *S. japonica* doubling time:

An estimation of *S. japonica*'s doubling time was measured prior to the "Effect of existing biofilm on colonization by *S. japonica*" experiment. This was done as a simple growth experiment measuring OD_{600} as a function of time. Only two replicates where made due to the simplicity of the experiment. Values are displayed in table 5 and plotted as a scatter plot and with mean values ± SD in figure 12.

OD_{600} Measurements were plotted as following

Time [minutes]	Flask 1 $[OD_{600}]t_{0\text{-}300\ min}$	Flask 2 $[OD_{600}]\ t_{0\text{-}300\ min}$	Mean [mean ± SD] $t_{0\text{-}300\ min}$
0	0.012	0.011	0.0115 ± 0.0007
60	0.019	0.018	0.0185 ± 0.0007
120	0.031	0.022	0.0265 ± 0.0064
150	0.052	0.04	0.0460 ± 0.0085
180	0.05	0.043	0.0465 ± 0.0050
210	0.081	0.097	0.0890 ± 0.0113
240	0.104	0.112	0.1080 ± 0.0057
270	0.163	0.173	0.1680 ± 0.0071
300	0.234	0.237	0.2355 ± 0.0021

Table 5: Data from Estimation of *S. japonica* doubling time experiment. Values for NH_4^+, TAN, NO_2 and pH for the entire experiment (0-28 days) are shown as mean values ± SD. TAN has been calculated from measurements of NH_4^+, temperature and pH.

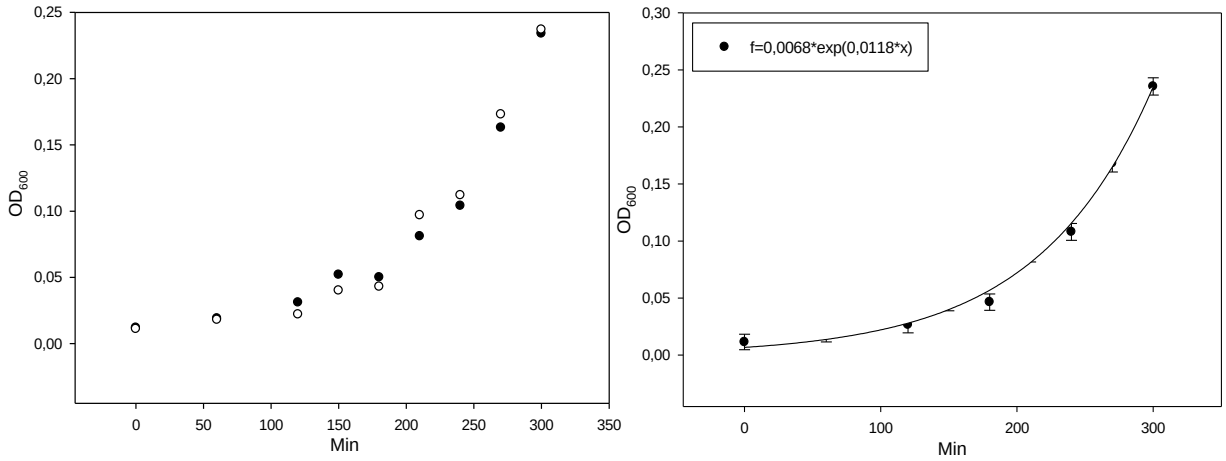


Figure 12: To the left is a scatter plot of flask 1 and flask 2. To the right the mean values ± SD of the experiment has been plotted. From the equation $f(x) = 0.0115 \cdot 1.0118^x$. The doubling time was calculated to be 59 min.

The standard deviations were very small and the plots seemed to represent growth of *S. japonica* very well. From the scatter the doubling time for S. japonica was calculated to be 59 min.

