Succession of marine fouling on experimental ship coatings in tropical waters, Oman

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Abstract

Surfaces submerged in the sea are rapidly covered by biofilm and later macrofouling. Biofouling on ship hulls spread exotic species to new habitats and are at the same time an economical burden. In the past paints with TBT has had great effect against fouling but also had have grave consequences for the marine life leading to an international ban in 2010 and a demand for environmentally friendly antifouling coatings has arisen.

In the present project coatings with different active compounds was prepared on microscope slides. The coatings, with both experimental and commercial paints, were submerged for 22 days in the Bay of Oman. The commercial paints had copper and a mixture of copper and zinc pyrithione as active compounds. The experimental paints had zinc oxide, zinc sulphide and aerogel with active compound x, zinc sulphide and aerogel without active compound, zinc sulphide without aerogel, zinc sulphide with commercial protease, zinc oxide without aerogel and titandioxide as active compounds. To harmonize the surfaces black nitrocellulose filters were attached on the slides for bacterial counts with DAPI staining. The filters were collected over time to determine bacterial and macrofouling succession.

The bacterial density increased over time for all treatments and most for the treatments with commercial paints. The lowest counts were observed in the Control samples and the engineered paints had counts in between. The commercial paints inhibited quorum sensing in reporter strain *C. violaceum* CV017.

Community analysis of microfouling by fatty acid analysis, in the first experiment, revealed when statistical analyzed by ANOSIM that the Control differed from most of the other treatments and that the commercial paint also differed from one of the engineered treatments. The statistically analysis SIMPER revealed that the fatty acid community was dominated by few fatty acids present in all the treatments. Differences between treatments were determined by the

density among the dominating fatty acids. At the same time a temporal succession in fatty acids was observed.

Contrary general assumptions and results the commercial antifouling paints with the highest fouling inhibition had the highest bacterial counts which must result from biofilm development of bacteria resistant to the active compounds copper and zinc pyrithione used in these treatments.

The poly unsaturated fatty acid (PUFA) C18:2ω9,12 was found in samples the Control, treatment MUZ and treatments T40 and T44 which have been connected to the groups of Cytophagales or Proteobacteria. Fatty acid profiles from several treatments had similarities to fatty acids profiles extracted from fluorescent *Pseudomonas*.

The surfaces with commercial paints had the lowest abundance of macrofouling recorded. The Control samples had the highest algae and invertebrate density and the engineered paints had fouling abundances within the range of the Controls and the commercial paints. The treatments with zinc sulphide without aerogel and zinc sulphide and aerogel with enzyme x had the poorest antifouling activity against macrofouling along with the Control. The commercial paints demonstrated both a broad fouling inhibition against all macrofouling organisms encountered.

Keywords; biofilm formation, marine fouling, bacterial community dynamics, quorum sensing

Abstrakt

Overflader nedsænket i havet dækkes hurtigt af en biofilm og senere af makro begroning. Biologisk forurening på skibs sider spreder eksotiske arter til nye habitater og er samtidig en stor økonomisk byrde. Før hen var maling med TBT meget brugt med stor effektivitet. De store konsekvenser for det omkringlevende marine dyre- og planteliv førte til et forbud mod brug af TBT i maling i 2010 og derfor er behovet for udvikling af miljøvenlig skibsmaling steget.

Mikroskopglas dækket med forskellige designede og to kommercielle skibsmalinger var nedsænket i havet i 22 dage i Bay of Oman. De to kommercielle skibsmalinger indeholdt kopper ag en blanding af kopper og zinkpyrithione. De designede skibsmalinger havde følgende stoffer som aktivt stof; zinkoxid, zinksulfid og aerogel med det active stof x, zinksulfid og aerogel uden det aktive stof x, zinksulfid uden aerogel, zinksulfid med den kommertielle protease, zinkoxid uden aerogel og titandioxide. Black filtre var fastgjort på mikroskopglassene til bakterie tællninger med DAPI farvning. Glassene blev indsamlet over tid for at bestemme succestion af bakterie og makro begroning.

Bakterie densiteten steg over tid i alle behandlinger og den steg mest for kommercielle malinger. De laveste tællinger blev observeret i kontrol behandlingerne medens tællingerne for de eksperimentielle behandlinger lå imellem de kommertielle og kontrol behandlingerne. De kommercielle malinger hæmmede quorum sensingen i reporter stammerne *C. violaceum* CV017.

Mikrobielle fedt syrer samfunds analyser i det første eksperiment afslørede ved statistisk ANOSIM analyse at kontrollen adskildte sig fra størstedelen af de andre behandlinger og den kommercielle maling adskildte sig også fra en af de eksperimentielle malinger. Den statistiske SIMPER analyse afslørede at fedt syre sammensætningen var domineret af få fedtsyres tilstedeværelse i samtlige behandinger og at forskelle mellem behandlinger var bestemt af densiteten af de individuelle fedtsyrer. Samtidig blev der også observeret en succession af antal og typer af fedtsyrer over tid.

I modsætning til den generelle overbevisning og dokumentation havde de kommercielle behandlinger der havde den bedste effect mod begroning også det højeste antal bacterier hvilket må skyldes biofilm dannelse af bacterie der var resistente overfor de active stoffer copper of zinc pyrithione brugt i disse behandlinger.

Den poly umættede fedtsyre C18:2ω9,12 blev fundes i prøver fra kontrol og MUZ behandlingerne og i behandlingerne T40 og T44. Denne fedtsyre er blevet forbundet med grupperne *Cytophagales* or *Proteobacteria*. Flere fedtsyre profiler fra behandlingerne havde også ligheder med fedtsyrer ekstraheret fra fluoresente *Pseudomonas*.

Mikroskop glassene med kommerciel maling havde den laveste observerede hyppighed af makro begroning. Kontrol behandlingerne havde de højeste observerede alge og invertebrat densiteter hvor de designede malinger havde makro forurenings densiteter der lå imellem Kontrollerne og de kommercielle malinger. Behandlingerne med zinksulfid uden aerogel and zinksulfid med aerogel og det aktive enzym x havde sammen med Kontrollen de laveste effekter mod makrobegroning. De kommercielle malinger udviste en bred vifte af inhibition af makrobegroning da de inhiberede all de typer makrobegroning de blev udsat for.

Nøgleord; biofilm formation, marin forurening, bakteriel samfunds dynamik, quorum sensing

Preface

I, Angelique Kozmér master student in Environmental Biology and Molecular Biology at Roskilde University, hereby certify that the work presented in this thesis is my own and that the work performed by others is appropriately cited.

This thesis deals with the problem of fouling of marine surfaces. Development of the engineered paints was performed by EnPro (Engineered Products) and CISMI (Centre for Interdisciplinary Studies of Molecular Interactions) and the testing of the paints and the experimental work took place at Sultan Qaboos University in Muscat, Oman.

To appreciate the master thesis the reader is assumed to have some background in natural science since the general acronyms, terms and methods used in environmental- and molecular biology are not explained in details. It should otherwise be possible for the non-professional to enjoy the introducing sections and the discussion.
The structure of this thesis is chronological and may be read from one end to the other as is the numbering of tables and figures. The tables placed in the appendix are marked with an "A" before their table number. In the back of the thesis under, fold-out map, named "bacterial maps" in the contents, to help give an overview of the relation between bacterial strains when reading the biofilm section. On the other side of the bacterial map, placed under "species and paints overview" in the contents, is there an overview of the species found on the engineered slides and a table with the treatments and their active compounds.

My thanks go first to my supervisors Professor Benni Winding Hansen, Assistant Professor Sergey Dobretsov and Assistant Professor Tove Atlung and thereafter to Management Director Eva Wallström at EnPro ApS and Professor Kjeld Schaumburg at CISMI which all made this project possible. Thank you for giving me the opportunity ☺.
An enormous thank to Assistant Professor Morten Foldager Petersen for helping me out with all the statistical work in this thesis.

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Last but not least, a big thanks to my family and friend for moral and economical support.

List of acronyms

AAP	–	Aerobic An-oxygenic Phototrophic
ACP	–	Acyl Carrying Protein
AHL	–	*N*-Acylated Homoserine Lactone
ANOSIM	–	ANalysis Of SIMilarities
ANOVA	–	ANalysis Of VAriance
BSA	–	Bovine Serum Albumin
CEM	–	Computer Enhanced Microscopy
COMSTAT	–	COMputer STATistics
CMP	–	Cypris Major Protein
DAPI	–	4',6-DiAmidino-2-PhenylIdole
DCOI	–	4,5-dichloro-2-*n*-octyl-4-isothiazolin-3-one
DGGE	–	Denaturing Gradient Gel Elecrophoresis
EPS	–	Extracellular Polymeric Substance
FAME	–	Fatty Acid Methyl Ester
FDA	–	Food and Drug Administration
FISH	–	Fluorescence In Situ Hybridization
GC-FAME	–	Gas Chromatographic – Fatty Acids Methyl Esters
GC - MS	–	Gas Chromatographic – Mass Spectrometer
HSL	–	HomoSerine Lactone
MDS	–	Multi Dimensional Scaling
MUFA	–	Mono Unsaturated Fatty Acids
PUFA	–	PolyUnsaturated Fatty Acid
RFLP	–	Restriction Fragment Length Polymorphism
SFA	–	Saturated Fatty Acid
SIMPER	–	SIMilarities PERcentages
TBT	–	Tri-Butyl-Tin
TBT-SPC	–	Tri-Butyl-Tin Self-Polishing Copolymer
WGS	–	Whole Genome Shotgun

Contents

Introduction

Settlement of organism on man-made surfaces suspended in the sea is known as marine biological fouling or just biofouling. Marine biofouling is an economical burden for several operations and especially in marine transportation (Yebra *et al*, 2004). Biofouling on ships also allows species to be transported to a location it would not inhabit normally. The introduction of exotic species can have grave biological consequences for native species (Montelli and Lewis, 2008). Surfaces submerged in the sea (figure 1), even the ones protected by antifouling paints, and biocides as copper and TBT-based paints, will be covered by biofilms in a short span of time (Cologer, 1991).

Figure 1: Fouling on ship hull and rudder, Oman

Biofilms can induce major problems both functional and economical by inducing increases in drag, metal corrosions and reduction in heat transfers (Dobretsov, 2009). Drag caused by an increase in hull roughness results in increased hydrodynamic drag as the ship moves through water. This drag leads to decreased maneuverability and an increased fuel use since the ship weights more. The drag can also lead to a need for heavier and more powerful machinery (Yebra *et al*, 2004). Further, the removal of fouling organisms and repainting of the hull leads to a second expense. Biofouling also favor metal corrosion which is induced by several mechanisms as cathodic and anodic depolarization, hydrogen protection, reduction of metals and the secretion of corrosive metabolites as organic acids and exopolymers (Dobretsov, 2009). All in all biofouling increases maintenance of ships considerable (Yebra *et al*, 2004). The fuel increase can be up to 40 % (Champ, 2000). The US Navy alone uses $ 1 billion per

year on marine biofouling (Callow & Callow, 2002). Overall the increase in fuel, cleaning materials and paints increases the emission of harmful compounds into the sea (Champ, 2003; Yebra *et al*, 2004).

Much time and effort has been devoted to solve this problem through time but not before the development of cobber oxide (Cu_2O) cold-plastic antifouling paints in the 20^{th} century a long term ship protection was an option (Yebra *et al*, 2004). The most well-known, successful and efficient paint was the tributyltin self-polishing copolymer (TBT-SPC) system. This system was used by up to 80 % of all ocean going ships up to the late 1990s (Champ, 2001; Yebra *et al*, 2004). Coatings containing organotin as for example TBT have been the most efficient to ever have been developed but unfortunately also a health hazard to the marine environment and humans. Sediments function as sinks for tributyltin (TBT) and the compound can therefore be absorbed and accumulated up through the food chain (Clare *et al*, 1992; Stewart & de Mora, 1990; Traas *et al*, 1996; Veltman *et al*, 1996). TBT has caused imposex (development of a penis or vas deferens on female genitals or development of a female genital opening (gonopore) or ovaries in male genitals) in several species. Wang *et al* (2010) discovered that female *Thais clavigera* whelk accumulated TBT and developed imposex when exposed to TBT by both dietary and aqueous and several similar cases have been reported in other species (Gravel *et al*, 2006; Horiguchi *et al*, 1995; Oehlmann *et al*, 1991; Ramasamy & Murugan, 2002; Ramón & Amor, 2001; Takahashi *et al*, 2000).
These side effects lead to a restriction of the organotins in 2001 (Yebra *et al*, 2004) and later to the global TBT ban in January 2008. The treaty states that by January 2008 no ship shall contain any paint containing organotin or shall bear a coating which forms a barrier that conceals the underlying organotin compound (AFS Convention; Regulation (EC) 782/2003).

The TBT-based paint restrictions and, latest in 2008, the ban has initiated major changes in the antifouling paint industry. Several tin free paints have been developed to prevent marine biofouling on ships (Yebra *et al*, 2004). Many of the earlier tin free paints were efficient because they contained other heavy metals as arsenic, copper, lead and mercury (Adkins *et al*, 1996). Today copper and zinc compounds are used but the metal amount is restricted and therefore the industry still seeks new and more

efficient alternatives to the existing antifouling paints. Because of the TBT ban development of new antifouling coatings have boomed since development of effective and safer paints are necessary.

The goal of this study is to investigate the bacterial and macrofouling succession over time and the effect of novel antifouling compounds on marine micro and macrofouling in laboratory and field experiments in the pier of Port Sultan Qaboos in Oman. The location of Oman was chosen on behalf of two reasons. The first was research network between Professor Benni Winding Hansen and Doctor Sergey Dobretsov. The second was the environmental conditions in Oman which compared to the Danish were much better concidering the time limith tied to this project. The Omani weather enabled the possibility of performing experiments in 2 – 4 week which would have taken approximately 2 – 4 months in Danish waters due to different weather conditions.
The following questions were investigated through the experiments in this thesis. The fouling succession of bacteria- and macrofouling performens was monitored over time and the differences of the bacterial and macrofouling succession between the different experimental slides were determined. Differences in the bacterial communities between experimenal slides and time were also investigated.

Biofilm

Biofilm distribution

Marine natural biofilm consists of bacteria, diatoms, protozoa and fungi. These organisms settle on surfaces and can colonize a submerged surface rather fast (Cooksey *et al*, 1995).

Biofilms are defined as bacterial populations which are enclosed in a matrix. The bacteria are adhered to each other and to a surface/interface. Microbial communities in porous spaces, floccules and aggregates are also a part of this definition (Costerton *et al*, 1995). Marine biofilms consist primarily of bacteria and diatoms. Since the marine environment is nutrient scarce microbial growth is found at interfaces mainly between solid surfaces and water. The solid surfaces accumulate nutrients as organic macromolecules and inorganic molecules which are required for microbial growth. Natural occurring surfaces are rocks, plants, the sea bed, animals, plankton and other biological materials where manmade surfaces found in the sea are ship hulls, bridge pillars, harbors and piers (Marshall, 1980).

All surfaces submerged in the sea will in short time be covered by biofilm. In the arctic almost all marine surfaces are covered with biofilm. Tests were made in a nutrient enriched river, and in a part of the river which was not nutrient enriched and functioned as a control area, on metal surfaces of stainless steel, copper-nickel and titanium. The biofilm development was alike on both stainless steel and titanium surfaces, in the nutrient enriched part of the river, where copper-nickel surfaces had a significantly lower biofilm development both in the enriched part of the river and in the control part. The biofilm development on stainless steel and titanium surfaces was very alike in both areas in the first 14 days of the experiment. After further 11 days the biofilm cell numbers grew continuously in the experimental area but not in the control (Ford *et al*, 1989).

In tropical waters all surfaces are also heavily covered with biofilms and other biofouling (Lau *et al*, 2002; Lee & Qian, 2003; Dobretsov *et al*, 2006; Dobretsov & Qian 2006; Huang *et al*, 2007; Huang, 2007a). Biofilms have also been reported to

develop on antifouling surfaces coated with biocide based antifouling paints (Dobretsov, 2009; Yebra *et al*, 2006).

Biofilm development

Colonization of an immersed surface can be divided into four phases. Phase one is molecular biofouling where dissolved organic molecules adhere to the immersed surface. In this initial stage, proteins, glycoproteins, proteoglycans, polysaccharides and lipids present in the seawater adhere to surfaces. Their absorbance to the substrate differs due to the surface material and structure. The adherence of molecules to an immersed surface in seawater takes place within minutes and continues as long as the surface is immersed in the water (Cooksey & Wigglesworth-Cooksey, 1995; Dexter, 1975; Wahl. 1997; Zobel, 1943). In phase two, surface colonization by prokaryotes begins. Depending on the molecular adhesion process in phase one the surface can inhibit or facilitate the bacterial settlement and also provide the settled microorganisms with nutrients to facilitate their growth. In phase three unicellular organisms settle and become a part of the growing biofilm. Finally in stage four larger eukaryotic organisms settle on the surface (Railkin, 2004).

The settlement of microorganisms is depending on factors as surface free energy (surface tension) (Dexter *et al*, 1975), properties of the substrate (Zobel, 1943) and water turbulence. Factors as protein and EPS production also affect settlement (McEldowney & Fletcher, 1986). The settlement of bacteria starts approximately a couple of hours after the surface is immersed into the water (Costerton *et al*, 1995; Wahl, 1997; Zobel, 1943).

In the second stage of biofilm development, where bacterial settlement takes place, the community composition is dependent on several factors. The presence of colonizers, the environment, the substratum and the physic- and chemical conditions of these all influence the biofilm structure and composition (Dobretsov, 2009; Sekar *et al*, 2004). The initial colonizers on marine surfaces are always rod-shaped chemotrophic bacteria. These bacteria appear and settle on submerged surfaces within hours. When these rod-shaped initial colonizers settle on surfaces they use a large amount of the carbon sources which were absorbed by the surface in stage one

(Dobretsov, 2009; Railkin, 2004). This carbon depletion opposes invading species and makes it hard for them to enter the existing biofilm community (Rao *et al*, 2006).

Phase three is recognized by adhesion of unicellular eukaryotes such as diatoms, flagellates, amoebae and ciliates. The organisms from phase three settle within days (Wahl, 1995). Phase four is where the colonization of macrofouling as invertebrate larvae and algae occurs (Dobretsov, 2009, Railkin, 2004; Wahl, 1997). Microalgae, diatoms and cyano-bacteriums settled with a higher density on titanium and stainless steel compared to aluminum, brass and copper. The attachment to the different materials and the time used on settling varied significantly. The roughness of the materials and in some cases the surface wettability also influenced the settlement. The diatom also adhered better to stainless steel and glass surfaces when the pH was above pH 7. Organic film and biofilm on the surfaces increased the settlement significantly (Sekar *et al*, 2004).

The four stages can happen simultaneously or overlap (Cooksey & Wigglesworth-Cooksey, 1995; Railkin, 2004) but in general phase one and two are important for the colonization of the larger organisms as larvae (Burgess *et al*, 2003; Dobretsov *et al*, 2006; O'Conner & Richardson, 1998; Qian *et al*, 2007; Wieczorek & Todd, 1997).

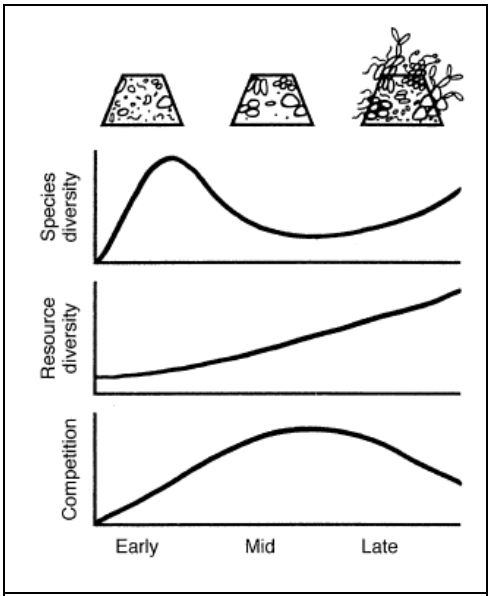


Figure 2: Model of biofilm development and diversity over time, by Jackson *et al* (2003). The collection days were 2, 7 (early), 15, 30 (mid), 60, and 90 (late).

Mature biofilms community and structural composition change dynamically according to the influence of the surrounding environment (figure 2). The abiotic factors as temperature, salinity, weather conditions, water turbulence, depth, light, water chemistry, nutrients availability, surface conditions and the biotic factors as colonizing species, intra and inter species competition (predation, grazing, parasitism, disease, mutualism, commensalism) and macroalgal canopy cover (Thompson *et al*, 2005) all influence and change the biofilms density, productivity, architecture, succession rate, metabolism (production of chemical compounds) and species composition (Dobretsov, 2009; Qian *et al*, 2007). Also

phenomena as biofilm sloughing and detachment change the biofilms architecture and give possibility of re-colonization, by planktonic bacteria and other microorganisms and already existing species in the biofilm, of the newly opened area (Underwood *et al*, 2005).

Several biofilm communities have different species composition through the year and some changed within hours (Dobretsov, 2009; Qian *et al*, 2007; Underwood, 2005). In low tide along the Colne estuary in Essex the biofilms are diatom rich. The diatoms migrate vertically in the biofilm relative to the light influence (time of the day) and species preferences (Underwood *et al*, 2005). Some microorganisms were present all year where others only were present in specific seasons (Dobretsov, 2009; Qian *et al*, 2007). In some locations diatom species diversity was lower when biomass on the site was high. In spring and summer when sites had a high biomass they were often dominated by a single or a low number of species where sites with low biomass, autumn and winter, had a diverse mix of species present. The number of dominant species was low and similar on different sites in spring (Forster *et al*, 2006). Bacterial community analysis in a subtropical estuary in Florida (East Sabine Bay) verified that bacterial groups in both benthic and floating biofilms changed through the year although some groups were present all year. *δ*- and *γ*-p*roteobacteria* were present all year and without a seasonal pattern. In January the dominating bacterial groups were *β*-, *δ*- and *γ-proteobacteria* and *Bacteriodes* where in April the dominating groups were from the phyla *Planctomycetales*, *Verrucomicrobia* and *Cyanobacteria* and in August the *Bacteriodes* and the *γ-proteobacteria* dominated the biofilms again. In general the genetic diversity decreased from winter to summer. The biofilms as communities showed tolerance against the seasonal changes in salinity and oxygen concentrations (Moss *et al*, 2006).
When biofilms are affected by all these factors they also reflect the key environmental factors (Dobretsov, 2009: Underwood, 2005).

