Quorum sensing and biofilm formation potential in Norwegian *Tenacibaculum dicentrarchi* isolates
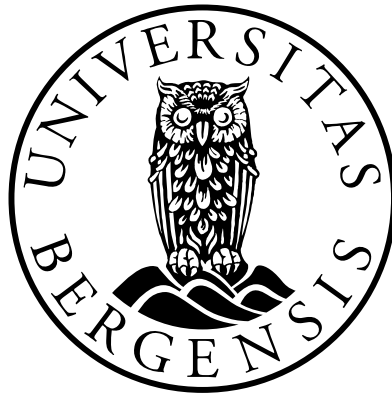
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Abstract

Fish farming has become one of the most important industries in Norway. To handle further growth, both in sea- and land-based facilities, challenges should be dealt with in a sustainable manner. Among these challenges are bacterial infections. New promising treatment methods are being developed, targeting the communication between the bacteria. This communication is referred to as quorum sensing, which enables the bacteria to act in a coordinated way, depending on cell density. The bacteria produce molecules called autoinducers that diffuse away from the cell, and at high autoinducer concentrations certain genes become up- or downregulated. This autoinducer regulated gene control includes biofilm regulating processes and other features often included in virulence. In this thesis, indications of autoinducer production in the *T. dicentrarchi* strains were found using *Vibrio harveyi* reporter strains. These reporters react to AHL and AI-2, two of the most common autoinducers. The *Tenacibaculum* strains did not show indications of producing short chain AHLs, which can be tested when a *Chromobacterium violaceum* reporter strain is used. Furthermore, bioassays showed indications that the *Tenacibaculum* strains are capable of biofilm production. Both quorum sensing and biofilm activity, and how they are connected, are important aspects to fully understand when targeting the virulence of bacteria in new methods as alternatives to antibiotics.

Abbreviations

AHL: Acyl homoserine lactone

AI: Autoinducer

Chl: Chlorampheniciol

QS: Quorum sensing

QQ: Quorum quenching

Km: Kanamycin

LB: Luria Bertani

MA: Marine agar

MB: Marine broth

OD: Optical density

TSB: Tryptic Soy Broth

VI: The Veterinary Institute

Contents

1. Introduction

1.1 Norwegian fish farming and new challenges

The Norwegian fish farm industry has been growing tremendously since its beginning in the 1970s, though low scale amateur production had occurred already since the 1800s (Gullestad et al., 2011). During the 1990s, the fish farm industry became one of the most important export industries in Norway. In 2006, the export value of farmed fish exceeded that of wild salmon for the first time. Over the last five years the sell value of salmon has doubled and salmon worth over 60 billion NOK were sold in 2017 (Baklien, 2018). At the same time, the produced volume of salmon in tonnes did not increase (Statistisk sentralbyrå, 2018). In total, 1.29 million tonnes of Norwegian fish were sold in 2017, of which 1.22 million tonnes were salmon.

Further growth in Norwegian fish farming can only occur at the biology's premise of sustainable development. Thus, political guidelines in form of a "traffic light"-system were implemented in 2017 (Lovdata, 2017). This system divides Norway into 13 graphical zones, based on environmental factors, in order to achieve a sustainable fish production. Additionally, the Norwegian institute for marine research gives annual reports about the environmental- and fish health risks linked to fish farming, by mandate from the Norwegian government. The latest report from 2018 focuses on fish lice, fish escape and impact on the genome of wild salmon, pathogens, and emissions from fish farms (Grefsrud et al., 2018). These are all challenges that arise when a high fish biomass is retained in cages, which can function as an infection point to the local environment. The fish farming industry is working together with research institutions towards reduced environmental impact, and new technologies like closed cages are under development. This will however lead to new challenges related to the water quality in closed systems.

1.1.1 Bacterial infections in fish farming

The level of disease outbreaks related to bacterial infections are reported to be at an acceptable low level, with winter ulcers being the most pressing problem in farming of salmonids (Norwegian Veterinary Institute, 2017). The diseases cold water vibriosis caused by *Vibrio salmonicida* and furuncolosis caused by *Aeoromodas salmonicida*, have

previously been serious threats to Norwegian aquaculture, but are now under control due to extensive vaccination. But *A.* salmonicida is still an important problem in cleaner fish cultivation.

Winter ulcers are cold water-associated skin lesions in salmonid species (Olsen et al., 2011), and normally appear at low sea temperatures. The report about the health situation in Norwegian aquaculture from the Veterinary Institute in Norway (Norwegian Veterinary Institute, 2018), states that winter ulcers were widespread along the whole coast of Norway in 2017. Bacteria associated with "typical" winter ulcers are *Moritella viscosa* (formerly *Vibrio viscosus*) and *Aliivibrio wodanis* (formerly Vibrio) (Norwegian Veterinary Institute, 2018). *M. viscosa* is the only bacterium confirmed to cause disease in challenge studies (Takle et al., 2015). The presence of *A. wodanis* in co-infection with *M. viscosa* has been shown to slow down the proceeding of the disease (Karlsen et al., 2014). Although *A. wodanis* has not been proven to be a direct agent for winter ulcers in challenge tests, the bacterium has for many years been reported in salmon with winter ulcers and has often been isolated in near pure culture. A third bacterial type also often found in winter ulcers with *M. viscosa* and *A. wodanis*, is *Tenacibaculum spp.* (Norwegian Veterinary Institute, 2018).

1.2 *Tenacibaculum* and its role in fish diseases

The genus *Tenacibaculum* was proposed in 2001, and consists of aerobe, Gram-negative rod shaped bacteria, which are able of gliding motility and form yellow colonies when grown on marine agar (MA) (Suzuki et al., 2001). Suzuki et al. first isolated *Tenacibaculum* from sponges around Japan and Palau, and from the surface of macroalgae. *Tenacibaculum* belongs to the Cytophaga-Flavobacterium-Bacteriodetes (CFB) group, which is known to be important for recycling of carbon in the oceans (Colin, 2011). Bacteria within the CFB group are considered among the dominant marine bacteria groups and have the ability to decompose cellulose, agar, and chitin (Suzuki et al., 2001). This also applies for *Tenacibaculum*, which can degrade large carbon molecules like cellulose and chitin (Takle et al., 2015). *Tenacibaculum* has an optimum temperature between 15 and 22°C, grows on marine agar and is resistant to oxolin acid (Takle et al., 2015).

As of today, there are 21 identified species of *Tenacibaculum*. There have been some changes regarding the taxonomy of the genus, which was earlier considered as a part of the *Flexibacter* genus. The *Tenacibaculum* genus is now placed in the *Flavobacteriaceae* family within the class Flavobacteria and phylum Bacteroidetes, and the type species is *T. maritimum* (NCBI). The phylogenetic tree (Figure 1) based on the GyrB protein and its coding gene *gyrB*, revealed a cluster of five isolates. In addition, the cluster was closely related to other isolates previously characterized as *Flexibacter ovolyticus* and *F. maritimus*, which were rooted in one separate clade (Suzuki et al., 2001). Four of the *Tenacibaculum* isolates were phenotypically characterized, and all of them displayed bright yellow colonies, not adhering to the agar, and gliding motility was observed. The strains are obligate aerobes, do not form gas vesicles, and are flexirubin-negative.
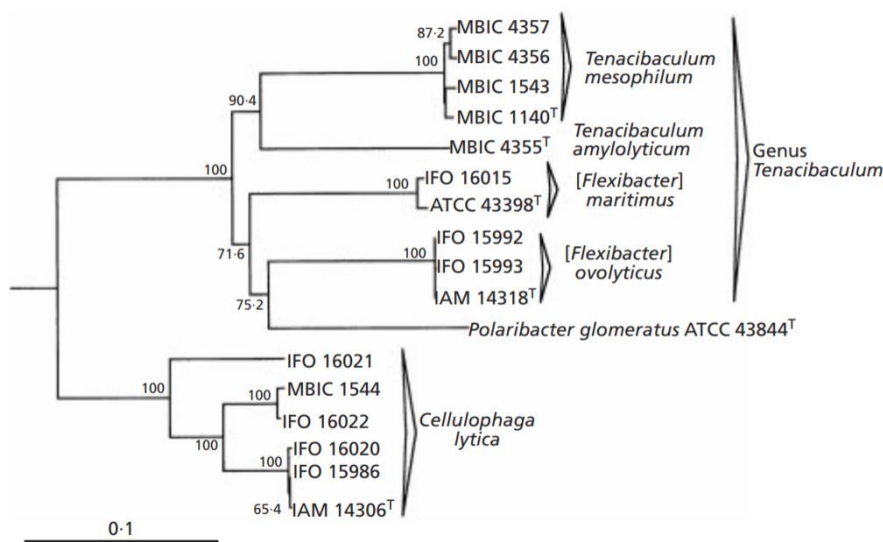
Figure 1. Phylogenetic tree from Suzuki et al., 2001 showing the genus *Tenacibaculum* and *Cellulophaga lytica* strains based on gyrB nucleotide sequences using the neighbour-joining method. *Cytophaga uliginosa* ATCC 14397T was used as the outgroup.

The type-strain *T. maritimum* is the agent for a fish disease referred to as tenacibaculosis. The disease has different names throughout the literature and is also referred to as atypical winter ulcers, gliding bacteria disease, or flexibateriosis, among others and is primarily associated with *Tenacibaculum spp.* (Norwegian Veterinary Institute, 2018). The disease is characterized by lesions on the side of fish on the head, jaw, tail and fins (Avendaño-Herrera et al., 2006, Norwegian Veterinary Institute, 2018). This bacterial

infection is of great concern in several areas of the world, for example in parts of Spain and Portugal, where it is one of the biggest threats for culturing of commercial fish species (Avendaño-Herrera et al., 2006). The fish species with most reported infections in this area are Atlantic salmon (*Salmon salar*), turbot (*Scophthalmus maximus*), sole (*Solea senegalensis* and *S. solea*) and gilthead seabream (*Sparus aurata*). Atypical winter ulcers are often associated with high mortality, and Tenacibaculum spp. can often be found in high numbers. Wounds on fishes, where *Tenacibaculum* is identified often have stress related causes, like non-optimal water temperatures, or the presence of other pathogenic bacteria (Norwegian Veterinary Institute, 2017). A study performed by Olsen et al. implies that winter ulcers caused by *Moritella viscosa* can predispose the fish for *Tenacibaculum sp.* infection (Olsen et al., 2011).

T. maritimum is often found in mixed infections, and may be difficult to obtain in pure culture because of the slow growth (Avendaño-Herrera et al., 2004). The difficulties in isolating and growing *Tenacibaculum* in a lab can lead to underestimations of these bacteria in infections (Norwegian Veterinary Institute, 2016).

As of today, little is known about the infection route of *Tenacibaculum maritimum* (Avendaño-Herrera et al., 2006a). In a mesocosm experiment performed by Avendaño-Herrera et al., *T. maritimum* remained culturable much longer in sterile water than in natural seawater (Avendaño-Herrera et al., 2006b). This suggests, according to the authors, that the sea water is probably not the most important route of infection. Instead, the bacterium needs protection in sediments or attached to the fish surface until favourable growth conditions occur.

Though *Tenacibaculum maritimum* is the strain primarily associated with tenacibaculosis, the gene for pathogenesis is shown to be scattered between separate taxonomical clades (Christophe et al., 2014). This suggests a parallel evolution of the pathogenesis in several groups within the *Tenacibaculum* genus. *Tenacibaculum spp.* in Norway have a great genetic variation (Olsen et al., 2017). A Multilocus Sequence Analysis (MLSA) of 89 *Tenacibaculum* isolates from disease outbreaks in Norwegian fish farms over a period of 19 years shows a total of four clades. *T. dicentrarchi was* one of the these four *Tenacibaculum* clades. This was the only major clade found in the study, and *T. dicentrarchi* comprised 19 of the total 89 isolates. *Tenacibaculum dicentrarchi* was first

isolated from a diseased sea bass in Spain in 2012 (Piñeiro-Vidal et al., 2012). The colonies were described as pale yellow, flat circles, with uneven edges when grown on MA plates. The cells are thin, filamentous rods with a length of 2-40µm, that can move by gliding motility. *T. dicentrarchi* isolated from lesions in Atlantic salmon from a disease outbreak in Chile in 2015, was fully sequenced in 2016 (Grothusen et al., 2016). Genes coding for metallopeptidases and collagen-binding proteins were found, which are often involved in pathogenesis of fish.

The high genetic variation found in Norwegian *Tenacibaculum* isolates indicates that *Tenacibaculum* already present in the environment are opportunistic and can infect the fish when the right conditions occur (Norwegian Veterinary Institute, 2016). Supporting this hypothesis is the ability of *Tenacibaculum maritimum* to change between a biofilm forming and planktonic lifestyle (Romero et al., 2010). Bacteria which undergo such transitions often coordinate via cell-to-cell communication or otherwise known as quorum sensing (Fuqua et al. (1994).

1.3 Quorum Sensing

The term "Quorum Sensing" (QS) was first used by Fuqua et al. (1994) to describe a form of communication between single cell organisms, that allows them to monitor and react to cell density. "Diffusion sensing" has been suggested as a more precise term, since QS involves information from the environment, rather than information of the quorum or bacterial community (Williams et al., 2007). The phenomenon was first identified in *Aliivibrio fischeri* (former *Vibrio fischeri,* or *Phopobacterium fisheri*), which is an bioluminescent marine bacterium (Nealson et al., 1970). The luminescence of *A. fisheri* is not proportional to the bacterial concentration, but during the exponential growth phase the light intensity suddenly increases strongly. Nelson et al. referred to this as "autoinducing" and concluded that the cellular control of this feature must happen on the transcriptional level. When cell-free supernatant from a culture in a phase with high bioluminescence is added to a culture with low cell concentration, luminescence is induced.

Bacteria with QS produce small molecules called autoinducers (AI), that diffuse (or are actively transported) out of the cell (Figure 2). When the concentration of AI reaches a threshold level, specific functional genes in the bacteria are either up- or downregulated.

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These genes can be involved in bioluminescence, biofilm production (Hentzer et al., 2002), virulence (De Kievit and Iglewski, 2000, Williams et al., 2000), flagella movement (Sperandio et al., 2002), extracellular enzymes, and other cell regulatory processes. At low bacterial concentrations, the processes controlled by QS are causing a higher energetically cost than benefit. The mechanism was earlier regarded as a rare phenomenon, but the recent increase in experiments and published papers in this field shows that QS is a common trait that might even exist in most bacteria. (Whitehead et al., 2001, Miller and Bassler, 2001).
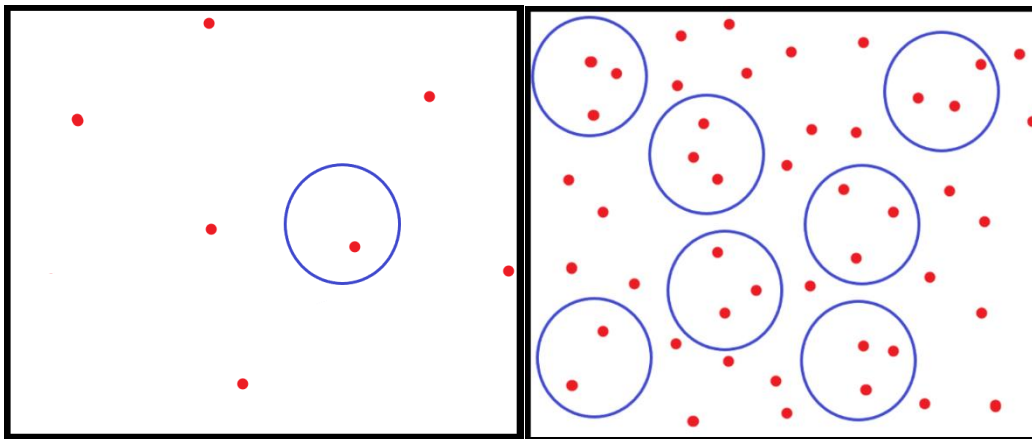
Figure 2. Illustration of cell-to-cell (blue circles) communication via quorum sensing, with autoinducers (red dots) at low (left) and high (right) cell concentrations.

1.3.1 Autoinducers

Acyl homoserine lactone (AHL)

The most common and thoroughly studied autoinducers in Gram-negative bacteria are acyl homoserine lactones (AHLs) (Camilli and Bassler, 2006). AHL is synthesised by LuxR or a LuxR-like protein from S-adenosyl methionine and has a homoserine lactone core (Figure 3). The number of carbon atoms in the acyl chain normally varies between 4 and 18 (Williams et al., 2007).

Autoinducer 2 (AI-2)

AI-2 was first described in *Vibrio harveyi* (Bassler et al., 1993). It was early discovered that cell-free medium from several other bacteria species could induce luminescence in *Vibrio harveyi* (Greenberg et al., 1979), one of the first bacteria found to have autoinduced

bioluminescence besides *A. fischeri* (Nealson and Hastings, 1979). The AI-2 molecule is produced and recognised by numerous different bacteria, and has therefore been suggested to be involved in interspecies communication (Bassler et al., 1994). The enzyme catalysing AI-2 production is identified as LuxS, and is highly conserved within the Bacteria domain (Xavier and Bassler, 2005). In 2002, the structure of AI-2 was identified as a boron containing molecule (Chen et al., 2002) (Figure 3).
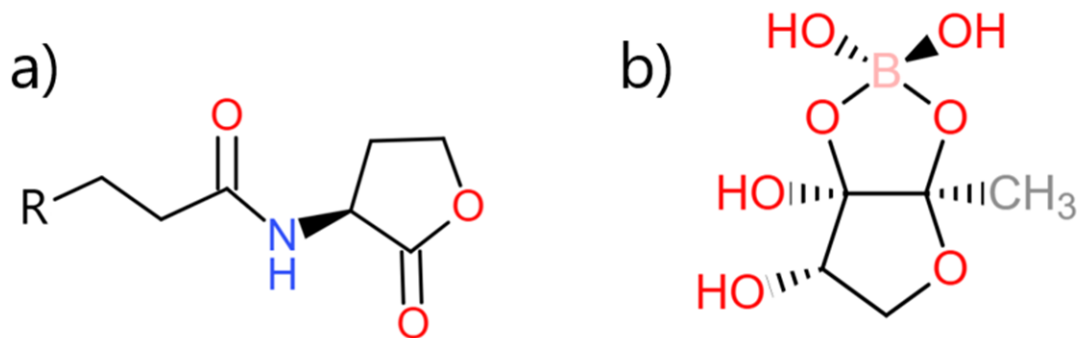
Figure 3. Chemical structure of a) AHL and b) AI-2 (Based on Williams et al. (2007)).

Both AHL and AI-2 are produced from S-adenosinmethionin (SAM), which is often used as a methyl donor in DNA, RNA and protein synthesis (Xavier and Bassler, 2003). AI-2 is synthesised from the toxic intermediate S-adenosylhomocysteine (SAH) derived from SAM (methyl donation). SAH is hydrolysed to S-ribosylhomoserine and adenine by the enzyme Pfs. LuxS then converts S-ribosylhomocysteine to 4,5-dihydroxy-2,3-pentanedione (DPD). DPD is rearranged spontaneously, and the derivates are collectively referred to as AI-2 (Xavier and Bassler, 2005). Crystalizing the sensor LuxP in a complex with AI-2 has revealed that the signal molecule is a furanosyl borate diester (Chen et al., 2002). The biosynthetic pathway described here uses a toxic metabolism intermediate to produce a signal molecule. This close link between metabolism and AI-2 allows the cells to measure the bacterial metabolic activity in the environment. AHL on the other hand is produced directly from SAM, and is not dependent on the level of cell metabolic activity (Xavier and Bassler, 2003). Therefore, the presence of AHL does not provide information about the growth or metabolism of a bacterial culture.

1.3.2 QS mechanisms

The proteins that are involved in AHL-mediated QS are normally members of the LuxR-LuxI family (Fuqua et al., 2001). AHL is synthetized by enzymes in the LuxI family, and the molecule diffuses freely through the cell envelope. When the concentration of AHL in the environment, and thus also inside the cell, reaches a threshold, AHL starts binding to the proteins in the LuxR family (Figure 4). The resulting LuxR/AHL complex will then activate or repress specific genes. The *luxI* gene is often among the upregulated genes, thereby causing a positive feedback (Williams et al., 2007).
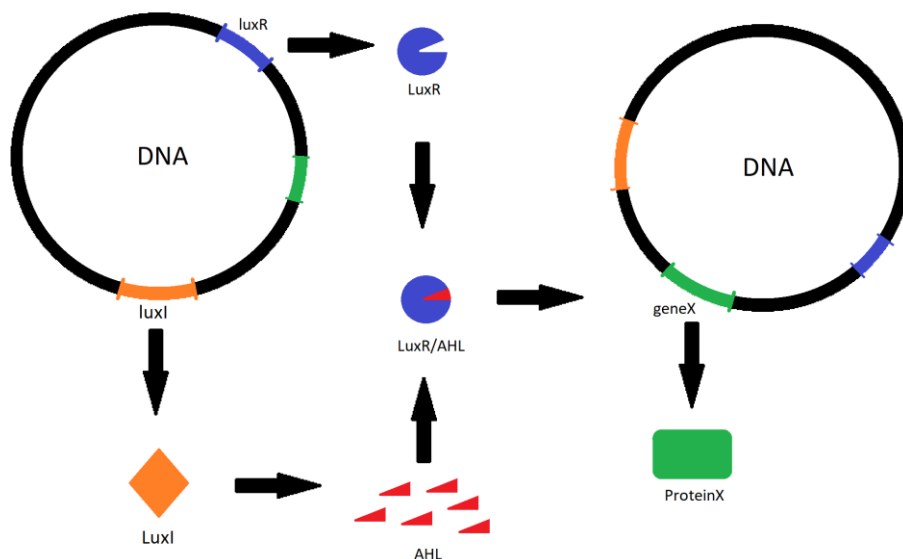
Figure 4. Schematic overview of AHL-mediated QS. *luxI* codes for LuxI, which produces AHL. LuxR is coded by *luxR*, and binds to the AHL molecule. The LuxR/AHL complex regulates the transcription of the target gene, *geneX*.