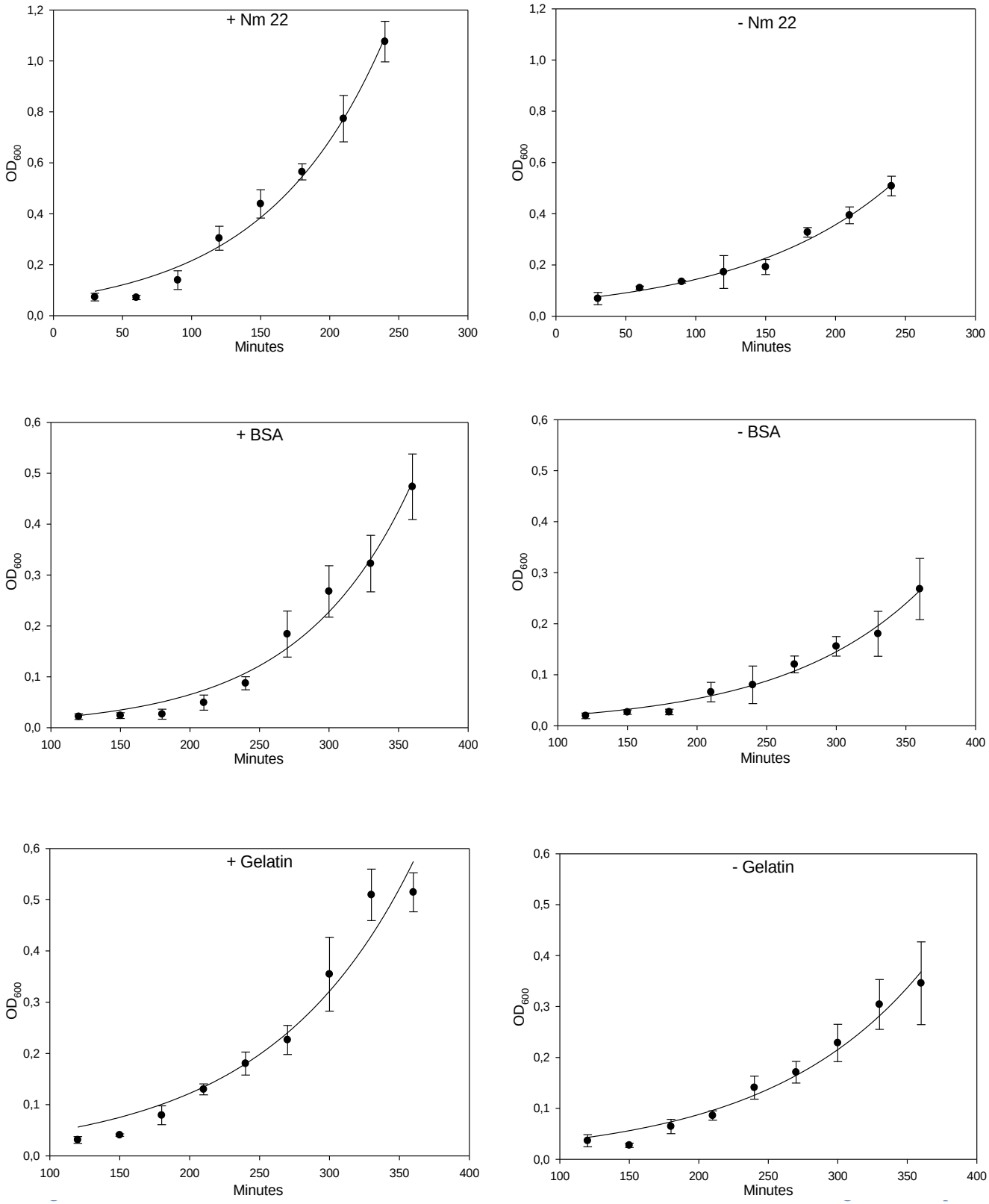
3.3 Effect of existing biofilm on colonization by *S. japonica*

In order to answer if pre-formed biofilm stimulates planktonic bacteria of *S. japonica* to form biofilm, three optically clear 96 well plates were coated with either biofilm from N. marina 22, Bovine serum albumin (BSA) or gelatin. Measurements of absorbance were used to determine the growth of S. japonica biofilm as a function of time (table 6). The experiments were divided into a negative group containing no preformed biofilm and a positive group containing preformed-biofilm. Each experiment had 4 replicates

Table 6: Data from the effect of existing biofilm on colonization by *S. japonica* experiment. Values for +Nm22, -Nm22, +BSA, -BSA, + Gelatin and - Gelatin. Data sets are shown as mean values ± SD. +Nm22 and -Nm22 were measured from 30 min to 240 min. +BSA, -BSA, +Gelatin and - Gelatin were measured at 120 to 360 min.

Time [min]	+ Nm 22 [Mean OD_{600} ± SD] $t_{30\text{-}240min}$	- Nm 22 [Mean OD_{600} ± SD] $t_{30\text{-}240min}$	+ BSA [Mean OD_{600} ± SD] $t_{120\text{-}360min}$	- BSA [Mean OD_{600} ± SD] $t_{120\text{-}360min}$	+ Gelatin [Mean OD_{600} ± SD] $t_{120\text{-}360min}$	- Gelatin [Mean OD_{600} ± SD] $t_{120\text{-}360min}$
30	0.0730± 0.0149	0.0688±0.0239	NA	NA	NA	NA
60	0.0713±0.0084	0.1103±0.0070	NA	NA	NA	NA
90	0.1395±0.0368	0.1345±0.0054	NA	NA	NA	NA
120	0.3040±0.0469	0.1725±0.0639	0.0693 ± 0.0049	0.0195±0.0055	0.0365±0.0119	0.0310±0.0066
150	0.4388±0.0555	0.1923±0.0293	0.0712 ± 0.0058	0.0268±0.0044	0.0275±0.0042	0.0405±0.0024
180	0.5645± 0.0315	0.3273±0.0188	0.0740 ± 0.0099	0.0270±0.0055	0.0645±0.0141	0.0793±0.0184
210	0.7735±0.0911	0.3936±0.0328	0.0968 ± 0.0149	0.0600±0.0190	0.0860±0.0093	0.1298±0.0107
240	1.0760±0.0796	0.5080±0.0385	0.1348 ± 0.0128	0.0802±0.0368	0.1407±0.0227	0.1803±0.0224
270	NA	NA	0.2315 ± 0.0452	0.1203±0.0166	0.1710±0.0212	0.2263±0.0283
300	NA	NA	0.3152 ± 0.0505	0.1558±0.0190	0.2285±0.0367	0.3545±0.0721
330	NA	NA	0.3700 ± 0.0556	0.1803±0.0440	0.3040±0.0489	0.5095±0.0502
360	NA	NA	0.5210 ± 0.0645	0.2680±0.0600	0.3458±0.0813	0.5145±0.0380