Biofilm architecture

All adhesion of microorganisms to surfaces, plastics, metals, tissue, glass and organic particles, is mediated by the secretion of extracellular polymers (EPS) which is a slimy glue-like mixture of polysaccharides, liposaccharides, proteins and nucleic acids (Celik *et* al, 2008; Dobretsov, 2009; Flemming *et al*, 2001; Long *et al*, 2009; Unz & Farrah, 1976). Production of EPS is depending on several factors as nutrient availability (Lee *et* al, 1997; Unz & Farrah, 1976) and preferences (Celik *et* al, 2008; Ko *et* al, 2000; Lee *et* al, 1997), pH (Ko *et* al, 2000; Lee *et* al, 1997), ionic concentrations (Saravanan & Jayachandran, 2008), and temperature (Ko *et* al, 2000).

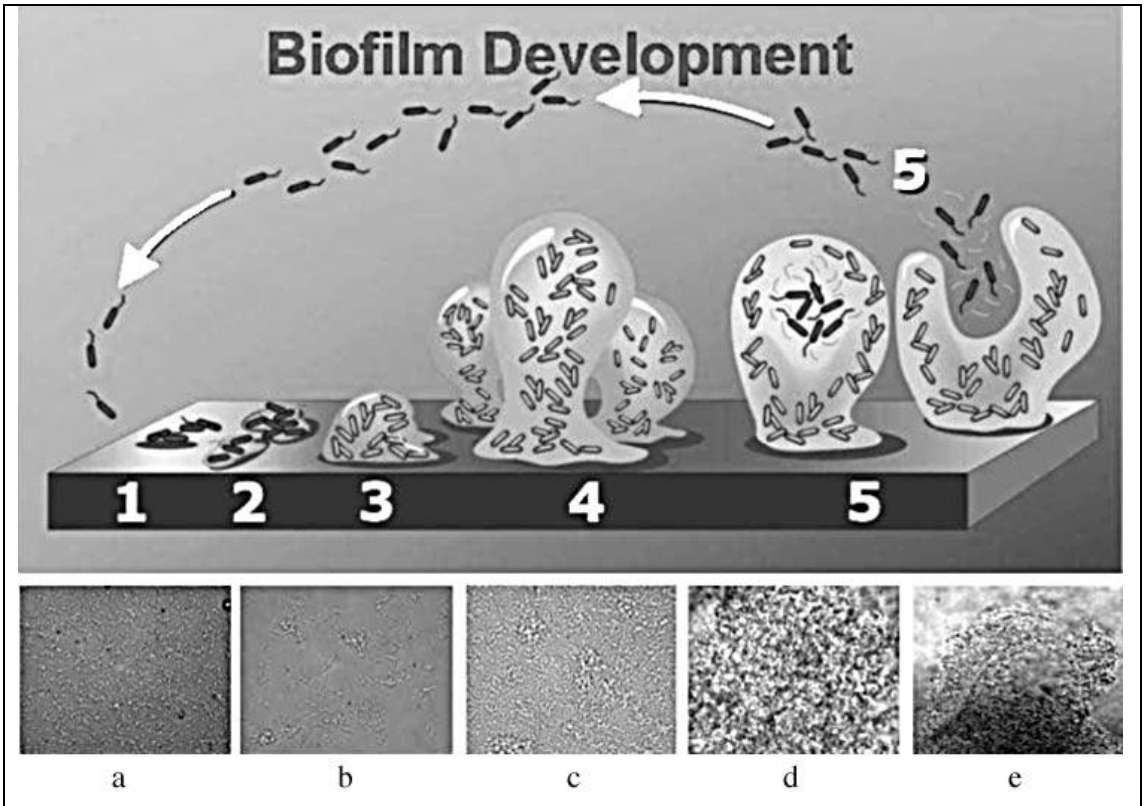


Figure 3: Schematic figure of biofilm development in five stages. 1; in stage 1 the single cell bacteria settle and attach to the surface. 2; the bacteria starts to produce EPS and the bacteria adheres permanently to the substrate. 3; the development of colonies and 3 dimensional structure of the biofilm starts. 4; developed mature biofilm. 5; dispersion of single cells from the biofilm by for example sloughing.
The five photos in the bottom are the five development stages in *P. aeruginosa* when grown on glass slides in a continous flow. Schematic figure by Stooley *et al* (2002) based on the work by Lawrence *et al* (1991) in the photos a-e.

Mature biofilms have a three dimensional structure (figure 3) (Cooksey *et al*, 1995; Heydorn *et al*, 2000; Lawrence *et al*, 1991). The architecture of the specific biofilms differs from each other because they are dependent on the species within them and the function of these. Biofilms are made of microcolonies which are divided by interstitial

cavities (Cooksey *et al*, 1995; Heydorn *et al*, 2000; Lawrence *et al*, 1991). The microcolonies are in general rounded and mushroom shaped with several layers of EPS within them. Lawrence *et al*, (2000) did CEM (computer-enhanced microscopy) analysis on two *Pseudomonas* strains and one *Vibrio* strain and found the primary position of the non cellular materials was placed differently. In the two *Pseudomonas* strains the non cellular materials were placed least at the base (substrate bacteria - interface), where the largest cell density was found, and increased towards the water - biofilm interface. This has also been observed by Pereira *et al*, (2002). The non cell materials in *Vibrio* biofilms were mainly placed at the surface - bacteria interface (Lawrence *et al*, 1991). There can also be significant structural differences in growth pattern between mono species biofilms. Heydorn *et al*, (2000) analyzed four mono species biofilm from *Pseudomonas* structures with COMSTAT (computer statistics) and found that the colonization structure went from strong micro colonies to flat uniform biofilms. Dalton *et al*, (1996) also observed differences in bacterial settlement behavior. One unidentified strain behaved differently depending on the surface wettability. When settling on a hydrophobic surface the cells formed a compact multi layered single cell biofilm where the cells when settling on a hydrophilic surface resulted in development of long multi cellular chains (Dalton *et al*, 1994; Dalton *et al*, 1996). Another unidentified strain showed proximal, vertical packing of the cells unaffected by the surface resulting in a honey comb like structure. *Vibrio* sp. S14 displayed a spreading behavior where some cells attached longitudinally and irreversibly which gave rise to development of microcolonies. *Pseudoaltermonas* sp S9 developed regular microcolonies on several substrates with single cells slowly migrating between the colonies (Dalton *et al*, 1996). Differences in cell surface characteristics were also observed by McEldowney & Fletcher in 1986 after culturing four marine bacteria under different nutrient conditions. Time spent in the culture before attachment also affected the bacteria attachment (McEldowney & Fletcher, 1986). Biofilm thickness increases in general over time (Pereira *et al*, 2002; Webster *et al*, 2004) but fluctuations can be observed (Pereira *et al*, 2002). The cavities between the microcolonies create an internal network in the biofilm in which a free water flow occurs (Costerton *et al*, 1995). Biofilms are highly hydrated and up to 95 % of the biofilm can be made by non cellular materials as EPS and pore spaces (Lawrence *et al*, 1991). *Pseudomonas fluoroscens* biofilms differed in thickness and total number of bacteria when grown under laminar and turbulent flow conditions.

The biofilm grown under the laminar flow was also less dense than the biofilms grown in a turbulent flow. There was observed fluctuations in biofilm mass, due to the sloughing off and re-growth phenomena, in the biofilms exposed to laminar flow (Pereira *et al*, 2002). Shallow water biofilms had a larger microbial biomass than in deepwater biofilms the first 2 – 4 weeks but evened out after 8 weeks of growth. There was also observed a larger diatom density in the shallow water biofilms (Webster *et al*, 2004).

Since bacteria multiply fast and they all produce exopolymers (Costerton *et* al, 1995; Dobretsov, 2009) they rapidly form a thin layer of organic matter which among other things hold the nutrients from the water column (van Loosdrecht *et al*, 1990). For example has the biofilm formation of *V. vulnificus* been found to be affected by nutrient content (glucose) and not by salinity (1 and 3.5%) or temperatures (20 and 37°C) the latter to mimic the environments of blood and seawater (McDougald *et al*, 2006). McEldowney & Fletcher (1986) observed changes in bacterial attachment when treated with different carbon:nitrogen ratios and sources. There are differences in biofilm structure between clinical and marine isolates and it is proposed that quorum sensing can have an effect on regulation of nutrient stress responses in relation to bacterial survival and maintenance in/of biofilm structures (McDougald *et al*, 2006).

Bacteria are the main dominating group found in biofilms and therefore they obtain larger densities than free floating cells. Because of the high bacterial density in biofilms, bacteria and other organisms in the film, must cope with all the by- products produced by the organisms in the film such as secondary metabolites, metabolic by products and other excretes (Costerton *et al*, 1995; Parsek & greenberg, 2005).

P. fluorescens biofilm structure was affected by the main carbon source available as nutrient. Aromatic hydrocarbons decreased growth rates and cell size and changed cell morphology, increased biofilm thickness and affected spatial relationships/topography as conical bacterial microcolonies, produced clusters of cocci, the biofilm were developed in 14 - 21 days. When an easier degradable carbon source (tryptic soy broth) was used, the biofilm was more homogeneous and was not as thick as observed when using aromatic hydrocarbons as nutrient source. There

were no clear cellular associations present when using the labile carbon source (tryptic soy broth). The uniform biofilm observed in growt with tryptic soy broth changed to the more non uniform pattern after 2 days exposure of diclofop (a herbicide) (Wolfaardt *et al*, 1994).

Bacteria which produce antibiotic or toxic compounds and, in general, quorum sensing molecules can have large influence or impact on biofilms. Bacteria as *Streptomyces*, *Altermonas*, *Pseudoaltermonas* and *Roseobacter* all belong to antibiotic producing genera and they produce a wide range of antibiotics (Dobretsov, 2009; Dobretsov *et al*, 2006). Antagonistic antimicrobial activity between bacteria and fungi was observed in marine water from a mangrove stand in Sai Kung (Miao & Qian, 2005). The bacteria *Pseudoalteromonas tunicata* can also out-compete some other bacterial strains during colonization by producing an antibiotic, unless the competing bacteria are insensitive to the antibiotic or they are producing a compound which inhibits the growth of *P. tunicata* (Rao *et al*, 2005).
The marine bacteria *Halobacillus salinus* produces a secondary metabolit which inhibits bioluminescence production by *Vibrio harveyi*, the violacein biosynthesis by *Chromobacterium violaceum* CV026 and the green fluorescent protein production by *Escherichia coli* JB525 which all are controlled by quorum sensing signaling (Teasdale *et al*, 2009).

Species composition in biofilms

The bacterial composition in a biofilm can be very dynamic over a short period of time. Natural subtidal biofilms from a fish farm located in eastern Hong Kong waters were examined over 9 days and there were found in total 68 different bacteria spread in 3 phylogenetic groups and in 15 genera. The majority of species was found in the genera of *Pseudoalteromonas* (18 isolates) and *Vibrio* (17 isolates). The composition of this biofilm, based on biofilm morphology, was very dynamic during the 9 days, many types of colonies were only present in the start, middle or in the end of the sampling period where only a very few were present during the whole period (Huang, 2007a).

Mature marine biofilms are in general dominated by bacteria from the groups of *α-proteobacteria*, *γ-proteobacteria* and the group of *Cytophaga-Flavobacterium* from the *Bacteroides* (Dobretsov, 2009; Dobretsov *et al*, 2007; Jones *et al*, 2006; Nocker *et al*, 2004; Webster *et al*, 2004).

Bacteria from the *α-proteobacteria* group mostly dominate the biofilms growing on hard substrates in an experiment using FISH (fluorescence in situ hybridization) probing on biofilms collected after 2, 4 and 8 weeks in both deep and shallow water at Davies Reef (Great Barrier Reef Australia). In general, on all sites and collection days, the *α-proteobacteria* and *γ-proteobacteria* dominated. *Bacteroides* was present in the microbial community in week 2 on shallow water and in weeks 4 and 8 in deep waters where *β-proteobacteria* only was found in considerable densities in the biofilm community on shallow waters in weeks 2 and 8 (Webster *et al*, 2004).
Differences in bacterial strains were observed between free living bacteria and settled bacteria in Delaware Bay estuary. The bacteria in biofilms settled on either stainless steel or polycarbonate was dominated by *α-proteobacteria* from the *Rhodobacterales* branch (58 – 64 %). The *α-proteobacteria* abundance was 30 – 43 % on stainless steel and 21 – 41 % on the polycarbonate respectively. They also observed that the biofilm community composition begun identical but changed over time but the total bacterial abundance in the biofilms did not differ significantly from each other (Jones *et al*, 2006). The most abundant bacterial group in biofilm, from Hong Kong water, was the *γ-proteobacteria* where *Vibrio* and *Pseudoaltermonas* dominated (Dobretsov & Qian, 2002; Dobretsov & Qian, 2004; Qian *et al*, 2007). Biofilm on the soft coral *Dendronephthya sp*. was dominated by *α*- and *γ-proteobacteria* and *Cytophaga-Flexibacter-Bacterioides* (Dobretsov & Qian, 2004). From biofilm on the green alga *Ulva reticulata* (Forsskal), which often is observed biofouling free, strains, identified by 16S rRNA analysis, of the genera *Alteromonas*, *Pseudoalteromonas* and *Vibrio* was dominating (Dobretsov & Qian, 2002). Biofilm from an oyster reef in Florida was dominated by bacteria from the *γ-proteobacteria* and *δ-proteobacteria* and the total species richness was estimated to 417 where biofilm from the muddy sand bottom at the same location, in contradistinction, was dominated by bacteria from the *Planctomyces* phyla and had lower species diversity only estimated to 60. Only 10.5 % of the species were found in both habitats even though the two habitats were not far from each other (Nocker *et al*, 2004).

Kjellerup *et al*, (2005) who worked with biofilms from freshwater found the dominating groups included *β-proteobacteria*, as the most dominant, *α-proteobacteria* and *δ-proteobacteria*. The *α-proteobacteria* group mainly consisted of bacteria from the families of Rhizobiaceae and Acetobacteriaceae and the *β-proteobacteria* was from the Comamonadaceae family.

Interactions between pro- and eukaryotes in biofilms

Wigglesworth-Cooksey & Cooksey have reported interactions between the bacterium *Pseudoaltermonas sp*. and the diatoms *Amphora coffeaeformis* and *Navicula sp*. The agglutination of the diatoms enhanced when they were grown in culture medium used by the bacterium. The used *Pseudoaltermonas sp*. media caused the diatoms first to decrease motility and thereafter detach and then to agglutinate. The cells started to lyse after 6 – 24 hours. The bacterium *Bacillus simplex* had the same effect on the diatoms as the *Pseudoaltermonas* sp. (Wigglesworth-Cooksey & Cooksey, 2005).

Grazing microorganisms as heterotrophic flagellates, amoebas and ciliates could influence and even control the density and diversity of microorganisms in biofilms (Dobretsov, 2009) but there were also studies where this was not the case. Heterotrophic flagellates removed approximately 2.5 % of the bacterial carbon present, in a study performed on mangrove tree roots, each day, and were in general considered among the major grazers, where ciliates consumed about 0.2 % and the amoebae only 0.3 % of the carbon present in biofilms located on mangrove trees roots. The amoebae were the only of the predators which were capable of growing while feeding on tightly attached bacteria. In this study the investigated part of the Protozoa population could not control the biofilm population (Maybruck & Rogerson, 2004). Kiørboe *et al*, (2003) measured the grazing rate of the heterotrophic *Bodonid* flagellates to between one and ten bacteria per flagellate per hour. The grazing rate of the flagellates increased with bacterial density and the presence of the grazing heterotrophic flagellates also changed the architecture of the biofilm. Species composition in the biofilm was also altered by the presence of the grazing flagellates resulting in lesser species diversity but more resistant to the grazing (Kiørboe *et al*, 2003). Grazing by ciliates, which preferred to grass on the bacterial EPS matrix and

selected yeast and planktonic *Pseudomonas* sp. cells over biofilm cells, actually increased the bacterial EPS production (Joubert *et al*, 2006). The bacteria *Pseudoaltermonas tunicata* required several other species of bacteria to be present to effectively colonize the surface of the alga *Ulva australis* (Rao *et al*, 2006). Limpets (Mollusca) which are large grazers could control both microbial biomass and species diversity in biofilms on rocky shores. The biofilm density increased by 20 % in two weeks when the limpet density was suppressed (Thompson *et al*, 2002). Copepod grazing affects biofilm densities both positively (because of dissolved organic matter) (Vargas *et al*, 2007) and negatively (Caron, 1987; Pearlmutter & Meyer, 1991) and could also alter microbial composition (Pearlmutter & Meyer, 1991). The particulate organic matter (POM) from copepod cultures, *Tisbe japonica* and *Nitocra spinipes*, increased bacterial density. Presence of either copepods or their particulate organic matter changed the community structure in the biofilms and reduced settlement of *Hydroides elegans* larvae (Damhs & Qian, 2005).

Biofilm induction and inhibition on larvae settlement

Microorganisms already adhered can affect, both positively and negatively, the adhesion of other planktonic microorganisms which again stimulate the establishment of multi species heterogeneous biofilms (Dobretsov, 2009; McEldowney & Fletcher, 1986).

Biofilms could induce or inhibit settlement and metamorphosis of marine larvae. This bacterial effect was clearly shown in monospecies biofilms (Maki *et al*, 1992; Lau *et al*, 2002; Dobretsov & Qian, 2004; Lee & Qian, 2003; Dahms *et al*, 2004; Dobretsov & Qian, 2006; Huang *et al*, 2007).

Maki *et al*, (1992) made settlement assays (22 hours) with cypris larvae of the barnacle *Balanus amphitrite* on biofilms from 2 strains of *Deleya marina*, which was phenotypically similar but had different cell surface free energy, where the bacteria used for biofilms, was obtained from either the stationary or the growth phase. They found that the surface free energy was not the reason for the inhibition of larval settlement and suggested therefore that the inhibition of larval settlement must be due to specific molecules in the extracellular polymers.

Lau *et al*, (2002) identified 38 bacterial isolates collected from the Clear Water Bay in Hong Kong and found that 58% of these had from an equally moderate to strong effect on settlement of the marine polychaete *Hydroides elegans* the remaining 42% were categorized as non-inductive. The bacterial isolates were obtained from 3 phylogenetic branches, γ*-proteobacteria* (26 isolates), gram positive (8 isolates) and *Cytophaga-Flexibacter-Bacteroides* (4 isolates) and most of the isolates belonging to the γ*-proteobacteria* (26 isolates) were from the 3 genera of *Vibrio* (7 isolates), *Altermonas* (8 isolates) and *Pseudoalteromonas* (8 isolates). It was also noticed that isolates from the same genus could have very different effect on larval settlement.

Pseudomonas fluorescens was considered a soil bacterium but could also be found both in marine and fresh waters (Anthoni *et al*, 1995; Kim *et al*, 2007; Kumaran *et al*, 2010).
Pseudomonas fluorescens had in contrast to *Pseudomonas aeruginosa* shown the ability to inhibit or retard, settlement and metamorphosis, of alga (Kim *et al*, 2007) and barnacle cyprids (O'Conner & Richardson, 1996), fungu (Manjula *et al*, 2004) and bacteria (Cabrefiga *et al*, 2007; Gram *et al*, 1999). *Pseudomonas aeruginosa* had in laboratory experiments been able to kill the nematode *Caenorhabditis elegans* by cyanide poisoning (Gallagher & Manoil, 2001) and inhibit growth of green microalgae and cyanobacteria (Dakhama *et al*, 1993) which had not been reported in *Pseudomonas fluorescens*. None of these two bacteria had proven the ability to inhibit *Bugula* spp. but together they covered a broad spectrum of the organisms found on the experimental slides in this thesis.

Bacterial communication

Quorum sensing

In gram negative bacteria where a quorum sensing system has been identified all, except *Vibrio harveyi* and *Myxoccus xanthus*, uses a quorum sensing system similar to the LuxI/LuxR-type quorum sensing of *Vibrio fischeri* and has as a minimum homologues of the two regulatory proteins LuxI and LuxR. The LuxI/LuxR bioluminescence system in *Vibrio fischeri* is the most throughly studied quorum sensing system and is therefore used as a model system (figure 4) (Miller & Bassler, 2001; Engebrecht *et al*, 1983; Engebrecht & Silverman, 1984; Waters & Bassler, 2005).

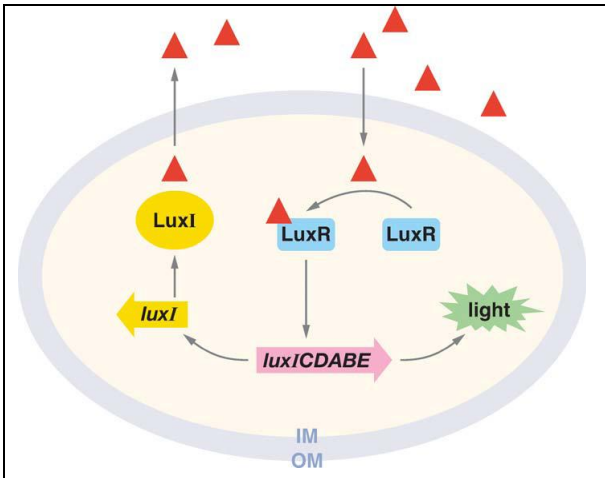


Figure 4: Model of the LuxI/LuxR quorum sensing circuit in V. Harvey. The red triangle indicates the autoinducer produced by LuxI and the IM and OM indicates inner and outer membrane (Waters & Bassler, 2005)

V. harvey uses a system where features from both gram negative and gram positive bacteria quorum sensing systems are present. They produce and respond to acylated homoserine lactones as all other gram negative bacteria but the transduction of the quorum sensing signal occurs via a two component circuit (Bassler, 1999; Miller & Bassler, 2001). *V. harvey* also has a signal molecule AI-2 (figure 5) (Bassler *et al*, 1993; Bassler *et al*, 1994) which has been found in both gram positive and negative bacteria and is speculated to be the evolutionary link between the two major quorum sensing systems (Surette & Bassler, 1998; Surette *et al*, 1999).

Gram positive bacteria secrete peptides as autoinducers for quorum sensing. The peptides are transported out of the cell through an ATP-binding cassette also known as an ABC-transporter. To detect the autoinducers the gram positive bacteria use a

two component adaptive response protein which is dependent on a phosphorylation/dephosphorylation cascade signal mechanism (Review: bassler, 1999; Kleerebezem *et al*, 1997; Lazazzera & Grossman, 1998).

The LuxI/LuxR bioluminescence system

LuxI homologous proteins are responsible of the biosynthesis of a homoserine lactone (HSL) or an *N*-acylated homoserine lactone (AHL) signal molecule (figure 5) (Miller & Bassler, 2001; Engebrecht *et al*, 1983; Engebrecht & Silverman, 1984; Waters & Bassler, 2005). This type of molecule is referred to as an autoinducer (Nealson *et al*, 1970).