The QS model organism *A. fischeri* becomes bioluminescent when they reach high cell concentrations. The genes controlling bioluminescence include *luxA-E, -G, -I*, and *-R*, and are found in two (bidirectionally transcribed) operons (Whitehead et al., 2001). The *luxA* and *luxB* genes code for subunits of the luciferase enzyme, and *luxC-E* codes for parts of a multi-enzyme complex producing aldehyde substrates used by luciferases. The *luxI* gene codes for LuxI which produces N-(3-oxohexanoyl)-L-homoserine lactone (OHHL), the autoinducer of *A. fischeri*. OHHL is produced at a basal level when the bacteria are in low concentrations. LuxR can bind to OHHL when it reaches a threshold concentration. The

LuxR-OHHL complex binds to the lux-box upstream of *luxI*, inducing transcription of *luxICDABEG*.

1.3.3 Quorum quenching

Quorum quenching (QQ) means disruption of QS, and can possibly become an important mechanism to control pathogenic bacteria in aquaculture (Defoirdt et al., 2004). By targeting the cell-to-cell communication, the virulence can be hindered without killing the bacteria, and therefore the selective pressure towards becoming resistant is lower (Rasmussen and Givskov, 2006). There is however a possibility for the bacteria to avoid QQ by overproducing QS molecules (Zhu et al., 1998). QQ can be done in several different manners, e.g. by inhibiting autoinducers or using autoinducer antagonists (Defoirdt et al., 2004). The opposite way has also been suggested, using agonists for the autoinducers and thereby activating the QS mediated virulence. This would allow the immune system of the host to react to the virulence already at low bacterial population density. A study performed by Bruhn et al. (2005) detected production of AI-like molecules in several Gram-negative fish pathogenic bacteria. AHLs were also found in bacterial infections of fish, indicating that QS has a role in virulence. A number of bacterial species can interfere with QS from other bacteria, by deactivating or metabolizing the autoinducer (Roche et al., 2004).

1.3.4 QS and QQ in *Tenacibaculum*

Similar to other Gram-negative fish pathogenic bacteria, potential for QS activity was also identified in *Tenacibaculum maritimum* (Romero et al., 2010). The experiments performed by Romero et al. showed production of the AHL molecule N-butyryl-L-homoserine lactone (C4-HSL) in *T. maritimum*. All the 9 *T. maritimum* strains tested in that experiment were positive for AHL production, indicating that QS might be a conserved trait in this species. However, the function of QS in *T. maritimum* is not yet fully known. It was also found that bacteria from this genus are capable of degrading C-10-HSL, but not C-6-HSL. QQ has also been shown in another *Tenacibaculum sp.* strain with 99% sequence identity to a *T. discolor* isolate (based on the 16S rRNA gene) (Romero et al., 2014)and the use of *Tenacibaculum* QQ has even been patented (Casal et al., 2013).

1.3.5 Reporter strains in bioassays to test for QS activity

Autoinducers are part of the QS mechanism and in order to test if a given bacteria species produces autoinducers, reporter strains can be used (Ravn et al., 2001). An appropriate reporter needs to have a clear phenotypically response regulated by QS, like bioluminescence or pigment production, to reveal if autoinducers are produced by the tested bacterium. A widely used reporter strain is *Vibrio harveyi*, which can be found naturally both free-living and symbiotic in the gut of animals (Bassler et al., 1997). *V. harveyi* can display bioluminescence at high concentrations, such as in the symbiotic state. Bioluminescence is one of the features controlled by QS in *V. harveyi*. Three QS systems have been found in *V. harveyi*, and these systems have a synergistic effect (Henke and Bassler, 2004). System 1 involves the AI molecule Harveyi Autoinduser 1 (HAI-1), LuxM, and LuxN. HAI-1 is a AHL type molecule (N-(3-hydroxybutanoyl) homoserine lactone) (Cao and Meighen, 1989) produced by LuxM, while LuxN detects the autoinducer. In system 2, AI-2 is produced by LuxS, and detected by a complex of LuxP and LuxQ.

LuxN from system 1 and LuxQ from system 2 autophosphorylate a protein called LuxU at low concentrations of autoinducers, i.e. low cell concentrations. LuxU then phosphorylates the response regulator LuxO, thereby activating it. The active LuxO leads to the destabilization of mRNA coding for LuxR, (not to be confused with the LuxR in *Aliivibri fischeri*) (Bassler et al., 1994). A smaller amount of LuxR in the cell results in decreased transcription of *luxCDABE,* which encodes the luciferase protein (Henke and Bassler, 2004). The third AI-molecule produced by *V. harveyi* is CAI-1 (*cholerae* autoinducer 1) (Henke and Bassler, 2004). CAI-1 is synthesized by the enzyme CqsA and detected by CqsS. This mechanism was first found in *Vibrio cholerae* (Miller et al., 2002). Due to the detailed knowledge regarding the regulation of QS in *V. harveyi,* it is often chosen to detect autoinducers in other bacteria. Thus, *Vibrio harveyi* was also one of the reporter strains used in this thesis. Another well studied QS regulated process in *V. harveyi* is biofilm formation, which is also considered to affect its pathogenicity (Karunasagar et al., 1996, Yang et al., 2014).

1.4 Biofilm

Biofilms are usually described as thin layers of living material. Different definitions have been given by several authors, and aggregates of microorganism are also referred to as biofilms (Flemming and Wingender, 2010). Microorganisms that can form biofilms include bacterial cells, but also archaea, fungi, and algae. Biofilm can grow on both biotic and abiotic surfaces, as a monolayer or multilayer film (Karatan and Watnick, 2009).

The biofilm is often anchored to the surface, and protected by a matrix of extracellular molecules called Extracellular Polymeric Substances (EPS) (Flemming and Wingender, 2010). The EPS consists of large molecules like polysaccharides, proteins, extracellular DNA, and lipids, and often accounts for over 90 % of the biofilm (dry weight). This provides a three-dimensional structure for the bacteria, often with a complex channel architecture. A biofilm is not a homogenous structure but is rather largely heterogenous and complex. This heterogeneity can be revealed by measuring the oxygen levels with microelectrodes (Flemming and Wingender, 2010). The composition of the EPS varies greatly and is dependent on both the types of microorganisms and environmental factors present. Polysaccharides, mostly long molecules and often branched, represent a large fraction of the EPS matrix. Though these can be homopolysaccharides, like cellulose or sucrose derived glucans or fructans, most of them are heteropolysaccharides. The heteropolysaccharides have a mixture of charged and neutral residues, and often contain organic or inorganic substitutes, which influence the structure of the EPS. The protein mass in the EPS can often exceed the polysaccharide mass. Among the proteins are enzymes that modify EPS polysaccharides, enzymes that degrade biopolymers, or that have a virulent function. Structural (non-enzymatic) proteins stabilize the EPS matrix. These proteins also link the bacteria to the EPS.

The EPS matrix of bacteria also involve eDNA (environmental DNA). This is supposedly an important structural component in biofilm of some bacteria, especially in waste-water. The eDNA is not only a biproduct from lysed cells but have been shown to be an integral part of the extracellular matrix.

The forming of biofilm include several stages, and is a cyclic process (Davey and O'toole, 2000). These stages include surface recognition and adsorption, attachment, reproduction, and detachment (Watnick and Kolter, 2000). The bacterium living in a

biofilm has a profile of transcribed genes that is distinct from the same bacterium in a planktonic stage. Many environmental signal molecules take part in this shift from free-living to biofilm forming, both molecules that are well characterized, and molecules that are unknown (Karatan and Watnick, 2009). The nutrient availability has an impact on the biofilm or free-living phenotype of the bacterium, with low or high levels inducing biofilm depending on bacterial species and the environment they inhabit.

The conditions for any bacterial species are completely different in a multispecies biofilm, compared to a mono species biofilm. Experiments that compare these two states have found that a synergistic effect occur in biofilms, increasing the resistance of antibiotics, and increasing biofilms growth (Burmølle et al., 2006). The majority of bacteria in aquatic systems occur on water-surface organized biofilms (Wietz et al., 2009), and the benefits of multispecies biofilms also apply here. The coexistence of heterotrophs and nitrifiers allows oxidation of both organic carbon compounds and ammonia (Hentzer et al., 2004). These are important functions in aquaculture, where an ammonium is toxic for most produced species at 0.05 to 0.2mg/L (Ruffier et al., 1981). Bacteria in fixed film biofilters often carry out these processes in recirculating aquaculture systems (RAS) (Malone and Pfeiffer, 2006). Autotrophic, nitrifying bacteria in the biofilters oxidize ammonia, and include ammonia oxidizing bacteria like *Nitrosomonas europa*, and nitrite oxidizing bacteria like *Nitrospira marina* (Blancheton et al., 2013). Despite the importance of the RAS biofilters, pathogen bacteria can also be protected within biofilm, which can lead to recurring infections of fish in the RAS facilities (Blancheton et al., 2013).

1.4.1 Biofilm and quorum sensing

The bacteria in a biofilm live in a dense community, in an environment kept stable by the EPS. Metabolites and enzymes produced by the cells are hindered from diffusing freely away. This leads to the assumption that quorum sensing might be an important contributor to the biofilm forming lifestyle (Parsek and Greenberg, 2005). Parsek and Greenberg introduced the term "sociomicrobiology" in 2005, discussing the link between QS and biofilm formation, and used *Pseudomonas aeruginosa* as a model bacterium. *P. aeruginosa*

inhibit three QS systems and up to 11% of the *P. aeruginosa* genome has been reported to be regulated by AHL-based QS (De Kievit, 2009). Among these are genes involved in

biofilm formation, like genes regulating extracellular polysaccharide synthesis, and lysing of cells leading to extracellular DNA. A third biofilm factor influenced by QS is the production of the biosurfactant rhamnolipid, which is necessary for maintaining the channel structure in the biofilm. Biosurfactants have also been shown to be regulated by AHL-based QS in other bacteria, like serrawettin W2 in *Burkholderia cepasia* (Huber et al., 2001*)* and a lipopeptide in *Serratia liquefaciens* (Lindum et al., 1998). *S. liquefaciens* was the first bacterium where surface-associated behaviour was found to be regulated by AHL-based QS (Eberl et al., 1996). This behaviour can involve e.g. swarming, twitching, and sliding motility (Hentzer et al., 2004)

A group of over 70 genes in *P. aeruginosa* are identified as invariably regulated by QS and are referred to as "the QS regulon" (Hentzer et al., 2004). The QS influence of biofilm formation was first reported by Davies et al. (1998), but this has later been a debated subject. Different opinions about the role of QS in biofilm formation are found in the literature, and this can partly be explained by a variation in both biofilm formation and QS conditions between bioassay setups (Hentzer et al., 2004).

1.4.2 Biofilm and antibiotics

Antibacterial treatments should be limited as much as possible in the fish farm industry, as well as in other fields, to avoid development of antibiotic resistant bacteria. The Norwegian Veterinary Institute conducts regularly investigations of antibiotic sensitivity of pathogens of salmonids (Norwegian Veterinary Institute, 2018). Two different antibiotics, consisting of florfenicol and oxolinic acid, are relevant for Norwegian fish farming today (Takle et al., 2015). Oxytetracylin is also being used, but to a much lesser extent (Norwegian Veterinary Institute, 2018). Reduced oxolinic acid sensitivity was reported in *Flavobacterium psychrophilum*, *Yersinia ruckeri*, *Vibrio anguillarum*, and *Aeromonas salmonicida* in 2017. The risk of horizontal transfer of the oxolinic acid resistance genes in bacteria is believed to be low, as it is related to chromosomal mutation. Biofilm bacteria are protected from environmental threats such as UV-radiation and dehydration (Hall-Stoodley et al., 2004). A slime layer functions as a physical barrier against biological threats like grazers or antibiotic substances. The cells in a biofilm have a different phenotype than its planktonic stage, and the biofilm phenotype has been shown to be more resistant against antibiotics. There is an activity gradient from the top layer to the inner layer in the biofilm, with the top layer being the most active (Høiby et

al., 2010). This mirrors the steep oxygen gradient, where the biofilm can be completely anoxic in the inner part. The activity and oxygen gradients can help explain the antibiotic resistance of biofilms. Low oxygen gradients can lead to increased oxidative stress, which can give a higher mutation rates. The high cell density can also increase the mutation rate, by facilitating horizontal gene transfer through conjugation (Burmølle et al., 2014). This can result in new genetic combinations. According to Costerton et al. (1995), some bacteria in biofilms are up to 500 times more resistant against certain antibiotic agents. The ability to create an advantageous microenvironment for themselves, the authors argue, can be viewed as the reason for the bacterial success. Both the role of biofilm formation in the regulation of the virulence of the bacteria and the increased antibiotic resistance of bacteria in biofilms make it highly relevant to study biofilm formation in potential pathogenic bacteria.

2. Aim of study

The aim of this study is to test five *Tenacibaculum dicentrarchi* strains for ability of quorum sensing and further test their ability to form biofilm.

- The five *T. dicentrarchi* strains, obtained from the Norwegian Veterinary institute, will be screened for autoinducer (AHL and AI-2) production in bioassays using *Vibrio harveyi* and *Chromobacterium violaceum* reporter strains. Presence of autoinducers may indicate the ability of quorum sensing in the tested bacteria.

- The biofilm formation of the *Tenacibaculum* strains will be tested using microwell plate assays. Analyses will be performed at different salinities and temperatures as this could influence bacterial growth and biofilm formation.

For these bacteria, both biofilm and quorum sensing activity can be involved in the pathogenesis of fish, and the understanding of these features can be important for finding preventive measures and development of treatment methods alternative to traditional antibiotics.

3. Materials and methods

This study was done partly at the University in Bergen (UiB), Norway, and partly at the University in Ghent, Belgium. Initial work was done at the Veterinary Institute in Bergen (VI).

3.1 Bacteria isolates

Five isolates of *Tenacibaculum dicentrarchi* were tested for quorum sensing (QS) activity. The isolates were provided by the VI and had been genetically confirmed as *T. dicentrarchi*. Four of these isolates were from the VI's archive, and one reference strain was from the National Collection of Industrial Food and Marine Bacteria (NCIMB) (Table 1). The names of the fish farms where the *T. dicentrarchi* strains were isolated are not listed, in accordance to VI's confidentiality agreement.

Table 1. Isolates used in this study, with information provided by the VI.

Isolate number	Information
S-I	Reference strain. NCIMB 14598
S-II	Isolated from salmon in an off-shore fish farm. Some wounds on the fish.
S-III	Isolated from lumpfish tailfin in a land-based fish farm. Salt water. Low mortality.
S-IV	Isolated from lumpfish in land-based fish farm. Salt water.
S-V	Isolated from salmon in off-shore fish farm. There had been an episode of higher mortality after sea lice treatment. Wounds were observed in most of the dead fish.

3.2 Media for bacterial growth

3.2.1 Bacterial cultivation

Marine Broth 2216 (Difco™, 279110) (MB) and Marine Agar 2216 (Difco™, 212185) (MA) were used for cultivation of bacteria at the University in Bergen and the Veterinary Institute. The dry powder media was mixed with Milli-Q water according to instructions given by the producer and autoclaved at 121°C for 15 minutes.

For the studies performed at the University in Ghent, Marine Broth (Carl Roth, CP73.1) and Tryptic Soy Broth (TSB) (Carl Roth, X938.1) were prepared following the manufacturer`s instructions. Lauria-Bertani liquid media (LB) was prepared with 10g/L tryptone (Carl Roth, 8952.2), 5g/L yeast extract (Carl Roth, 2363.2), and 5g/L NaCl. Agar (Biokar diagnostics, 0001097) was added to concentrations specified bellow. Stock solutions of Kanamycin disulfate salt (Km) (Sigma-Aldrich, K1876-5G) and Chloramphenicliol (Chl) (Sigma-Aldrich, C0378-25G) were made by dissolving the antibiotics in Milli-Q water, and subsequent filtration through a 0.2µm Whatman filter. The solutions were stored at 4°C. All media were prepared with Milli-Q water and autoclaved for 20 minutes at 121°C.

3.3 Cultivation and storage of *Tenacibaculum dicentrarchi*

The *T. dicentrarchi* isolates were cultivated with MA or MB at 15°C. When the bacteria were cultivated in broth, flasks were incubated with 200rpm (if not otherwise mentioned). For long term storage, the isolates were first grown in broth until they reached the exponential phase. Then cultures were mixed with glycerol to a final concentration of 15% glycerol, allocated to cryotubes (Thermo SCIENTIFIC, Nunc Cryotube Vials) in 1ml portions and finally stored in a -80°C freezer. This procedure was used both in Bergen and in Ghent. All the bacteria stored in Ghent were destroyed after the completion of the experiments.

3.4 Bacteriological tests

Various initial bacteriological analyses were performed at the Veterinary Institute in Bergen during the autumn of 2017. Different growth temperatures were tested, and Gram-staining, oxidase and catalase tests, API-zym, and oxylin acid resistance tests were performed.

To test the growth at different temperatures, the five isolates were inoculated on Marine Agar at 4°C, 15°C, 22°C, and 30°C. Two plates were used for 15°C, one with bacterial material from a single colony, and one with several colonies. The isolates were also grown on blood agar at 15°C. The growth on all plates was examined after 3 days.

API-zym (bioMérieux) was performed following the manufacturer`s instructions. All the bacteria prepared in tubes for API-zym were also spread on MA plates and incubated at 15°C with the API-zym strips. The strips were incubated until the next day, and a drop of zym-A and zym-B was added to each well. The strip was placed in bright light for 10 minutes before analysis. The colours of the wells were compared to a reference picture and given a score. The scores were listed in a table, showing the specific profile for all tested bacterial strains. The five isolates were also tested for resistance against oxolinic acid by spreading them on MA and placing an oxolinic acid wafer in the middle of the petri dish. The inhibition zones around the wafer were measured after 3 days.

3.5 Growth curve and cell number

Growth curves for the isolates were made by measuring optical density (OD) at a wavelength of 600nm. The cell numbers were estimated by using CASY, counting colony forming units (CFU), and by using a microscope counting chamber (hemocytometer). Cell count results were then correlated with OD measurements.

3.5.1 Cultivation of bacteria

Marine Broth (MB) (Difco) was prepared as described above, and 50ml was transferred into Erlenmeyer bottles before autoclaving. Two parallels were prepared for each isolate. Every flask was inoculated with one colony grown on Marine Agar (MA) (Difco) at 15°C. The cultures were incubated at 15°C and shaken at 200 rpm overnight.

The main cultures were inoculated by transferring 1ml of the starter culture to a 1L Erlenmeyer flask containing 200ml MB. These were incubated at the same conditions as described above and grown for 2-3 days.

3.5.2 Growth curve based on optical density (OD)

The growth of the cultures was monitored by measuring the optical density at 600 nm (OD_{600}) with a spectrophotometer at two to three hours intervals for a total of 31 hours. For every measurement 1ml culture was used, and 1ml un-inoculated medium was used as a blank. The numbers were plotted against time using GraphPad prism 6 (Figure 6).

3.5.3 Colony forming units (CFU) and CASY

CFU was estimated for the *Tenacibaculum* isolate S-I at five different time points. Start and main cultures were prepared as described above. Ten ten-fold dilutions were made using PBS and 100 µl of each dilution were added to MA plates. This was done after 10, 13, 17, 19, and 23 hours, and the OD was measured at the same time. The petri dishes were incubated at 15°C, and colonies were counted after three days of incubation.

Cell numbers were estimated using CASY (INNOVATIS, CASY model TT 150 µl) for the same culture of the isolate S-I as used for testing CFU. The measurements were done at the same time points as listed above. Dilutions of the cultures were made by adding 10µl culture to 10ml filtered CASY-ton (OLS OMNI Life Science, 5651808). If the test results were too high for the instrument to read, the bacterial culture was diluted by an additional ten-fold.

3.6 Screening for Quorum Sensing molecules

The screening work to test for autoinducers was performed at the University in Ghent. Reporter strains were used both to detect AHL and AI-2.