+ Nm 22 and -Nm 22 are measured at different times than +BSA, -BSA, +Gelatin and -Gelatin but that should not affect the estimated exponential growth rate. The mean values ± SD were plotted and fitted with an exponential growing function (fig 13). All positive samples are displayed next to their negative sample to easier visualize difference in growth. In order to show if there is any significant difference statistical comparison were done. To determine if preformed biofilm has a significant effect a one-way ANOVA test employing the $P< 0{,}001$ was done testing each of the coatings against an uncoated sample. Before this however a test to determine normality and Equal variance had to be past. If one-way ANOVA test showed statistical significance the Holm-Sidak was used as Post Hoc test.



the left show production on pre-formed biofilm made from *N. marina sp.* 22 and production on synthetic pre-formed biofilm made of bovine serum albumin (BSA) and gelatin. Displayed to the right is biofilm production without any pre-formed biofilm. Plotted mean value +-SD. (n=4)

Each positive group was tested against its negative group. All statistical analysis was done using Sigmaplot 11.0

The different growth rates estimated from mean plots are shown in figure14. A one-way ANOVA and post hoc Holm-Sidal shows a significant difference. It shows that + Nm 22 and + BSA are

significantly different from their counterparts and gelatin is not. This indicates that colonization by *S. japonica* is accelerated when grown on a surface treated with biofilm from *N. marina sp 22* and with *BSA.*

Table 7: Calculated exponential growth rates ± SD from one-way ANOVA test. n=4

+ Nm 22 [Mean exp growth rate ± SD]	- Nm 22 [Mean exp growth rate ± SD]	+ BSA [Mean exp growth rate ± SD]	-BSA [Mean exp growth rate ± SD]	+ Gelatin [Mean exp growth rate ± SD]	- Gelatin [Mean exp growth rate ± SD]
0.0117±0.0006	0.0093±0.0169	0.0128±0.0009	0.0101±0.0003	0.0097±0.0007	0.0096±0.0010

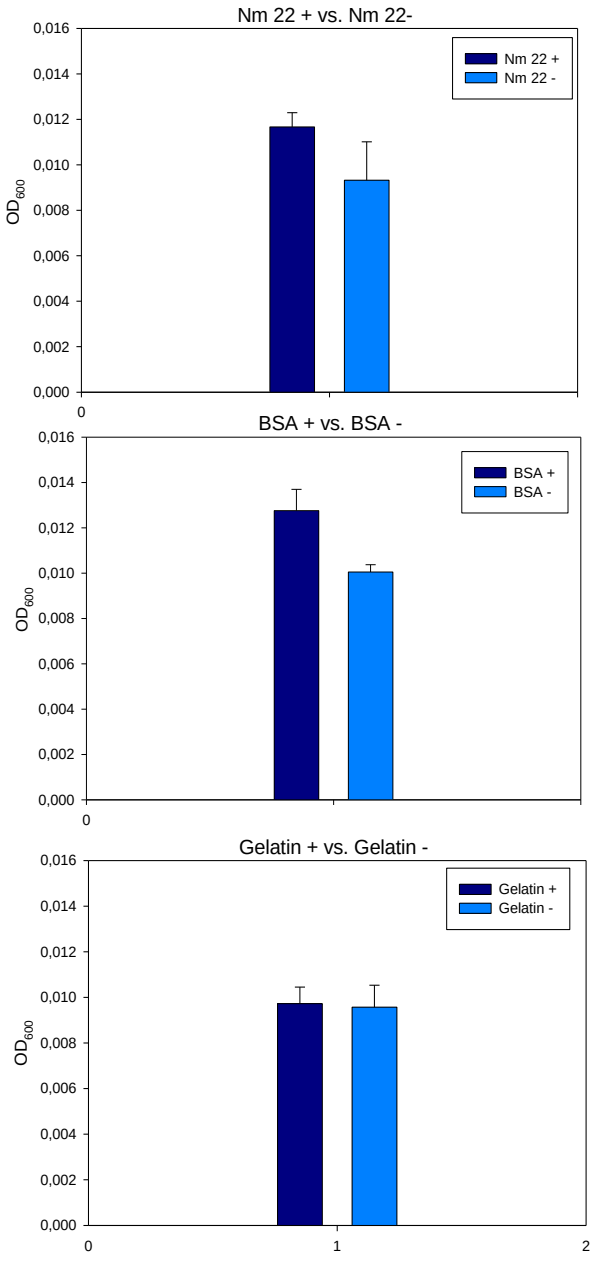


Figure 14: Mean values of growth rates ± SD, n=4. One-way ANOVA test showed a significant difference on growth rate, when incubated with BSA or Nm 22 biofilm.

From figure 14 it can be seen that both +Nm 22 and +BSA has a positive effect on the exponential growth rate of biofilm from *S. japonica* where gelatin does not have any effect. Statistical data for one-way ANOVA test is shown in table 7 confirms that there is a significant difference when a surface is coated with biofilm from *N. marina sp. 22* or BSA. Gelatin does not show any significant difference from an untreated surface.

Table 8: Statistical data from a one-way ANOVA test on the exponential growth rate of NM 22+ vs. NM 22-, BSA+ vs. BSA-, Gelatin+ vs. Gelatin- .