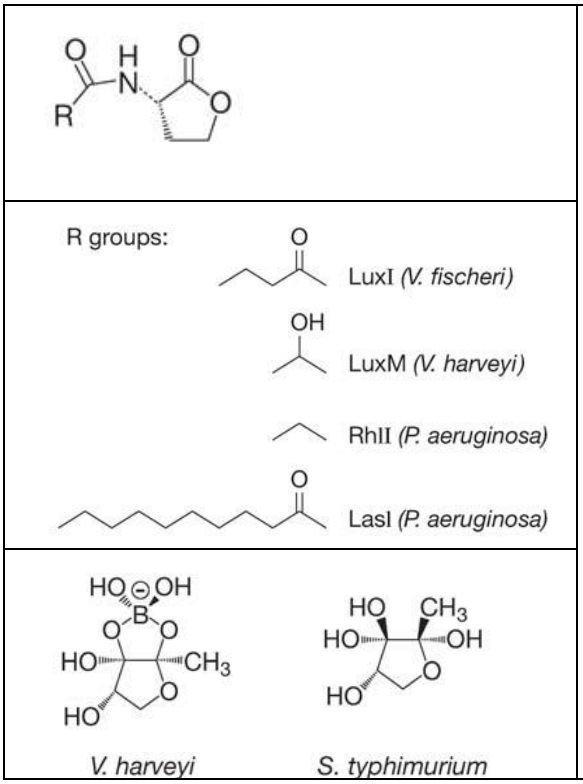


Figure 5: The autoinducer acyl homoserine lactone core molecule (upper). Some examples on side chains also called R groups (middel). Examples on AI-2 autoinducer compounds from the model organism *V. harvey* and *Salmonella typhimurium* (bottom) (Waters & Bassler, 2005).

The concentration of the autoinducer will increase with cell density. The LuxR homologous proteins bind to related AHLs when these reach a threshold concentration and they also activate target gene transcription. This type of quorum sensing system allows bacteria to react on alterations in population density by coupling gene expression to a protein which concentration is density dependent (Engebrecht *et al*, 1983; Engebrecht & Silverman, 1984; Miller & Bassler, 2001). Seven genes dispersed in two operons cloned from *V. fischeri* were required for light production in recombinant *Escherichia coli* were identified by Engebrecht &

Silverman, (1984). These genes coded for the regulatory functions and activities which are required for a light reaction. The genes named *luxA* and *luxB* codes for the α- and β subunits of luciferase. The three genes *luxC*, *luxD* and *luxE* code for the synthesis and reuse of the aldehyde substrate. The genes *luxI* and *luxR* regulated the light production (Engebrecht & Silverman, 1984).

LuxI directs synthesizes of the autoinducer *N*-(3-oxohexanoyl)-homoserine lactone (a HSL, also known as N-(3-oxohexanoyl)-3aminohydro-2(3H)-furanone) (Eberhard *et al*, 1981). LuxI uses S-adenosylmethionine (SAM), a methionine product, as the amino acid component and acyl-ACP, an intermediate generated from thioesters in the fatty acid synthesis, for synthesis of the autoinducer (Hanzelka & Greenberg, 1996; Val & Cronan, 1998).

LuxR responds to the autoinducer by binding it in the autoinducer-binding domain located in the N-terminal region (Hanzelka & Greenberg, 1995) and also activates transcription of the *luxICDABE* (Engebrecht *et al*, 1983; Nealson *et al*, 1970; Stevens *et al*, 1994) by binding to these with the C-terminal (Choi & Greenberg, 1991) upstream in a region containing the *lux* box and the *luxI* -35 and -10 regions and the *crp* box (cAMP receptor protein). Together with RNA polymerase and through a synergistic binding they bind to the promoter and protect a region including the *lux* box and *lux* operon from DNase I digestion (Stevens *et al*, 1994). LuxR demands a minimum length of side chain of autoinducer compounds, derived from 3-oxohexanoyl HSL (3-oxo analogs), of five carbons to bind and can bind autoinducer compounds with 14 carbons in the side chain. The most efficient binding requires autoinducer compounds with side chains lengths of 6-8 carbons. The carbonyl group in position 3 increases the potency as a luminescence inducer (Schaefer *et al*, 1996). The autoinducer can diffuse freely over the bacterial cell membrane and therefore the intra- and extracellular concentrations of the HSL molecule are alike when the bacteria are enclosed as in *in vitro* experiments. When extracellular autoinducer concentration increases the intracellular concentration also rises. Operon *luxR* activates by autoinducer concentrations as low as 10 nM which is approximately 2 molecules per cell (Kapland & Greenberg, 1985).

Quorum sensing in CV026 and CV017

In order to determine if a given bacterial species have the LuxI/LuxR type quorum sensing system, the first step is to test for production of acyl homoserine lactones (AHLs) signal molecules (Blossor & Gray, 1999; Winson *et al*, 1998).

The gram negative *Chromobacterium violaceum* grows in soil and water (Vasconcelos *et al*, 2003) and produces the purple pigment violacin which is water soluble and have antibacterial activity (Lichstein & Van De Sand, 1945).

The *C. violeceum* AHLs reporter system uses this bacterium's ability to produce violet pigment in respons to *N*-hexanoyl homoserine lactone (C6HSL) (Blossor & Gray, 1999; Lichstein & Van De Sand, 1945; McClean *et al*, 1997).

The designer strain CV026 is a Smr mini-Tn*5* Hgr *cviI*::Tn*5xylE* Kmr mutant of *C. violaceum* ATCC 31532 (CV0) and does not produce any autoinducer itself but produces violacein in respons to added external *N*-acyl C6HSL and other short chain *N*-acyl AHLs from C_4-C_8 in lenght. In return longer *N*-acyl AHLs chains from C_{10} to C_{14} inhibit the production of violacein (McClean *et al*, 1997).

C. violaceum CV017 is a designer strain with a Sm^r mini-Tn*5* Hg^r mutation. As the parental strain *C. violaceum* ATCC 31532 (CV0) it produces *N*-acylhomoserine lactone (AHL) *N*-hexanoyl-homoserine lactone (C6-HSL). It carries an unknown genetic mutation which depresses the AHLs inducable violacein production when the bacterium is grown above 30°C and produces therefore more violacein compared to the parental strain (Chernin *et al*, 1998; Matz *et al*, 2004).

Fatty acids in bacteria

Fatty acids

Fatty acid compositions in bacterial cultures are affected by different environmental factors. Of the nutrient factors presence are availability of acetate, glycerol, carbohydrates, lipids and nitrogenous substances and their internal ratios of importance. Other factors as oxygen concentration, pH, atmospheric pressure (Yano *et al*, 1998), salt concentration (Monteoliva-Sanchez & Ramos-Cormenzana, 1986; Monteoliva-Sanchez *et al*, 1988), culture age also affect the fatty acid composition (Bas *et* al, 2003; Bååth *et al*, 2003; Hunter & Thirkell, 1971; Kanfer & Kennedy, 1963; Mansbridge & Blake, 1997; Nordström & Laakso, 1992; O'Leary, 1962; Walker *et* al, 2005) and the most important, temperature (Kamimura *et al*, 1992; Kamimura *et al*, 1993; Monteoliva-Sanchez & Ramos-Cormenzana, 1986; Monteoliva-Sanchez *et al*, 1988; Nordström, 1993).
It is important to remember that since there is difference in the artificial media laboratory cultures are grown in and the nutrients available in a natural environment that this can affect the fatty acid composition. For example differences were observed in cells from *Mycobacterium tuberculosis* grown in lung tissue and in laboratory media both in relative lipid content and in utilization of some fatty acids (O'Leary, 1962; Segal & Bloch, 1956).

The fatty acids in bacteria cells are mainly a part of the cell membrane as the acyl component of the phospholipids. The bacterial fatty acids in the membranes can be divided in two groups based on their biosynthesis; the straight chain fatty acids and the branched fatty acids. The straight chain fatty acids are mostly found in bacteria. They are synthesized from acetyl coenzyme A (acetyl-CoA) as a primer and with malonyl-CoA as the chain extender. The chain can be further modified after basic synthesis. The straight chained fatty acids include fatty acids as palmitic, staeric, hexadecenoic, octadecenoic, cyclopropanic, 10-methylhexadecanoic and 2- or 3-hydroxyl fatty acids. The branched fatty acid chains are not as common as the straight fatty acids but are present and have significant functions. They are synthesized from iso, anteiso or cyclic primers and malonyl-CoA can be further modified and or have

unsaturated or hydroxylated parts (Kanada, 1977; Kaneda, 1991; Kanada & Oku, 1988; Smirnova & Reynolds, 2001).

Biosynthesis of fatty acids

Fatty acid synthesis is divided in two systems based on their physical structure; type I and type II. Type I is a multifunctional system which synthesize fatty acids in animals and yeasts where type II consists of a soluble system composed by several individual enzymes which synthesize fatty acids in higher plants and bacteria (Kaneda, 1991; Marrakchi *et al*, 2002; Schweizer & Hofmann, 2004). The principle in the fatty acid synthesis is the same for both systems. Acetyl-CoA is used as the primer and its carbon chain is elongated by adding malonyl-CoA to the primer by condensation. Hexadecanoic acid (palmitic) acid is the major product. In the bacterial (type II) synthesis system an acyl carrying protein (ACP) is required for the synthesis of hexadecanoic acid where in the type I system an ACP domain is included in the molecule (Byers & Meighen, 1989; Heath & Rock, 1996; Kaneda, 1991; Smith *et al*, 2003).

The straight chain fatty acid biosynthesis pathway, which is basic for all bacteria, describes the pathway and enzymes used in the model organism *E. coli*. The pathway starts with carboxylation of acetyl-CoA by the four subunits AccABCD to produce malonyl-CoA. Malonyl-CoA is transferred to ACP by the malonyl-CoA:ACP transacelylase FabD. The elongation of the fatty acid is done in cycles and is initiated by condensation of acetyl-CoA with malonyl-ACP catalysed by FabH (*β*-ketoacyl-ACP synthase III) to *β*-ketoacyl-ACP and CO_2. This step is repeated with two carbon units per cycle until a saturated fatty acid with 16-18 carbons is made. *β*-ketoacyl-ACP are reduced to *β*-hydroxyacyl-ACP by the NADPH dependent reductase FabG (*β*-ketoacyl-ACP reductase). *β*-hydroacyl-ACP is dehydrated to *trans*-2-enoyl-ACP by either FabA (*β*-hydroxydecanoyl-ACP dehydratase/isomerase) or FabZ (*β*-hydroxylacyl-ACP dehydratase) and is then further reduced to acyl-ACP by enoyl-ACP reductase (FabI) which also is NADPH dependent. The elongation, and subsequent elongation rounds, is initiated by the condensing enzymes FabB (*β*-keto-acyl-ACP synthase I) and FabF (*β*-keto-acyl-ACP synthase II) and their substrate specificities and expression of these controls the structure and distribution of fatty acids (Marrakchi *et al*, 2002: White *et al*, 2005).

The genes coding for the enzymes in this pathway are in general conserved but there are some differences between the *E. coli* model system and the pathway in other bacteria. The genes *fabA* and *fabB* are in many bacteria which produces unsaturated fatty acids (UFA), for example in *Pseudomonas aeruginosa*, co-transcribed together in a *fabA-fabB* operon (Cronan *et al*, 1969; Hoang & Schweizer, 1997; Marrakchi *et al*, 2002). In *E. coli* only two reactions are needed to produce UFA's. *β*-hydroxy-C10:0-ACP (the number before the colon refers to the number of carbon atoms in the fatty acid chain and the number after the colon refers to the number of double bonds) is dehydrated to *trans*-2-C10:1-ACP by either FabA or FabZ but FabA can also isomerize *trans*-2-C10:1-ACP to *cis*-3-C10:1-ACP which diverts the nascent acyl chain into the UFA pathway where it is further elongated by the FabB enzyme. Gram positive bacteria, some anaerobic bacteria as Streptococci and Clostridia lack the *fabA* and *fabB* genes and therefore also the enzymes and use other enzymes to synthesize UFA's but the pathway is similar. For example in Streptococci pneumoniae where the FabA and FabB enzymes are not present the FabZ enzyme alone dehydrates the *β*-hydrox-C10:0-ACP to *trans*-2-C10:1-ACP. The isomerization of *trans*-2-C10:1-ACP to *cis*-3-C10:1-ACP is performed by the FabM enzyme and the elongation of the growing UFA is done by FabF where the elongation of the nascent SFA is performed by the FabK enzyme (Clark *et al*, 1983; Cronan *et al*, 1969; Marrakchi *et al*, 2002: White *et al*, 2005).

Fatty acid profiles in bacteria

Bacteria can be identified with gas chromatography by analyzing their fatty acids. The method is called GC-FAME (Gas Chromatographic – Fatty Acids Methyl Esters). The analysis uses short chained fatty acids which are volatile. A short chained fatty acid is between 9 and 20 carbons in length. With profiles of the fatty acids it is possible to identify genera and strains of bacteria (Sasser, 1990).

Fatty acid composition from a study of 12 strains of marine aerobic anoxygenic phototrophic (AAP) bacteria was characterized by saturated and monounsaturated fatty acids ranging from C_{12} to C_{18} and C_{12} to C_{19} where the *cis*-11-octadecenoic acid (*cis*-vaccenic or 18:1ω7) was the dominating fatty acid present. All of the analyzed bacteria contained 11-methyloctadec-12-enoic acid which was rare but had been

found in other marine bacteria (27-29) before. 3 strains had small quantities of 12-methyloctadec-11-enoic acid which had not been reported elsewhere. 9 out of the 12 strains also contained octadec-5,11-dienoic acid which was a polyunsaturated fatty acid (PUFA). Since bacteria normally do not contain PUFA this could be a useful indicator of AAP's from the marine environment. All strains from the genera of *Erythrobacter* and *Citromicrobium* contained 2-hydroxyacids and all from the *Roseobacter*-like strains contained 3-hydroxyacids (Rontani *et al*, 2005).
Gharaibeh & Voorhees (1996) investigated the cellular fatty acid composition of 10 bacteria strains of which 5 were gram positive and the other 5 gram negative. They observed a different fatty acid distribution between the two gram types. The saturated branched fatty acids (*i*C14:0, *i*C15:0, *a*C15:0, *i*C17:0 and *a*C17:0 (*i* stands for iso and *a* stands for anteiso)) were only found in gram positive bacteria except for a trace in *P. fluorescens*. Cyclopropyl (cyC17:0 and cyC18:0) and unsaturated straight chained fatty acids (C16:0 and C18:0) were mainly detected in the gram negative bacteria. The straight chained saturated fatty acid (C18:0) was only detected in gram positive bacteria and in large quantities where the C16:0 fatty acid (straight chained saturated) was observed in both types of bacteria and was a major contributor (Gharaibeh & Voorhees, 1996). Cis-9-octadecenoate (oleic acid) had been detected in streptococci (Hofmann & Tausig, 1955). In *Vibrio* species the major fatty acids were 16:1, 16:0, and 18:1 (Bertone *et al*, 1996).

Community analysis in bacteria

Community analysis by FAME analysis is an easy and fast way to determine how similar communities are. Fatty acids in bacteria are in general saturated- (SFA) and monounsaturated (MUFA) fatty acids which are ranging from C10 to C20. Poly unsaturated fatty acids (PUFA) are quite rare. Fatty acid biomarkers in bacteria are typically odd-numbered, branched *trans*-unsaturated and cyclopropyl fatty acids such as 15:0, 17:0, *iso*- and *anteiso*-branched saturated fatty acids and mono unsaturated fatty acids as 10-methyl-16:0 (Bergé & Barnathan, 2005).

Bacteria with the ability to produce PUFA is limited to five well-known marine genera distributed in two domains of bacteria the Cytophagales (Flexibacter, Psychroflexus) and the Proteobacteria (Shewanella, Colwellia, Moritella) (Nichols &

McMeekin, 2002). PUFA-producing ability has been preserved within the (generally) psychrophilic and halophilic species of Shewanella (Russell & Nichols, 1999) but the poly unsaturated fatty acid C18:2ω9,12 could also indicate the presence of microeukaryotes (Su & Yang, 2009).

Li *et al*, 2007 investigated sediment cores near a cold methane seep and gas hydrates in the Mexican gulf. The branched fatty acids (i.e., aC15:0, iC15:0, Me10C16:0) could be largely attributed to sulfate-reducing bacteria and the significant amount of cyclopropyl fatty acids found at all sites indicated the stress adaptation of the bacteria in the extreme environments (a cold methane seep and gas hydrates) (Li *et al*, 2007).

Olsson & Persson, (1999) investigated soil fractions from barley roots by FAME analysis. They found a proportional connection between soil adhesion to the barley roots and the fatty acid profiles. The higher degree of adhesion observed the higher relative proportions of 15:0, *i*15:0, *i*15:0-3OH, *i*15:1, *i*17:0-3OH and *i*17:1 fatty acids were observed. In another study of soil bacteria originating from barley roots grown in three different soil types, two clay and on silt loam, the dominating bacteria groups were Pseudomonas, Cytophaga and gram positive bacteria. The fatty acid profiles from the clay samples contained almost only straight chained fatty acids where in the silt loam branched fatty acids were dominating (Olsson *et al*, 1999).

Marine aggeregate bacteria collected from phytoplankton communities contained α-*Proteobacteria*, γ-*Proteobacteria* and *Cytophaga-Flavobacter* complex. The fatty acid profiles revealed that different aggeregates had a different dominating group or a contribution of several groups. As the aggregates aged a shift in the bacteria communities towards the *Cytophaga-Flavobacter* complex occurred and simoultanious a decrease in *Proteobacteria* was seen (Blažina *et al*, 2005).

Fouling species in Omani waters

The species and groups of organisms described in this section are all fouling organisms found on the experimental slides used in this thesis. The organisms found were *Bugula* sp., *Hydroides elegans*, barnacles (Cirripedia) and a variety of algae.

Fouling species

The *Bugula* belongs to the family of Bugulidae and the order of Cheilostomata. In common speak they are known as moss animals or bryozoans and are often mistaken for plants and are therefore also known as air ferns. *Bugula* is mostly found in marine tropical and temperate harbors.

Figure 6: *Bugula* sp. Oman

Bryozoans grow in colonies (figure 6) which are composed of individuals called zooids. The zooids are less than 0.5 mm. The colonies grow attached to a substrate as rocks or macro algae or again on ship hulls and other man made constructions which have labeled them as a fouling organism. All bryozoans are suspension feeders. They have a u-shaped retractable ring of tentacles which bears cilia which creates a current which brings food particles closer towards the zooids and the particles are brought to the mouth by movements of the tentacles and the cilia.

Bryozoan reproduction can both be sexual and asexual. A new bryozoan colony begins with a sexually produced zooid. This single zooid performs asexual budding and creates daughter cells which again also perform asexual budding and the colony is created by this process. In most bryozoans each zooid is hermaphroditic and is capable of producing both eggs and sperm (Hawaii Biological Survey, 2001; Lander University).

Barnacles belong to the infraclass of Cirripedia which is in the subphyla of Crustacea. The infraclass Cirripedia is divided into three superorders Acrothoracica, Thoracica and Rhizocephala (Martin & Davis, 2001).

They are small sessile filter feeding crustaceans which attach to a surface as rocks, pilings, ship hulls (Anderson, 1994) and living organism as whales (Nogata &

Matsumura, 2006). Because of their attachment to manmade structures barnacles are viewed as major biofoulers (figure 7).

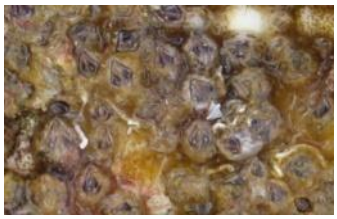


Figure 7: Barnacles, Oman

Barnacles are hermaphroditic (Anderson, 1994). The larva (nauplius) is released from the barnacle and goes through 6 stages before molting into the cyprid stage (for *Balanus amphitrite*). In this stage the larva becomes competent and chooses a place to settle and metamorphosis. In general the overall lifecycle of barnacles can be divided into three phases; 1) the six nauplius stages where it is plantonic and feeding, 2) a planktonic cyprid larva which are non-feeding and lives on stored energy reserves and is searching for a place to settle, 3) a benthic juvenile and adult which is permanently sessile (Hentschel & Emlet, 2000; Zhang *et al*, 2010).

The term algae are a common term which covers seven divisions of photosynthetic, eukaryotic organisms. They can be either unicellular (microalgae) or multicellular (macroalgae) organisms and their development in biofilms has impact on the microbial community and is suggested as "ecosystem engineers" (Besemer *et al*, 2007).
Algae are found both in fresh and salt water and can be free floating (unicellular) or attached to soil, rocks, larger animals or other structures as pillars and ship hulls. They are classified on behalf of their color, green, brown and red, which can be difficult since some green algae looks like brown algae and the brown algae can be mistaken for red algae and opposite. Algae reproduction can both be sexual (isogamy) or asexual (sporulation) (Allen & Hollenberg, 1976).

The class of Chrysophyceae covers the golden-brown algae and the diatoms which are unicellular algae though diatoms can be found in chains or colonies. Most species are marine but occur also in freshwater. Their cell walls are made of cellulose and a pectic material. In diatoms the cell walls are rigid because of silica impregnation. The golden-brown algae locomotes with the help of a flagellae, where diatoms move through the exopolysaccharide matrix they produce. These algae have chlorophyll a and c as photosynthetic pigments (Allen & Hollenberg, 1976; UCMP).

Chlorophyta is also known as green algae. Most green algae are microscopic and live in fresh water but there are both marine and macroalgae in this division. The cell walls are constructed of mostly cellulose but also contain hemicellulose. Some species also have calcium carbonate in their cell walls. Some of the unicellular algae have flagella for movement. The main photosynthetic pigments are chlorophyll a and b (Allen & Hollenberg, 1976; UCMP).
The class of Phaeophyceae is commonly known as brown algae and are found both as micro-and macroalgae. The cell walls consist of cellulose and the polysaccharides alginic acids. Brown algae have complex differentiated tissues which are used for securing the organism to substrates, air bladders for buoyancy, a supporting stalk, large wide leaves which provides a large surface for nutrients exchange and photosynthesis and spore producing reproductive tissue. The main photosynthesis pigments are chlorophyll a and c. The reproductive tissue produce cells which are freed into the water column where they locomote with their two flagella (Allen & Hollenberg, 1976; UCMP).

Most of the seaweed in the ocean are from the division (Phyla) of Rhodophyta and nearly all species in this division is marine species. Red algae are all multi cellular and are much branched compared to other types of macroalgae but do not have any complex differentiated tissue. The inner cell wall consists of cellulose and the outer of a mucilaginous (gelatinous) layer. The spores of red algae are not flagellated. The primary photosynthetic pigment is chlorophyll a (Allen & Hollenberg, 1976; Cole & Sheath, 1990; UCMP).

The *H. elegans* belongs to the family of *Serpulidae* which again is in the order of Canalipalpata. The worms produce a small calcified tube which is irregular in shape. The worm is approximately 20 mm in length and 3 mm in diameter but the tube can be up to 75 mm. The worm extends feather like radioles with cilia attached (figure 8) which it used to trap small organic particles. The cilia transfer the organic particles from the radioles to the mouth of the worm.