3.6.1 Reporter and control strains

Chromobacterium violaceum CV026 was used as a reporter strain to screen for AHL-production. *C. violaceum* is a Gram-negative bacterium that is normally found in soil (McClean et al., 1997), and reacts mainly to AHL with a length between C4 and C8. The phenotypic response to AHL is production of the purple pigment violaceum. *C. violaceum* CV026 is a Tn-5 mutant that does not produce AHL itself and can thereby be used as a reporter strain. The positive control used with reporter strain *C. violaceum* was *Yersinia ruckeri*, which produces at least eight different AHLs, with N-(oxoheptanoyl)-L-homoserine lactone being the most abundant (Kastbjerg et al., 2007).

Yersinia ruckeri was used as a positive control when *C. violaceum* CV026 was used as reporter strain. *C. violaceum* CV026 was grown on LB plates (1.5%) with 20ppm Km at 28°C, and *Y. ruckeri* CCUG14190 was grown on TSA (1.5%) at 28°C. The bacteria were incubated at 28°C with 130rpm when grown in the corresponding broth.

Six different *Vibrio harveyi* strains were used to screen for AI-2 and AHL (Table 2). The strains were grown on MA (1.5%) at 28°C, or in MB with 130rpm at 28°C. A final concentration of 20ppm Chl was used for the *Vibrio harveyi* strain JMH634.

Table 2. *Vibrio harveyi* strains used as reporters and controls.

Strain	Mutation	Phenotype
BB120	Wild type	
MM30 (Surette et al., 1999)	*luxS*::Tn5	AI-2 (-)
BB152 (Bassler et al., 1997)	*luxM*::Tn5	AHL (-)
JMH634 (Henke and Bassler, 2004)	Δ*luxM* Δ*luxS* $cqsA_{Vh}$::Cm^r	AHL (-), AI-2(-) (dark strain)
BB886 (Bassler et al., 1997)	*luxPQ*::Tn5	AI-2 receptor (-)
BB170 (Bassler et al., 1997)	*luxN*::Tn5	AHL receptor (-)

3.6.2 Bioassay with *Chromobacterium violaceum* CV026 as reporter strain

Bioassays with *Chromobacterium violaceum* CV026 were used both for detection of AHL production (QS) and AHL degradation (QQ). The agar plates with embedded *C. violaceum* were prepared by first adding 18ml LB agar (1%) with 20ppm KM to 50ml falcon tubes that were placed in water baths at 45°C. Before the agar was poured into petri dishes, 2ml over-night culture of CV026 was added and carefully mixed. The agar was dried in a fume hood for about 20 minutes, and wells were made with the backside of pipette tips. One well was made in every agar plate for the QS bioassay, and four in every plate for the QQ bioassays. One additional plate with five wells was made to test the sensitivity by adding artificial AHL in different concentrations in every test.

To prepare supernatant, Erlenmeyer flasks with 20ml MB were inoculated with the *Tenacibaculum* strains. The flasks were incubated at 16°C with shaking at 130rpm for 48 hours, until the stationary phase was reached. *Y. ruckeri* was grown in TSB and incubated at 28°C with 130rpm over night. Both from the *Tenacibaculum* cultures and the *Y. ruckeri*

culture, 10ml were allocated to falcon tubes and centrifuged at 4000G for 10 minutes. The supernatants were filtered through 0.2µm Whatman filters. 10ml MB was treated the same way and used as negative control. A 100mM stock solution of N-Hexanoyl-DL-homoserine lactone (AHL) (Sigma-Aldrich, 106983-28-2) was prepared using DMSO. Ten-fold dilutions were made with sterile Milli-Q water.

The test for AHL production was performed by adding 50µl of the supernatant from the *T. dicentrarchi* cultures to the wells in the LB agar plates with embedded *C. violaceum* CV026 reporter strain. 50µl of supernatant from *Y. ruckeri* was used as a positive control and 50µl MB was used as a negative control. A further control was prepared by using AHL solutions (N-Hexanoyl-DL-homoserine lactone) with concentrations of 10nM, 100nM, 1µM, 10µM, and 100µM, which were added to five wells in one agar plate. All plates were checked for violacein after 48 hours of incubation at 28°C.

A similar bioassay was done with *Chromobacterium violaceum* CV026 to investigate if the *Tenacibaculum dicentrarchi* strains degrade the AHL N-Hexanoyl-DL-homoserine lactone. Supernatant from the five *Tenacibaculum* strains were prepared as described above but additionally AHL was added to the supernatant to a final concentration of 10µM. The same was done with uncentrifuged *Tenacibaculum* cultures. Both the cultures and the supernatants containing AHL were incubated at 16°C and shaking at 130 rpm for 24 hours. The incubated samples were used in a bioassay with CV026 embedded in 1% LB-agar. 50 µL of the supernatant containing AHL and 50 µL of the culture with AHL were added to two wells each in one agar plate, with two parallel plates. The plates were incubated for 24 hours at 28°C, and the purple zones around the wells were measured and compared.

3.6.3 Bioassay with *V. harveyi* reporter strains

The *V. harveyi* strains BB120, MM30, BB152, and JMH634 were cultivated in MB as described above (3.6.1) for 24 hours. These strains are all mutated in genes involved in production of one or several autoinducers, but react to both AHL and AI-2 (Table 2). The *V. harveyi* cultures were diluted 10 times by adding 2 ml of culture to 18 ml MB. This was further incubated for 4 hours at 28°C and shaking at 130 rpm, and then diluted to an OD_{550} of 0.1. Supernatant from the *Tenacibaculum* isolates and *V. harveyi* strains BB120 and

JMH634 were prepared as described above. The supernatant from the *Tenacibaculum* isolates were tested with the *V. harveyi* reporter strains MM30, BB152, and JMH634. Supernatant from BB120 was used as a positive control, and un-inoculated MB and supernatant from JMH634 were used as negative controls. 100 µl of the diluted reporter strains and 100 µl of the supernatant from *Tenacibaculum* strains or controls were added to four parallel wells in a black 96-well microwell plate. The luminescence was measured using a Tecan microplate reader every hour for six hours. This experiment was performed three times, and the OD_{600} of the *Tenacibaculum* cultures was noted every time.

The same procedure was followed for an experiment with *V. harveyi* BB170 and BB886 as reporter strains. These two strains are mutated in genes involved in AI receptors (Table 2). *V. harveyi* strain BB170 does not react to AHL, and BB886 does not react to AI-2. This time the measurement was done for 12 hours, and MB was used as the only negative control. The experiment was done twice; the first time with reporter strain cultures diluted to an OD_{550} of 0.1, and the second time with an OD_{550} of 0.05.

3.7 Biofilm assays

Biofilm formation was measured with bioassays using microwell plates (Nuclon™ Delta Surface, Thermo Scientific) and salt water solutions with concentrations of 25%, 50%, 75%, and 100% sea water. The salt water solutions were prepared by mixing autoclaved sea water with autoclaved Milli-Q water. Bacterial cultures were prepared as described above (3.5.1 Cultivation of bacteria. 175 µl of the salt water and 25 µl culture or 25 µl sterile Marine Broth as negative control were added to each well in microwell plates, with up to five parallels for every tested combination. Three variations of the bioassay with different parameters were tested, including differences regarding plate setup, incubation time, and nutrients added to the wells. For every bioassay the plates were incubated at 10°C, 15°C, and 20°C. The second variation (Biofilm test 2) was repeated three times.

3.7.1 Preparation of microwell plates

Biofilm test 1:

Three microwell plates were prepared with four parallels for each combination of salt concentration and strain and incubated for 2 weeks. Four parallel wells were used for every strain tested.

Biofilm test 2:

The outmost wells were filled with 200 µl water in order to avoid biased result due to drying effects at the edge of the well plate. The plates were incubated with moist paper underneath them, in plastic bags. The salt water solutions were prepared as described in paragraph 3.7, and two different salt concentrations were tested together on one plate. Five parallel wells were used for every combination of salt concentration and bacterial strain, and plates were incubated for two weeks.

Biofilm test 3:

This test was changed in regard of nutrients available for the bacteria. Marine Broth (Difco) at half strength was prepared by doubling the amount of water added. Four different salt concentrations adding up to 10, 18, 26, and 35g NaCl/L were prepared by adding NaCl. In the outermost wells 200 µl water was added, as done for Biofilm test 2, and the plates were incubated in plastic bags with moist paper. Five parallel wells were used, and the plates were incubated for three weeks. After one and two weeks 100 µl of the content of the wells was discarded, and 100 µl of sterile medium was added.

3.7.2 Reading of microwell plates

The OD_{595} of the culture in the wells was measured using a Tecan microplate reader, before rinsing and colouring of the plates. A microplate washer was used to empty and rinse the wells three times with 250µl Milli-Q water. The plates were than tapped against paper to remove extra water. To stain the wells, 200µl crystal violet (5%) was added to each well, accept the outermost wells when these were only filled with water. Extra care was taken to avoid droplets of crystal violet on the edge of the wells, because this easily drained into the wells when ethanol-acetyl was added. After 30 minutes, the plates were again rinsed three times with 250 µl Milli-Q water using the microplate washer. Then 200

µl ethanol-acetone (70%-30%) was added to the wells. After 15 minutes the plates were measured again and the OD values at 595nm were recorded.

3.8 Statistics

All statistical analyses were performed in Excel. A two-sided (students) t-test was done to test for statistical significance (p<0.05). Graphs were made by using GraphPad Prism v6.01 for Windows (GraphPad Software, CA, USA).

4. Results

 Five *Tenacibaculum dicentrarchi* strains obtained from the Norwegian Veterinary institute were characterized, examined for their quorum sensing activity and biofilm formation potential, by using bioassay studies. Classical bacteriological tests and growth analyses were done to characterize standard microbial features of the five *T. dicentrarchi* strains.

4.1 Bacteriological tests and growth analysis

A variety of microbiological tests were applied in order to characterize the strains provided by the VI. Bacteriological tests were performed for the five different strains and the growth at 15°C was monitored for over 30 hours. During the growth experiments, sub-samples were taken to measure bacterial abundance using three different methods, a microscope counting chamber, plating for CFU, and CASY. The five *Tenacibaculum* isolates, S-I to S-V, all stained Gram-negative. Bacterial growth on MA was analysed at four temperatures (4, 15, 22, and 30°C). The highest growth was observed at 15°C and 22°C. At 4°C all strains showed only minimal growth, and at 30°C no growth could be observed. The colonies had a weak yellow colouration. All five *Tenacibaculum* strains were oxidase positive, and strains S-IV and S-V were weakly catalase positive, indicated by minimal bubbling observed using a stereo microscope with low magnification. The strains were tested positive for alkaline phosphatase, leucine arylamidase, valine arylamidase, phosphatase acide, and Naphthol-AS-BI-phosphohydrolase (Figure 5). Weak positive results with a score under 3 (5 being the highest) were observed for esterase (C4), esterase lipase (C8, lipase (C14), and cystine arylamidase. All strains had similar scores for all enzymes tested, including the reference strain from NCIMB (S-I). No inhibition zone could be observed around the oxolinic acid wafer in any of the five strains.
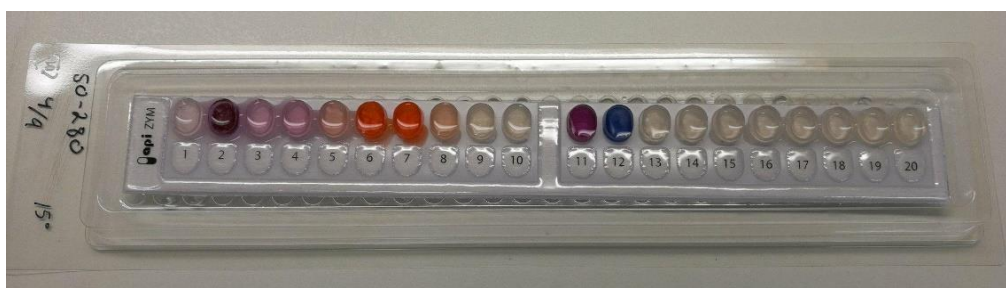

Figure 5. Picture of the API-zym strip, which was used to test the *Tenacibaculum* isolate S-I.

4.1.1 Growth curve

There was a clear and consistent difference in the growth curves for the five *T. dicentrarchi* isolates (Figure 6). The isolates S-I, S-III, and S-IV (reference strain from NCIMB) all had similar patterns. They entered the exponential phase after 5-10 hours and reached the stationary phase after about 25 hours. At this point the optical densities at 600nm were around 2.5. The isolates S-II and S-V displayed a slower growth and reached the exponential phase after approximately 15 hours. Both isolates reached the stationary phase after more than 30 hours, with an OD_{600} below 2. Isolate S-II showed a slower growth than S-V and had a lower optical density.
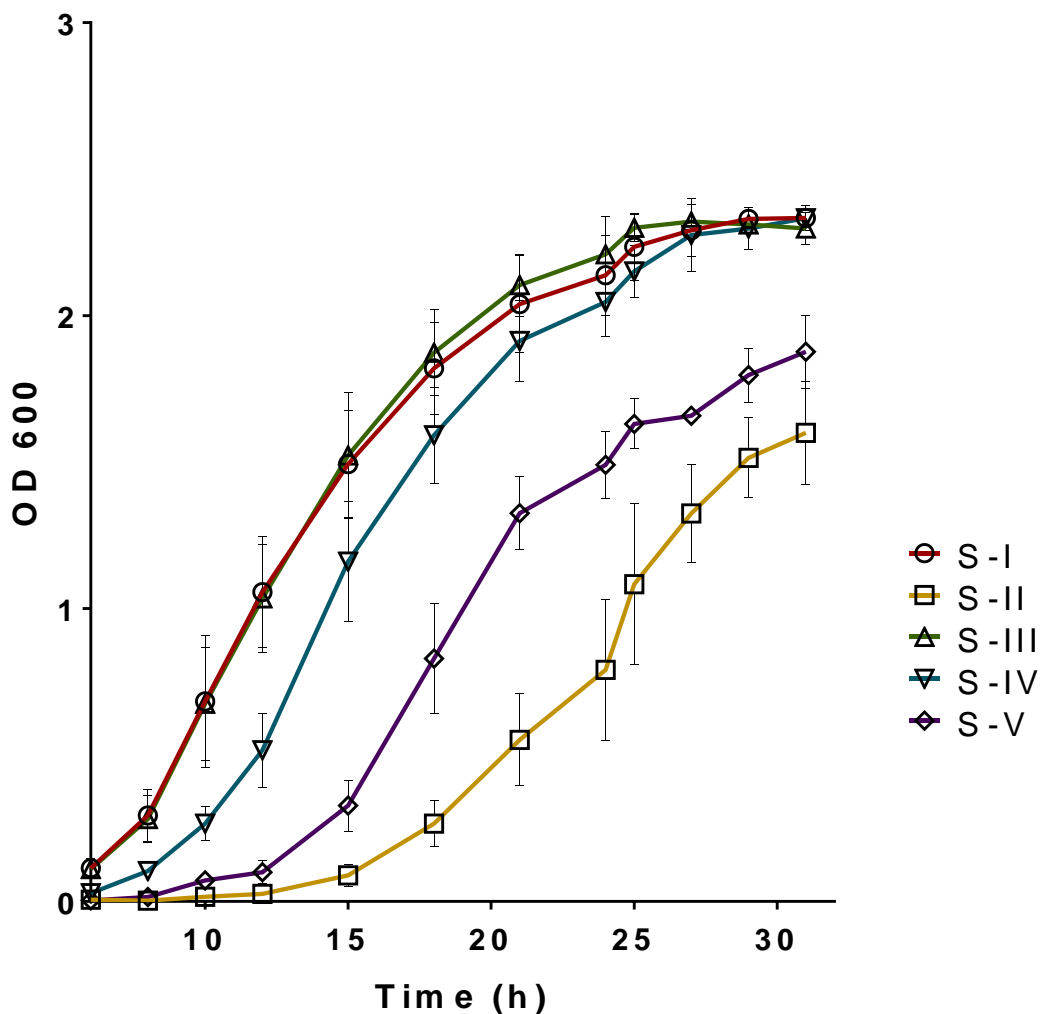

Figure 6. Growth curves for the five *Tenacibaculum dicentrarchi* isolates measured as OD_{600} for 31 hours. Data is presented as average of two parallels.

4.1.2 CFU and numbers from counting chamber

Cell number estimations were done for the strains S-I, S-III, and S-IV from the same cultures as used for growth curve analysis in 4.1.1 (Figure 6). The cell counts obtained from counting chamber ranged from 1×10^8 in the *Tenacibaculum* strain S-IV, to 3×10^8 in S-I after 21 hours (Figure 7a). The OD of the cultures at this point was highest for the strain S-III (Figure 6), and the relationship between OD and cell number is included in Figure 7b. When cells were counted with the microscope, differences in cell size for the different strains was observed and cell numbers at given densities were different in the three cultures.
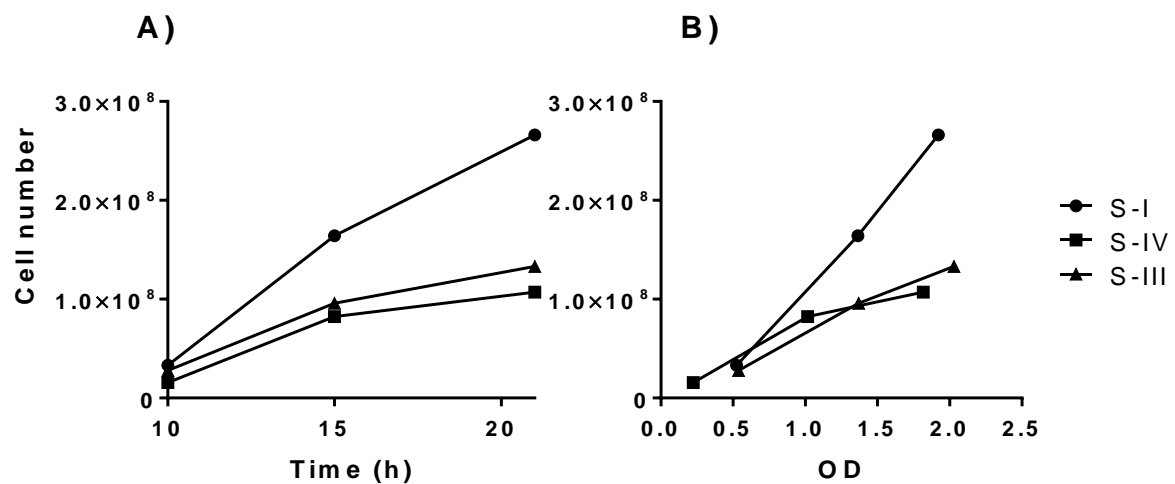

Figure 7. Bacteria number obtained for 3 *T. dicentrarchi* strains using a counting chamber in the microscope and plotted against incubation time (A) and OD (B).

Because the *Tenacibaculum* strains did not grow as colonies on the MA plates for the CFU measurements the first time, an additional test was done with strain S-I. For this test, cell number was estimated using CASY, and the culture was also plated on MA plates at corresponding timepoints. The OD_{600} was measured, and the cell number estimates at the different densities are represented in Figure 8. Due to slimy growth on the MA plates, the CFU was difficult to estimate precisely. The growth resulting from the different dilutions are listed in Table 3. At an OD_{600} of 1.6, one colony was observed on the MA plate with a 10^{-4} dilution, giving a CFU estimate of about 10^4 cells per ml. Measurements with CASY at the same OD gave a cell number of about 2.4×10^9. The cell

number obtained from CASY for the strain S-I (Figure 8) was about ten times higher than the number counted in counting chamber (Figure 7).
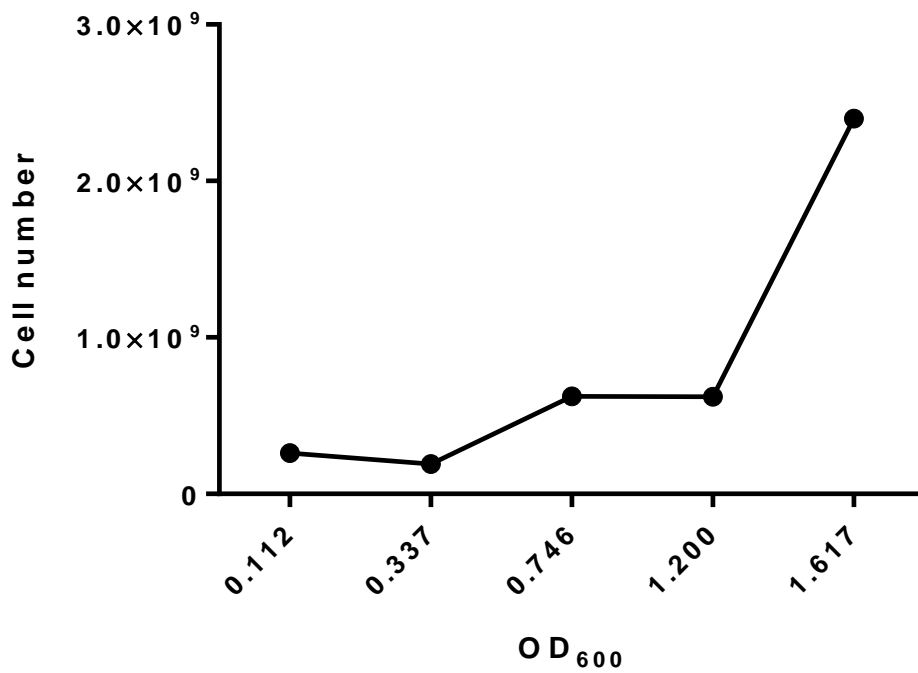
Figure 8. Cell number obtained using CASY and plotted against OD_{600} for a culture of the *T. dicentrarchi* strain S-I.