NM 22+ vs. NM22- (one-way ANOVA: $F_{1,6}$= 6,788; P=0,04 , Post Hoc Holm-Sidak)	Significant difference
BSA+ vs. BSA- (one-way ANOVA: $F_{1,6}$= 29,695; P=,002 , Post Hoc Holm-Sidak)	Significant difference
Gelatin+ vs. Gelatin- (one-way ANOVA: $F_{1,6}$= 0,0627; P=0,811	No significant difference

3.4 Live/ dead staining:

In an effort to visualize how *S. japonica* affects *N. marina* sp. 22, two batch cultures were stained using the live dead staining where one of the batch cultures where inoculated with *S. japonica* for 24 hours. From figure 15 it is quite clear that a lot more dead cells are present when *N. marina* sp. 22 and *S. japonica* are grown together and heavier biofilm seems to be formed when *S. japonica* is present.

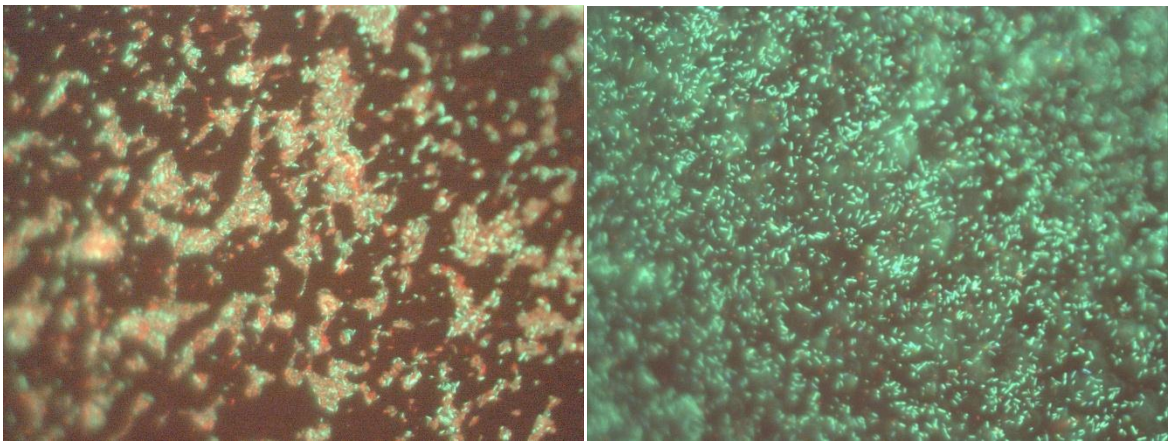


Figure 15: Pictures of live/ dead staining. Red bacteria are dead and green are living. To the left is a culture of *S. japonica* and *N. marina sp 22* that have inoculated together for 24 hours. To the right is a pure culture of *N. marina sp 22*.

3.5 Inhibition of nitrification:

3.5.1 Ammonium measurements

In an effort to describe how development of nitrification is affected by heterotrophic growth *N. marina sp. 22* would be inoculated in 450 ml media containing 2 mg L^{-1} NH4 and with and without *S. japonica*. Readings of NH_4 would be compared to the amount amoA expressed.

Table 9: Readings of NH_4 and pH over 12 days. Setup 1 top table contained only *N. marina sp. 22*. Setup 2 bottom table contained both N. marina and *S. japonica.* C/N=1. n=3

Time [days]	Nm 22; 1 [NH_4 mg L^{-1}]	Nm 22; 1 [pH]	Nm 22; 2 [NH_4 mg L^{-1}]	Nm 22; 2 [pH]	Nm 22; 3 [NH_4 mg L^{-1}]	Nm 22; 3 [pH]
3	2.2	8.1	2.2	8.2	2.2	8.2
8	2.3	8.2	2	8.2	2.2	8.2
12	2	8.2	2.1	8.1	2.1	8.2

Time [days]	Nm 22. Sj: 1 [NH_4 mg L^{-1}]	Nm 22. Sj; 1 [pH]	Nm 22. Sj: 2 [NH_4 mg L^{-1}]	Nm 22. Sj: 1 [pH]	Nm 22. Sj [NH_4 mg L^{-1}]	Nm 22. Sj; 3 [pH]
3	1.9	8.0	2.0	8.0	2.1	8..0
8	1.9	8.1	2.1	8.0	1.97	8.1
12	2	8.0	2.0	8.0	2.2	8.0

No change in NH_4 was observed in any of the setups. Most likely due to a crash in the batch culture added meaning that the culture added did not contain any living or a very small amount of *N. marina* sp. 22.

3.5.1 Quantitative real-time PCR

In an attempt to quantify the ratio between *N. marina* sp. 22 and *S. japonica*, I attempted to establish a quantitative real time PCR protocol. To determine the efficiency of PCR a standard curve was made from an overnight culture of *S. japonica* and a batch culture of *N. marina* sp. 22. From several attempts of optimization a fitting standard curve arose but only for the Sj 16S rDNA primer. The melting peaks of the PCR products in the standard curve of Sj 16S rDNA are all the same temperature (fig 16) indicating the same product was formed in all samples. This is a criterion for a successful standard curve. Had other melting points been present it would mean that the primers had bound to a different cDNA and formed other PCR product. The standard curve would then have flawed since it is impossible to determine which melting point that represents the intended target.