The worm settles on rocks and pilings in tropical and sub-tropical waters and on ship hulls which has labelled it as a fouling organism (MBL; Smithsonian Marine Station). Settlement on ship hulls are the largest transport vector for *H. elegans* with transport on bivalves as the secondary introduction source to non native locations (NIMPIS, 2002).

			Figure 8: Photos of *H. elegans*. A: Close-up of the radioles and cilia. B: Worms settled on antifouling coating. C: Close-up of worm settled on antifouling coating.
A: *H. elegans* (SMS)	B: *H. elegans*, Oman	C: *H. elegans*, Oman	

H. elegans reproduces sexually by the release of gametes which are fertilized externally. The worms are mature after 14 to 15 days. The fecundity of the females varies between 1100-9050 oocytes per female. The worm fecundity was not affected by temperature in range 15°C – 30°C but was reduced in low salinity 20 ‰. The survivorship, growth and maturation of juvenile worms were also significant influenced by salinity (Qui & Qian, 1998).

Settlement of organisms

Shipping traffic transports fouling and thereby also exotic species to ports in coastal cities (Farrapeira *et al*, 2007). The most dominant species in Eastern harbor of Alexandria, Egypt, were the polychaete *Hydroides elegans*, the barnacle *Balanus amphitrite*, the amphipods *Corophium acutum* and *Elasmopus pectenicrus* (Ramadan *et al*, 2006). A study in Humboldt Bay, California, found that approximately 35 % of the fouling species was introduced. The panels were dominated by colonial and solitary ascidians, bryozoans and hydroids (Boyle *et al*, 2006).

Laboratory and field experiments showed that the higher the surfaces elastic modulus (elasticiy) was, the higher the larvae settlement. In a field experiment the settlement density of ciliates (*Zoothamnium* sp., *Astylozoon* sp. and *Folliculina* sp.) had a significant positive correlation with the elastic modules. There was also found a significant correlation between settlement and elastic modules for the tube dwelling polychaete *Polydora ligni*. In the laboratory where *B. neritina* was used as model organism the same observations were made (Gray *et al*, 2002). *Bugula* larva had also a lower settlement and less successful metamorphosis for the longer period they were forced to swim (Hunter *et al*, 1998).

Several studies confirm that barnacle demonstrates gregarious behavior when choosing settlement location (Dreanno *et al*, 2006; Dreanno *et al*, 2007; Head *et al*, 2004; Jeffery, 2000). Head *et al* (2004) found that as few as three larvae were enough to have a significant effect on settlement in *Balanus amphitrite* and Dreanno *et al* (2006) found that a α_2-macroglobulin-like protein is the settlement cue. A study by Larman & Gabbot (1975) confirms that adult barnacles or extracts from these work as settlement cues. Hills *et al*, (1998) also found that cyprid larva settled in larger densities when the surface was treated either by chemicals or by a crushed cyprid larvae but cyprids used longer time to investigate the area before settlement in the treated areas. The settlement of the whale barnacle, *Coronula diadema*, is mediated by settlement cues in the skin from the whale (Nogata & Matsumura, 2006). Barnacles, of the species *Balanus balanoides*, prefer to settle in places where other individuals already have settled but held a territorial distance of approximately 2 mm from the earlier settled ones. This separation distance is reduced as the population density increases but will still exist in some form to allow the cyprid larva time to

settle and establish itself before it grows into the edge of another barnacle (Crisp, 1961). *B. amphitrite* cyprid larvae preferred to settle on hydrofilic (NH_2 terminated glass) surfaces compared to hydrophobic (CH_3 terminated glass) (Phang *et al*, 2009).

Dahms *et al,* (2004) studied *B. neritina* larvae settlement on bacterial and diatom biofilms and how different biofilm densities affected settlement. They found *B. neritina* larvae settlement on different monospecies bacterial biofilms was significantly different from each other and *B. neritina* settled in general with a higher percentage on diatom mono-species biofilm compared with bacterial mono-species biofilm. They also found that in mixed biofilm the biofilm density affected the settlement of *B. neritina*. Biofilms consisting of the bacteria and diatoms had a low effect on inhibiting larvae settlement, that showed to be rather indiscriminating, but the different biofilm compositions and biofilm density still had a significant effect on larvae settlement (Dahms *et al,* 2004).

In double dish settlement experiments *B. neritina* larvae preferred to settle on the biofilmed side of the container compared to the sterile side. When biofilms contained bacteria from the subtidal zone the larvae, significantly, preferred to attach to the biofilmed side of the container and the opposite was observed when the biofilm contained bacteria from the intertidal zone (Dobretsov & Qian, 2006).
Larvae settlement was also tested on the sub- and intertidal biofilms from day 7, 14 and 28 against sterile dishes and larvae preferred to settle on biofilmed surfaces and on subtidal when subtidal and intertidal was tested against each other. They also found that it is bacteria and not diatoms that drove larval attachment and that the larvae could differentiate between multi-species biofilms from different habitats. They also suggested that the biofilm composition and not bacterial and diatom density was important for the induction of larval settlement (Dobretsov & Qian, 2006).

Cyprid larva of the barnacle *Balanus amphitrite*, nectochaete larva of the tube worm *Hydroides elegans* and coronate larva of the bryozoan *Bugula neritina* were observed and videotaped when swimming besides clean and biofilmed surfaces. The larvae of both *H. elegans* and *B. neritina* swam close to the clean surfaces and straight along them where both larvae stopped swimming and crawled onto biofilmed surfaces when

encountering these. It was also observed that larvae when swimming close to biofilmed surfaces turned and circled and started to crawl on them. This behavior was not observed if the biofilmed surface was covered with a mesh to prevent larval settlement but still allowed dissolved chemicals to diffuse from the biofilm surface. This last observation could indicate that no soluble cues from the biofilm stimulated the settlement (Hadfield, 2006).

The daily settlement of barnacles, Chthamalus spp., was followed in Scripps bay in San Diego California and a significantly cross correlation was found between settlement and the maximum daily tidal range. The maximum settlement tended to occur one to four days before the spring tide (Shanks, 1986).
The larvae from *H. elegans*, *B. neritina* and *B. amphitrite* adhered tighter to a biofilmed surface than to a clean surface when the larvae were exposed to a boundary of shear stress at 75 Pa for 5 minutes in a turbulent channel-flow tank (Hadfield, 2006).

Resistance to antifouling coatings

Several groups of sessile marine organisms have been found to have significant tolerance against copper; calcious tube worms (Dafforn *et al*, 2008; Johnston & Keough 2003), bryozoans (Floerl *et al*, 2004; Piola & Johnston, 2006), barnacles (Weiss, 1947) and hydroides (Stepping, 2002).

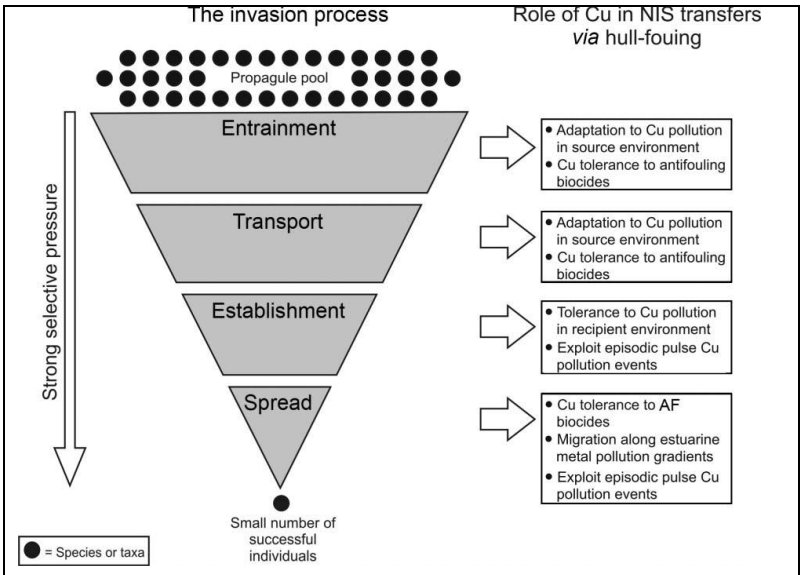


Figure 9: Model of fouling invasion by resistance to antifouling compounds used on ship hulls by Piola *et al*, (2009).

Organisms with adaptations are making them resistant to antifouling compounds as for example copper can use this adaptation to outcompete other organisms in an exotic environment (figure 9). The invasion process can be divided in four phases. (1) Entrainment, contact between fouling organism and human vector, for example an organism settling on a ship hull. (2) Transport, the organism is transported to a habitat outside its normal range. (3) Establishment, the invading organisms establishe a population in the new environment. (4) The established population starts to migrate from the initial point of fouling (Floerl & Inglis 2005; Richardson *et al*, 2000).

B. neritina collected in both recreational and commercial estuaries was considered a dominating fouling species when grown under copper and TBT treated conditions even if there was a negative relation to growth under some concentrations. The *B. neritina* was not affected by the other antifouling coatings in the experiments containing copper and Diuron, copper and zinc pyrithione and TBT (Dafforn *et al*, 2008, Dafforn *et al*, 2009). Piola & Johnston, (2005) found that *B. neritina* from copper polluted areas had a larger resistance against copper compared to populations from non copper polluted areas but the growth and survival benefit of the copper resistance population was only a benefit in copper polluted areas.

B. amphitrite settlement was severely inhibited by zinc- and copper pyrithiones and 10-100 times more inhibiting than polymeric 3-alkylpyridinium salts, both zinc- and copper pyrithiones affected the nauplii swimming abilities and caused 100 % mortality in the cyprid II nauplii at high concentrations (Faimali *et al*, 2003). Copper pyrithione was tested on *B. amphitrite* nauplii from four locations and the LC50 values differed from between sites (Romano *et al*, 2010) but were still in the same range as found by Faimali *et al*, (2003). Zinc pyrithione also inhibited the swimming speed of the brine shrimp *Artemia* sp. (crustacea) and the rotifer *Brachionus plicatilis* (Garaventa *et al*, 2010).

Bao *et al*, (2008) tested toxicity of zinc pyrithione and copper on the diatom *Thalassiosira pseudonana*, the polychaete *H. elegans* and the amphipod *Elasmopus rapax* (a crustacean). The zinc pyrithione was more toxic to all three species than the copper. A synergetic effect was observed when the two antifouling compounds were mixed. *H. elegans* had also in other experiments showed a positive growth in copper treated environments specially in areas where there already was high copper background concentrations in the water (Dafforn *et al*, 2009) and in another experiment showed higher or no difference in percentage cover of copper and TBT treated coatings (Dafforn *et al*, 2008).

Antifouling coatings

Biological adhesion on surfaces

The primary biofouling on water-solid inter-phases will be by bacteria, first as single cells and later if survival and multiplying is possible, as a biofilm. The cells will arrive to the interface by diffusion (Whitehead & Verran, 2008). Adhesion of bacteria to a surface takes from a few seconds to a few minutes (Davies et al, 1993).

When cells are close to the surface physiochemical forces like Lifshitz-van der Waals forces, electrostatic forces and hydrogen bonds will influence the attachment to the surface. Also cell and surface topography and chemical properties will have an effect on attachment. Cell adhesions to surfaces have been reported to have a positive effect on survival and an up regulation of alginate (an anionic polysaccharide which with water forms a fluidly gum) synthesis in a strain of *Pseudomonas aeruginosa* (Davies *et al* 1993). Bakker *et al* (2004) demonstrated differences in bacterial adhesion both between strains but also between the environments they were collected from. Three strains collected in a medical environment had a decreasing affinity for a substrate when the surfaces free energy of the substrate increased where the opposite was observed in the three marine strains used in the experiment. The marine strains adhesion was also positiv related with an increase in substratum elasticity where the same relation was absent in the strains from the medical environment (Bakker *et al*, 2004).

Biofilm has been defined as "a microbially derived sessile community characterized by cells that are irreversible attached to a substratum or interface or to each other that are embedded in a matrix of extracellular polymeric substances that they themselves have produced. The cells in the biofilm may exhibit an altered phenotype with respect to growth rate and gene transcription" (Donlan & Costerson 2002).

Cell surfaces are complicated designed concerning chemical properties and topography. These will not be even distributed and will therefore support several island environments with different properties which also will change accordingly to the environment surrounding them. Surfaces dispersed in water will also be covered

by different organic materials and will also influence settlement (Whitehead & Verran, 2008).

Bacteria are not the only organisms found in marine biofilms, vira, fungi, algae and protozoa are often also present and then macrofouling, which can be made from barnacles, worms, seaweeds, bryozoans and mollusks (Davies *et al* 1993). Microbial attachment to surfaces and the physiochemical properties involved is a complex process (Whitehead & Verran, 2008) and there are therefore several examples in the literature where results are different and sometimes conflicting (Dewi *et al*, 2004; Pringle & Fletcher, 1986; Zhao *et al*, 2009).

In experiments with larval settlement on different dimethyl silicones (DMS) surfaces in both laboratory and field experiments, it was found that the higher the elastic modulus in the DMS surfaces the higher the settlement of organisms. In the field experiment the settlement density of ciliates (*Zoothamnium* sp., *Astylozoon* sp. and *Folliculina* sp.) had a significant positive correlation with the elastic modules. There was also found, in the field experiment, a significant correlation between settlement and elastic modules for the tube dwelling polychaete *Polydora ligni* after the data were quadratic regressed. In the laboratory experiment there was a statistic significant positive correlation between the settlement of *B. neritina* and the elastic modules (Gray *et al*, 2002).

Coating composition in general

Paints or coatings consist of three major components; a binder, which in the litterature also is reffered to as vehicle, medium, resin, film former and polymer, a pigment or extender and a solvent. The binder and the pigment or extender form the final coating. The solvent is only used to apply the paint and evaporates after application.
The binder is the part of the paint which forms the film and dertermines therefore also the physical and chemical charateristics of the paint. The binder function is to provide an adhering stable continious permanent coating which contributes to the surfaces restance against environmental factors.

Paint binders are divided in two clases, the convertable and the non convertable. These classifications are based on the paint's film forming abilities while drying.
A convertable coating will chemically differ between liquid and solid state where a non convertable paint will have rather similar chemical features. In a liquid convertable paint with a solvent the process of drying is concidered a two stage process. The two processes actually occur simultaniously but in differnt rates. In the first process the film loses the solvent by evaporation and the coating will feel dry if touched. In the second process the coating becomes more chemical complex by either reacting with oxygen (oxidation), by reacting with a chemical compound added to the film, reacting with water (moisture in the atmosphere), by artificial heating or by radiation curing (for example ultra violet radiation). These processes result in a paint which is chemically different from the original binder and can not redissolve in the original solvent. Types of coatings made from these processes are oleoresinous varnishes, oil modified alkyd resins (dries by oxidation), urethane oil/alkyd resins (air drying resins), epoxy ester resins (air drying resins), two component epoxy resins (dries by chemical cuing), two component polyurethane resins (dries by chemical cuing), moisture cured polyurethane resins (dries by water absorption) and organic silicate resins (dries by water absorption) (Candries, 2000).
The non convertable coatings are made from a mix of binders as resins or polymers dissolved in a suitable solvent. These paints dry as the solvent evaporates and since this is a physical process no chemical changes occur. These paints can therefore dissolve in the original solven and soften when heated. Coatings with these features are chlorinated rubber resins, vinyl resins, bituminous binders and cellulose derivates (Candries, 2000).
The pigments or extenders are compounds added to the paints to improve or give the coating the desired properties. The pigments are added to the binder as fine powders with particle size around 5-10 microns (for finishing paints) and approximately 50 microns when used for primers. The materials used as pigments have different features as anticorrosive pigments which prevent chemical and electrochemical corrrosion, barrier pigments which increase the paints impermability, different coloring pigments and other extending pigments which give the coating other desired properties (Candries, 2000).

The solvents are used to dissolve the binder to a level where application is optimal. Since the solvent evaporation has no part in the finished coating and is therefore concidered an expensive waste material.
Solvents are divided in three groups based on their features as a solvent. True solvents are liquids which will dissolve the binder totally because they are compleately compatible with the binder. Latent solvents are liquids which are not true solvents since they are not compleately compatible with the binder but when mixed with a true solvent, give stronger dissolving properties than the true solvent would have given alone. Diluent solvents are also not true solvents but are mixed with the true solvents or the latent solvents to reduce the cost of the true solvents. The down-side of diluents solvents are that the binders can only cope with a certain amount (Candries, 2000).

Prevention of attachment

The optimal antifouling coating has a smooth surface during sailing. It has a thin stable leaching layer which has a continuous and constant biocide release over time and a polishing rate which ensures the antifouling compounds activity even under stationary conditions (Yebra *et al*, 2004).

The key mechanisms in the performance of the biocide based antifouling (for example the now banned TBT self polishing copolymer systems) paints are chemical reactions and diffusion. The system is based on the release of several biocides which are encased in a film forming organic matrix. The sea water has to erode some of the paint surface to release the biocides and then diffuse out in the bulk phase between the ship and the sea again. To maintain stabile release rates of the biocides the organic matrix is designed to dissolve slowly, within the pores created when the sea water reacts with the paint. When a certain conversion at the water paint interface is reached the binder phase is released and the polymer matrix is degraded by the movement of the water. The binder phase (for example rosin a solid form of resin) controls the paint thickness of the pores and thereby the biocide depleted layer and ensures that biocide release rates do not decrease. The performance of the biocide based antifouling paints can be affected by the water conditions (Kill *et al*, 2002; Yebra *et al*, 2004). The antifouling paints are also affected by temperature (Kill *et al*, 2002), salinity (Ferry & Carritt, 1946) and pH (Kill *et al*, 2002; Yebra *et al*, 2004).

After the TBT ban a need for tin free technology has arisen and there has been a boom in development of tin free antifouling systems. The products which have reached to commercial market are in the literature divided in two systems, the biocide and the foul-release systems (FR systems). There are two main coating types within the biocide systems. Controlled depletion systems (CDPs) and tin free self polishing copolymers (tin free SPCs or TBT-free SPC).
The self polishing systems hold different pendant groups attached to a polymeric backbone in an acrylic matrix which releases when in contact with water, very similar to the hydrolysis reaction in the former TBT self polishing copolymers paints (Candries, 2000; Yebra *et al*, 2004).

The control depletion polymer (CDP) coatings are developed from the traditional soluble matrix. These coatings work by slowly dissolving into the water and thereby releasing the active compounds. The coatings ability to dissolve are formed from a mixture of resins, traditional made from rosin but today also by several different polymers.
There are several drawbacks with these paints, (1) polymer control; the mixture of resins is very crusial for the paint activity since polymers are insoluble in water and to high an amount will inhibit the active compound release and to low an amount will release the active compound to fast. (2) In these paint seawater will penerate deep into the coating and a leaching layer will develop which will slow the release of active compound (Candries, 2000).
The TBT free self polishing copolymers mimic the TBT systems. They are made from acrylic polymers with active compounds as copper, silyl and zinc. The polymers produce a soluble micro layer at the paint surface which graduately polishes away. This polishing mechanism counteracts the buildup of a leaching layer and ensures the continuet release of biocide (Candries, 2000).
The foul foul-release systems are based on silicone elastomer technology and works on a non-stick principle. Their effency is based on a hydrophobic surface with low surface energy and smoothness (Candries, 2000).

Thesis aim

The overall aim of this thesis is to study micro- and macrofouling development on novel antifouling coated slides and plates to determine, both time frames for biofilm establishment and to resolve the effectiveness of the antifouling coatings.
The experiments took place at the pier of Sultan Qaboos harbour in Oman where the Sultan Qaboos University had a site for research. The reason for the location was partly based on network opportunities since the project superviser here in Denmark, Professor Benni Winding Hansen, has collaboration with Dr. Sergey Dobretsov which has a research position at Sultan Qaboos University. The second reason for the choise of Oman was the environment which permits fast growth of micro- and macrofouling, due to tropical climate, and thereby enables several experiments in a short time frame without large temperature fluctuations.

The main aim is, through the experiments performed, to answer the following questions; how does the bacterial and macrofouling perform over time, how does the antifouling treatment perform and how is the biofilm community dispersed between treatments and over time?

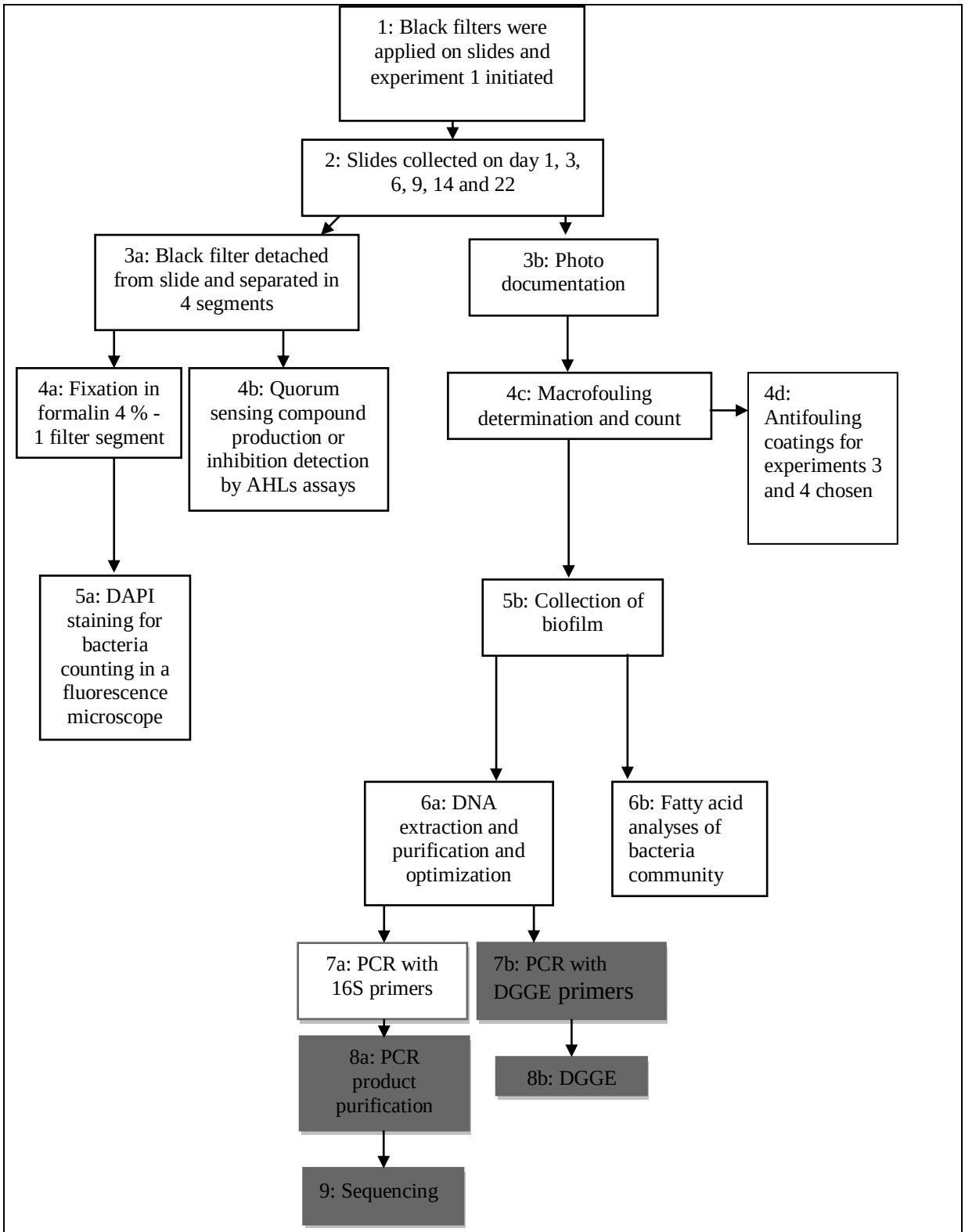
The study was divided into foue experiments; experiments 1 and 2, figures 10 and 11, which were performed on microscope slides and experiments 3 and 4 which were performed on large plates, figure 12.
Experiments 1 and 2 were designed for several reasons. The first reason was to give information abouth the biological succession over time. The second was to decide which treatments performed better against the recorded succession. The bacterial succession over time and between treatments was determined by counting bacteria on DAPI (4',6-diamidino-2-phenylindole**)** stained black filters. The last reason for experiments 1 and 2 was also to decide which antifouling coatings to proceed with in the following experiments 3 and 4.
The purpose of experiments 3 and 4, besides to follow the bacterial and macrofouling succession as in experiments 1 and 2, was to perform larvae settlement assays. The assays were performed both in vivo and in lab. The purpose was to determine both the

coatings effect on settlement density but also to investigate larva settlement behaviour and if the behaviour was affected by the different antifouling paints.