Table 3. Colony forming units (CFU) per ml for the isolate S-I at five different optical densities

Dilution	OD 0.112	OD 0.377	OD 0.756	OD 1.200	OD 1.617
10^-2	Overgrown	Overgrown	Overgrown	Overgrown	Overgrown
10^-3	163	471	800	Overgrown	Overgrown
10^-4	0	0	0	8	1
10^-5	0	0	0	0	0
10^-6	0	0	0	0	0

4.3 Quorum sensing results

To test whether the *Tenacibaculum dicentrarchi* strains (Table 1) were potentially capable of cell-to-cell communication via quorum sensing, screening for autoinducers were performed using *Vibrio harveyi* strains (Table 2) and *Chromobacterium violaceum* CV026.

4.3.1 Bioassays with *Chromobacterium violaceum* CV026

The five *Tenacibaculum dicentrarchi* isolates were screened for AHL-production by using reporter strain *Chromobacterium violaceum* CV026 embedded in agar. Supernatant from *Yersinia ruckeri* was used as a positive control. The sensibility of the experiment was tested with five concentrations of artificial AHL (N-Hexanoyl-DL-homoserine lactone), in five wells in the same agar plate. Purple areas were observed around wells with artificial AHL concentrations down to 1μM (Figure 9). The supernatant from *Y. ruckeri* was used as positive control and induced a purple circle comparable with the colouration induced by 1μM artificial AHL (Figure 9). When the five *Tenacibaculum* isolates were tested, no purple colouration was observed, and all strains were therefore tested negative for AHL production.
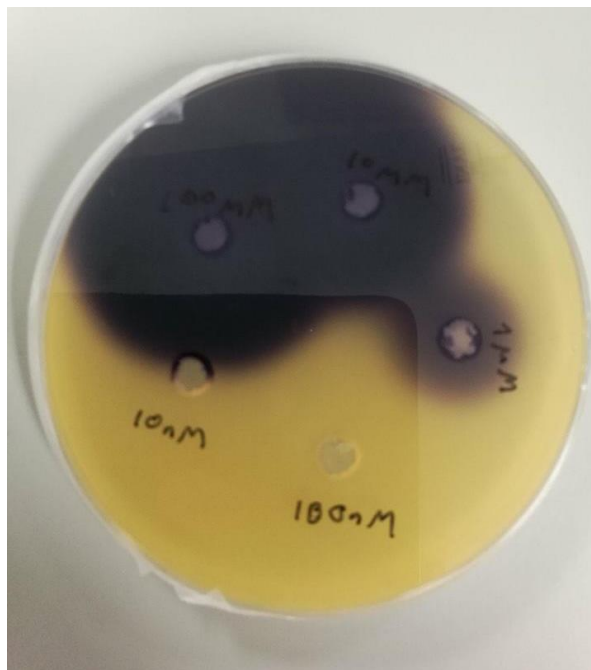

Figure 9. The sensitivity of reporter strain *Chromobacterium violaceum* CV026 embedded in agar was tested by using artificial AHL at five concentrations (100μM, 10μM, 1 μM, 100nM, and 10nM). Purple colouration was observed around the wells with AHL concentration down to 1μM.

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4.3.2 Degradation of AHL by the *Tenacibaculum* strains

Artificial AHL was added to cultures and to supernatant from the five *Tenacibaculum* strains and incubated overnight. Later, this was added to wells in agar plates embedded with the reporter strain *Chromobacterium violaceum* CV026. The size of the purple zones induced in the reporter strain by the AHL between the two treatments was similar (Figure 10). Both zones were the same size as the zone induced by the negative control (MB containing AHL, not shown), and the *T. dicentrarchi* strains thereby tested negative for degradation of this AHL molecule.
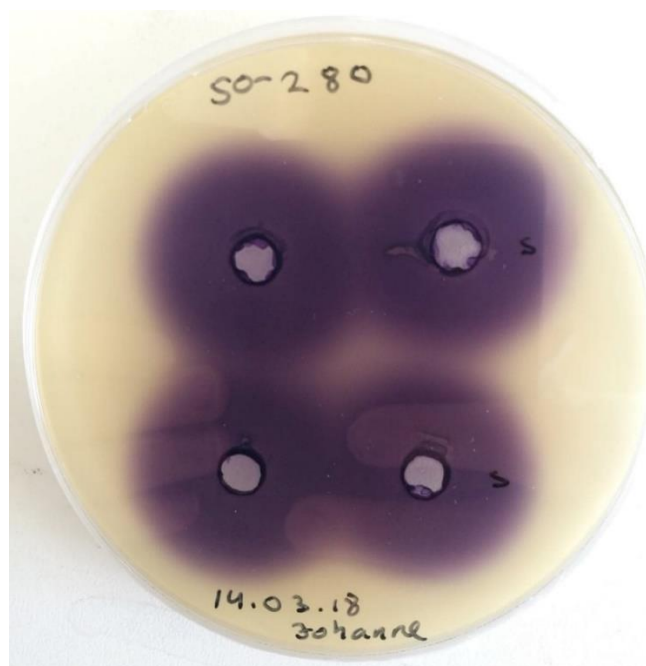

Figure 10. Picture of an agar plate from the bioassay testing the *Tenacibaculum* strains for degradation of AHL. Artificial AHL was added to culture and supernatant from the *Tenacibaculum* strains and incubated overnight. The two wells on the left side on this picture contained culture from the *Tenacibaculum* isolate S-I, with added AHL. The two wells on the right side contained supernatant supplemented with AHL.

4.3.3 Bioassays with *V. harveyi* reporter strains MM30, BB152, and JMH634

Screening for production of either AHL or AI-2 was done by adding supernatant from the *Tenacibaculum* strains to a 96-well plate with *Vibrio harveyi* JMH634, MM30, and BB152 as reporters. The response in luminescence was measured for six hours. A t-test was performed to examine whether there was a significant difference between the luminescence measured for the negative control (MB) compared to the five

Tenacibaculum strains. This experiment was performed three times, using cultures at different densities. The results are presented as three separate tests because different cultures of both reporter and test strains are used, resulting in different ODs, AI concentrations and reporter protein concentrations. These are referred to as Test 1, Test 2, and Test 3 throughout this paragraph and results are listed in Table 4 a-c.

Four of the five *Tenacibaculum* strains tested positive with all the three reporter strains in at least one of the tests (Table 4). The only exception was isolate S-V, which was only tested positive with the *V. harveyi* MM30 reporter strain. This *Tenacibaculum* strain did not reach a cell density as high as the other strains in Test 1 (Table 5). Isolate S-II was the only one that was tested positive for all reporter strains in all tests except BB521 in test 2. This reporter strain had a low density in Test 2 (Table 6), and none of the tested *Tenacibaculum* strains had significantly different results from the negative control, and neither did the positive control (Figure A.1 in appendix). All the *Tenacibaculum* isolates were positive in all three tests with the *V. harveyi* reporter strain MM30, which is incapable of AI-2 production. Supernatant from the *V. harveyi* strain JMH634 was used as an additional negative control in these experiments. This strain is a so called "dark strain" that does not produce autoinducers. Supernatant from cultures of these strains should therefore not contain AHL or AI-2. All the three *Tenacibaculum* strains that tested negative when compared to MB in Test 2 (Table 4), were significantly different from JMH634 supernatant. The positive control *V. harveyi* BB120 induced about 9 to 18.5 times as much luminescence in reporter strain *V. harveyi* MM30 compared to MB as negative control (Table 4). With reporter strain JMH634, BB120 induced 3 to 20 times the luminescence of the negative control. For reporter strain BB152, about 1.1 times the induced luminescence was observed in the positive control compared to the negative control.

Table 4. Luminescence in three *Vibrio harveyi* reporter strains induced by supernatant from the five *Tenacibaculum* strains (S-I to S-V) measured after six hours in three separate tests (Test 1-3). *V. harveyi* strain BB120 was used as positive control. The luminescence is given in percentage compared to marine broth (MB) as negative control, and the standard deviations (Std) are listed. Green colour indicates significant difference from MB used as negative control ($p < 0.05$). No colouration indicates no significant difference. The OD_{600} of the *Tenacibaculum* cultures used in the three tests are listed in Table 5.

a) Test 1:

Strain:	MM30 %	Std	BB152 %	Std	JMH634 %	Std
S-I	624.21	231.50	65.03	7.26	256.72	12.01
S-II	576.61	211.44	120.78	47.57	410.29	26.40
S-III	522.39	203.76	139.97	6.99	253.50	32.08
S-IV	681.76	349.84	118.91	5.44	247.21	32.96
S-V	339.49	137.29	36.57	5.37	43.71	2.72
BB120	1525.04	628.61	114.01	7.70	2073.31	418.18

b) Test 2:

Strain	MM30 %	Std	BB152 %	Std	JMH634 %	Std
S-I	1193.14	183.17	123.35	106.97	324.63	90.63
S-II	1055.07	180.79	95.38	59.50	240.43	88.10
S-III	903.86	158.05	72.41	43.62	162.63	60.99
S-IV	572.14	253.01	73.78	46.59	146.97	44.37
S-V	183.26	119.00	40.19	56.18	64.28	44.88
BB120	894.33	18.58	150.81	95.90	691.51	214.02

c) Test 3:

Strain	MM30 %	Std	BB152 %	Std	JMH634 %	Std
S-I	880.98	492.98	213.82	20.30	466.37	150.02
S-II	567.28	336.01	143.81	25.17	333.73	150.44
60-1003	500.42	294.97	122.66	9.00	208.78	108.16
S-IV	441.23	240.28	112.30	11.43	177.62	105.96
S-V	334.00	186.20	94.32	12.77	106.86	44.92
BB120	1860.16	1046.31	113.46	12.95	318.83	155.47

The supernatant from the *Tenacibaculum* strains started inducing luminescence in the reporter strains after two to three hours. For the experiments with *V. harveyi* MM30, BB152, and JMH634 reporter strains, the luminescence was measured for six hours. Based on the measured luminescence values (Figure 11, and Figure A.1 and A.2 in appendix), the luminescence is still increasing at the last time point of the experiment. This is most obvious for reporter strain JMH634 in test 3, where the luminescence did not increase until 3 to 4 hours after the start of the measurement, both in the test strains and in the positive control (Figure A.2 in appendix). The differences between the luminescence induced by the tested strains and the negative control is also increasing. Because of this, the luminescence was measured for 12 hours in bioassays with *V. harveyi* BB886 and BB170 as reporter strains (Figure 13 + Figure A.3 in appendix).

Table 5. Table of OD$_{600}$ measurements for cultures of the five *Tenacibaculum* strains (S-I to S-V) used in the tests screening for AI-production with reporter strains *Vibrio harveyi* MM30, BB152, and JMH634. All *Tenacibaculum* strains were used for all three tests.

Strain	Test 1	Test 2	Test 3
S-I	1.394	0.430	1.248
S-II	1.313	0.350	1.315
S-III	1.339	0.410	1.253
S-IV	1.370	0.420	1.280
S-V	0.590	0.410	1.235

Table 6. Tables with ODs of cultures used in the bioassay with *Vibrio harveyi* BB886 and BB170 as reporter strains. The OD was measured at 600nm for the cultures of *Tenacibaculum dicentrarchi* strains S-I to S-V, and at 550nm for the *Vibrio harveyi* control strains MM30, BB152, and BB120.

Strain	Test 1	Test 2
S-I	1.300	1.380
S-II	1.430	0.313
S-III	1.245	1.378
S-IV	1.277	1.341
S-V	1.100	0.687
MM30	1.445	NA
BB152	1.239	NA
BB120	1.522	1.490

A)

BB152

B)

MM30

Legend:
- S-I
- S-II
- S-III
- S-IV
- S-V
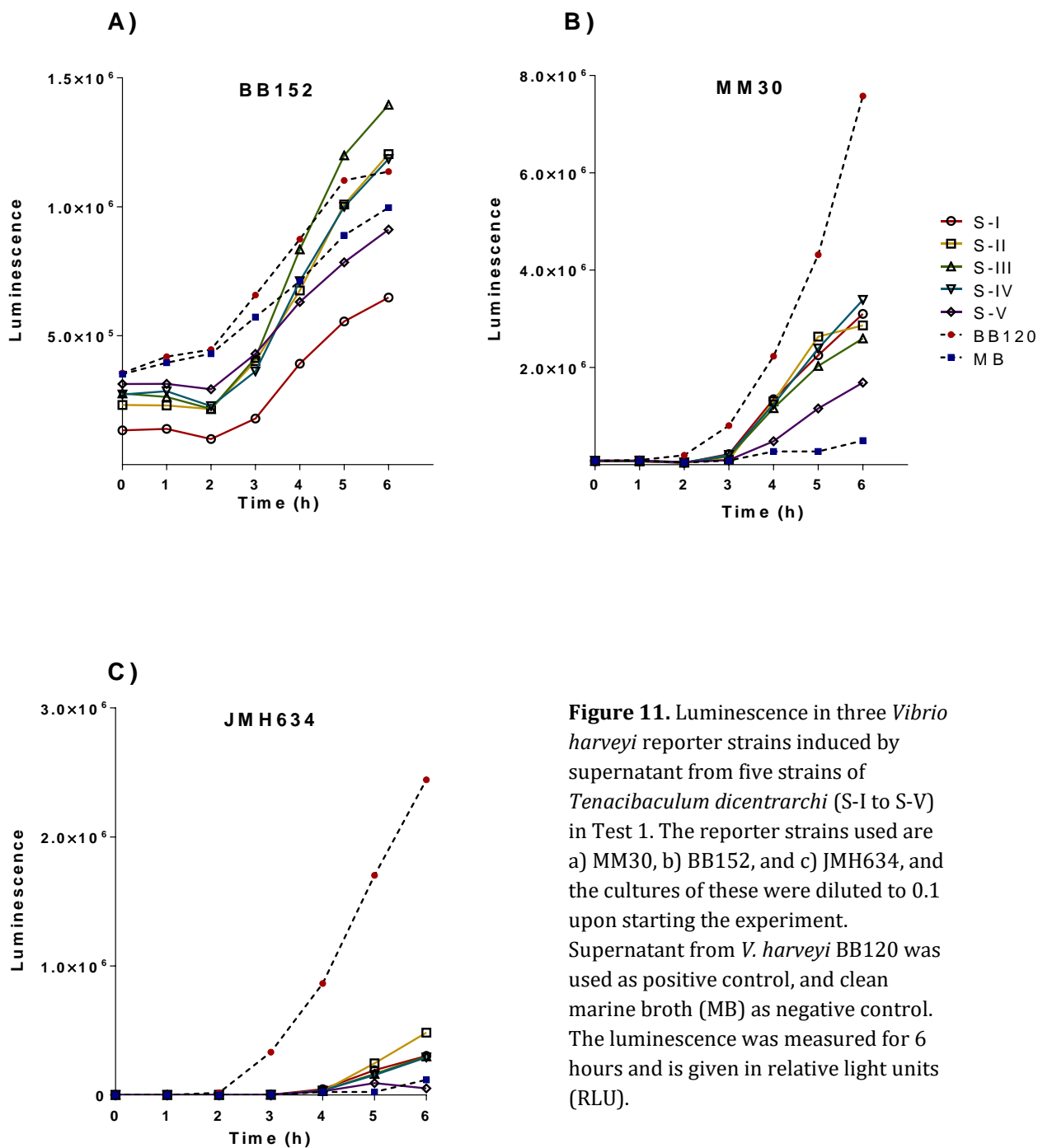- BB120
- MB

C)

JMH634

Figure 11. Luminescence in three *Vibrio harveyi* reporter strains induced by supernatant from five strains of *Tenacibaculum dicentrarchi* (S-I to S-V) in Test 1. The reporter strains used are a) MM30, b) BB152, and c) JMH634, and the cultures of these were diluted to 0.1 upon starting the experiment. Supernatant from *V. harveyi* BB120 was used as positive control, and clean marine broth (MB) as negative control. The luminescence was measured for 6 hours and is given in relative light units (RLU).

4.3.4 Bioassays with *V. harveyi* BB170 and BB886 as reporter strains

A screening for autoinducers discriminating between AHL and AI-2 was done in a similar way as the bioassay described above (4.3.3). Supernatant from the *Tenacibaculum* strains was added to cultures of the reporter strains in microwell plates and luminescence was measured over time. The reporter strain *V. harveyi* BB170 has a mutation in the AHL receptor and was therefore used to detect AI-2 production and *V. harveyi* BB886 has a mutation in the AI-2 receptor and was used to detect AHL production.

V. harveyi BB170 and BB886 were diluted to OD_{550} 0.1 in test 1 (Figure A.3 in appendix), and OD_{550} 0.05 in test 2 (Figure 13), and the luminescence was measured every hour for 12 hours. The ODs of these cultures before centrifuging and filtering are given in Table 5. The luminescence induced in the first test with reporter strain BB170 was too high to detect after eleven hours for all *Tenacibaculum* strains tested, and for some strains already after seven hours (Appendix A.3).
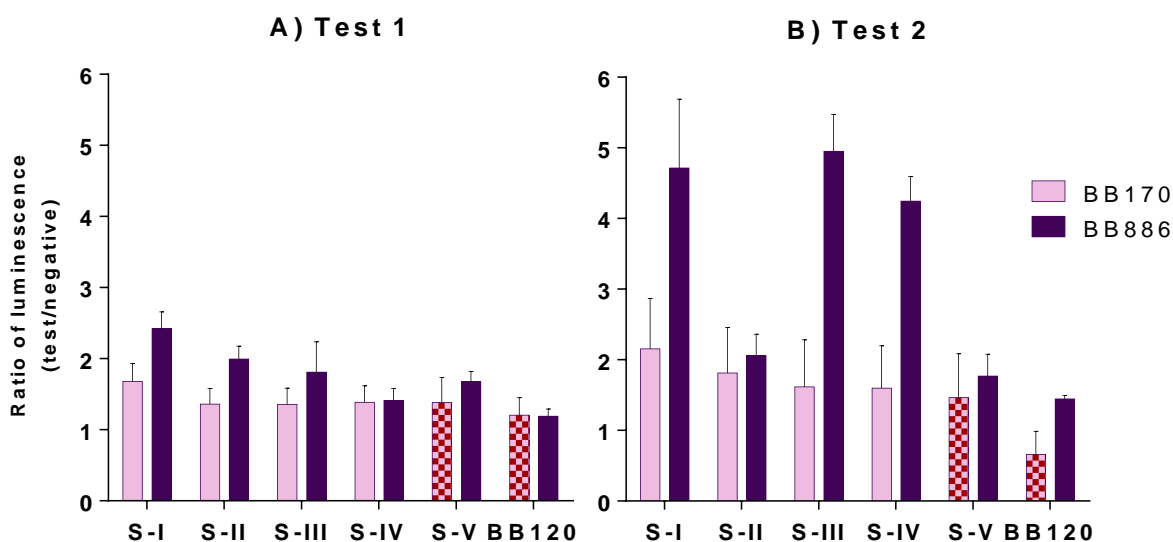

Figure 12. Luminescence measured in *Vibrio harveyi* BB170 and BB886 after the addition of supernatant from the *Tenacibaculum dicentrarchi* strains (S-I to S-V) and incubation of A) 8 and B) 12 hours in two separate tests. The reporter strains were diluted to an OD_{550} of 0.1 in Test 1 (A), and 0.05 in Test 2 (B). The luminescence is given related to that induced by MB used as negative control (test/negative). *V. harveyi* BB120 was used as positive control. The bars with a red pattern indicate no significant difference from the negative control, while no pattern indicates a significant difference ($p<0.05$). The results are shown as average of four replicates for each strain.

All five *Tenacibaculum* strains induced luminescence that was significantly different (p<0.05) from the negative control (MB) when tested with *V. harveyi* BB886 in both the tests performed (Figure 12). The *Tenacibaculum* strains S-I, S-II, S-III and S-IV were also positive when tested with reporter strain *V. harveyi* BB170.