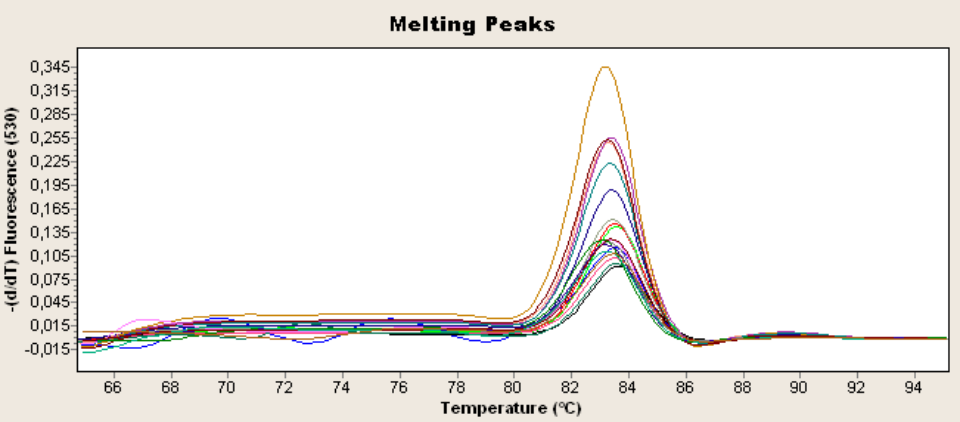


Figure 16: The melting peak of PCR product shown for the Sj rDNA primer standard curve. All the melting peaks are on top of each other and only a single spike for each sample indicates that only one product is amplified.

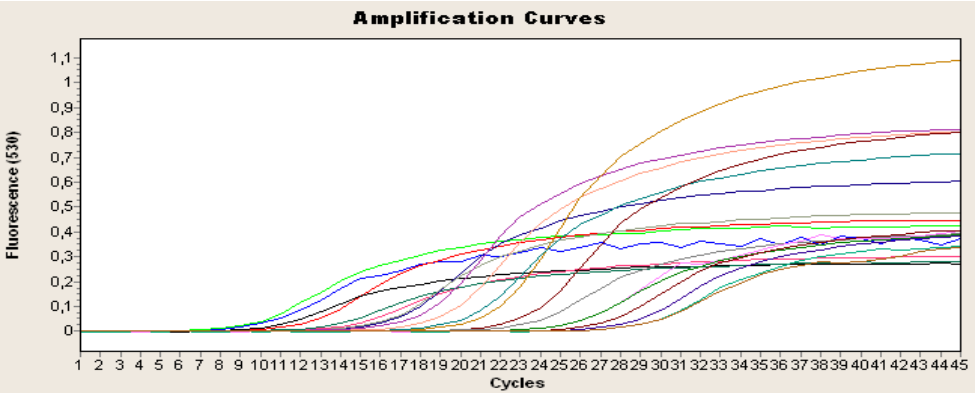


Figure 17: The amplification curve of the SJ rDNA 16S standard curve. It can be seen how readings of fluorescence is happening at different cycles indicating different start concentrations of the target cDNA. The more cycles needed for a fluorescent signal to show the less cDNA is present at the beginning of the run. By having a horizontal line pass through the curves in their exponential line, crossing point values (cp) can be determined.

Crossing points of the amplification curve indicates the start concentration of the target cDNA. By having Log concentration in the x axis and crossing point in the y axis a straight line must hit all measured points in order for the standard curve to be precise. The standard curve is used as a reference for the actual samples. The standard curve of Sj 16S rDNA (fig 16) was successful and can be used as a reference for the SJ 16S rDNA results.

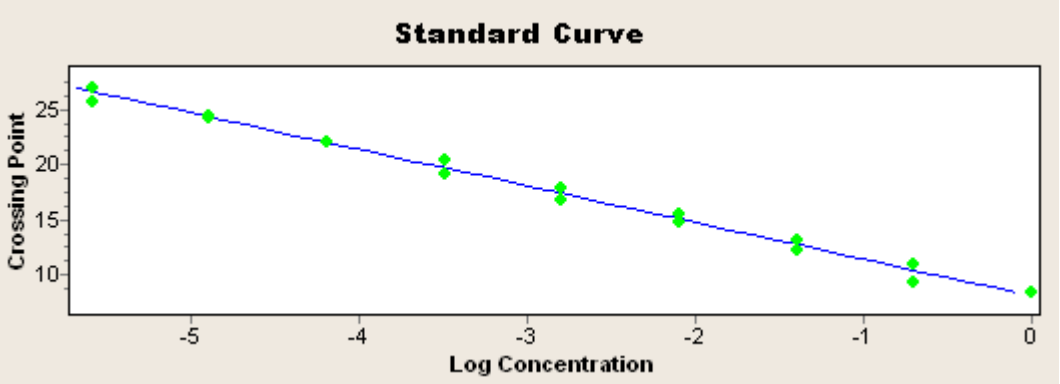


Figure 18: The standard curve of the primer Sj rRNA. The standard curve is made from double determination of each sample. With Log concentrations in the x axis and crossing points in the y axis a fitted line must hit each sample for it to be precise.