In the experiments 1 and 2 there were applied black filters (24 mm in diameter and 0.2µm pore size) on all slides to facilitate counting of bacteria under a light microscope, to create an identical physical surface for the bacteria to settle on, regardless of antifouling coatings and to use for screening for production or inhibition of bacterial quorum sensing by AHLs assays. The black filters, besides creating a uniform surface for biofilm establishment, allow the compounds from the antifouling coatings to penetrate and influence biofilm formation.
The experiments 1 and 2 were used to set a time frame for biofilm and macrofouling development, to determine which antifouling coating was relevant for further work and to verify production and inhibition of bacterial quorum sensing compounds. Macrofouling organisms were counted, species present were determined and there were taken photos of the slides. Biofilm from the slides were collected and used for fatty acid analysis and for DNA extraction to perform PCR and Denaturing Gradient Gel Elecrophoresis (DGGE).

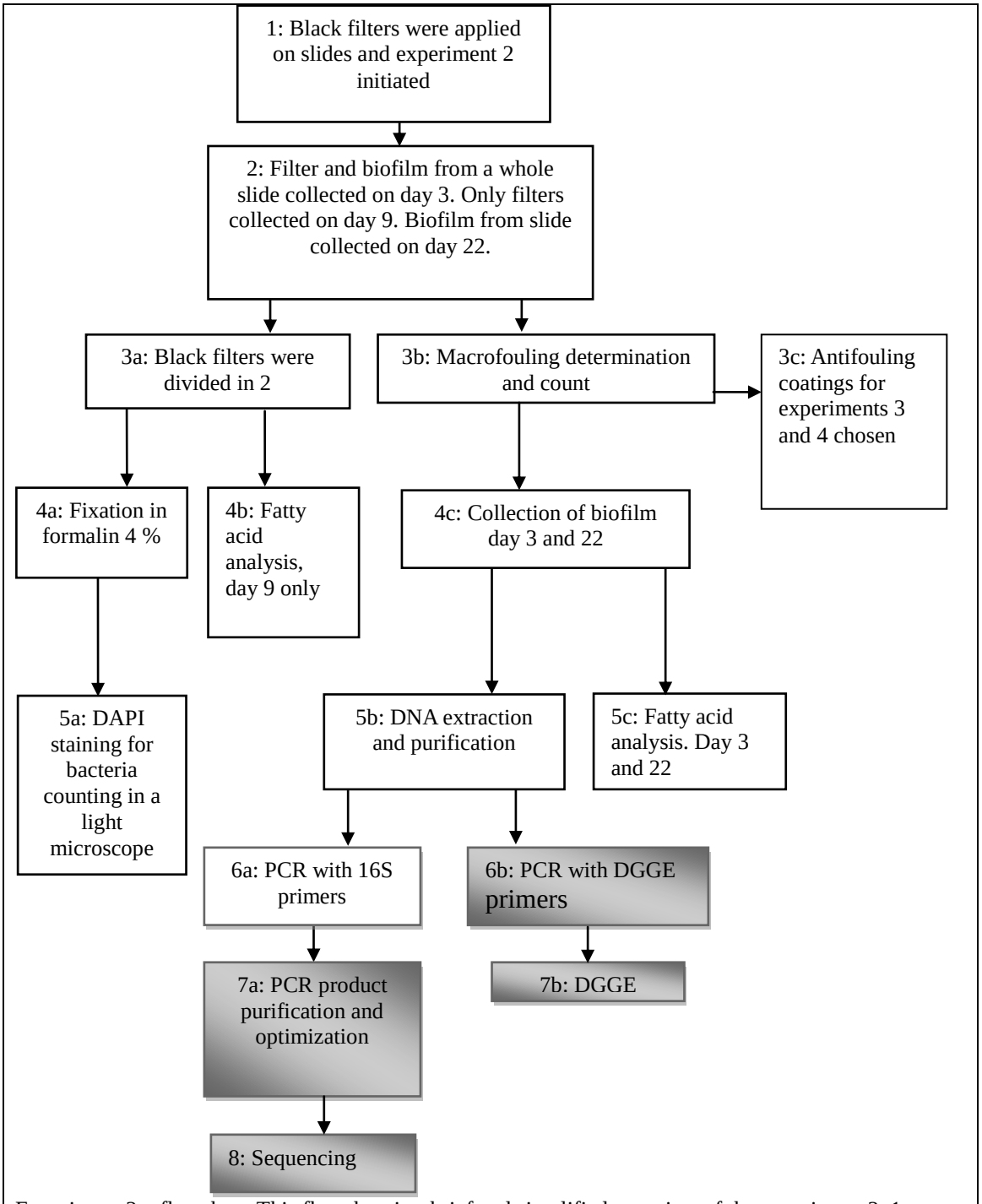
There were applied two black filters and one membrane filter (9 cm in diameter and 0.2µm pore size) on each plate in the experiments 3 and 4. The black filters used were similar to the ones used in the experiments 1 and 2 and were used for counting bacteria on two, on behalf of the experiments 1 and 2, selected days. The larger membrane filters were used to create a uniform and detachable substrate for larval settlement and also permitted penetration of antifouling compounds from the coatings. This ensured similar conditions for the video surveillance of larval behaviour no matter if recorded *in situ* or *in vivo*.
The presence of macrofouling was documented by photos.

Figure 10: Experiment 1 – flowchart



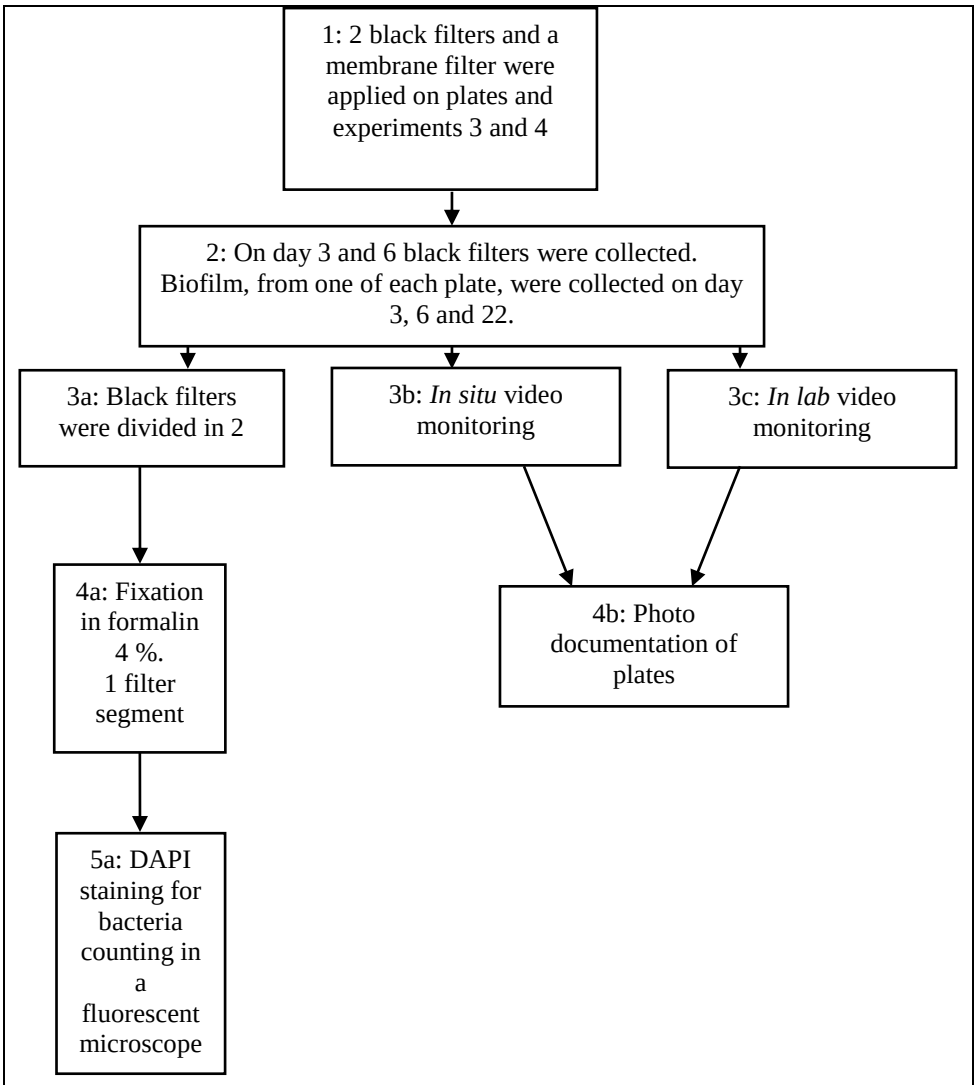
Experiment 1 – flowchart. This flowchart is a brief and simplified overview of the experiment 1.
1, start of experiment 1. 2, slides were collected on selected days. 3a, black filters were divided in 4 segments, 3b, all slides with macrofouling were photo documented. 4a, segment D was fixed in 4% formalin. 4b, segments A, C and S were used for quorum sensing assays. 4c, when macrofouling was present, these were indentified and counted. 4d, on behalf of the data obtained from 4c the main experiment was designed. 5a, the filters fixed in formalin (4a) were stained with DAPI. 5b, after 3b biofilm was collected and frozen for later use. 6a, DNA was extracted and purified from the collected biofilms. 6b, fatty acids were extracted from the collected biofilm and analysed on a GC-MS. The grey boxes 7b – 9 are future work.

Figure 11: Experiment 2 – flowchart



Experiment 2 – flowchart. This flowchart is a brief and simplified overview of the experiment 2. 1, start of preliminary experiment 2. 2, slides were collected on selected days. On day 3 a whole slide was collected, on day 9 a black filter and on day 22 the slide which had the black filter removed on day 9. 3a, black filters were divided in 2 segments. For day 3 one half was used for bacteria counting and the other half frozen. For filters collected on day 9, half the filter was used for bacteria counting and the other half for fatty acid analysis. 3b, when macrofouling was present, these were indentified and counted. 3c, on behalf of the data obtained from 3b the main experiment was designed. 4a, one filter segment was fixed in 4% formalin. 4b, fatty acids were extracted from the collected biofilm and analysed on a GC-MS. 4c, after 3b biofilm was collected and frozen for later use. 5a, the filters fixed in formalin (4a) were stained with DAPI. 6a, DNA was extracted and purified from the collected biofilms. 5c, fatty acids were extracted from the collected biofilm and analysed on a GC-MS. The grey boxes 6b – 8 are future work.

Figure 12: Experiments 3 and 4 – flowchart



Experiments 3 and 4 – flowchart. This flowchart is a brief and simplified overview of the experiments 3 and 4.
1, filters were attached to plates with super glue and submerged in the water. 2, the black filters for bacteria counting were collected on day 3 and 6. There was collected biofilm on day 3, 6 and 22. 3a, the collected black filters were divided in two where one segment was used for counting bacteria and the other were stored in -20°C. 3b + 3c, as the biofilm developed *in situ* and *in lab* monitoring was initialised. 4a, one filter segment was fixed in formalin 4 %. 4b, after larvae monitoring and biofilm collection on day 22 there were taken photos of all plates. 5a, after fixation in formalin the filters were stained in DAPI for bacteria counting.

Methods

The experiments in this thesis consist of 4 experiments. Experiment 1, consists of two series with 5 treatments which were repeated twice (referred to as experiments 1a and 1b). Experiment 2 had 6 treatments and was only performed once. Experiments 1 and 2 were run in February, March and April. Experiments 3 and 4 were run in May and June and had 4 treatments.

Experiments 1 and 2

The main purpose of the experiments was to get an overview of the time range of biofilm and macrofouling development rates in Omani waters and to get a picture of the effectiveness of the treatments towards fouling.

The experiments 1a and 1b consisted of 5 and 6 treatments, using microscope slides (76 mm x 26 mm) (Knittel Glaser & Menzel-Glaser). On all microscope slides was placed a black filter (0.2 µm Ø25 mm) (Micropore GTBP02500), se figure 13 and figure 18. The treatments consisted of 1 control, a microscope slide with a black filter, and the residual treatments were 4 different antifouling paints.

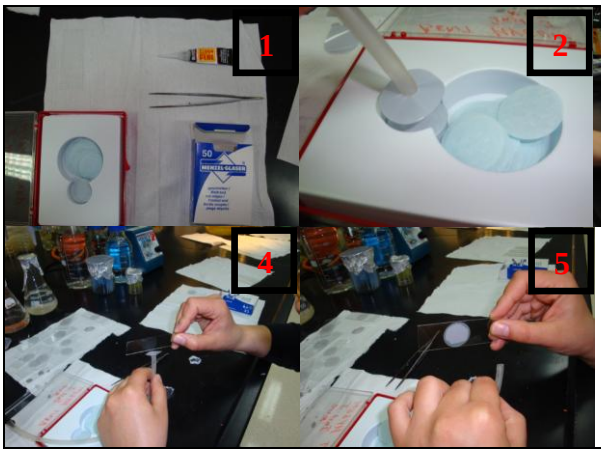


Figure 13: Materials and tool used to assemble black filters to microscope slides; black filters, microscope slides, super glue and forceps, picture 1. In picture 2 a filter is prepared for gluing by removing it from the box by a clean vacuum tube to prevent any contamination of the filter. In picture 3 the glue is carefully applied to the rim of the filter. Picture 4, the filter is placed gently on the cleaned microscope slide. The finished slide with attached filter, picture 5.

In experiment 1a; each treatment had 42 samples. These were divided between 7 racks. In each rack were 6 samples from each treatment (30 slides). The 6 samples in each rack were divided in two groups of 3 in each (5 treatments, 10 groups) and placed randomly in the rack (figure 14).

The groups were placed 3 cm from each other both horizontal and vertical and there was also 3 cm from the top/bottom groups to the top/bottom of the rack. There were

used 7 racks, and they were placed from the pier of Port Sultan Qaboos with ropes at a length of 1 meter giving the slides in the racks a depth of 103 – 117 cm.

In experiment 2; each treatment had 12 samples (figure 15). These were divided between 6 racks. In each rack were 2 samples from each treatment (12). The two samples from each treatment were kept in one group and the groups were placed randomly in the racks. The groups were placed 6 cm from each other horizontal and 3 cm vertical and there was also 3 cm from the top/bottom groups to the top/bottom of the rack. There were used 6 racks, and they were placed from the pier of Port Sultan Qaboos with ropes at a length of 1 meter giving the slides in the racks a depth of 103 – 117 cm.

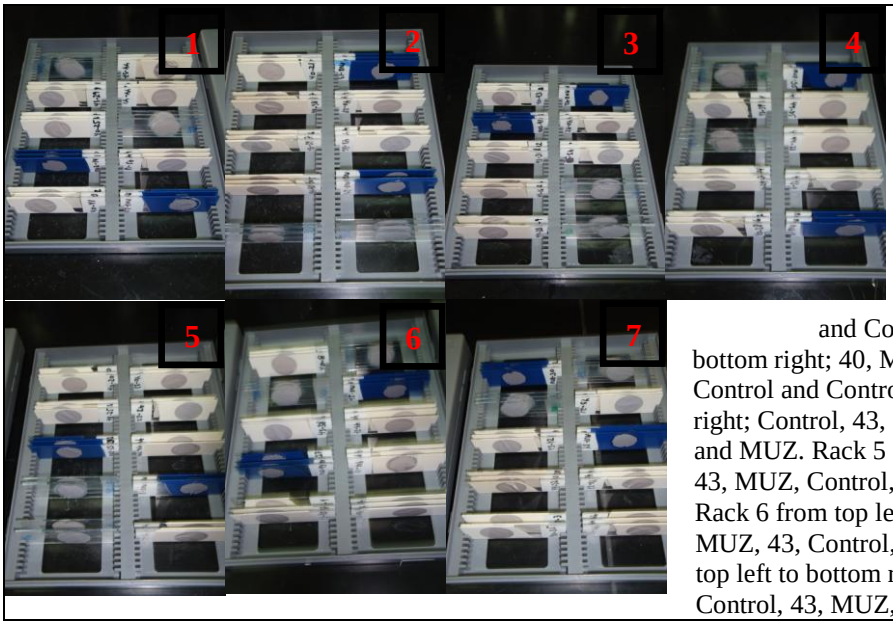


Figure 14: Slide placement in experiments 1a and b, rack no. 1-7. There were 5 treatment groups in each rack and they were placed group wise randomly. Rack 1 from top left to bottom right; Control, 43, 40, MUZ, 40, 44, 44, C, 43 and MUZ. Rack 2 from top left to bottom right; 40, 44, 43, 44, Control, MUZ, 40, 43, MUZ and Control. Rack 3 from top left to bottom right; 40, MUZ, 43, 44, 44, MUZ, 40, 43, Control and Control. Rack 4 from top left to bottom right; Control, 43, Control, 40, 40, MUZ, 44, 44, 43 and MUZ. Rack 5 from top left to bottom right; 40, 43, MUZ, Control, Control, 40, 43, 44, MUZ and 44. Rack 6 from top left to bottom right; 40, Control, 43, MUZ, 43, Control, MUZ, 44, 44 and 40. Rack 7 from top left to bottom right; MUZ, Control, 43, 44, 40, Control, 43, MUZ, 40 and 44.

1	
C	MUZ
49	MU
46	53

2	
46	MU
53	C
49	MUZ

3	
46	53
MUZ	MU
49	C

4	
C	46
49	MUZ
MU	53

5	
46	C
49	MU
MUZ	53

6	
46	MUZ
C	MU
49	53

Figure 15: Slide placement in experiment 2, rack no. 1-6. There were 6 treatment groups in each rack and they were placed group wise randomly. The C represents the Control treatment.

In experiments 1a and b samples were set out, figure 16, on day 0 and collected on day 1, 3, 5, 7, 10, 14 and 22. There was collected 1 sample pr treatment from each rack (7 samples pr treatments) on every collection day. The samples collected from each treatment were the samples closest to the top of the rack and from the group placed in the left side of the rack, if both sample groups were in the right or left side of the rack the sample closest to the top was collected, figure 17. The samples were transported in a rack similar to the test racks and placed in treatment groups for not affecting each other. The rack was transported in a box with seawater collected on site and the box was placed on ice in a camping cooler during transport. There was also measured temperature at 1 m depth, in the surface water, in the shade on land and salinity at every collection day.

In experiment 2 samples were set out on day 0 and collected on day 3, 9 and 22. On day 3 the whole slide was collected. The filter was used for bacterial counting and the slide for biofilm collection for Fatty Acid Methyl Ester (FAME) analysis. On day 9 the whole filter from the other slide was collected one half for bacterial counting and the other half for FAME analysis. On day 22 the last slide without filter was collected, there was taken photos of the macrofouling and biofilm was collected for FAME analysis.

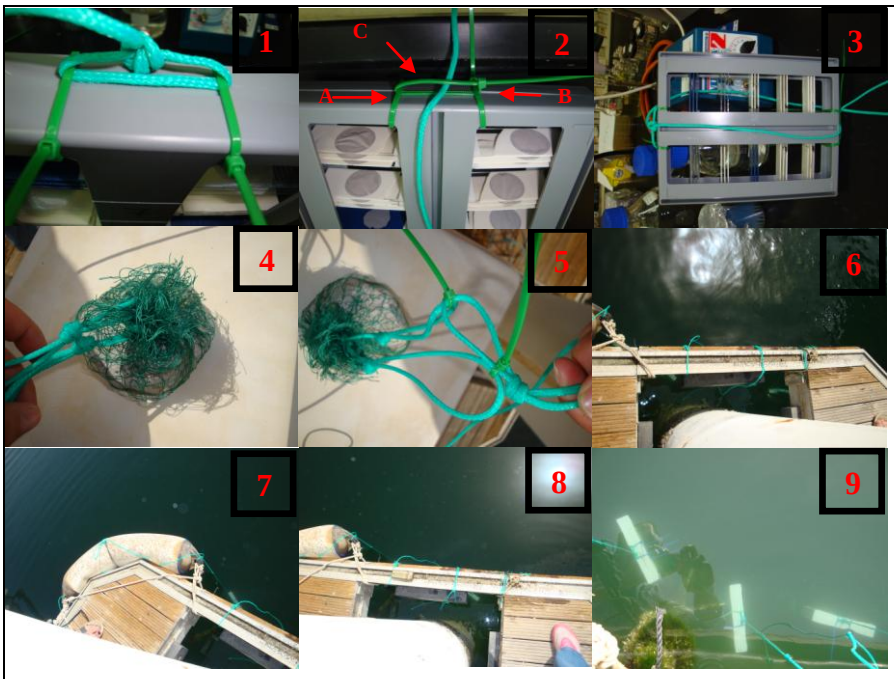


Figure 16: Assembling of lock mechanism on racks, stabilizing weight to counteract movement from current and passing boats, and placement of racks on the pier. The racks were hold together in bottom and top by cable ties. 1, permanent attachment of rack to cord from pier. 2, reversible lock mechanism in bottom of rack. The cable ties A and B holding the two parts of the rack together were cut and the lid part of the rack were lifted (still hold together in the top by the cable ties) to remove samples. The cord running through the cable tie C was connected to the weight stabilizing the rack. 3, whole rack with assembled cord system, where one end of the cord is used to attach the rack to the pier and the other to the stabilizing weight. 4 + 5, The stabilizing weight was made from a rock wrapped in fishing net and connected to the cord (and rack) by cable ties. 6 + 7, placement of racks on the pier. 9, view of racks in the water taken from the pier.