The positive control *V. harveyi* BB120 did not induce luminescence in *V. harveyi* BB170 in any of the two tests. Though it was positive when tested with BB886, it is clear from Figure 14 that it peaked after about 10-11 hours, at $2x10^6$ (RLU). The *Tenacibaculum* strain S-I in comparison, peaked at about $6x10^6$ (RLU) after 10 hours.
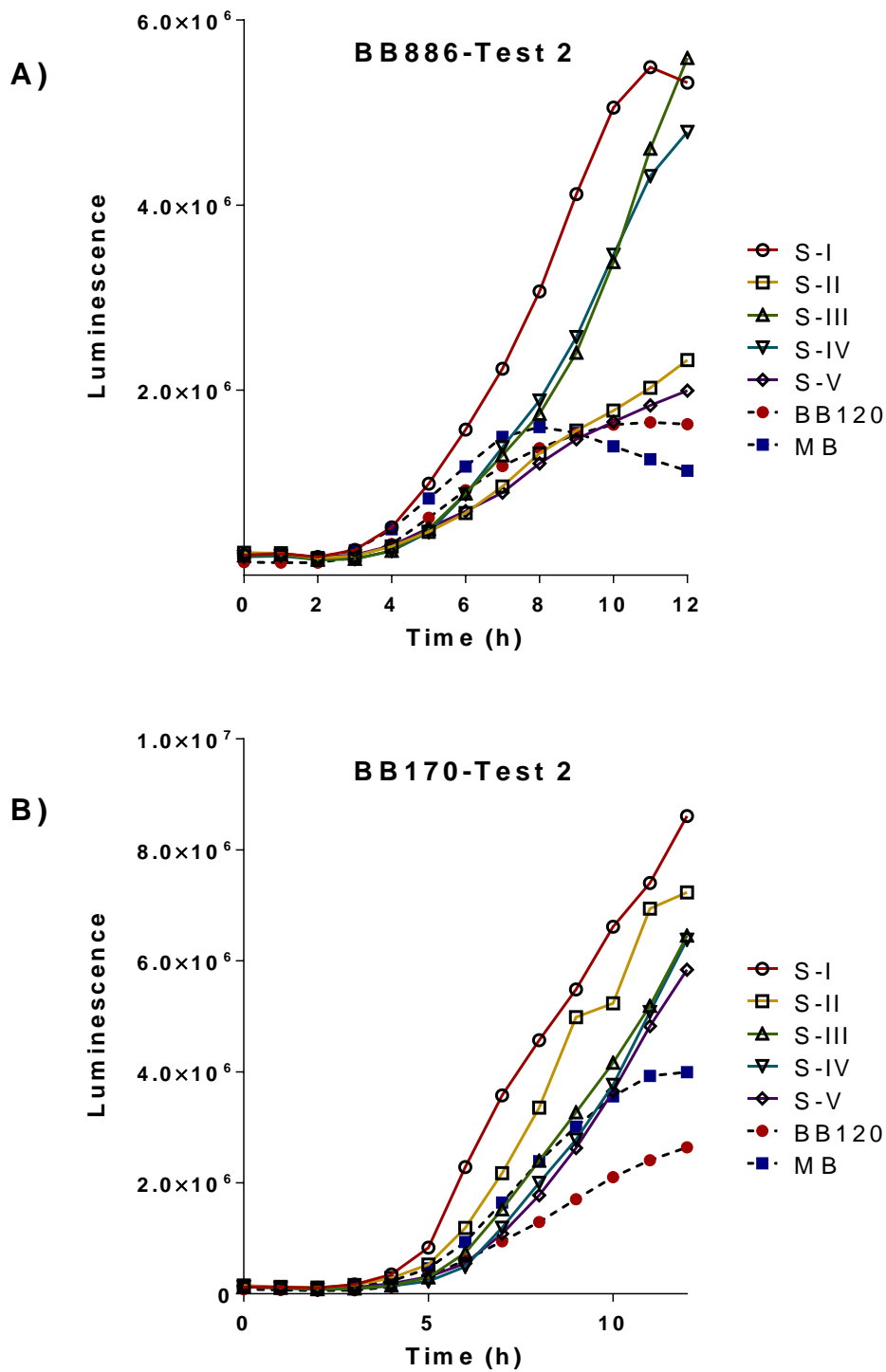
Figure 13. Luminescence induced the in two reporter strains A) *Vibrio harveyi BB886*, and B) *V. harveyi BB170*, after the addition of supernatant from the five *Tenacibaculum* strains (S-I to S-V). The luminescence was measured every hour for 12 hours. The reporter strains were diluted to an OD_{550} of 0.1 in Test 1 (A), and 0.05 in Test 2 (B). V. ha*rveyi* BB120 was used as positive control, and MB as negative control. Four replicate wells were used for every strain tested. Luminescence values in wells with strain S-II after 9 hours, and strain S-I after 11, hours were above the detection limit and therefore not included in the graph. Thus, the points in the graph representing these time points are assumed to be lower than the actual luminescence. Luminescence is given in relative light units (RLU).

In the first bioassay with *Vibrio harveyi* strains BB886 and BB170 as reporters, supernatant from *V. harveyi* MM30 and BB152 were used as additional controls. With both these reporter strains, MM30 and the positive control BB120 followed about the same pattern (Figure 14 and 15), right above the negative control MB. The supernatant from *V. harveyi* BB152 induced the most luminescence in both the reporter strains. Note that the test with the two reporters were performed with supernatant from the same control cultures.
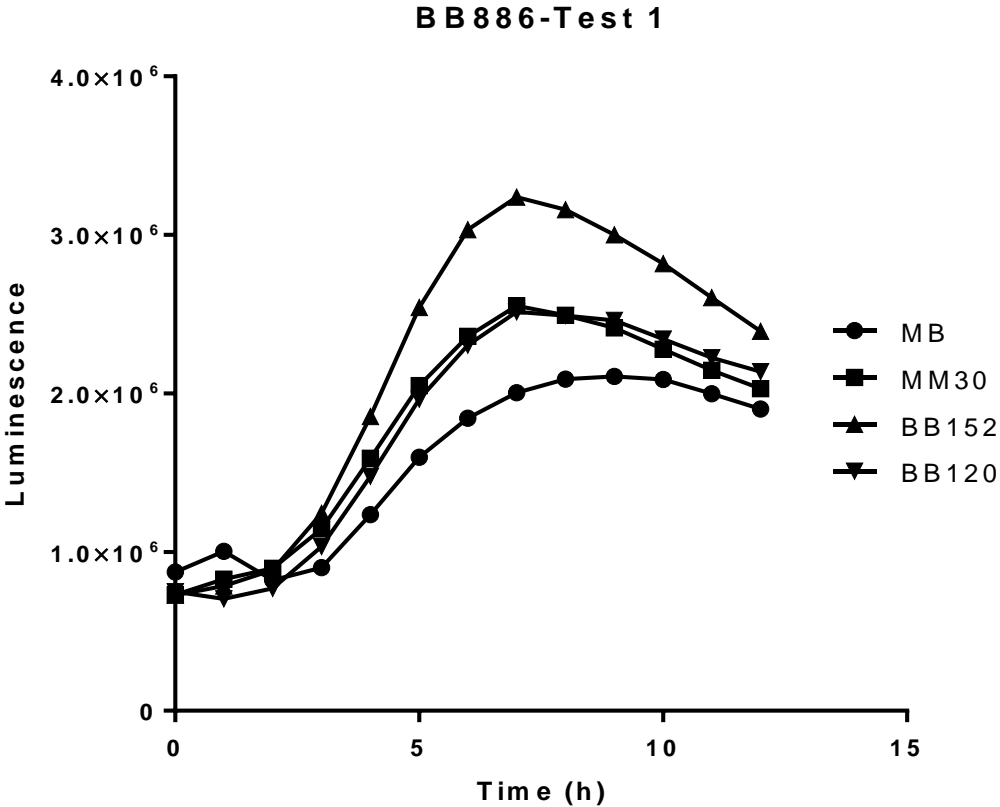

Figure 14. Luminescence induced in the first test with reporter strain *Vibrio harveyi* BB886 by supernatant from the *V. harveyi* strains BB120, BB152, and MM30. MB was used as a negative control. The luminescence was measured for 12 hours and is given in relative light units (RLU). The culture of the *V. harveyi* reporter strain BB886 was diluted to an OD_{550} of 0.1.
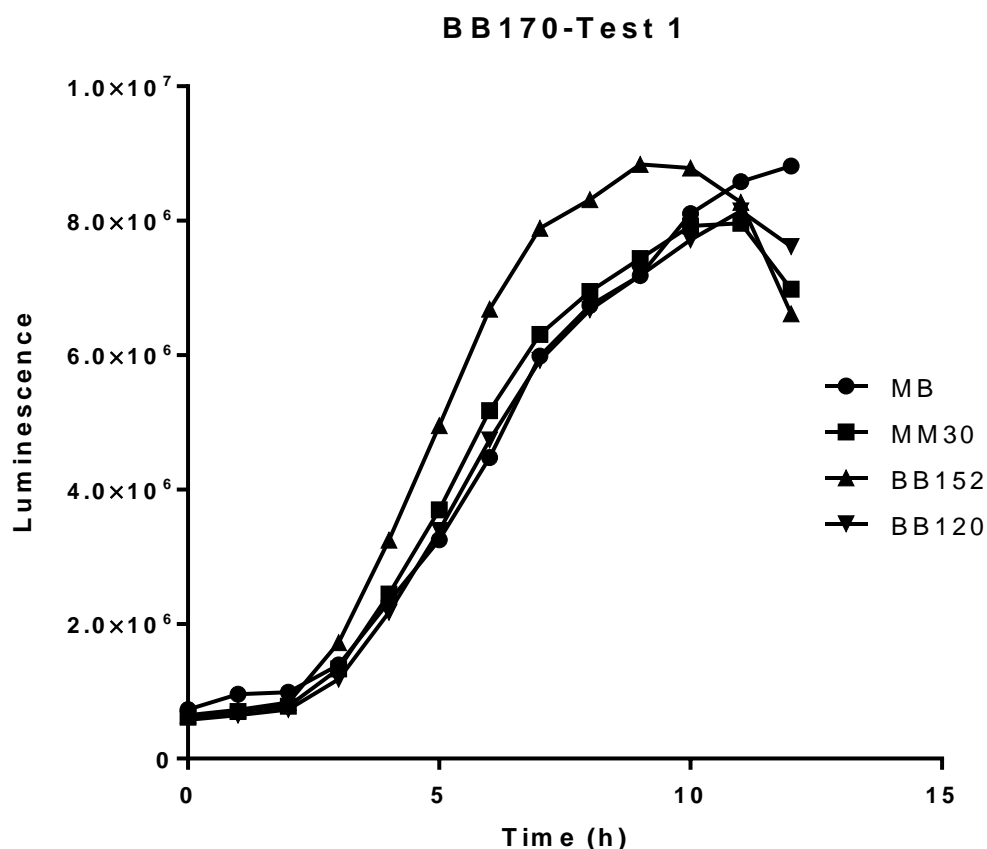
Figure 15. Luminescence induced in first test with the reporter strain *Vibrio harveyi* BB170 by supernatant from the *V. harveyi* strains BB120, BB152, and MM30. MB was used as a negative control. The reporter strain culture was diluted to an OD_{550} of 0.1, and the luminescence was measured for 12 hours and is given in relative light units (RLU).

4.4 Biofilm assay

The five *Tenacibaculum* strains were tested for their biofilm formation potential in three different variations of a biofilm assay (Biofilm test 1-3). In Biofilm test 1, 175 µl salt water solution with different concentrations of sea water and 25 µl of culture was added to each well in a microwell plate, and these were incubated for two weeks. The analysis of Biofilm test 1 revealed that the outmost wells dried out faster than the inner wells and thereby increased the colour strength obtained from the staining procedure. Because of this weakness, the outmost wells were filled with 200 µl of clean water instead of culture in the next two assays (Biofilm test 2 and 3). Biofilm test 3 was performed with half strength of marine broth in four salt concentrations, instead of salt water. Due to limitations of the staining procedure, additional colour residue from the well edges occasionally entered

the well when ethanol-acetone solution was added. These outliers were removed from the dataset before statistical analyses.

The viability of at least one strain was tested for every bioassay, by plating culture from one well of every salt concentration to MA plates. Although it was not possible to obtain a CFU number due to a lack of growth in colonies on agar plates, growth was observed at all salt concentrations, including 25% sea water.
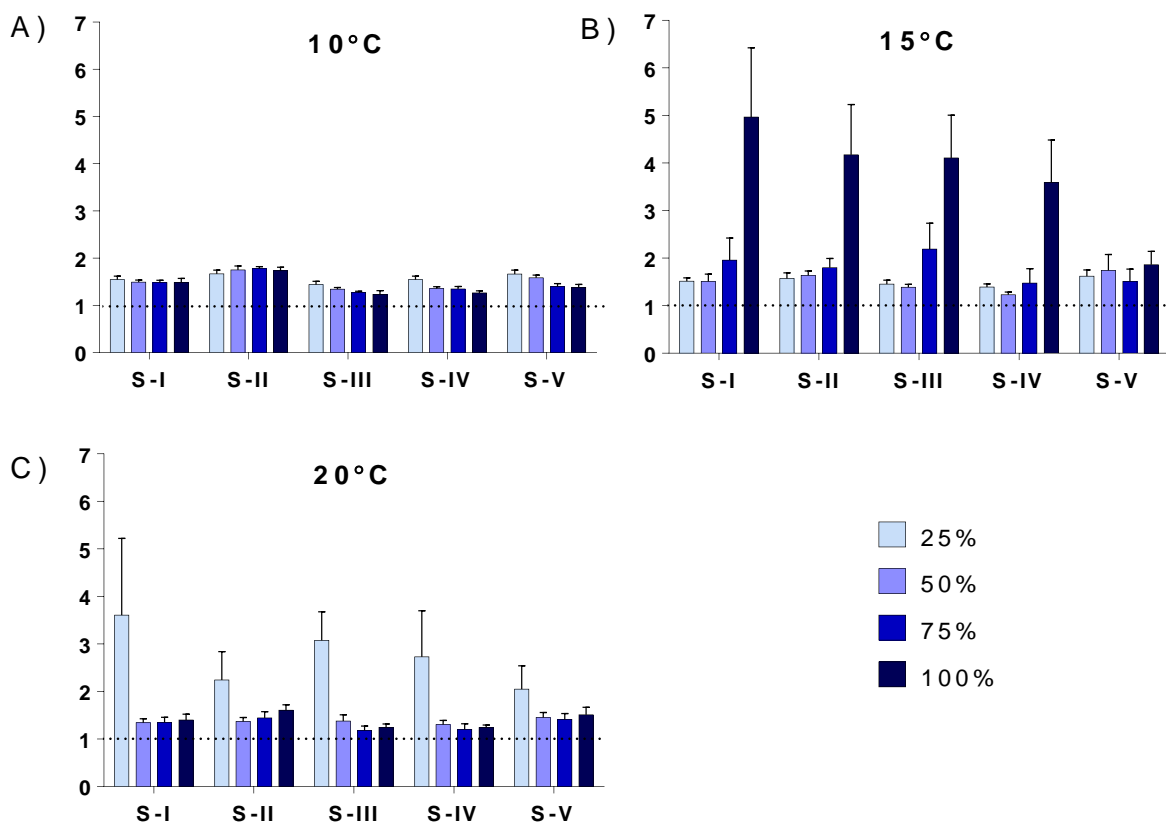

Figure 16. Biofilm test 1. Biofilm growth of the *Tenacibaculum dicentrarchi* strains (S-I to S-V) was tested in microwell plates incubated at A) 10°C, B) 15°C, and C) 20°C. The plates were prepared with 25%, 50%, 75%, and 100% concentrations of sea water mixed with Milli-Q water, shown in the different shades of blue. The OD_{595} was measured after colouration with crystal violet, and the amount of biofilm is given in relation to the negative control (test/negative control). The level of the negative control is indicated by the dotted line at position 1 at the y-axis. Biofilm levels for all tested strains and under all temperatures were significantly different from the negative control ($p < 0.05$).

All the five *Tenacibaculum* strains investigated in Biofilm test 1 were tested positive for biofilm formation at the four salt water concentrations, and the three temperatures tested (Figure 16 and Figure A.4 in appendix). The increased levels of biofilm formation for all strains grown at 100% salt water at 15°C and with 25% salt water at 20°C are most likely biased due to the drying effect in the outmost wells ("border zone effect"). This bias applies to the results represented both in Figure 16 and Figure A.4. Row G and H horizontally on the microwell plate were both treated with 100% salt water, but the results indicated that biofilm production was significantly different between the two rows (Black rectangle in Figure 17). The same effect could be seen in row A and B with 25% salt water, for the plates incubated at 20°C.
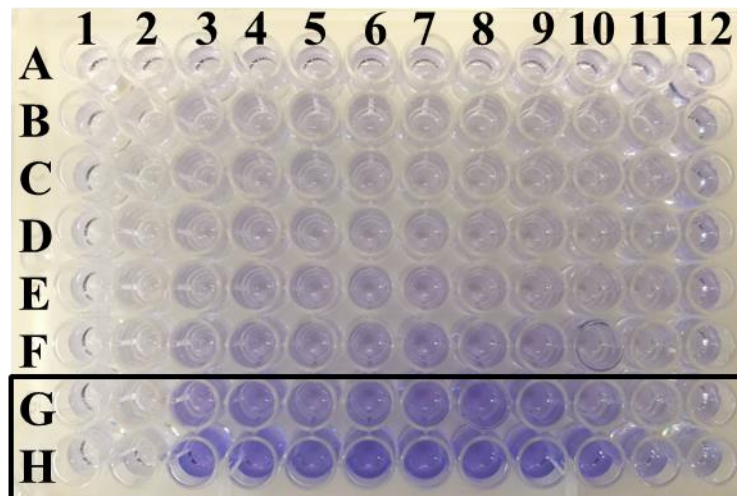

Figure 17. Biofilm test 1. Picture of microwell plate from Biofilm test 1, incubated at 15°C. The wells in horizontal columns (A-H) were filled with 175 µl 25% (A and B)), 50% (C and D), 75% (E and F), and 100% (G and H) salt water. The wells in vertical columns (1-12) were additionally filled with 25 µl of culture from the different *Tenacibaculum* strains or marine broth (MB) as negative control. 1 and 2 are the negative control, and the strains S-I, S-II, S-III, S-IV, and S-V are added in two vertical columns each, in the listed order. The black rectangle highlights the "boarder zone effect".
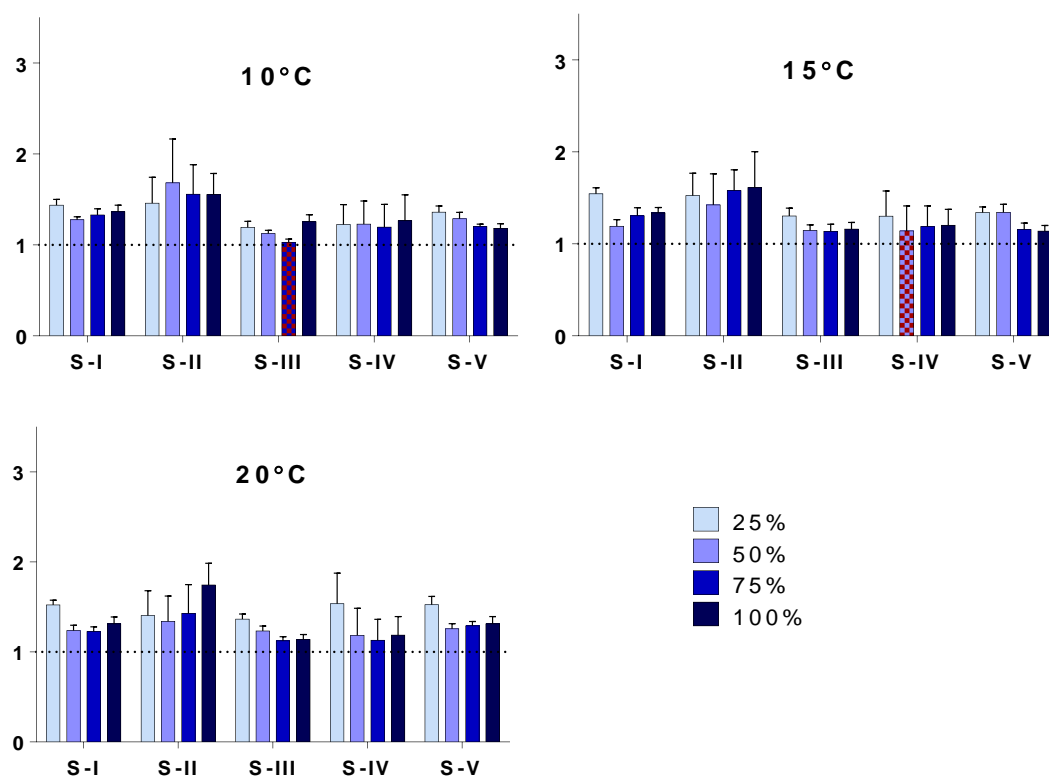
Figure 18. Colouration of wells in a 96-well microplate from Biofilm test 2, where the five *Tenacibaculum* isolates (S-I to S-V) were incubated at A) 10°C, B) 15°C, and C) 20°C for two weeks. The OD$_{595}$ was measured after colouration with crystal violet, and the amount of biofilm is given in relation to the negative control (test/negative control). The dotted line in 1 on the y-axis indicates the level of the negative control. The strains were tested with sea water was diluted to four concentrations, indicated by the different shades of blue. Bars with a red pattern indicate no significant difference (p<0.05), while all the other results were significantly different compared to the negative control. The outmost wells in the microplate were filled with water, to prevent drying.