In order to be able to measure the expression of Nm rDNA and Nm amoA standard curves for these primers needed to be prepard as well. This however was not successful and further optimization was needed.

The defrosted samples were analyzed even though only one standard curve was successful. All samples were prepared by synthesizing cDNA from isolated RNA from each sample. Samples would normally be diluted 1/10 but undiluted samples were used because of the presumed low concentration of cDNA. Figure 19 and 20 shows the amplified measurements of fluorescence of PCR product from primer NM 22 16S rDNA and NM 22 amoA. None of the samples showed any measurable product indicating that no *N. marina* sp. 22 was present.

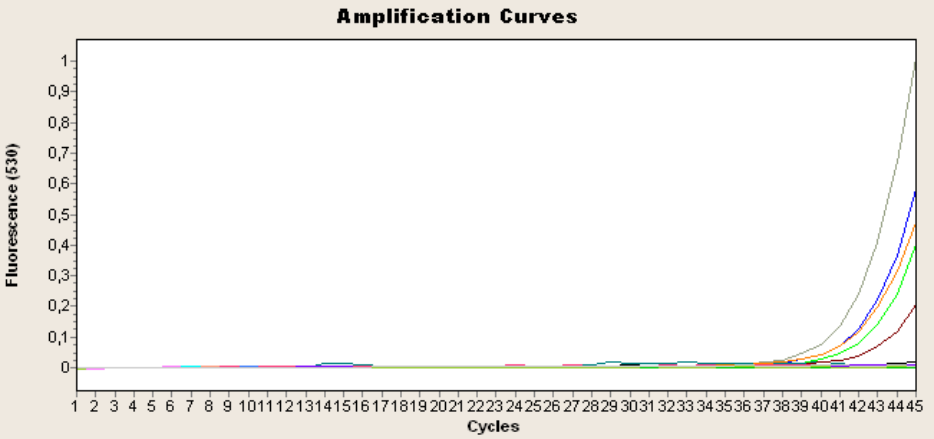


Figure 19: The amplification of the measurements from qPCR using the Nm amoA primer. No bands are present indicating that no product is synthesized. The bands forming at cycle 39 are the primers binding with themselves creating a false product.

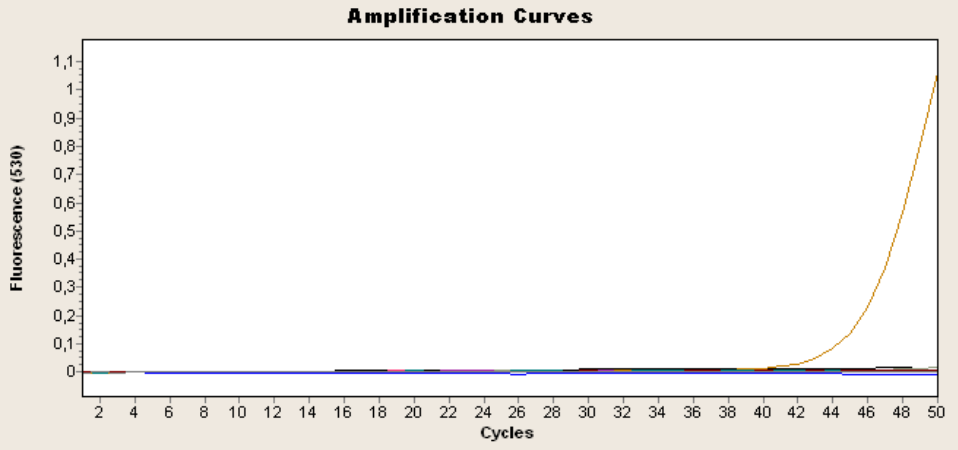


Figure 20: The amplification of measurements from qPCR using nm rDNA Primer. No bands were detected indicating that no product was synthesized.

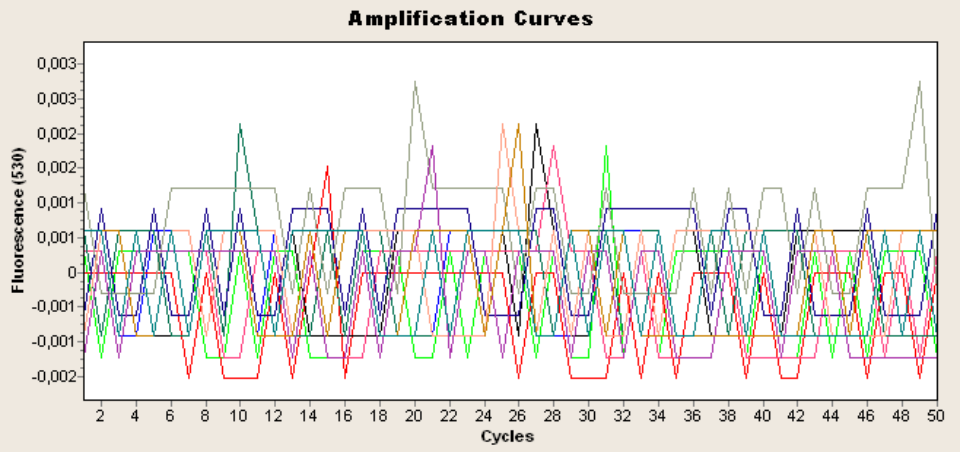


Figure 21: The amplification of measurements using Sj rDNA primer. No bands were detected indicating that no product was synthesized. The small bands seen of the figure is simply noise. The bands seen are only visible because the fluorescence value is very low

No product was formed in any of the experiments indicating no growth in any of the flasks. The poor results may also be because of very low concentrations of the target cDNA making qPCR impossible.