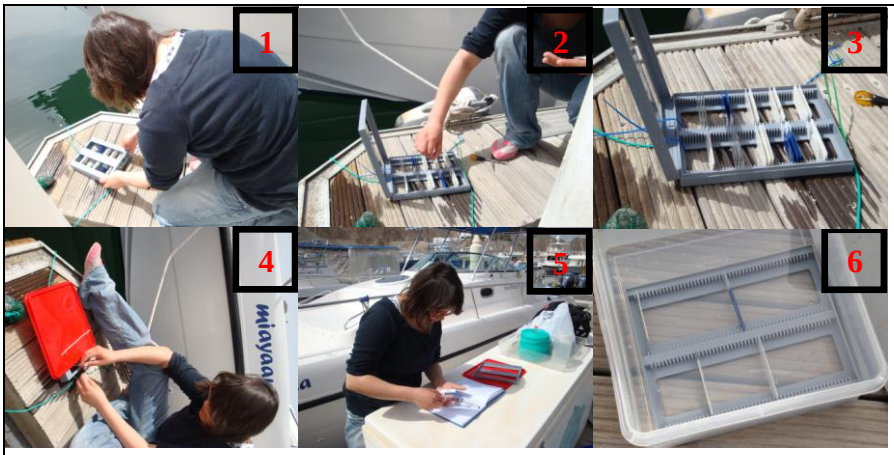


Figure 17: Collection of slides and placement in transport rack. 1, the two cable ties are cut and the rack is opened. 2, the samples are collected. 3, rack after collection. 4, the samples in the rack are protected by a lid while it is closed with cable ties. 5, before the rack was placed back in the water the samples were controlled to prevent any mistakes. 6, picture of transport rack and placement of the different treatment groups.

The filters from the samples were divided in 4 segments (figures 18 and 19). Segments A_1, A_2 and A_3 were used for AHLs assays with respectively *Chromobacterium violaceum* CV026 and *Chromobacterium violaceum* CV017. Segment D was used for DAPI staining for bacteria counting. After separation, filters A_1, A_2 and A_3 were stored in -20°C. Segment D was fixed in formalin 4% (Fluka chemika 02880) over night and stained with DAPI for 15 min before use.

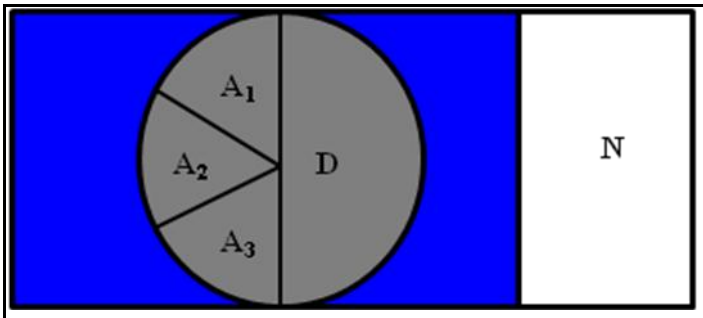


Figure 18: Example of sample from experiments 1 and 2. A microscope slide (76 mm x 26 mm) (Knittel Glaser & Menzel-Glaser) covered with antifouling paint (EnPro & CISMO) and a black filter (0.2 µm Ø25 mm) (Micropore GTBP02500). Each microscopy slide was numbered with treatment number and sequentially numbered (N). The black filters were divided into 4 segments. The largest segments D were fixed in formalin 4% (Fluka chemika 02880) and thereafter stained with DAPI 0.1% (SIGMA). Bacteria and diatoms were counted on the D segment under a fluorescence microscope Axiostar plus Fluorescence generator HBO 500/AC with power generator mbq 52 ac. After bacteria counting the D segments from same treatment and collection day were pooled and frozen at -20°C. The other part of the black filter was divided into 3 smaller segments, A_1, A_2 and A_3 which was frozen treatment and collection wise at -20°C.

After segmentation of filters the remaining slide surfaces were swabbed with sterile cotton buds and frozen separately at -20°C in Eppendorf tubes. Biofilm collections were only done for experiment 1a on day 9, 14 and 22 and for all collection days in experiments 1b and 2. These biofilm collections were used for FAME analysis (Sasser, 1990) and for DNA extraction (Zhou *et al*, 1996).

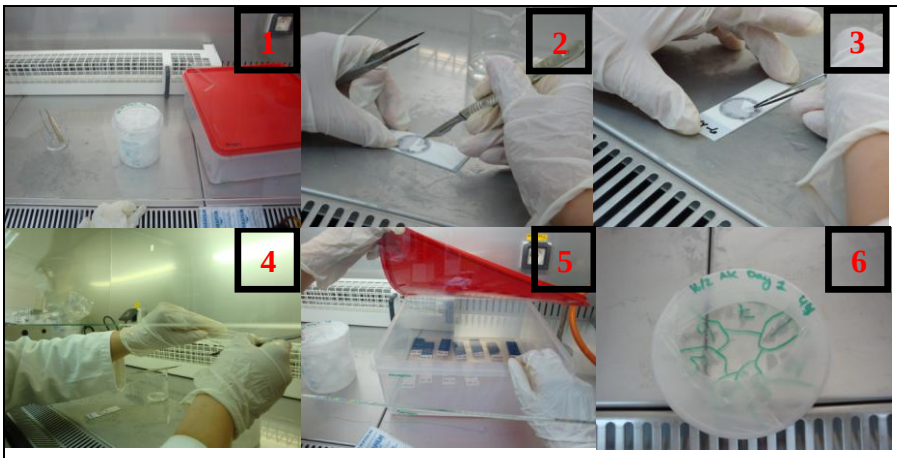


Figure 19: Preparation of filters for quorum sensing assays and for fixation in formalin. 1, material for filter preparation; ice, scalpel, forceps, gloves, Para film and box with formalin. 2, the filter is divided. Black filters are fragile and the easiest way to divide them is to make the segment cuts A_1, A_2 and A_3 and D first and then cut the segments A_1, A_2 and A_3 free from the glue at the rim of the filter. 3, the segments were removed with a forceps and placed (4) in a Petri dish on ice. 5, the segment D, still attached to the slide, was fixed in formalin. As biofilm formation increased on the filters and more paint was released because of the polishing effect the background fluorescence increased and made bacteria counting difficult and in some cases impossible, and the segments D were therefore completely removed from the slides and fully suspended in formalin treatment and collection wise. 6, The segments A_1, A_2 and A_3 were placed treatment wise in a Petri dish which were closed with Para film, and marked with treatment and collection day, before freezing in -20°C

Bacteria and diatoms were counted in a fluorescence microscope Axiostar plus Fluorescence generator HBO 500/AC with power generator mbq 52 ac. On each filter 10 fields were counted and for each replicate a filter average was made, after which a group average was decided.

When counting bacteria from day 3 and forward it was noted that there were background fluorescence present and it increased with time. At the first counting, sampling days #1 and #3, filters were prepared by formalin fixation where the whole slide, with the filter attached, was submerged into formalin for 24 hours. Because background fluorescence made counting difficult it was decided to separate the filters from the slides and fix the filters group wise for up to a month. This gave better counting conditions but also the disadvantage that the individual filter could not be traced back to its original positioning in the rack. This method was used in all experiments.

Treatments for experiments 1a and 1b

The antifouling paints used in experiment 1a were named T40, T43, T44 and MUZ. The paint treatments were selected in cooperation with the companies EnPro and CISME.

Treatment T40 was a water-borne paint with zinc-sulphide and aerogel with the enzyme (x) inserted in the aerogel, treatment T43 was a water-borne paint with zinc-sulphide without the aerogel and worked as a control of the effect of zinc-sulphide. Treatment T44 was water-bourne paint with zinc-oxide without aerogel and treatment MUZ was Mille Ultimate as 2-component with copper and zink-pyrrothione (table 1).

Table 1: Antifouling coatings used in experiment 1a and 1b.

Treatment	Explanation surface	Number of slides	Recipe
Control	Silane prep glass	42	None
T43	Water-borne paint with zinc sulphide without aerogel	42	Engineered
T40	Water-borne paint with zinc sulphide and aerogel with enzyme x	42	Engineered
T44	Water-borne paint with zinc oxide without aerogel	42	Engineered
MUZ	Mille Ultimate as 2-component with copper and zinc pyrithione	42	Commercial paint

The experiment 1 had the purpose to explain the results from experiment 1 and to test other compounds and thereby clarify which paint to continue working with in experiments 3 and 4.

Treatments for experiments 2

Experiment 2 consisted of 4 antifouling paints and a control (table 2). The four treatments were named MU, MUZ, T46, T53 and T49.

Treatment MU was designed to study if the initial bacteria growth and the inhibition of macrofouling on the sample MUZ from the experiment 1 were caused by zincpyrrothione. This paint had the same features except the zincpyrrothione.

Treatment T46 was a water-borne paint with zinc sulphide and an aerogel with an active component (x) inserted. This sample was designed to test another enzymes function concerning the initial stages of bacteria growth and macrofouling compared to the one used in treatment T40. Treatment T53 was used to monitor the influence of a water-borne paint with zinc sulphide and a commercial protease on the growth of micro- and macrofouling and to serve as a link to previous research. Treatment T49 was a water-borne paint used to monitor the effect of titanoxide which is a pigment used in paint on bacteria growth and macrofouling (pers. comm. Eva Wallström).

Table 2: Antifouling coatings used in experiment 2.

Treatment	Explanation surface	Number of slides	Recipe
Control	Silane prep glass	12	None
MUZ	Mille Ultimate as 2-component with copper and zinc pyrithione	12	Commercial paint
MU	Mille Ultimate with copper but without zinc pyrithione	12	Commercial paint
T46	Water-borne paint with Zinc sulphide and aerogel with active compound x	12	Engineered
T53	Water-borne paint with zinc sulphide with commercial protease	12	Engineered
T49	Water-borne paint with titandioxide (anatas)	12	Engineered

Experiments 3 and 4

The purpose of experiments 3 and 4 was the further investigation of the connection between micro- and macrofouling and the connection between fouling and antifouling compounds.

The experiments consisted of two replications of the same setup referred to as experiment 3 and 4. There were used 3 treatments and 1 Control (table 3). The experiments were made on larger panels, 15 × 20 cm and there were 10 replicates in each treatment (figure 20).

Table 3: Antifouling coatings used in experiment 3 and 4.

Treatment	Explanation surface	Number of panels	Recipe
Control	PVC plate	20	None
MUZ	Mille Ultimate as 2-component with copper and zinc pyrithione	20	Commercial paint
MU	Mille Ultimate with copper but without zinc pyrithione	20	Commercial paint
UV	Zinc pyrithione and pigments x	20	Engineered

The treatments used were all antifouling coatings painted on to PVC plates. They were named MU, MUZ, UV and a control C.
The treatments MUZ and MU were Mille Ultimate as 2-component with and without zink-pyrrothione added. The treatment UV was paint with non soluble pigments and zinc pyrithione. The Control was a non painted PVC plate.

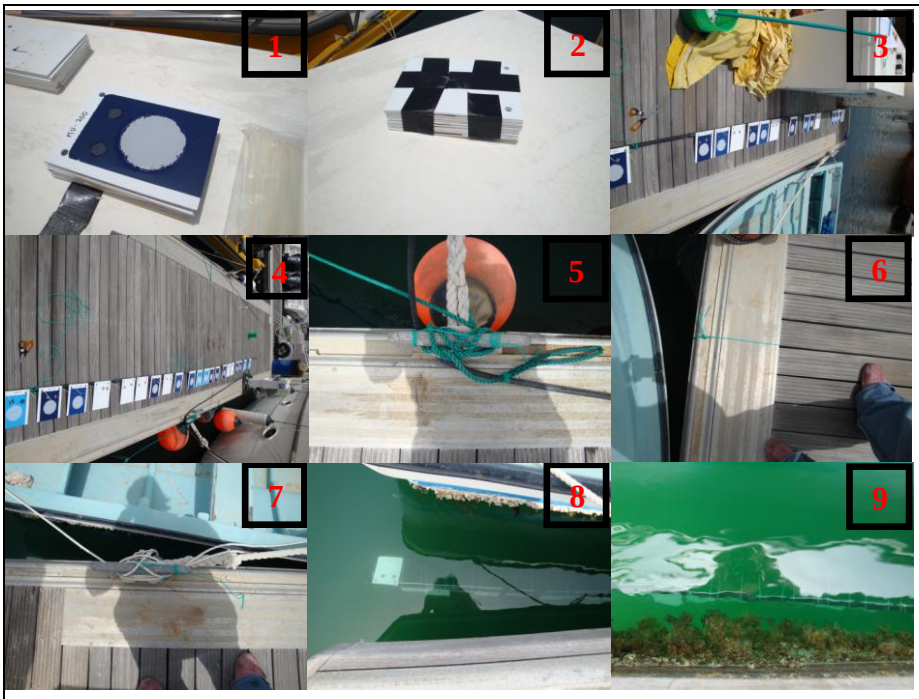


Figure 20: Setup experiments 3 and 4. 1, there were glued two black filters (0.2 µm Ø25 mm) (Micropore GTBP02500) and one large filter (0.2 µm Ø90 mm) (Micropore GTTP09030) on the plates (example shown for treatment MUZ). The two black filters were for bacteria counting on day 3 and 6. The large filters were for settlement surveillance to ensure the same substrate surface for both in vivo and in vitro recordings. 2, for transportation the plates were stacked with ice-lolly sticks between them and the top plate was faced toward the other and the plates were then duck-taped together. 3, the plates were fastened on to plastic pipes with cable ties. 4, plates ready to be lowered into the water. 5 + 6 + 7, the pipe was tied to different places on the pier. 8 + 9, the setup lowered 1 m down into the water.

There were placed two black filters ((0.2 µm Ø25 mm) (Micropore GTBP02500)) and one large filter ((0.2 µm Ø90 mm) (Micropore GTTP09030)) on each panel with super glue. The black filters ((0.8 µm Ø25 mm) (Poretics no. 11053)) were used for bacterial counts on day 3 and 6. The large filter ((0.2 µm Ø90 mm) (Micropore GTTP09030)) was used for larvae settlement monitoring, *in situ* and *in lab*.
The experimental setup was constructed by connecting plastic tubes with matching connector pipes. These were bought at a general builder's merchant. The panels were placed randomly and fasten with cable ties. The whole setup was lowered into the water, so the panels were submerged to 1 m depth, and tied to the pier in four different places.

At collection days the whole setup was raised to water level with the panels still submerged in water. Each panel was individually flipped above water when filters or biofilm were collected on days 3, 6 and 22.

At larvae monitoring days the whole setup was raised to water level with the panels still submerged in the water and the sample monitored was cut loose and placed in the video setup.

Video surveillance of larvae settling

To investigate larval settlement, *in situ* and *in lab*, the large filters which were placed on panels used in experiments 3 and 4 were recorded with a video camera (SonyHDV Handycam HDR-HC1E PAL) placed in a water proof casing (NIMAR). There were developed (Guillaume Drillet) and build (Jan-Ole Nielsen) a devise for holding panels stable in front of the underwater camera for *in situ* recording.

In situ monitoring: To perform the larvae monitoring the camera was placed in the water proof case. The slide holding device positioned and weights to pull the camera down was attached. The pipe holding the panels was pulled up so the panels were just beneath the water and still covered. The selected panel was cut free and positioned in the panel holding device and the camera focus was also adjusted here. The monitoring setup was carefully lowered into the water and submerged to -1 m. The monitoring ran for approximately 45-50 min depending on the length of the video tape. After monitoring the camera was gently pulled up and the panel was reattached with cable ties on its former position on the pipe.
The monitoring took place in the morning after sunrise since it was the time with most larvae activity.

In lab monitoring: The in vivo monitoring was preformed as a double-dish experiment where larvae had the choice to settle between a biofilm covered surface and a plastic surface (top cover of Petri dish). To perform the larvae monitoring the filters were cut free from the panels and transported back to the lab in Petri dishes with seawater cooled on ice. The pipe was raised so the panels were just under water and the selected panel was lifted above water and the filter was cut free, with a scalpel, and transferred to a Petri dish. There were also collected water samples with a plankton collection net, 50 μm.
At arrival in the lab the filters were glued to new Petri dishes. These were done by adding glue to the rim of the bottom of the new Petri dish and then place the filter in the Petri dish. After letting the glue dry for at few seconds, so the filter did not dry out, the collected water was added to the top of the Petri dish, the lid was put on and the Petri was hold together by Para film. The Petri dish was placed in a holding device and then larvae settlement was monitored by video camera for approximately 45-50 minutes depending on the length of the video tape.

Reporter strain assays

To investigate the biofilms on the filters for the presence of quorum sensing- or quorum sensing inhibitory compounds there was made reporter strain assays. There were used 2 different reporter strains; *Chromobacterium violaceum* CV026 (detects short chain AHLs C_4-C_8) and *Chromobacterium violaceum* CV017 (detects AHLs). *C. violaceum* CV026 respond to the presence of acyl homoserine lactone compounds by producing a blue color where *C. violaceum* CV017 register the presence of extern acyl homoserine lactones (AHLs) compounds which is observed by blue coloration of the biofilm.

The strains were grown in Petri dishes and the filters collected were placed biofilm-side down on the cultures. The Petri dishes were cultured in 37°C for 24 and 48 hours.

1: An inoculation needle swap of CV017 was taken from frozen sub culture at -20°C and inoculated in 15 ml LB medium for 24 hours. 1 ml stock was plated on an agar Petri dish and incubated at 35°C for 24 hours. After 24 hours incubation at 35°C, an inoculation needle swap was re-inoculated in 15 ml LB medium and incubated at 32°C for another 24 hours. 2 ml of the CV017 stock was mixed with 10 ml LB agar (Sigma) in a Petri dish and after settling a filter, segments from each treatment (5 replicates from experiment 1a) were placed biofilm side down on the bacteria/agar mixture. For each collection day there was also made a CV017 positive control Petri where there were added 20 µl 6C-AHL (Sigma).

2: An inoculation needle swap of CV026 was taken from frozen sub culture at -20°C and inoculated in 15 ml LB medium for 24 hours. 1 ml stock was plated on an agar Petri dish and incubated at 35°C for 24 hours. After 24 hours incubation at 35°C, an inoculation needle swap was re-inoculated in 15 LB medium and incubated at 32°C for another 24 hours. 2 ml of the CV026 stock was mixed with 10 ml LB agar (Sigma) in a Petri dish and after settling a filter, segments from each treatment (5 replicates from experiment 1a) were placed biofilm side down on the bacteria/agar mixture. For each collection day there was also made a CV026 positive control Petri where there were added 20 µl 6C-AHL (Sigma).

FAME analysis

Short chain fatty acids, fatty acids with chain lengths between 9-20 carbons, can be used to identify genera and species of bacteria and can thereby give a characterization of communities and their differences. In this assay the term fatty acids cover over compounds as aldehydes, hydrocarbons, dimethyl acetals and methyl esters.

The procedure consists of 5 steps and 4 reagents to cleave the fatty acids from lipids.

Step 1: Harvesting

Approximately 40 mg of bacteria are needed. If bacteria are grown on Petri plates then it is approximately half a plate. In this thesis bacteria were removed from the antifouling coated microscope slides with cotton buds and transferred to Eppendorf tubes. The cotton buds were then frozen at -20°C until use. The cotton buds were then transferred to 13 × 100 culture tubes (Chromacol LTD no. 132 – 125x10-SV).

Step 2: Saponification

1.0 ml of reagent 1 was added to the culture tubes with cotton buds and the tubes were then transferred to a sonication bath for 30 min at 30°C to release the collected biofilm from the cotton buds. After sonication the cotton buds were removed from the culture tubes and discarded. The culture tubes were heated at 100°C in a water bath (Clifton unstirred water bath 72815) for 5 min, vortexed thoroughly and then heated for 30 min at 100°C. After heating the tubes were cooled in a water bath.

Reagent 1: 45 g sodium hydroxide
150 ml methanol
150 ml distilled water

Step 3: Methylation

2.0 ml reagent 2 were added to the cooled tubes. The tubes were vortexed briefly and then heated in a water bath (Grant T14) at 80°C for 10 min. It is important to be very precise with temperature and time in this step. The tubes were cooled in a water bath after heating. Reagent 2 causes the solutions pH to drop to below 1.5 and the fatty

acids to methylate which makes the fatty acid methyl esters very insoluble in the water phase.

Reagent 2: 325 ml certified 6.ON hydrochloric acid
275 ml methyl alcohol (methanol)

Step 4: Extraction

1.25 ml of reagent 3 were added to the cooled tubes and the tubes were placed in a clinical rotator (Bibby – Stuart Scientific – Blood tube rotator SB1 – R000102238) for 10 min and slowly rotated. After rotation the water phase (bottom phase) was removed and discarded. Reagent 3 will extract the fatty acid methyl esters to the organic phase.

Reagent 3: 200 ml hexane
200 ml methyltert-butyl ether

Step 5: Base wash

3 ml of reagent 4 were added to the remaining top phase in the tubes and again gently rotated on the clinical rotator for 5 min. After rotation the upper 2/3 of the top phase was transferred to GC vials, capped and stored at -20°C. Reagent 4 reduces impurities in the injection port liner, the column and in the detector.

Reagent 4: 10.8 sodium hydroxide
900 ml Milli-Q water (Millipore – F6AM97369T)

Injection of samples on the GC-MS (PerkinElmer Clarus 600 gas chromatograph 665N8030302 and PerkinElmer Clarius 600 mass spectrometer 664N8021604) was performed by Mr. Jamal Al-Sabahi. The analysis of results was performed in Turbo Mass version 5.4.0.

Extraction of DNA

There was extracted DNA from cotton bud swabs from collection day #22 from both experiments for later DGGE runs. There were prepared 3 replicates from each collection day. There was extracted DNA from experiment 1 collection day #9 with 3 different methods to decide which one gave the best results. The methods used were from Zhou *et al*, (1996), Lueders *et al*, (2004) and Qiagen DNA extraction kit. The method with the best result was used to extract the replicates from experiment day 22-1 and 22-2. The DNA extracts were loaded on 1 % agarose gels and the best extraction method was decided from gel pictures. The success criteria were the method with highest number of successful DNA extraction and with the clearest bands and the least smear in the lanes, se figures 21 and22.