Biofilm test 2 was performed three times (Figure 18, and Figure A.5 and A.6 in appendix). All the tested strains showed a colouration that was up to two times as strong as that of the negative control when stained with crystal violet. The only exception was strain S-II at 100% salt water, incubated at 20°C, which was just above two times the strength of the colouration of the negative control (Figure A.6 in appendix). In the same test, all the strains showed less colouration than the negative control when tested with 75% and 100% salt water. These two salt concentrations were tested on the same microwell plate, and the results are most likely biased due to contamination of the negative control. In the two replicates of Biofilm test 2, the same cultures of the five *Tenacibaculum* strains were used for two tests. Therefore, these must be viewed as technical replicates rather than biological replicates (Figure 18, and Figure A.5 in appendix). Almost all the strains were

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tested positive at all temperatures and salt concentrations in these assays, except for two strains at either 50% or 75% salt water (indicated by a red pattern in Figure 18 and A.5. All the strains were tested positive for all salt concentrations at 20°C, in the three repetitions of this experiment and strain S-II showed the highest biofilm formation, especially at salt concentrations of 75% and 100%. This can be seen from Figure 18 and could also be observed on the microwell plates, both before and after colouration (Figure A.7 and A.8 in appendix).

Biofilm test 3 was performed only once, and several of the plates seemed to have a contaminated negative control (Figure 19). This was especially apparent in the plates with 75% and 100% salt water, incubated at 15°C and 20°C. Biofilm test 2 was the least biased in terms of contamination and "edge effect", and was therefore repeated three times.
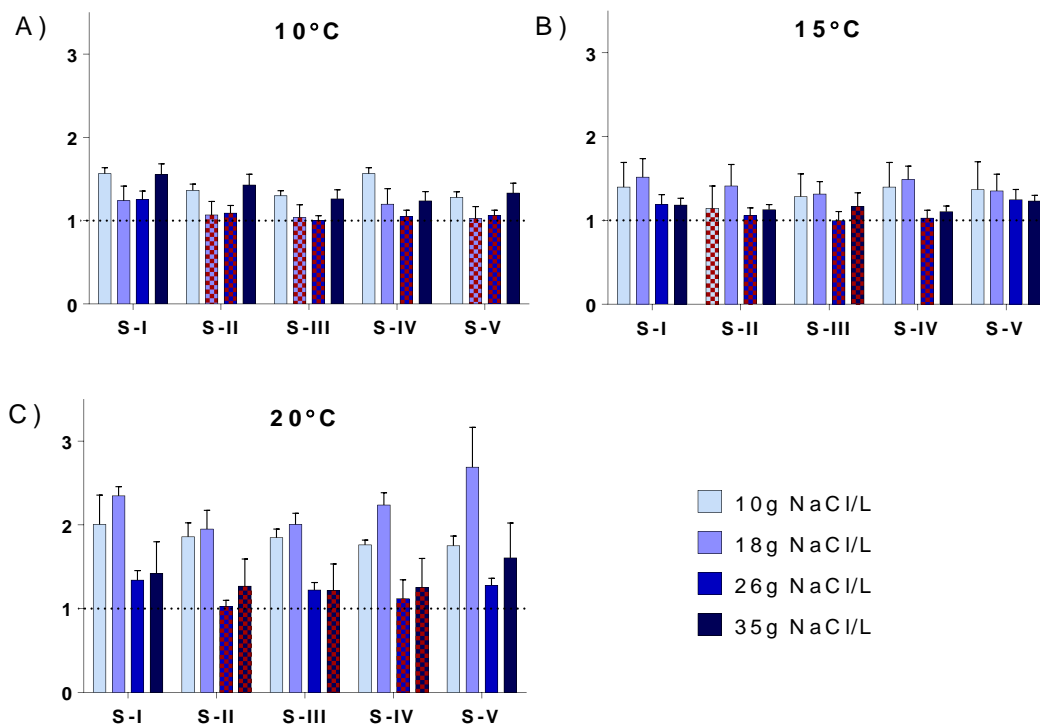

Figure 19. Biofilm formation in the five *Tenacibaculum dicentrarchi* strains (S-I to S-V) incubated at A) 10°C, B) 15°C, and C) 20°C, in Biofilm test 3. Microwell plates were prepared with Marine Broth (Difco) made to half strength. The broth was added with salt at four different concentrations, shown in different blue colours. The OD_{595} was measured after colouration with crystal violet, and the amount of biofilm is given in relation to the negative control (test/negative control). A dotted line at position 1 on the y-axis indicates the level of the negative control. The red pattern indicates no significant difference from the negative control ($p < 0.05$), while all other results were significantly different compared to the negative control.

5. Discussion

The quorum sensing (QS) and quorum quenching (QQ) experiments conducted for this thesis are all *in vitro* studies, like most QS studies performed until today (Defoirdt et al., 2008). Identification of a potential regulation of genes involved in pathogenesis via QS would require *in vivo* studies. However, *in vitro* studies have the advantage to be conducted under controlled conditions. Factors that can be regulated *in vitro*, e.g. pH, are beyond the control of the investigator in *in vivo* experiments. A high pH of a bacterial culture is known to destabilize the AHLs present (Byers et al., 2002). Taga and Bassler (2003) hypothesized that the multiple AI signals present in *Vibrio harveyi* are helping the bacteria to hinder the disruption of quorum sensing *in vivo*. If one of the three AIs is degraded by chemical or biological factors, the presence of the two other AIs could balance out the negative effect of this.

The virulence by *V. harveyi* in larvae of the brine shrimp *Artemia franciscana* has been shown to be decreased by altering the QS through an inactivation of the AI-2 system, but not by inactivating the AHL system (Defoirdt et al., 2008). QS mediated regulation by the three AIs in *V. harveyi* is dependent on the same master regulator, but interestingly regulations of some genes require the presence of all three autoinducers. This could be due to different affinities for the activated LuxR protein in the gene promotors. The reason for the necessity of a functional AI-2 system could be a disturbance of the AHL molecules in the host, making the total AI concentration too small for *V. harveyi* to express virulence factors. To stop virulence by *Vibrio harveyi* in gnotobiotic rotifers (*Brachionus plicatilis*), both the AI-2 and the AHL system must be inactivated, indicating that the host is not capable of interfering with these autoinducers. These examples show that it is not enough to understand the *in vitro* nature of the bacteria in question, the impact from the environment and specific hosts must also be taken in consideration. However, testing for the presence of autoinducers as done in experiments for this master thesis, might provide a promising first step in understanding a possible role of bacteria found in association with diseases, but where the virulence is not yet fully understood, such as *Tenacibaculum spp.* Besides quorum sensing activity, biofilm formation can also be involved in the pathogenesis of fish, and thus the biofilm potential of the *Tenacibaculum* strains isolated from different Norwegian fish farms was also investigated in course of this thesis.

Understanding both can be helpful for the development of treatment methods as an alternative to traditional antibiotics.

Quorum Sensing

Chromobacterium violaceum CV026

Supernatant from the *Tenacibaculum* strains were added to wells in agar with embedded *Chromobacterium violaceum* CV026, to test for AHL production. No pigments were observed in the CV026 reporter strains, and therefore this test did not indicate production of AHL. The major AHL in *C. violaceum* has been identified as N-hexanoyl-L-homoserine lactone, where the R in Figure 3a would be CH_3CH_2 (McClean et al., 1997). The CV026 strain was shown to react to AHL with N-acyl side chains with a length of 4-8 C atoms. The AHLs tested with a side chain of 10C-14C did however not induce and even inhibit the pigment production. Results from the experiment performed for this thesis can thus not be informative regarding potential production of these AHLs. The control chosen for this bioassay in addition to supernatant from *Yersinia ruckeri* was artificial N-hexanoyl-DL-homoserine, which induced pigment production in concentrations down to 1µM. In experiments profiling the AHLs in *Y. ruckeri*, at least 8 different types were found (Kastbjerg et al., 2007). N-(oxooctanoyl)-L-homoserine lactone was the most abundant AHL, and N-(3-oxoheptanoyl)-L-homoserine lactone and N-(3-oxononayl)-L-homoserine lactone were also found. The supernatant from *Y. ruckeri* induced therefore as expected a small purple zone around the well in the CV026 embedded agar in this experiment, which was comparable to the zone induced by artificial AHL at a concentration of 1µM.

Vibrio harveyi reporter strains

The reporter strains used in the QS bioassays have different mutations making them unable to either produce or react to specific autoinducers. In the first bioassay, reporter strains were chosen to investigate whether the *Tenacibaculum* strains produce autoinducers or not. *Vibrio harveyi* MM30, BB152, and JMH634 used in this bioassay are all mutated in different genes involved in autoinducer production. MM30 does not produce AI-2 (Surette et al., 1999), and BB152 is incapable of producing AHL (Bassler et al., 1994). JMH634 produces neither of the two autoinducers, and has additionally a mutation in the gene coding for CqsA, the synthase responsible for the CAI-1 autoinducer

(*cholerae* autoinducer 1) (Henke and Bassler, 2004). Using an experimental setup with these reporter strains allows only detecting the presence of autoinducers but does not give information about the type of autoinducer being produced. As all the *Tenacibaculum* strains induced luminescence in at least one of the reporter strains, they most likely produce some type of autoinducer that *V. harveyi* can detect. The three reporter strains displayed different sensitivities to the supernatant of the tested strains, and different levels of basal luminescence (measured with marine broth as negative control). Being a triple mutant, the *V. harveyi* reporter strain JMH634 would in theory be expected to have the lowest bioluminescent level in the negative control. In this experiment, this was not observed for *V. harveyi* JMH634, but instead reporter strain *V. harveyi* BB152 showed the lowest basal luminescence. The cultures of the three reporter strains were all diluted to an OD_{550} of 0.1, and by doing so an equal bacterial concentration can be assumed. The genes coding for autoinducer sensor proteins in these reporter strains are also the same, and therefore there should not be a noticeable difference in the sensitivity. Why the basal luminescence was not the lowest in the triple mutant remains unclear. As mentioned above the assumption regarding which strain has the lowest level of basal luminescence is based on the number of mutated autoinducer genes in the different reporter strains. This information is based on the literature, and the reporter strains used for the experiments presented in this thesis have not been additionally genetically tested for type and number of autoinducer genes present.

In the second bioassay, *V. harveyi* reporter strains BB886 and BB170 were used, which are both mutated in genes coding for autoinducer sensor proteins. These strains therefore only react to the two remaining AI's found in *V. harveyi.* Because the third AI found in this species (CAI-1) is believed to be specific for the *Vibrio* genus, it can be assumed that CAI-1 is not responsible if luminescence is induced by the *Tenacibaculum* supernatants. Bassler et al. (1997) tested the luminescence in *V. harveyi* BB170 and BB886 induced by supernatant from several other *Vibrio* species. In their experiments, the supernatant from *V. harveyi* BB152 induced only 1.0% of the luminescence induced by the positive control *V. harveyi* BB120 in the reporter strain *V. harveyi* BB886. When BB170 was used as reporter strain, BB152 induced 120% of the luminescence compared to BB120. BB170 is a *luxN* mutant that lack the ability to response to AHL, while BB886 has a mutation in the *luxPQ* gene, and is therefore not reacting to AI-2 (Bassler et al., 1994). The strain BB152

does not produce AHL, and therefore only induced luminescence in BB170 in the experiment performed by Bassler et al. (Bassler et al., 1997).

The QS bioassay with the *V. harveyi* reporter strains BB170 and BB886 was performed twice for this thesis. In the first experiment, additional controls in form of supernatant from *V. harveyi* BB152 and MM30 were used. BB152 induced luminescence significantly different ($p < 0.05$) from the MB as negative control, both when *V. harveyi* BB170 and BB886 were used as reporter strain (after six and eight hours of measuring, respectively,). The supernatant from MM30 only induced a significantly different strength in luminescence when BB886 was used as reporter, which is consistent with the literature, as MM30 produces only AHL and not AI-2. The positive control *V. harveyi* BB120, however, did not induce significantly different luminescence in reporter strain BB170 when these bioassays were done. It must be noted that the ODs of the reporter strains were too high in the first bioassay experiment, and this could be a part of the reason for these unexpected results. The background luminescence in the reporter strains cultures with high density masks the luminescence induced by the supernatant from the strains tested, which makes it unlikely to obtain significant results even if there are autoinducers present in the test strains. Still, four of the five *Tenacibaculum* strains tested positive for AI-2 production when BB170 was used as reporter strain.

Unexpectedly, BB152 induced bioluminescence in reporter strain BB886, indicating that some features in one of these two bacterial strains are not in consensus with the literature. The results from BB152 in the first QS bioassay with *V. harveyi* was also not as expected, and there were difficulties in growing the bacteria to desired density. I therefore suggest that aspects with the strain used as *V. harveyi* BB152 might have been the reason for the unexpected results, although the addition of supernatant from this strain to the BB886 reporter strain was only done once, and not tested further.

In summary, the results from this quorum sensing experiment showed that all the *Tenacibaculum* strains were tested positive for AHL production when BB886 was used as reporter, and three *Tenacibaculum* strains were tested positive for AI-2 production when BB170 was the reporter strain. None of the strains were tested positive for AHL production with reporter strain *C. violaceum* CV026, which is most sensitive to short-chain AHLs with a side chain of 4-8 C atoms. Altogether, this indicates that some of the

tested *Tenacibaculum* strains might produce both autoinducer AHL and AI-2, and that the AHL molecules did not have a side chain of 4-8 C atoms.

A previous study on QS i *Tenacibaculum maritimum* shows production of short-chain AHLs (Romero et al., 2010*).* This is the first observation of AHLs in a pathogen in the Cytophaga-Flavobacterium-Bacteriodetes (CFB) cluster. These results were obtained with bioassays using *Escherichia coli* JM109 reporter strain, and subsequent thin layer chromatography (TLC) analysis. The presence of N-butyryl-L-homoserine lactone was confirmed using liquid chromatography-mass spectrometry (LC-MS).

The results from bioassays must be carefully interpreted and should be supported by chemical analysis to confirm the findings. Other factors than production of autoinducers in the test strains, such as media condition or growth stage of the reporter strain, may interfere with the phenotypic response defining the positive or negative test results.

Quorum Quenching

To test quorum quenching (QQ) potential of the *Tenacibaculum* strains, the AHL molecule N-hexanoyl-DL-homoserine lactone was added to the different *Tenacibaculum* cultures. The test was performed with both culture and supernatant from the *Tenacibaculum* strains, to identify whether quorum quenching was based on extra- or intracellular processes. AHL was also added to MB as a negative control. No difference could be observed regarding the extent of the purple zones around the wells added *Tenacibaculum* culture or supernatant compared to the negative control in this QQ experiment, and therefore it cannot be concluded that the *Tenacibaculum* strains have the ability of quorum quenching.

A similar bioassay with *Chromobacterium violaceum* reporter strains has been performed to screen for AHL degradation in *Tenacibaculum maritimum* (Romero et al., 2010). *C. violaceum* CV026 was used to detect degradation of short-chain AHL (C6-HSL), and *C. violaceum* VIR07 for degradation of long-chain AHL (C10-HSL). The different AHLs were added both to *T. maritimum* cultures, and to cell free *T. maritimum* supernatant, to a final concentration of 2 µg/mL and incubated for 24 hours. Sterile medium and medium supplemented with the two mentioned AHLs were used as controls. After 24 hours, the C10-HSL was completely degraded, but no degradation of the C6-HSL could be observed.

Because this did not occur in the spent culture supernatant, the degradation of C10-HSL was regarded as cell-bound. Romero et al. however emphasize the uncertainty of these types of bioassays, as several variables may interfere with the results.

Phylogeny of the QS genes

The phylogeny of QS genes is of great importance if QS is to be targeted in new medicinal alternatives to antibiotics. A phylogenetic analysis was published in 2004, discussing the LuxS system and the LuxI/R system, that in fact consists of two separate families (Lerat and Moran, 2004). These two subgroups of the *luxI* and *luxR* genes could be paralogs considering the similar activity, but according to Lerat and Moran the divergence is too great to include both families in a single phylogenetic analysis. A total of 55 sequences of LuxI and LuxR proteins were included in the analysis, where the first subgroup was found in alpha- beta- and gammaproteobacteria, and the second group was only found in gammaproteobacteria. These phylogenetic groups were the only ones where *luxI/luxR* related genes were examined, though AHL production, which is regulated by *luxI* type genes, have been found in other bacteria than Proteobacteria. An AHL producing isolate belonging to the Bacteroidetes phylum was found in a screening experiment using biosensors, and identified as a Flavobacterium sp. from sequencing the 16S rRNA gene (Wagner-Döbler et al., 2005). Another study showed that three Bacteroidetes isolates could activate the AHL reporter strain *Agrobacterium tumefaciens* A136, further indicating the potential of AHL production in the phylum Bacteroidetes (Huang et al., 2008).

Biofilm formation tested with bioassay

Quorum sensing has been shown to be involved in the formation of biofilms and experiments showed that degradation of signalling molecules can prevent or hinder biofilm formation (Li and Tian, 2012). The potential for biofilm formation was tested for the five *Tenacibaculum* strains investigated in this thesis, by growing them in microwell plates and staining the wells after two weeks of incubation. All five strains were positive in at least one of the tests performed. This implies that the bacteria can likely form biofilms on this type of material, although the information provided by this experimental setup inform only about whether the cells stick to the wall of the wells or not. The negative

control used was sterile marine broth, and naturally had a lower density than the cultures when the experiment was started. This might be the reason for the significant difference. However, the assumption that the higher colouration is a result of actively produced biofilm by volatile cells, and not only clusters of dead cells, is supported by the fact that the bacteria from the wells grew on MA.

The method of staining with crystal violet to quantify biofilm was first described by Christensen et al. (1985). Crystal violet binds to all negatively charged surface molecules and will therefore stain polysaccharides in the EPS (Peeters et al., 2008). Because it does not discriminate between dead and living cells, it is not an appropriate method to examine e.g. the efficiency of antibiotics against cells in biofilm. Instead, methods that are based on the metabolic activity in the cells could be used. The crystal violet staining method is however cheap, less time consuming and has been used to test biofilm production potential in a broad range of bacteria. Peeters et al. (2008) tested the method by growing two or three strains of several bacterial species, such as *Burkholderia cenocepacia*, *Staphylococcus aereus*, *Candida albicans*, and *Propionibacterium acnes*. They reported a high repeatability of the method, since the average colouration had high similarities in the six experiments performed. However, the results were not equally reliable for all the species tested, and large variations of the biofilm biomass produced by *P. aerugionosa* were observed both within each experiment, and between the experiments. It was proposed by the authors that this was an effect of large amounts of water in the *P. aeruginosa* biofilm matrix, which could have impeded the fixation of the biofilm.

Growing the bacteria in microwell plates to examine biofilm production is a static approach, where drawbacks such as nutrition limitation can decrease the ability of biofilm production (Merritt et al., 2011). In addition, limited aeration can be a problem, and especially with the *Tenacibaculum dicentrarchi* strains tested, where aeration, in form of incubation with rotation, is important for the bacterial growth. In the description of the microplate assay with crystal violet staining by Merritt et al. (2011) it was noted that if the experiment was to be repeated several times with the same isolate over a time span, an increase of biofilm biomass, followed by a decrease caused by nutrition limitation could be expected. The authors also mention that the staining results would depend on the species being tested, as noted by Peeters et al. in 2008. In their description, Merritt et

al. (2011) state further that 48 hours would be sufficient for the examination of biofilm production in many commonly studied microorganisms. The time length used in the biofilm assays for this thesis were two to three weeks. An initial assay with only one week of incubation was done, but the observed biofilm production was very minimal (this could not be shown by statistical analysis due to problems in the staining procedure). Hence, the incubation time in the experiments were extended.

Bioassay experiments require a strong positive control in addition to the negative control, because of the large variation between the tests (Merritt et al. 2011). A positive control was not included in the biofilm assays performed for this thesis. Instead, to minimize the effects of variation between assays, the results were shown in relation to the negative control. Besides the duration of the assay and the lack of a positive control, the protocols are very similar. An important improvement of this method is that using a microplate washer, as done in this thesis, is a much better alternative than the original setup where the planktonic cells and the colour are removed by pouring the fluid into waste trays and rinsing the plate in water trays. Another method frequently used to test for the ability to form biofilm, is growing the bacteria in biofilm flow-cell chambers (Crusz et al., 2012). This allows the biofilm forming cells to grow without the planktonic cells remaining in the medium, and with supply of fresh medium.

Biofilm and sociomicrobiology

Pseudomonas aeruginosa has been used as a model organism in sociomicrobiology, and both the biofilm and QS traits have been thoroughly studied (Parsek and Greenberg, 2005). The connection between QS and biofilm in this bacterium has been interpreted differently by several authors. Davies et al. (1998) reported that the QS mutants (mutation in *lasI*) displayed a biofilm structure distinct from the wild-type strain biofilm. This was contradicted in 2002 by Heydorn et al., who found a uniform and densely packed biofilm in both the wild-type strain and the *lasI* mutant (Heydorn et al., 2002). It was shown in 2006 that the QS impact on the *P. aeruginosa* biofilm is nutrition-dependent (Shrout et al., 2006). *P. aeruginosa* wild-type strain and QS mutants were grown in biofilm flow-cell chambers with three different carbon sources. The biofilm of QS mutant strains differed from the biofilm formed by the wild-type strain when the bacteria were grown with succinate. Furthermore, the QS mutants showed a reduced swarming motility with succinate as carbon source. This shows the importance of investigating how different

factors, like the carbon source, can affect bioassays on QS or biofilm formation, or the connection between the two.