4. Discussion:

The competition between heterotrophic and nitrifying bacteria in RAS is of major practical importance in biofiltration. Most studies done on the microbial community in RAS has been done with the primary focus on ammonia oxidizing microbes since these are the ones that contribute directly to the removal of potentially harmful bi-products (Andersson et al., 2008) . Studies that focus on other areas of the heterotrophic microbial community tend to focus on pathogenic bacteria, that might infect the fish and reduce the net-gain of the operation (Leonard et al., 2000). It has been observed though that many biofiltrations are affected by heterotrophic growth and that this can potentially reduce the removal of TAN.

In the first part of the study I wanted to investigate how heterotrophic bacteria would influence development of a nitrifying biofilm and to do this I monitored TAN. The results demonstrated a decline in TAN over time. Though the results showed a significant change (Fig 10), a very large SD was observed which might be due to the difference of AOB in the 50 ml batch culture that were added to the tanks. The rate of TAN conversion was shown as an exponential decay (Fig 10). This was expected since the *Nitrosomonas* lineage is growing exceptionally slow, with a doubling time of approximately 8 to 11 hours (Skinner and Walker, 1961). With decrease in TAN concentrations, NO_2 was expected to rise similarly. This was however not the case. The NO_2 increase very little and was saturated at 0.0350 ± 0.0160 mg L^{-1} NO_2 (table 4) . TAN however went from 1.9875 ± 0.1458 mg L^{-1} TAN to 0.0863 ± 0.0741 mg L^{-1} TAN (table 4). However under optimal conditions the end concentration of NO_2 should match the start concentrations of TAN. Thus my results may indicate that NO_2 was being converted into something else, possibly NO_3, almost immediately after being formed. Although the seawater used in this experiment had been sterilized beforehand by autoclaving and the experiment was carried out with the intention to keep it as sterile as possible it cannot be excluded that other bacteria were present converting the NO_2.

In order to examine the relationship with ammonium oxidation and the expression of the amoA gen with and without the presence of heterotrophic bacteria six closed systems were set up (fig 9). This would show how the presence of heterotrophic bacteria would influence the ammonium oxidation process and correlate this to the expression of amoA by qPCR. Because a pilot experiment showed ammonium oxidation without the supply of oxygen and a live/dead staining showed living cells (fig

15) it was not deemed necessary to supply the flasks with oxygen. Measurements indicated that no ammonia oxidation had taken place (Table 7). Measurements from qPCR on *N. marina* sp. 22 16-S rRNA, *S. japonica* 16-S rRNA and *N. marina* sp. 22 amoA did not give any reading either (fig. 18, fig. 19, fig. 20). The low concentrations of substrate and dissolved oxygen may have stopped growth entirely. The low biomass resulted in very small concentrations of synthesized cDNA making PCR impossible. The two sets of primers used for both *N. marina* sp. 22 16-S rRNA and amoA were unable to form standard curves and did not show any readings on any of the samples. The primers used for *S. japonica* 16-S rRNA was able to form a standard curve from a positive control but no readings were made from setup 4,5 or 6 (fig 18, fig 19). In order to make a standard curve for *N. marina* sp. 22 16-S rRNA and amoA more optimization of primers was needed. It was not possible to determine if the presence of heterotrophic bacteria had any effect. Previous studies indicate that when the C/N ratio is increased to 0,5 and above ammonia oxidation is decreased. In wastewater management especially were the loads of organic carbon is much bigger than what is seen in RAS (Leonard et al., 2000). Zhu and Chen (2001) showed that when a series of submerged biofilm reactors with no organic carbon the TAN removal rate was at 1500 mg m^{-2} d^{-1} but with an increase of sucrose carbon ratio to C/N=1.0 or 2.0 a decrease of TAN removal followed to 481 mg m^{-2} d^{-1} a decrease of around 66% (Zhu and Chen, 2001). The same was observed by Michaud et al. (2006) all though they only experienced a decrease of 30% at C/N=0.5 and 50% at C/N= 1.0 or 2.0 (Michaud et al., 2006). Both studies conclude that the reason for the decrease in ammonia oxidation is due to the competition for oxygen. Also that when the C/N ratio is above 1 nitrification gets less sensitive to C/N. The studies only describe how ammonia oxidation gets affected but leave out what is happening with the expression of genes involved in ammonium oxidation. In the present study it was meant to describe how the expression of amoA correlates to the measured reduced nitrification and if at all amoA could be applied as an indicator for nitrification when dealing with low substrate concentrations. The results from this study was unfortunate inconclusive. When comparing the method used in this study with other studies a lot of improvements could be made. In other studies they used a flow through system with steady-state flow of fresh media maintaining a fully oxidized stable substrates concentration in each reactor (Zhu and Chen, 1999, Michaud et al., 2006, Zhu and Chen, 2001). This ensured that a stable biofilm was formed and maintained. This also insures that pH is constant insuring available ammonium. In the present study the oxygen exchange between the surface of the media and the air was the only oxygen supplied; which ultimately was insufficient. When compared with the setup of the 25 L tanks nitrification did succeed with a calculated