DNA extraction by Zhou *et al*, (1996)

Step 1: Lysis of bacterial cells – approximately 3 hours

Eppendorph tubes with cotton buds stored at -20°C were thawed on ice and 0.4 ml extraction buffer was added. The tubes were frozen at -20°C and then thawed at 60°C in a heat incubator. This was repeated twice.

Extraction buffer:	100 mM Tris-HCl (pH 8)
	100 mM Na_2-EDTA
	100 mM Sodium phosphate buffer
	1.5 M NaCl

Sodium phosphate buffer:	0.58 g NaH_2PO_4 pr 100 ml
	0.82 g Na_2HPO_4 pr 100 ml

8 µl Proteinase K was added to each tube and the tubes were incubated in a water bath at 37°C for 30 min. The tubes were slowly inversed every 10 min.

Proteinase K:	10 mg/ml in TE buffer
TE buffer:	10 mM Tris-HCl
	1 mM EDTA (pH 8)

80 µl 20 % SDS were added to each tube which was shaked and vortexed briefly and then incubated at 60°C for 2 hours. The tubes were inversed slowly every 20 min.

Step 2: Extraction of DNA – approximately 2 hours

After the 2 hours heat incubation the tubes were vortexed and then shaked gently with an even volume of chloroform-isoamylalcohol in a 24:1 vol/vol ratio. The aqueous top phase was very carefully pipetted in to new tubes. This step was repeated 2 times. 0.6 ml of cold (4°C) isopropanol were added to the tubes containing the previously mentioned aqueous top phase, mixed well and incubated at room temperature for 15 min.

After incubation the tubes were centrifuged at 10000 g for 15 min with the hinges out to give a better control over where the invisible DNA pellets would collect in the tubes. The supernatants were decanted very carefully. There was added 1ml of cold (-20°C) 70 % ethanol to the tubes and they were vortexed. The tubes were then centrifuged again at 10000 g for 15 min with the hinges out and the supernatant was again very carefully removed and the tubes were dried head down on a paper towel for the ethanol to evaporate.

There were added 100 µl sterile Milli Q water to each tube to dissolve the DNA pellets and the tubes were stored at -20°C.

DNA extraction by Lueders *et al*, (2004)

750 µl 120 mM $NaPO_4$ buffer pH 8 and 250 µl TNS were added to Eppendorph tubes with cotton buds and then vortexed vigorously.

$NaPO_4$ buffer:	112.87 mM Na_2HPO_4
	7.12 mMNaH_2PO_4
	The solution was filter sterilized and autoclaved
TNS:	500 mM Tris-HCl pH8
	100 mM NaCl
	10 % SDS (w/v)
	pH was adjusted with HCl. The solution was filter sterilized and autoclaved.

The tubes were then spun down at 13000 rpm at 4°C for 4 min. 900 µl of the supernatants were transferred to new 2 ml tubes placed on ice. The DNA was then extracted with 1 volume (900 µl) phenol/chloroform/isoamylalcohol in ratio 25:24:1 and pH 8 and the tubes were spun down at 13000 g for 4 min at 4°C.
800 µl supernatants were placed in new 2 ml tubes and extracted with 1 volume (800 µl) chloroform/isoamylalcohol in ratio 24:1 and spun down at 13000 g for 4 min at 4°C.
650 ml supernatants were then mixed well with 2 volumes (1300 µl) PEG and precipitated by spinning for 30 min at 13000g and 4°C with the hinges out.

PEG: 30 % (w/v) polyethylene glycol 6000 in 1.6 M NaCl
The solution was prepared in baked glassware with RNase free water in microwave and autoclaved.

The liquid was carefully removed and 500 µl cold (4°C) 70% ethanol were added to wash the DNA pellet. The tubes were then spun down 13000 g for 4 min at 4°C with the hinges out.
The ethanol was removed carefully, not to disturb the DNA pellets, and the tubes were dried at room temperature for 5 min for the ethanol to evaporate. The DNA pellets were eluted with 50 µl elution buffer.

Elution buffer: 10 mM Tris-HCl pH 8
RNase free water
Solution was filter sterilized and autoclaved.

DNA extraction by DNeasy Tissue Kit (Qiagen cat. No. 69504)
Step 1: Purification of genomic DNA from Gram negative bacteria (DNeasy Tissue handbook p. 32)
The colon buds in Eppendorph tubes were suspended in 180 µl ATL buffer and vortexed vigorously.

Step 2: Purification of total DNA from animal tissue (DNeasy Tissue handbook p. 19, step 2)
20 µl Proteinase K were added to the tubes, vortexed and incubated in a water bath at

55°C until all tissue/bacteria/material in the tubes were completely lysed. This took 1-3 hours. The tubes were vortexed every 10 min.

After incubation the tubes were vortexed for 15 sec and the cotton buds were removed. 200 µl AL buffer were added and the tubes were vortexed and then incubated at 70°C for 10 min.

200 µl ethanol (96-100 %) were added to the tubes and they were vortexed. The mixture from the tubes were transferred to DNeasy mini spin columns which were placed in 2 ml collection tubes and centrifuged at 6000 g for 1 min. The flow through was discarded.

The spin tubes were placed in new 2 ml collection tubes. 500 µl AW1 buffer were added to the spin columns and spun for 1 min at 6000 g. The flow through was discarded.

The spin tubes were placed in new collection tubes and 500 µl AW2 buffer was added. The columns and tubes were spun for 3 min at 20000 g and the flow through was discarded.

The spin columns were placed in Eppendorf tubes and the DNA was eluted with 50 µl autoclaved Milli Q by adding the water directly on the membrane and then spinning the tubes at 6000 g for 1 min.

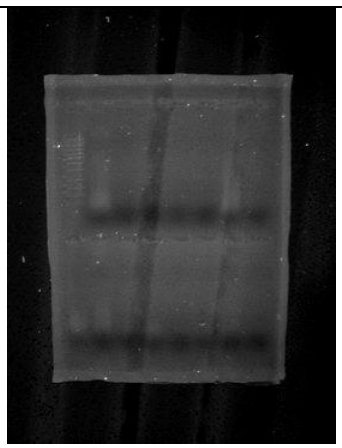


Figure 21: DNA extraction from experiment 1 sample day #9 and #22 with Zhou *et al*, (1996). The numbers after treatment indicates collection day. Gel was loaded as follows from top and from left to right; 1 kb ladder (Fermentas), C-9, T40-9, T43-9, T44-9, MUZ-9, C-22, T40-22, T43-22, T44-22, MUZ-22, TC-9, T40-22, T43-9, T44-9 and MUZ-9 (last sample was a redone from the cotton buds used in the first day 9 series). There was DNA in the following lanes; C-9, C-22, T43-22, T44-22 and C-9 (redone). The gel was documented on light board and with a Sony DSC-W170 camera.

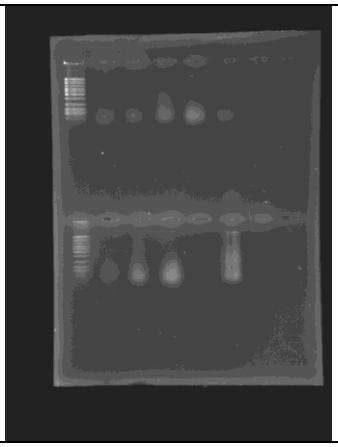


Figure 22: DNA extraction from experiment 2 sample day 22 with DNeasy Tissue Kit (Qiagen cat. No. 69504) (top) and Lueders *et al*, (2004) (bottom). The numbers after treatment indicates collection day. Lanes were loaded as follows from top and left to right; 1 kb ladder (Fermentas), C-22, T40-22, T43-22, T44-22, MUZ-22 and 1 kb ladder (Fermentas), C-22, T40-22, T43-22, T44-22, MUZ-22.
There was DNA in the following lanes; C-22, T40-22, T43-22, T44-22, MUZ-22 and 1 kb ladder (Fermentas), C-22, T40-22, T43-22 and MUZ-22.
The gel was documented in a light box.

There was not obtained DNA from all treatments with the extraction method from Zhou *et al* (1996) (figure 9) and this method was therefore rejected. Because of no success with obtaining DNA from treatment T44 collection day #22 with the method from Lueders *et al*, (2004) (figure 21) and because there was observed more smear in the lanes loaded with DNA extracted by the method from Lueders *et al*, (2004) it was decided to extract the rest of the replicates with the DNeasy Tissue Kit (Qiagen cat. No 69504) (figure 22).

There were prepared 3 replicate series from experiment 1 and 2 (33 DNA extractions). The DNA content and the 260/280 ration were measured by Nanodrop-1000 spectrophotometer (not shown).

PCR- polymerase chain reaction

There was run PCR on the extracted DNA with universal cloning primers GM3F and GM4R (provided by Dr. Raeid M. M. Abed, Sultan Qaboos University) (Muyzer *et al*, 1995). The primers are 16S primers with a length of approximately 1500 base pairs (table 4).

Table 4: The universal 16S cloning primers used in the PCR.

Name	Primer length and annealing points	Primer sequence
GM3F	8–24	5´-AGAGTTTGATCMTGGC-3´
GM4F	1492–1507	5´-TACCTTGTTACGACTT-3´

There were used two PCR systems, the first was from Promega (cat. no M2861) which did not give any results and the following protocol (tables 5 and 6) from Fermentas.

Table 5: PCR mix for 1 reaction in 25 µl.	
Water DNase free	15.25 µl
10 × buffer	2.5 µl
DNTP	2.0
Forward primer	0.5 mM
Reverse primer	0.5 mM
$MgCl_2$	2.0 µl
Taq polymerase (1.25 unit / reaction) (5unit / µl)	0.25 µl
Template	2 µl

The samples used for PCR was samples from experiment 1 collection day #9 and #22 because the biofilm on these samples represented a biofilm under development (day #9) and a mature biofilm (day #22). There was performed five unsuccessful PCR with the Promega system before the switch to the Fermentas system. The standard protocol from Fermentas (tables 5 and 6) was used without success and there was thereafter also run several PCR where there was manipulated with the amounts of template and temperature, still without success.

Table 6: Basic PCR program. The loading buffer was Blue/Orange 6X (Promega, G190A 24267214), the ladder was a 1 kb ladder from Promega (Cat # M-DNA-1kb) (figure 23), the cycler was an AB (Applied Bioscience) 2720 Thermal Cycler, the agarose gel (1g / 100 ml TAE buffer) was run on a Cleaver Scientific Ltd with a Cleaver Scientific Ltd mini 150 power unit and the gel was stained with ethidium bromide (10 µl (10 µg / ml) in 100 ml H_2O, Promega).

Program	Cycles	Denaturation		Annealing		Elongation	
		Temperature	Time	Temperature	Time	Temperature	Time
1	1	94°C	5 min				
2	25	94°C	1 min	42°C	1 min	72°C	1 min
3	1					72°C	10 min

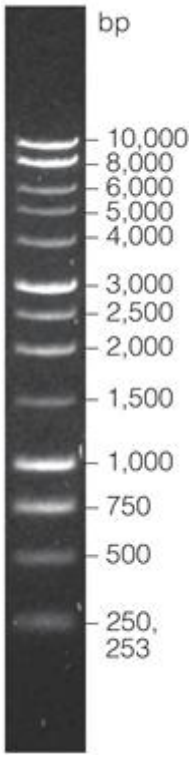


Figure 23: 1 kb ladder from Promega (Cat # M-DNA-1kb).

Since the DNA content and purity varied between replicates from both collection days #9 and #22 (figures 21 and 22), and there had been no success with the standard protocol from Fermentas in table 5, where minor manipulations also had been tried there were further manipulated with the content of the reaction mix, as amount of primers, Taq polymerase, magnesium chloride and dilution of the templates (×10 and ×100). To some reactions there were also added 0.8 μg / μl BSA (Bovine Serum Albumin enhances the polymerase activity) (Kreader, 1996, Fermentas) to the PCR water or 5 % v/v DMSO (dimethylsulfoxide) (Frackman *et al*, 1998) or both to some of reactions. DMSO can optimize uniform amplifications of DNA in PCR since it facilitates separation of DNA strands by disrupting base pairing.
There were also manipulated with the denaturation and elongation temperatures in some reactions. When all changes still proved unsuccessful touch-down PCR with Master Mix (Fermentas) was also applied without any success (table 7).

Table 7: Touch-down PCR program. The temperature in the touch-down decreases with 0.375°C / sec.

	Cycles	Annealing		Elongation		Denaturation	
		Temperature	Time	Temperature	Time	Temperature	time
Program 1	1					94°C	2 min
Program 2 Touch -down	15	65 - 50°C	40 sec	72°C	40 sec	94°C	30 sec
Program 3	40	50°C	40 sec	72°C	40 sec	94°C	30 sec
Program 4	1	42°C	60 sec	72°C	5 min		

In general the DNA contents were low and the purity also differed between the samples.

Results

An introduction to the statistics used in this project

All statistics made in this thesis was made with the statistical programs SYSTAT 13 and PRIMER 5.

The analysis made in the SYSTAT 13 program was preferable 1 and 2 factor variance analysis otherwise Kruskal-wallis- and Conover-Inman post hoc tests. Certain conditions had to be fulfilled before running an ANOVA (analysis of variance) test. The datasets residual values had to be normal distributed and the variances could be significant different from each other. There was used Kolmogorov-Smirnov analysis to test the data sets for normal distribution and Levene test for testing equality of the variances. If the dataset did not fulfill the required expectations the residual values were transformed by logarithm or square root. If the data still not fulfilled the requirements there was used the non-parametric Kruskal-Wallis test for the data which should have been analyzed by a 1 factor ANOVA and the 2 factor ANOVA was run with the transformation which gave the best basis to fulfill the requirements for the ANOVA test.

Both the ANOVA and the Kruskal-Wallis analyses determined if there were differences between the treatments in the dataset but did not inform between which treatments there were differences. The Tukey test explained the information generated from the ANOVA test and the multiple comparison test Conover-Inman for the Kruskal-Wallis test.

For the analysis made in the PRIMER 5 program which was MDS (multi dimentional scaling) plots (multidimensional scaling) and ANOSIM analysis (analysis of similarities) the biological collected data was square root transformed and a similarity matrix was made from Bray-Curtis coefficients. From this matrix the similarity coefficients were presented in MDS plots, ANOSIM. SIMPER (similarities percentages) were done without the similarity matrix and the data were both square root transformed and standardized.

The data in this project consisted of macro- and microfouling samples and fatty acid samples. The fatty acid analysis samples and the macrofouling samples were multivariate samples since each sample consisted of different species and fatty acids. These data were placed in n columns (samples) with p rows (species or fatty acids). The microfouling data were also placed in n columns (samples) with p rows (replicates).

The data were square root transformed to alter the relative distances between data points to improve the normality of the data sets. Square root transformation also down weighted the influence of dominating species in the data set so less abundant species also counted in the similarity determination between the data sets.
All community analyses (SIMPER analysis) data were also standardized to get a better comparison between the data sets. Standardization changed the actual values in a data set to percentages of the data set and thereby allowed comparison between data sets with a focus on the community composition.

Similarity coefficients were calculated and a similarity matrix was made. In a similarity matrix the similarities are determined between each pair of samples and the calculated values are presented in a triangular matrix. Similarity coefficients of 0 mean there is no similarity between the datasets where coefficients of 100 mean the data are 100% identical. It is from this matrix the ANOSIM analysis was performed and a MDS plot was made. The ANOSIM is an analysis of similarities between data set consisting of community data. This analysis tells which data sets are different from each other.

The SIMPER analysis was made on the datasets without the similarity matrix was made. The data were square root transformed and standardized before the analysis. In general the SIMPER analysis describes the similarity internal in each treatment between replicates and the dissimilarity between treatments and which species drive the differences observed in the ANOSIM.

In the fatty acids dataset two fatty acids dominated the results from the experimental slides exceptionally. More than 50 % of the SIMPER analysis results were made from

Methyl-hexadecanoate and Methyl-octadecanoate when the data were standardized and square rooted. Therefore it was decided to remove these two dominant fatty acids from the SIMPER analyses since they covered the effect of the other fatty acids present. A 90 % cut-off limit was also chosen to limit the fatty acids which were only present in very small quantities (traces).
The MDS plots were, as the analyses, made from treatment averages to get a better overview of treatment placement compared to each other.

Environmental description – All experiments

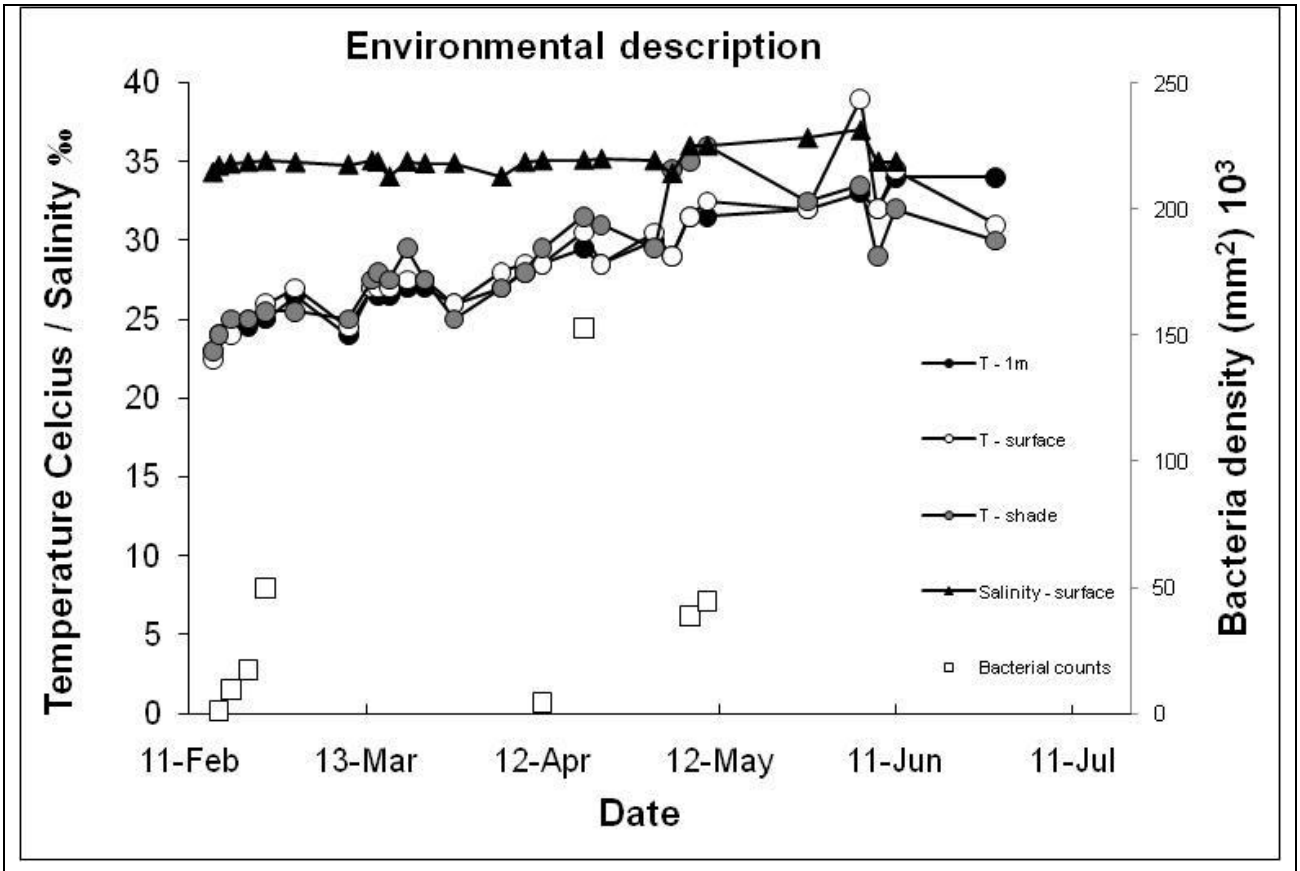


Figure 24: Basic description of the environment wherein the experiments took place. Salinity and temperatures (surface, - 1m and in shade) were measured on each collection day. The bacterial counts were from the Control treatment. The temperature was measured with a thermometer and the salinity with an YSL measuring meter. Since the temperature through the experimental period and the salinity had similar values these measurements shared axis where the bacteria counts which had much higher values were placed on the secondary y-axis.
In experiment 1 (February) there was collected bacteria on day 1, 3, 6 and 9. In experiment 2 (April) the bacteria were collected on day 3 and 9. In the third experiment (May) the bacteria were collected on day 3 and 6.

Figure 24 is a presentation of the basic environmental data collected through the four experiments. Data for salinity and temperatures were measured on each collection day through the whole experimental period. The bacterial counts are the Control treatment from experiment 1 – 3, there were not any usable bacterial counts from the Control treatment in experiment 4. The salinity measurements were stabile through the whole experimental period and were roughly measured to approximately 35 ‰ on each collection day. The temperatures were raised with approximately 10°C, from 22°C to 32°C, through the experimental period ranging from mid February to end of June. The bacterial counts from experiments 1 – 3 increased with time and with the temperature rise over the experimental period.

Bacterial results

Bacteria counts – experiment 1

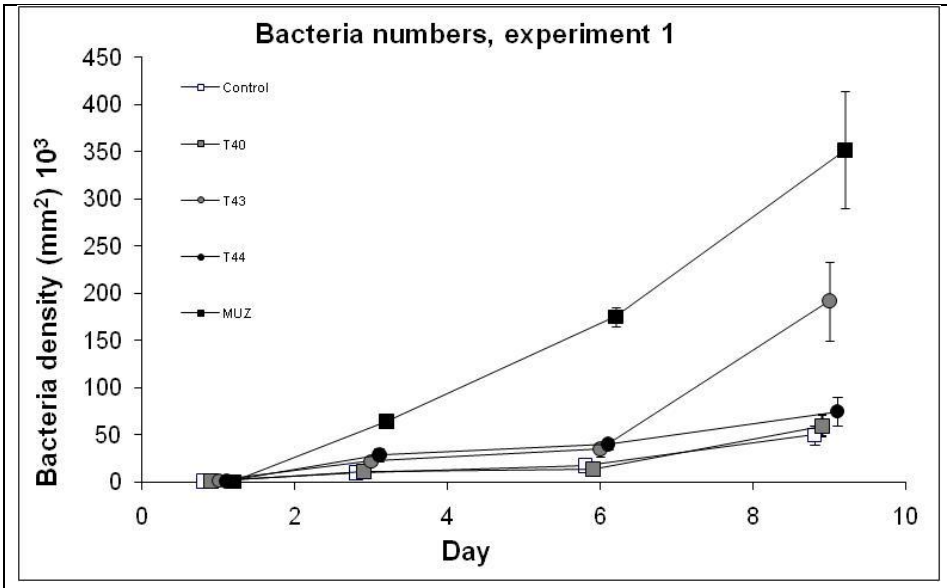


Figure 25: Number of bacteria from experiments 1 (sampling days 1, 3, 6 and 9). The abundance is mean values ± SE given in 10^3 mm^2. Note that data points from the same date are slightly displaced from each other along the x-axis to give a better representation of the error bars.