Virulence in *Tenacibaculum*

Tenacibaculum isolated from diseased fish in Norway grouped phylogenetically into four clades, where one clade belongs to *T. dicentrarchi* (Olsen et al., 2017). From the 89 isolates analysed by Olsen et al., 19 were *T. dicentrarchi* or *dicentrarchi*-like. Most of these were isolated from wrasse, and three out of four isolates obtained from salmon were from asymptomatic fish. This can indicate that the strains are more pathogenic towards non-salmon fish. The name of the strains comes from the European sea brass (*Dichentrarchus labrax* L), from which it was first isolated (Piñeiro-Vidal et al., 2012). Contradictory to the findings from Olsen et al., the strain has been shown to be a pathogen to Atlantic salmon (*Salmon salar*) by Avendaño-Herrera et al. (2016). Atlantic salmon and rainbow trout were treated with *T. dicentrarchi* in a bath challenge, which resulted in 65% and 93% mortality, respectively. The infection route of this bacterium is however still uncertain, though water is a likely infection source.

The virulence mechanisms of *T. maritimum* have been more thoroughly examined. *T. maritimum* produces a slimy substance of extracellular polymers that allows it to adhere to hydrophobic surfaces (Avendaño-Herrera et al., 2006). The extracellular substances produced by the strain has a very high proteolytic activity and has been shown to have a positive cytotoxic activity in some fish cell lines. An iron-uptake mechanism that could interfere with the iron-binding protein of the host could also be a virulence mechanism. All these possible virulence mechanisms involve the production of extracellular molecules that have been shown to be regulated by QS in other bacteria and highlights the importance of studies such as this master thesis, where QS activities in potential pathogenic bacteria are investigated.

QQ as a weapon against pathogenic bacteria in aqua culture

Pathogenic bacteria often form biofilms and communicate via QS. To make an anti-virulence drug that is not overcome by evolving and spreading of resistance mechanisms, the drug should target non-beneficial virulence factors (Allen et al., 2014) (here resistance

refers to the ability of expressing virulence in presence of the drug, not only survival). After the realization of QS influencing the virulence of bacteria, the idea of targeting QS has been a popular topic. In an article written by Defoirdt et al. (2010), the possibility of evolving resistance towards QS disruption is addressed. For a trait to be selected upon, it must influence fitness, and there must be variation present in the gene pool. Defoirdt et al. state that autoinducer production has been found to be variable between strains, both in amount and type of molecule produced. This can be important when treating with QS interrupting substances such as autoinducer antagonists. Examples of bacteria overcoming this by overproducing autoinducers have already been seen. There can also be variations in the number of autoinducer receptors present, and studies have implied that also these can be overproduced to overcome a QS blockade.

Although not many experiments so far have shown the presence of AHL based quorum sensing in bacteria outside the Proteobacteria phylum, there are some indications of cheating, i.e. an individual bacterium stops producing either autoinducers or the proteins coded by QS regulated genes, within other phyla too. An *in silico* analysis was performed by Barriuso and Martinez (2018) to screen for quorum sensing protein coding genes in environmental biofilms. A total of 22 metagenomes were analysed by searching for QS related terms, and by using 37 reference proteins. According to the authors a limited number of references proteins from the different quorum sensing systems are available. Phylogenetic trees were constructed based on the results from the two searching strategies. In the search for proteins in the LuxI/LuxR family, hits were found in the phyla Nitrospirae and Bacteroidetes, in addition to Proteobacteria. Within Bacteroidetes, taxa with LuxR genes and *not* LuxI genes were found. This can be due to unidentified LuxI genes in these organisms, coding for undescribed AHL synthases that lacks homology to the reference protein used. Another possible explanation is that these genes are "LuxR solos", that are believed to be found in "cheating" bacteria.

No experiments were performed to find LuxR homologues or detect any other autoinducer receptors for this thesis. To eventually identify a QS system, not only the possibility of autoinducer production, bioinformatic analysis, including genome sequencing could be performed. To detect any connection between QS and biofilm

formation in the *Tenacibaculum dicentrarchi* strains, QS inhibiting experiments could be done.

6. Concluding remarks and future work

Four of the five *Tenacibaculum dicentrarchi* strains were tested positive for the presence of the autoinducers AHL and AI-2 in bioassays with *Vibrio harveyi* reporter strains. *Chromobacterium violaceum* CV026 did not show any AHL-production, which may indicate that the AHLs produced by the *Tenacibaculum* strains are outside the response spectrum of this reporter strain. Although AHL was previously considered to be restricted to the Proteobacteria phylum, both the autoinducer itself and homologues of the reporter genes have been observed in Bacteroidetes, off which *Tenacibaculum* spp. are members. This supports the findings in this thesis.

The bioassays used in this thesis indicate that AHL and AI-2 autoinducers are present, but the findings should be verified by identifying the molecule itself. Proteomics and mass spectrometry could further confirm that these molecules are actually produced and whether their increase is connected to diseases in fish. Molecular methods, such as metatranscriptomics of biofilm samples from healthy and infected fish, and genome analyses of isolates, such as the *Tenacibaculum* strains investigated in this thesis, should be performed to confirm the genetic requirements to produce autoinducer molecules.

All five *Tenacibaculum* strains also tested positive for biofilm formation. The biofilm bioassays were not able to provide information about the impact of the salinity on the biofilm potential. To examine a possible connection between QS and biofilm formation, biofilm assays using quorum quenching molecules could be used.

In summary, the *Tenacibaculum dicentrarchi* strains showed both production of autoinducers and biofilm formation.

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Appendix

Bioassay with reporter strains *Vibrio harveyi* MM30, BB152, and JMH634
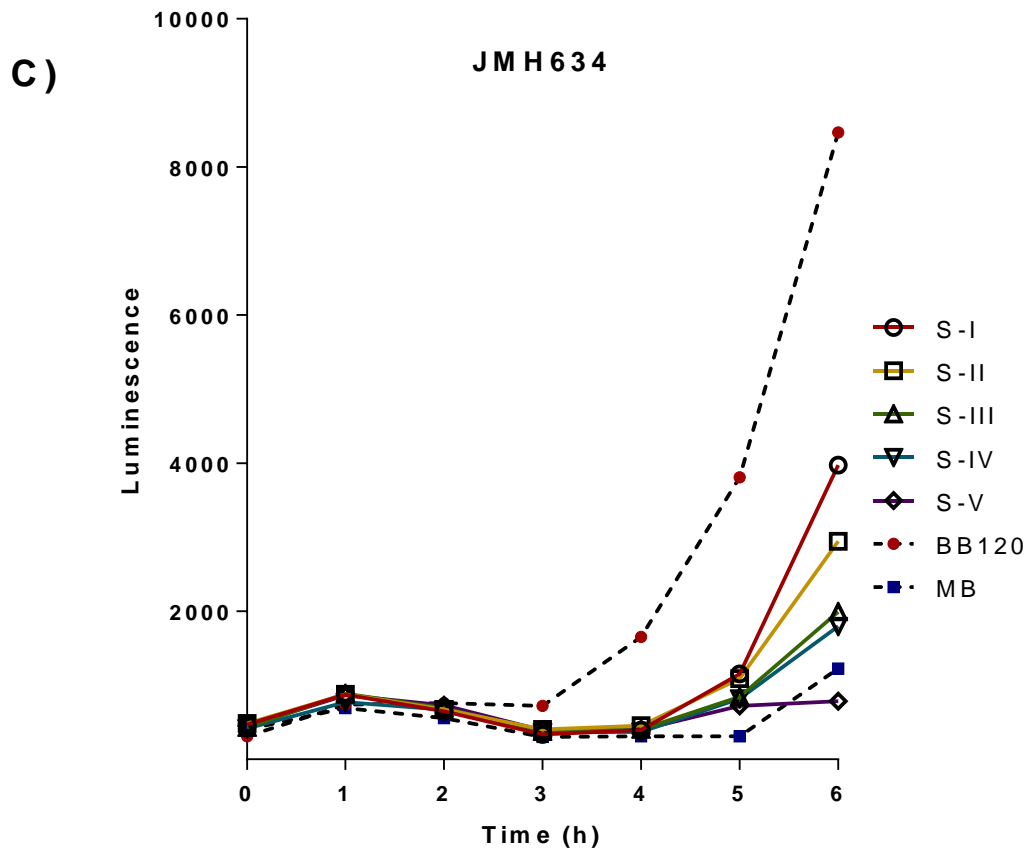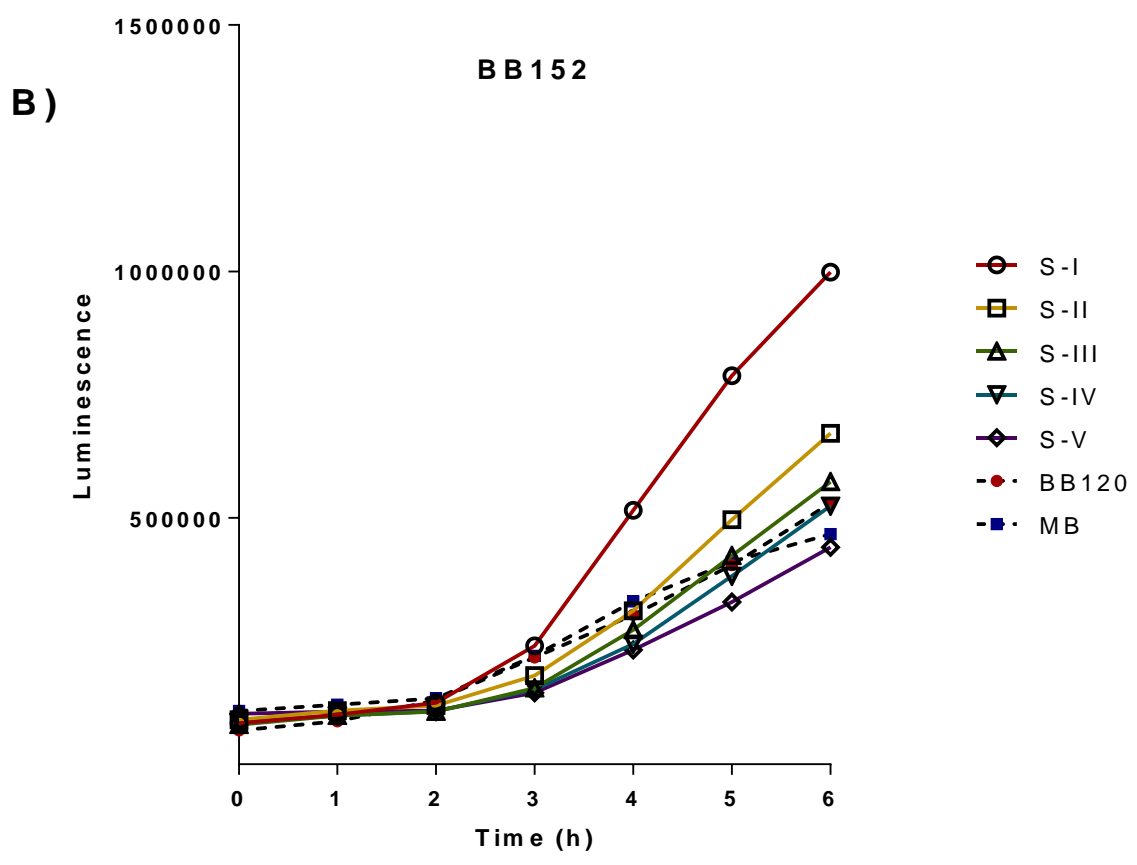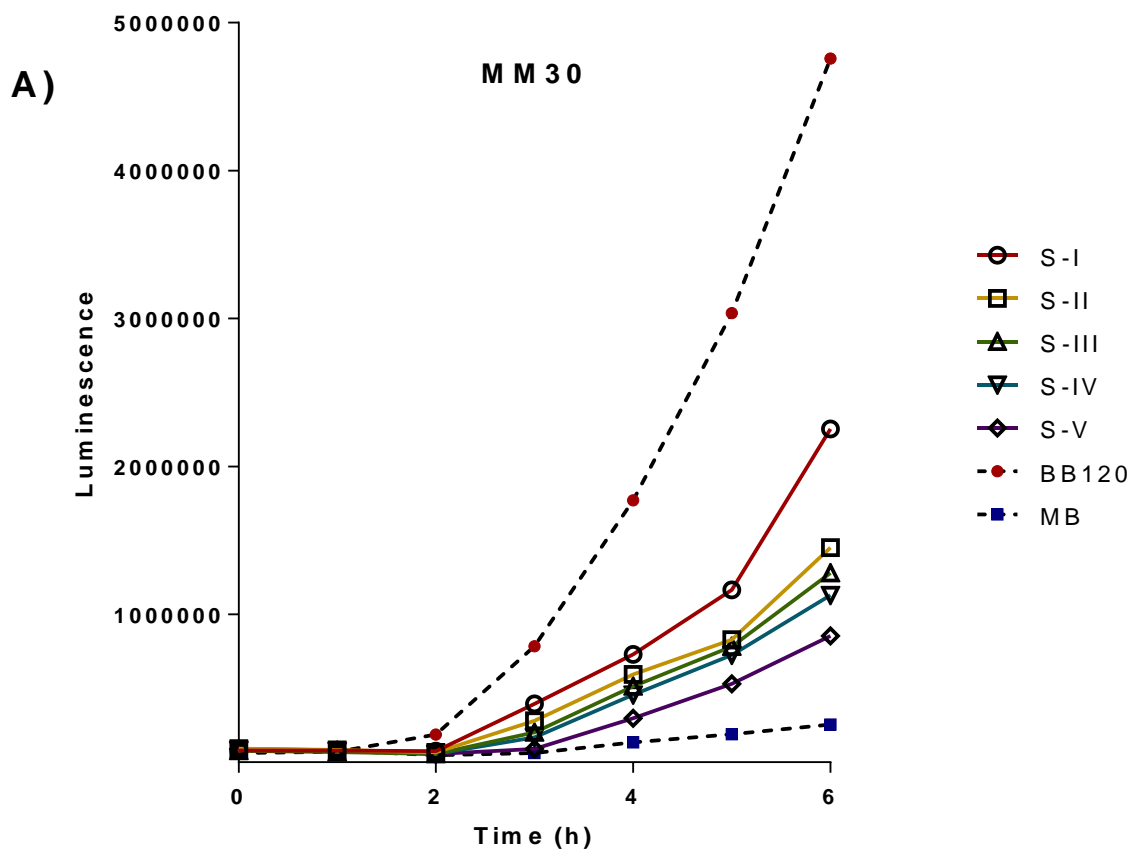
A)

B)
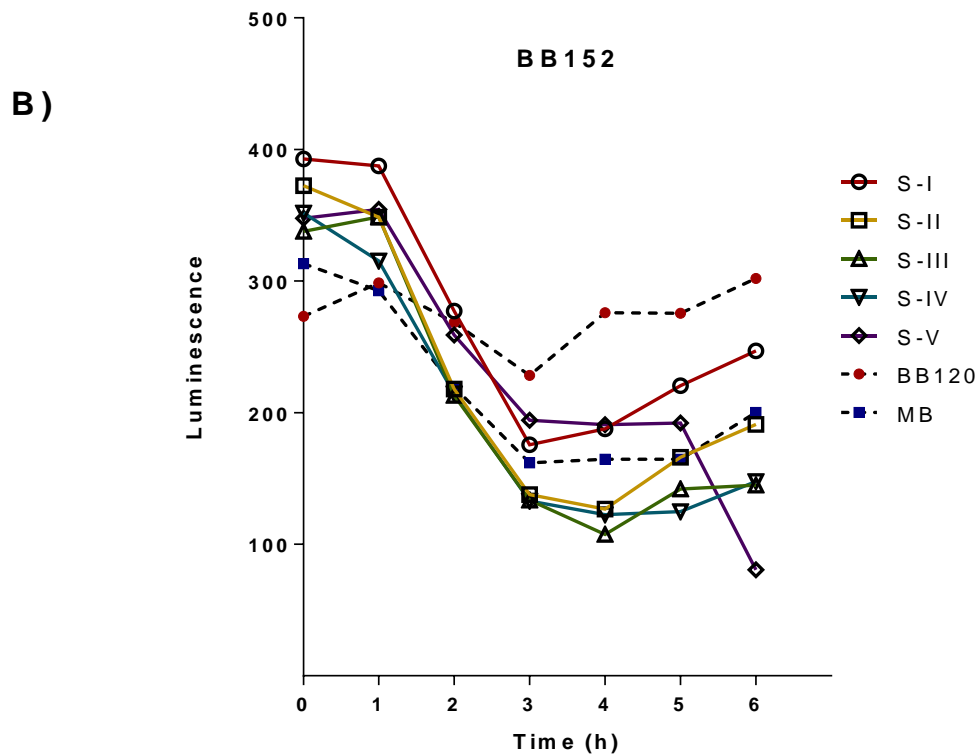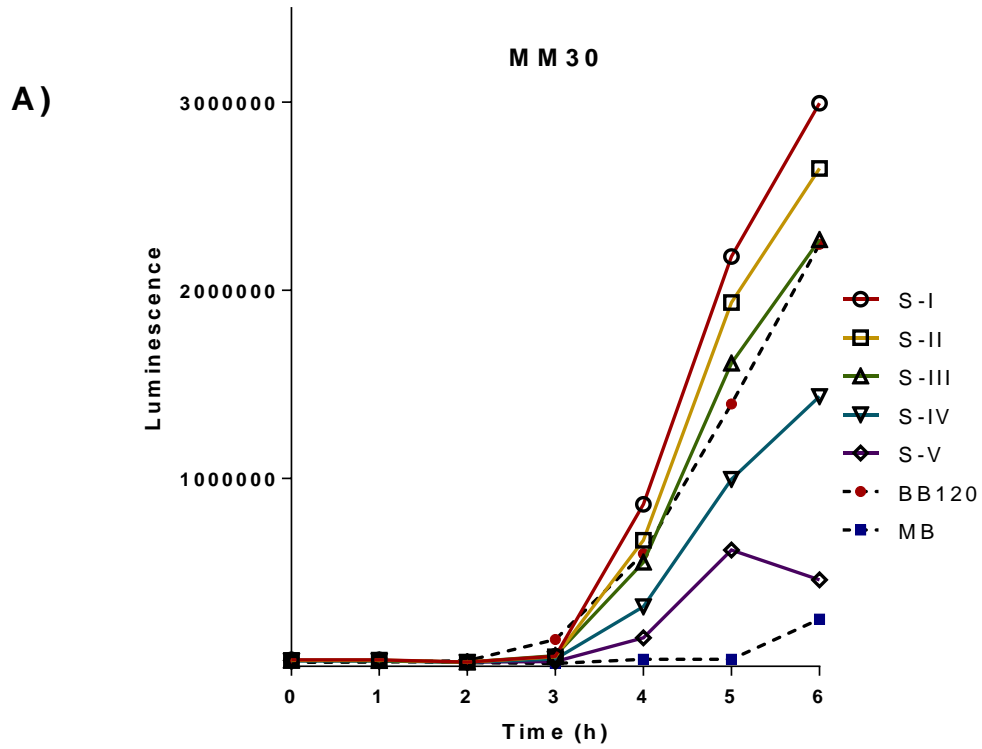
Figure A.1. Luminescence measured for six hours in the second test performed with *Vibrio harveyi* reporter strains a) MM30, b) BB152, and c) JMH634, Test 2. The graphs show luminescence induced in the reporter strains by supernatant of the five *Tenacibaculum* strains (S-I to S-V). MB was used as a negative control, and *V. harveyi* BB120 was used as positive control. The luminescence was measured for six hours. The reporter strains were diluted to an OD_{550} of 0.1, but the cell density of *V. harveyi* BB152 was too low to observe induced luminescence.