exponential decay of -0.0721(fig 10). This was done however with an oxygen supply and 100 RK BioElements bio filters. In the small scale experiment no bio filters were added to the flasks either. The small surface area may have contributed to the poor results as well. The constant re-suspension of the *N. marina* sp. 22 batch cultures for 10 months may have washed out all *N. marina* sp. 22 from the batch cultures leaving a very small concentration in the end. Many batch cultures crashed during inoculation causing the healthy cultures to be thinned in order to maintain a large enough stock. If a crashed culture of *N. marina* sp. 22 was transferred in the start of an experiment no ammonium oxidation could be measured. Had this succeeded it might have given a better understanding of the amoA gen and how this is affected by competition between AOBs and heterotrophic bacteria.

In order to clarify if nitrifying biofilm may stimulate heterotrophic bacteria to colonize a biofilm, I examined the effect of pre-formed biofilm on the exponential growth rate of biofilm by *S. japonica*. Previous studies show that the production of biofilm can be stimulated between species and strains (Reisner et al., 2006, Andersson et al., 2008, Saito et al., 2008). Studies done on the bacterial community on bio filters in RAS and wastewater facilities shows a wide range of both autotrophic and heterotrophic bacteria (Purkhold et al., 2003, Foesel et al., 2008). That the different bacteria have an effect on each other's biofilm production is therefore highly possible. By using *S. japonica* as a model for heterotrophic bacterium I tried to describe the effect of biofilm production when grown on a living biofilm, BSA and gelatin. From the calculated exponential growth rates of biofilm from *S. japonica* it shows that there is a significant difference when inoculated with biofilm from *N. marina* sp. 22 (one-way ANOVA: $F_{1,6}$= 6,788; P=0,04 , Post Hoc Holm-Sidak) or BSA (one-way ANOVA: $F_{1,6}$= 29,695; P=,002 , Post Hoc Holm-Sidak) when compared to the growth rate without any coating (table 8). The mean growth rate when grown on *N. marina* sp. 22 was 0.0117±0.0006 and when grown with BSA 0.0128±0.0009 (table 7). *S. japonica* grown under optimal conditions at 28˚ C has an exponential growth rate of 0.0118 (figure 12) very close to what is seen when grown with *N. marina* sp. 22 or with BSA indicating that biofilm is produced equally fast as cell division of *S. japonica* when grown under optimal conditions. The rugged surface of BSA biofilm may have contributed to the colonization making it easier for bacteria to settle. Wells treated with BSA or gelatin was supposed to simulate the structural matrix of a biofilm. But only BSA was able to stimulate biofilm production (fig 14). This may be because of the difference in the structure the film made by BSA and gelatin. BSA is very sticky and more cells may have gotten stuck than the gelatin which tends to be smoother in its structure. The outer matrix of the *N. marina*

sp. 22 biofilm might have different signaling molecules that stimulate bacteria from *S. japonica* to colonize the surface and start multiplying into micro colonies. The difference in biofilm production when compared with the uncoated wells indicate that *S. japonica* is in fact stimulated by living biofilm and might indicate a form of selection process when settling on different surfaces. This indicates that active bio filters in RAS may actually stimulate colonization by heterotrophic bacteria.

5. Conclusion

Unfortunately the influence by heterotrophic bacteria on the development of nitrifying biofilm was not able to be described. From what can be seen on the live/ dead staining it seems to have some effect although this needs further clarification before anything can be concluded. Nitrification was successfully obtained in 25 L tanks with oxygen supply and expanded surface area by having bio filter tablets present. In the small scale experiment optimization by having oxygen supplied as well as bio filters is required if the experiment is to be repeated. In order to run a successfully qPCR it is imperative that enough of the target cDNA is present. One of the problems with the qPCR results in this study was most likely lack of cDNA in the samples. There is clear indication that pre-formed biofilm from *N. marina* sp. 22 and BSA stimulates biofilm production by *S. japonica*. If it is stimulation by quorum sensing or the rugged surface of the biofilm has not been clarified. It is clear however that a contaminated bio filtration accelerate the rate by which further recruitment of heterotrophic bacteria happens. It is therefore important to replace bio filter overgrown with heterotrophic bacteria since this will only amplify the decrease of nitrification observed in bio filtration. A convenient and efficient way of replacing the bio filtration in a RAS without stopping production would be invaluable if developed.

7. Future perspectives

An optimization of the experiments from the present study should be carried out by the using a flow through system with fully oxygenated media. Besides describing the relationship between the expression of amoA and ammonia oxidation a description of the development of the biofilm structure itself would be obvious. By cloning a fluorescent plasmid into a heterotrophic bacterium the use of confocal microscopy could describe how nitrifying biofilm changes and develops when colonized and why ammonia oxidation is decreased during different stages of colonization. Cloning could also be done on the nitrifying bacteria but because of the very high generation time it could prove to be very difficult. The experiment could be done at different C/N ratios, oxygen and pH levels describing how different environments may influence colonization. This would further enlighten the relationship of nitrifying bacteria and heterotrophic bacteria in bio filtration which so far is still poorly understood.

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