A)

MM30

B)
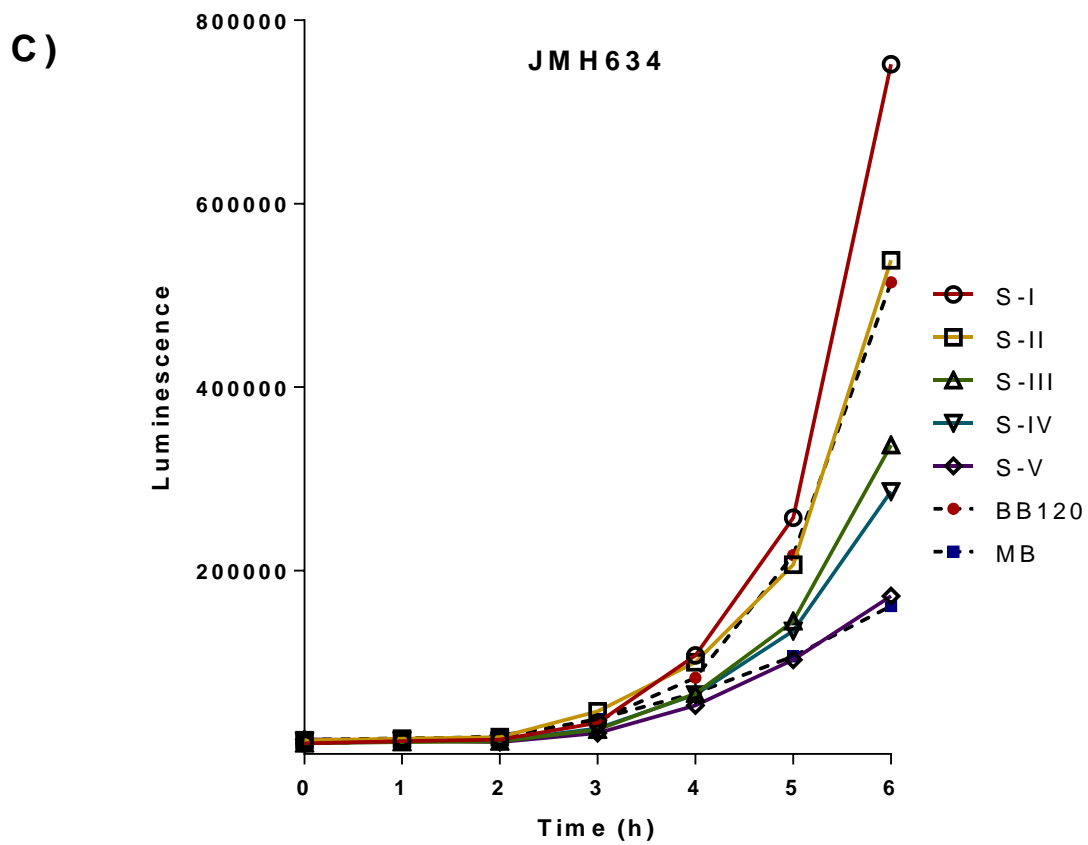
BB152

Figure A.2. Results from the third bioassay with *Vibrio harveyi* reporter strains a) MM30, b) BB152, and c) JMH634 (Test 3). The graphs show luminescence induced in the reporter strains by supernatant of the five *Tenacibaculum* strains (S-I to S-V). MB was used as a negative control, and V. harveyi BB120 was used as positive control. The luminescence was measured for six hours, and the reporter strains were diluted to an OD_{550} of 0.1 upon the start of the experiment. Luminescence was measured for six hours.

Bioassay with Vibrio harveyi reporter strains BB170 and BB886.
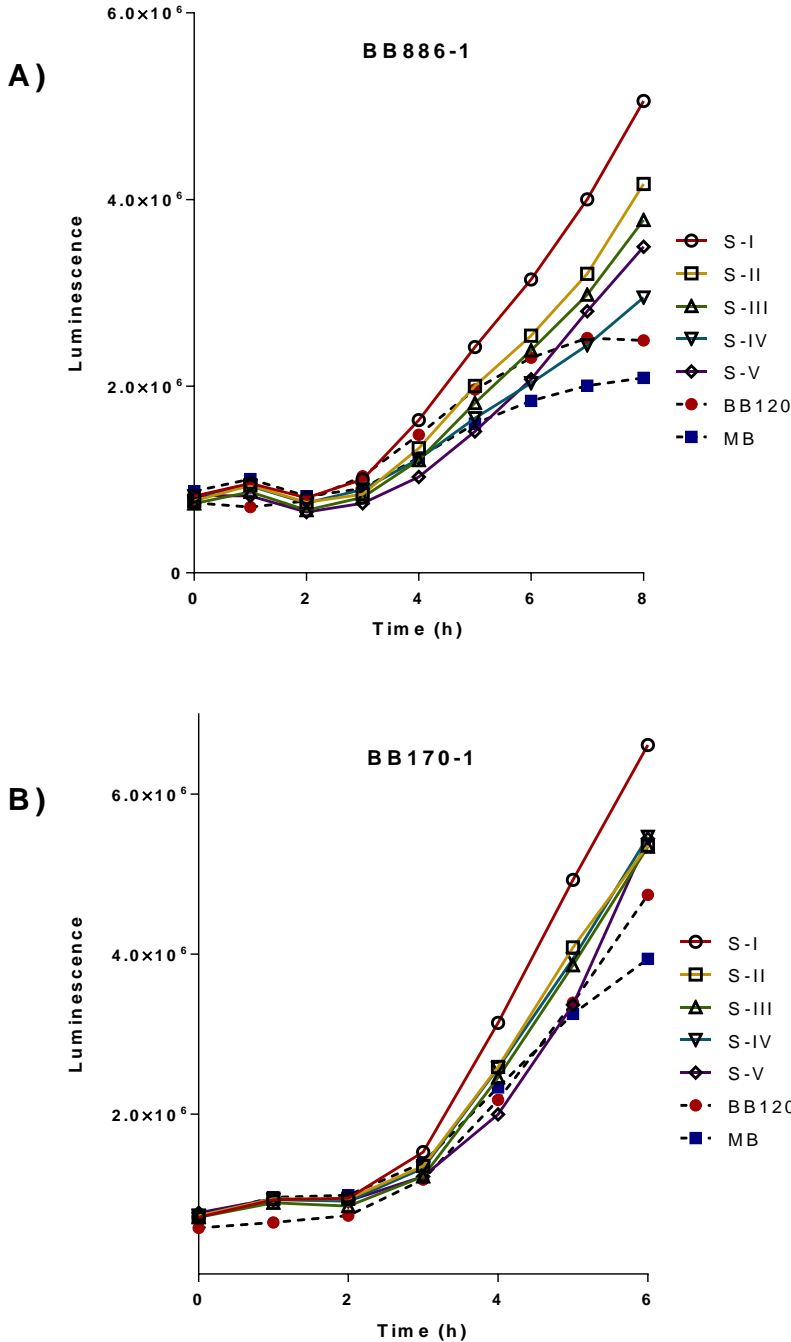
Figure A.3. Luminescence induced in the first test with reporter strains Vibrio harveyi a) BB886 and b) BB170 by the five Tenacibaculum strains (S-I to S-V). The reporter strains were diluted to an OD_{550} of 0.1. MB was used as a negative control, and V. harveyi BB120 was used as positive control. Luminescence was measured for six hours.
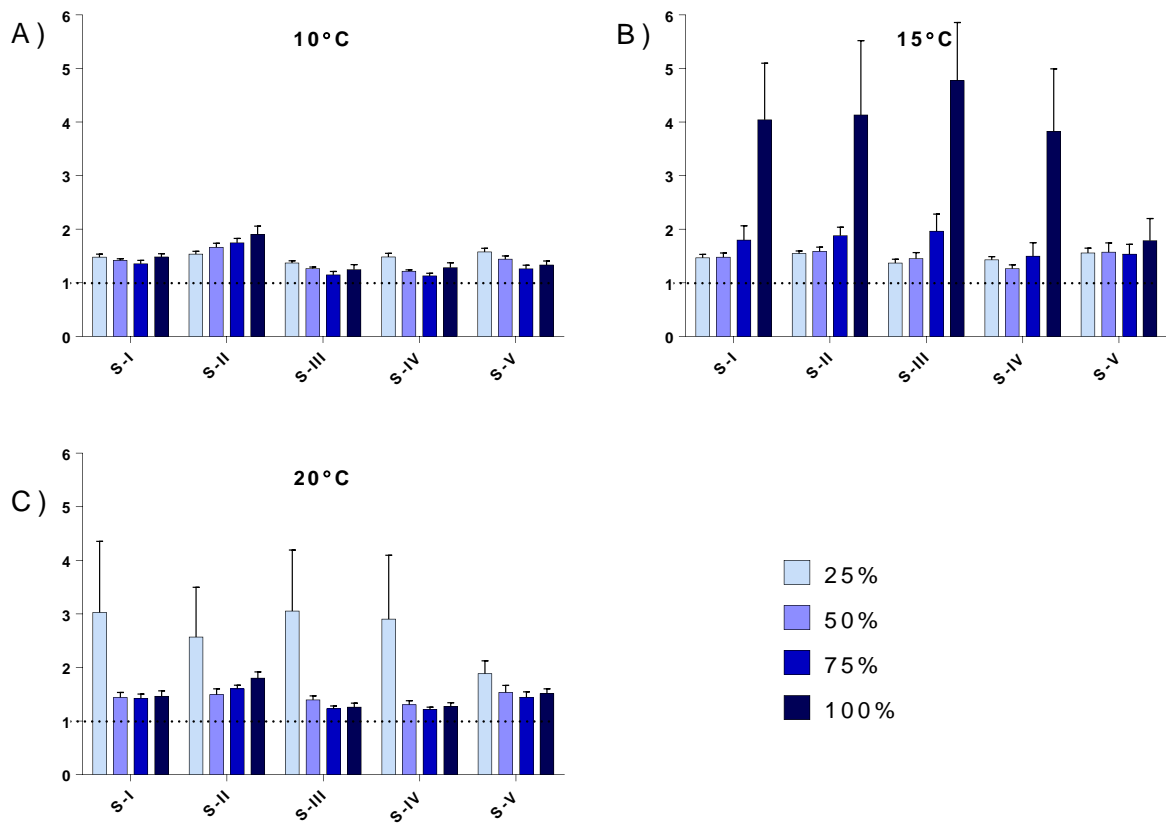
Biofilm test 1

Figure A.4. Biofilm produced by the five *Tenacibaculum dicentrarchi* strains (S-I to S-V) in Biofilm test 1 by incubating the bacteria in microwell plates at A) 10°C, B) 15°C, and C) 20°C, and subsequently colouring the microwell plates with crystal violet. The OD_{595} of the tests strains is given in relation to the negative control (MB) (test/negative control). The outmost wells were not filled with water, and therefore the colouration in these wells may be overestimated. Biofilm formation was tested with sea water diluted to four salinities, shown by different shades of blue.
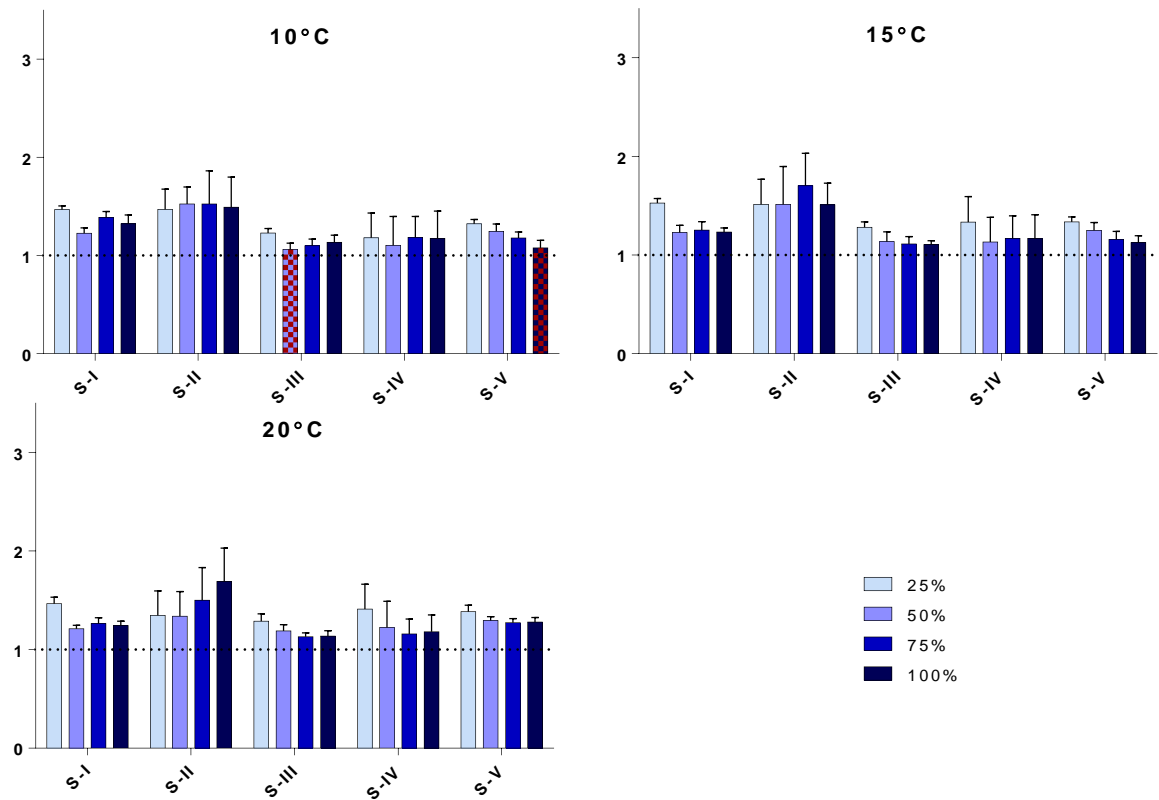
Biofilm test 2

Figure A.5. Biofilm produced by the five *Tenacibaculum dicentrarchi* strains (S-I to S-V) in Biofilm test 2. Microwell plates incubated with the *Tenacibaculum* strains were emptied and coloured with crystal violet. The OD$_{595}$ was measured after colouration with crystal violet, and the amount of biofilm is given in relation to the negative control (test/negative control). The outmost wells were filled with water to prevent drying.
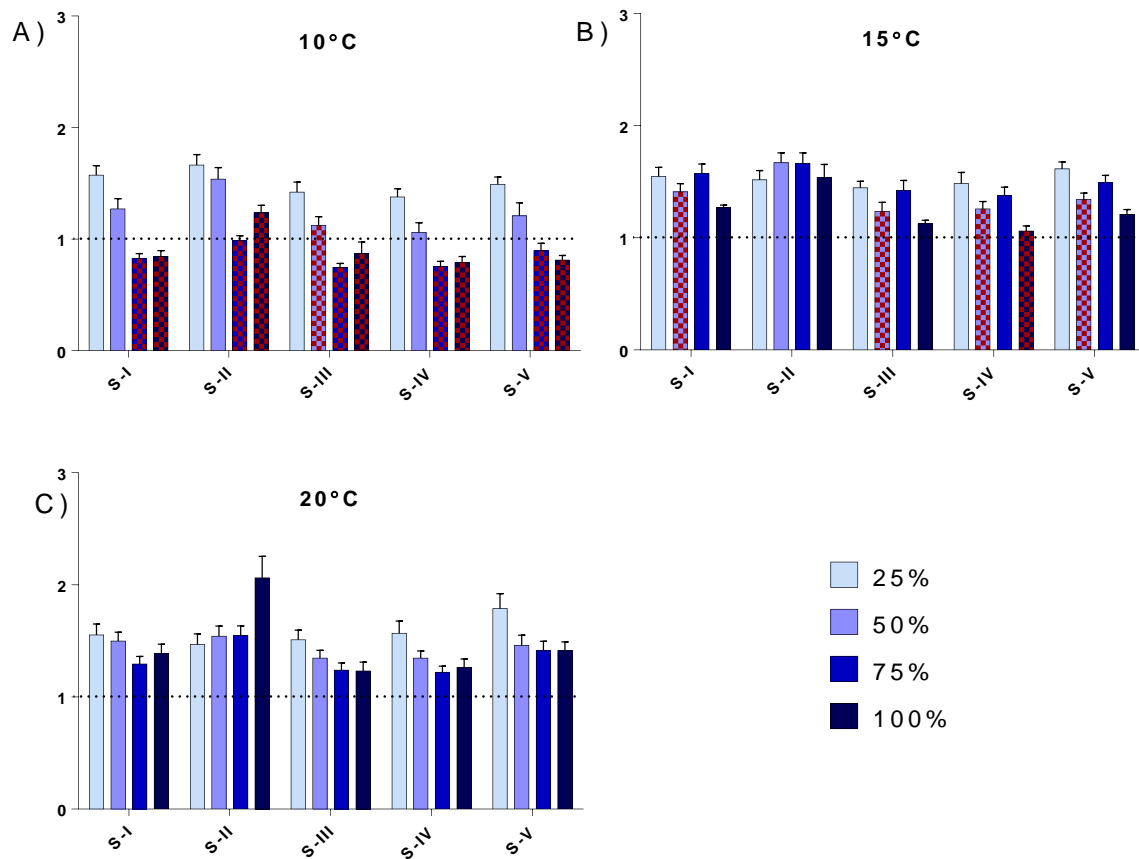
Figure A.6. Results from Biofilm test 2. The *Tenacibaculum* strains (S-I to S-V) were grown in microwell plates where the outmost wells were filled with water. The results of the microwell plate coloration measured in OD_{595} are given relative to the negative control (MB) (test/negative control), which level is indicated by a stippled line. Biofilm formation was tested at four salt concentrations, indicated by different shades of blue. The low coloration of the plates with 75 and 100% salt water incubated at 10°C and 50% incubated at 15°C are most likely due to contamination of the negative control.

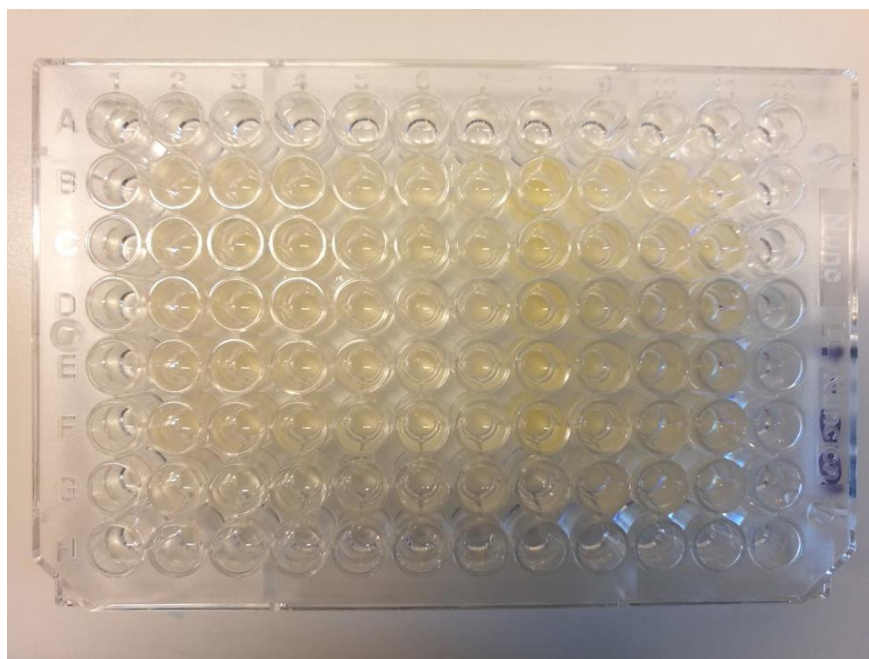**Pictures of microwell plate used in biofilm bioassays**

Figure A.7. Picture of microwell plate from Biofilm test 3, before colouring with crystal violet. The outmost wells were filled with water and are not a part of the experiment. This plate was used for 25% (vertical rows 2-6) and 50% salt water (vertical rows 7-11), incubated at 10°C. Note that the vertical row 8 has higher density. The *Tenacibaculum* isolate S-II was tested in vertical row 3 and 8.
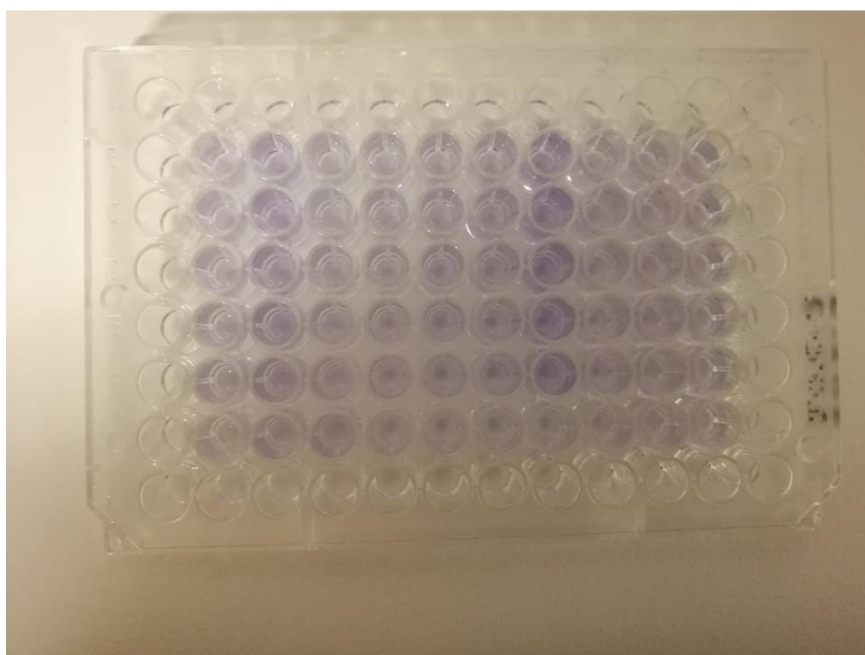

Figure A.8. Picture of microwell plate from Biofilm test 2, after colouring with crystal violet. This plate was incubated at 20°C, and the wells were filled with 75% (vertical rows 2-6) and 100% (vertical rows 7-11) salt water. The *Tenacibaculum* isolate S-II was tested in the vertical rows 3 and 8.