Bacterial counts from the various treatments in the experiments 1 are presented in figure 25.

ANOVA – Bacterial counts experiment 1

Bacterial numbers increased over time in all the five treatments (ANOVA, $p<0.001$, table 8). The Control, treatments T40 and T43 followed approximately the same growth pattern where-as the treatment MUZ had a more bacteria than the other treatments from day 3 and forward. Treatment T43 followed the growth pattern of the control, and treatments T40 and T44 until day 6 where the bacteria numbers increased faster (figure 25). Bacterial numbers were similar in all treatments at day 1 but became more different from each other over time. Bacterial counts on day 9 were approximately 60.000 mm^{-2} on the Control, T40 and T44, 200.000 mm^{-2} on T43 and 350.000 mm^{-2} in the MUZ treatment.

The density of bacteria on treatment T40 and on the Control was not different from each other (Tukey, $p=0.999$, table A1), but had less bacteria than the other treatments (Tukey, $p<0.001$, table A1). The treatment MUZ had a larger amount of bacteria than any of the other treatments. Treatment T43 and T44 had more bacteria than the Control and T40 and less bacteria than the MUZ treatment (table A1).

Table 8: ANOVA test results from experiment 1. The significance limit was p=0.05.

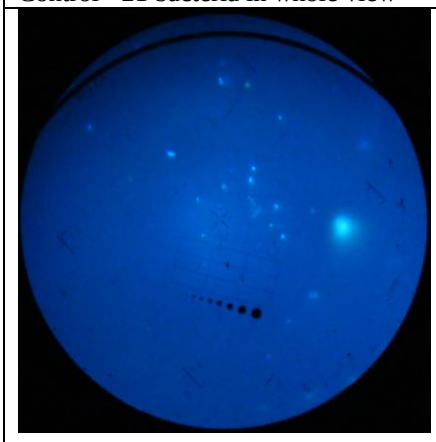
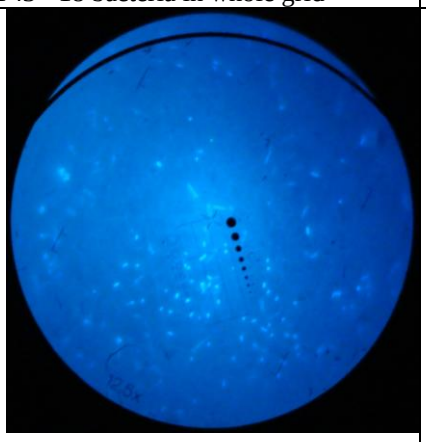
ANOVA	df	MS	F-ratio	p
Treatment	4	8.83	29.82	0.001
Time	3	147.62	498.56	0.001
T × T	12	2.78	9.40	0.001
Error	108	0.30		

Photo documentation experiment 1

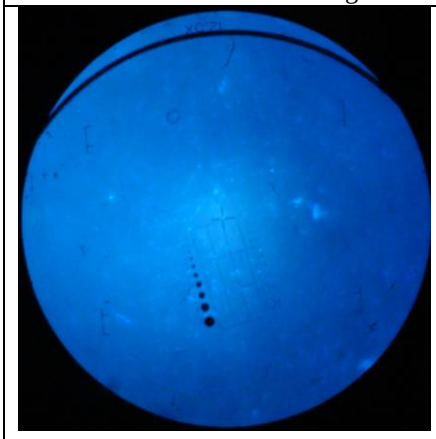
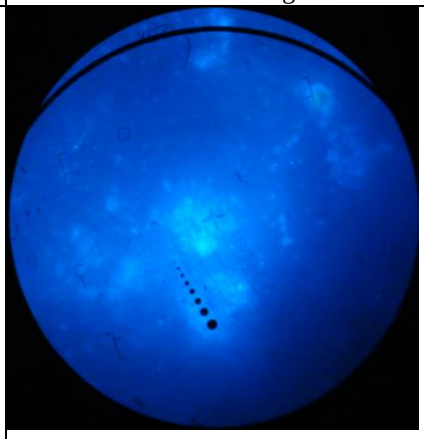
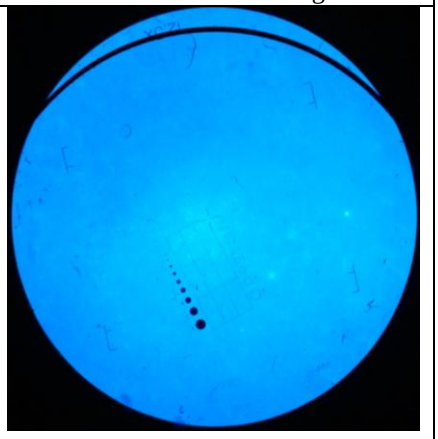
There was through the bacterial counts taken photos of the DAPI stained filters and a representative photo from each treatment group can be seen in figure 26.

Figure 26: Photos from black filters on experimental slides on sample days #1 and #3 from experiment 1.

Photos of bacterial counts from experiment 1 sample day 1. All photos are in ×1000 magnification.

Control - 21 bacteria in whole view	T43 - 18 bacteria in whole grid	
		

Photos of bacterial counts from experiment 1 sample day 3. All photos are in ×1000 magnification.

Control - 30 bacteria in whole grid	T43 - 71 bacteria in one grid	MUZ - 30 bacteria in whole grid
		

The photos are presented to give an idea of how the bacterial counts changes over time and how the bacterial clustering and biofilm develop. All treatments, except the Control, were affected by background fluorescence (the cloudiness in the photos). Especially treatments T43 and MUZ were affected by back ground fluorescence. The background fluorescence was generated by a rise in concentration of antifouling compounds in the biofilm. This phenomenon has been experienced before when working with bacterial counts on antifouling paints (pers. comm. Kjeld Schamburg).

Bacteria counts – experiment 2

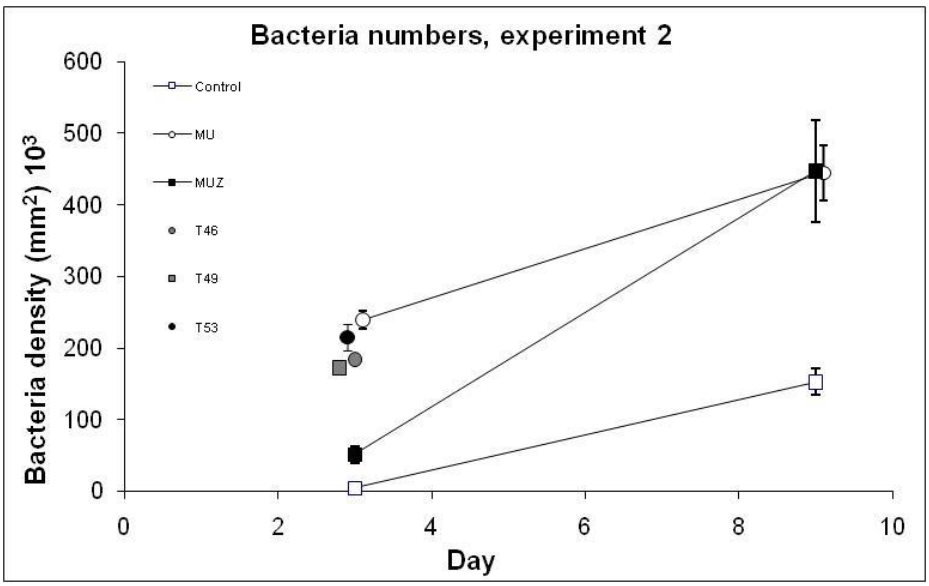


Figure 27: Bacterial counts from experiments 2 (sampling days 3 and 9). The abundance is mean values ± SE given in 10^3 mm^2. Note that data points from the same date are slightly displaced from each other along the x-axis to give a better representation of the error bars.

The bacterial counts from experiment 2 are presented in figure 27.

ANOVA – Bacterial counts experiment 2

The bacterial counts increased from day 3 to day 9 in all treatments (ANOVA, $p<0.001$, table 9). The density of bacteria were slightly different from each other on sample day 3 (treatments Control<MUZ<MU). MUZ had on day 3 a lower bacteria abundance than MU but not on day 9 (Tukey, $p=0.038$, table A2). At day 9, the MU and MUZ had approximately the same density (450.000 mm^{-2}) where the Control had much lower density (150.000 mm^{-2}) (figure 27). Overall the Control had lower density than both the MU and MUZ treatments (Tukey, $p< 0.001$ and $p=0.003$, table A2). The treatments T46, T49 and T53 were only countable on day 3, on collection day #9 the treatments were uncountable* due to biofilm structure and background fluorescence (figure 28). When comparing all 6 treatments on day 3, were there difference between the treatments (Kruskal-Wallis $p=0.001$). The bacterial abundance in treatment MU was larger than that in all other treatments except for treatment T53 (Conover-Inman, table A3). The Control- and MUZ treatments had approximately the same bacterial density (table xa3) which were lower than for T46, T49 and T53 (Conover-Inman, $p<0.000$, $p=0.001$, table A3, figure x2) which had the same density. Very few diatoms were observed on the control treatment and therefore not included in the results.

Table 9: ANOVA test results from experiment 2. The significance limit was sat to p=0.05.

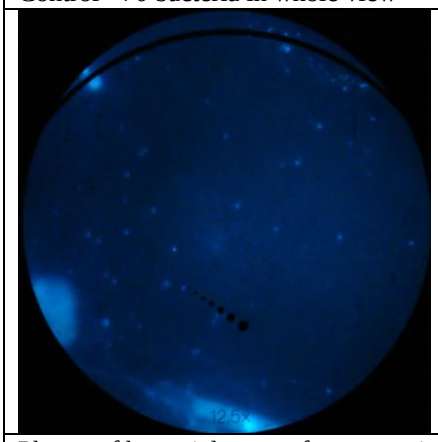
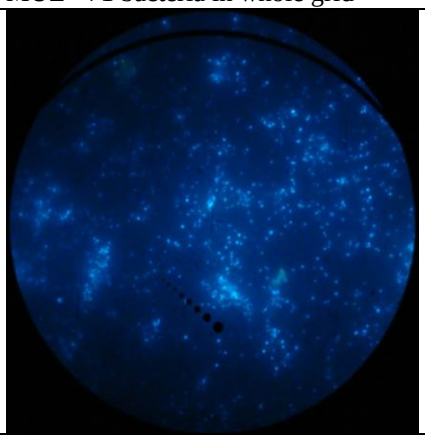
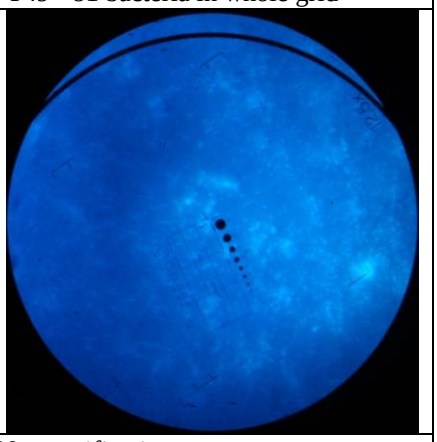
ANOVA	df	MS	F-ratio	p
Treatment	2	$1.198 \cdot 10^{11}$	17.03	0.001
Time	1	$3.668 \cdot 10^{11}$	52.12	0.001
T × T	2	$3.677 \cdot 10^{10}$	5.22	0.014
Error	22	$7.037 \cdot 10^{9}$		

*Uncountable: The treatments reffered to as uncountable had much background fluorescence and could therefore not be counted manually by DAPI staining under a fluorescence microscope.

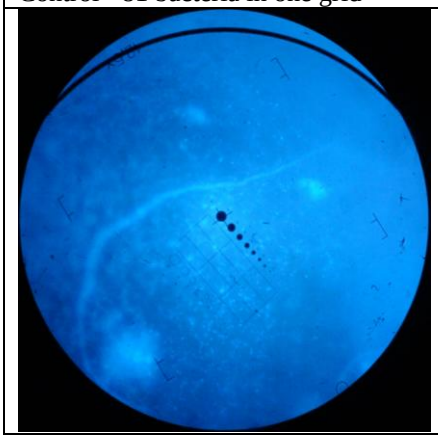
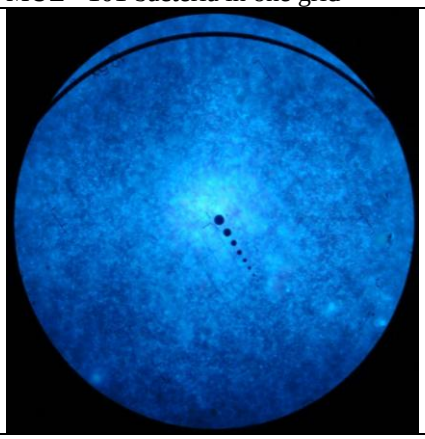
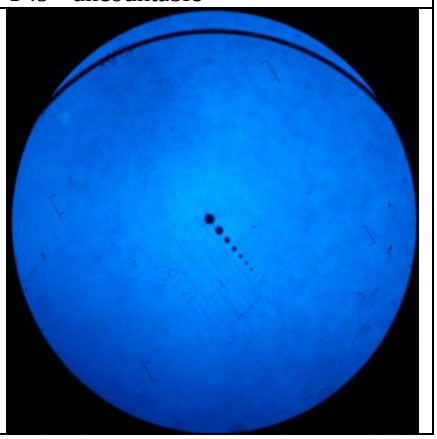
Photo documentation experiment 2

There was through the bacterial counts taken photos of the DAPI stained filters and a representative photo from each treatment group can be seen in figure 28.

Figure 28: Photos of bacterial counts from experiment 2 sample day 3. All photos are in ×1000 magnification.

Control - 70 bacteria in whole view	MUZ - 71 bacteria in whole grid	T49 - 81 bacteria in whole grid
		

Photos of bacterial counts from experiment 2 sample day 9. All photos are in ×1000 magnification.

Control - 81 bacteria in one grid	MUZ - 101 bacteria in one grid	T49 - uncountable
		

The photos presented in figure 28 are DAPI stained black filters from treatments Control, MUZ and T53 from collection days 3 and 9. The photos show how the biofilms in each treatment changed over time. I general all treatments except the Control were affected, and some strongly affected, by background fluorescence. Treatments T46, T49, T53 were strongly affected specially on sample day #9 (the cloudiness in the photos). Background fluorescence is generated by an up concentration of antifouling compounds in the mature biofilm. This phenomenon has been experienced before when working with bacterial counts on antifouling paints (pers. comm. Kjeld Schaumburg).

Summary

Bacterial density increased over time in both experiments in all treatments. In experiment 1 the Control, treatments T40 and T43 followed the same growth pattern where treatment MUZ had both a higher bacteria density and a steeper growth pattern (figure 25). Treatment 43 only followed the bacterial growth of treatments T40 and the Control until day 6. There after treatment T43's bacterial number increased faster than in treatments T40 and the Control.

In experiment 2 the highest bacterial abundance was observed in the treatments MU and MUZ and the lowest in the Control. At day 3 MU had larger bacterial counts than the other treatments except from treatment T53 which it did not differ from (Conover-Inman, table A3). Treatments T46, T49 and T53 had all larger bacterial density than the Control and treatment MUZ but were not different from each other (Conover-Inman, table A3). Treatment MUZ which had lower bacteria density in the beginning of the experiment (day 3) had the highest in the end of the experiment (day 9) (Tukey, $p=0.038$, table A2).

The bacterial counts on sample day #9 were lower for the Control and treatment MUZ in experiment 1 (49.682 and 351.923 bacteria mm^2) compared to experiment 2 (152.962 and 447.196 bacteria mm^2). There were no differences in the growth patterns for the treatments between the experiments. The MUZ treatment had a steeper growth curve than the Control both in experiment 1 and 2.

Bacteria counts – experiment 3 and 4

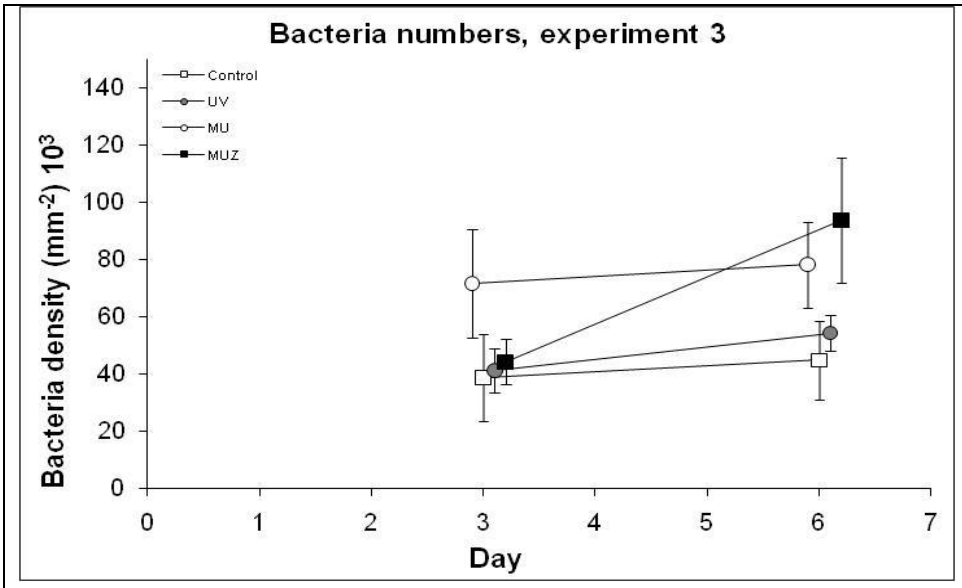


Figure 29: Number of bacteria from experiment 3 (sampling days 3 and 6).
The abundance is mean values ± SE given per 1 mm^{-2} in 10^{-3}.
Note that data points from the same date are slightly displaced from each other along the x-axis to give a better representation of the error bars.

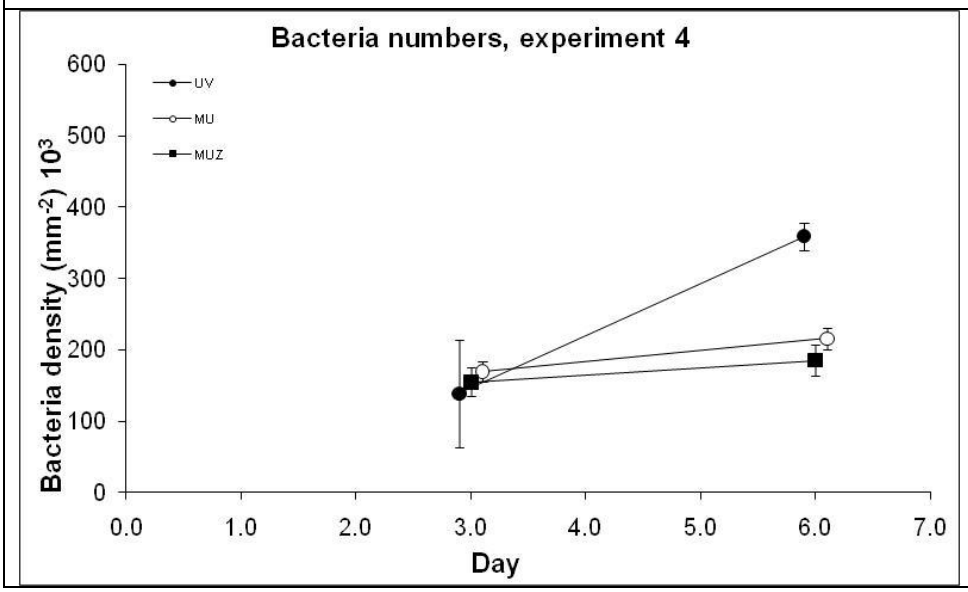


Figure 30: Bacteria counts from experiment 4 (sampling days 3 and 6).
The abundance is mean values ± SE given per 1 mm^{-2} in 10^{-3}.
Note that data points from the same date are slightly displaced from each other along the x-axis to give a better representation of the error bars.
The Control treatment was uncountable in experiment 4.

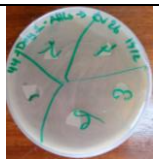
Bacterial counts from the various treatments in the experiments 3 and 4 are presented in figure 29 and 30. Biofilms on filters were very developed on both sampling days in experiment 3 and 4 and therefore the counts are misleading due to forced counts on top of biofilm and in "low density" areas of the filters. These data are therefore not treated further in this project.

Reporter strain assays

Chromobacterium violaceum CV026 detects if any short chain *N*-acyl homoserine lactones (AHLs) compounds are present by producing violaceum (blue coloration). The bacterium *C. violaceum* CV017 produce violacein and when grown above 30°C the violacein production increases due to its mutation. If the collected filters with biofilm contain compounds which inhibit AHLs quorum sensing compounds the violacein production will cease and blank/light spots can be detected.

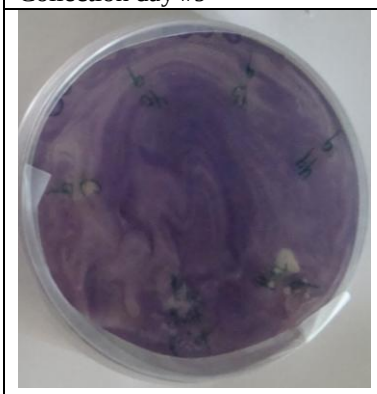
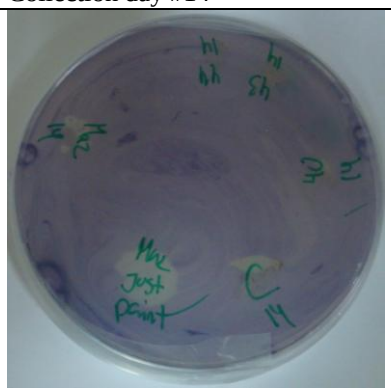
There was not observed any quorum sensing compounds in any of the treatments from experiment 1 collection day #1 (figure 31) but there was blue coloration from the violaceum in the positive control. Some paints had an inhibitory effect on the growth of *C. violaceum* CV026 and is seen as blank areas surrounding the filters in treatment MUZ (figure 3, all filters) and in T43 (figure 31).
This was the only time that *C. violaceum* CV026 was grown successfully.

Figure 31: *Chromobacterium violaceum* CV026 reporter strain assay from experiment 1a collection day #1.

Positive control	Control	T40	T43	T44	MUZ
					

Chromobacterium violaceum CV017 assays were run with filters from collection days #9 and #14 (figure 32). There was only observed inhibition in treatment MUZ and in the MUZ paint which suggests that it is the paint which inhibits the bacteria in the biofilm and maybe also the reporter strain.

Figure 32: *Chromobacterium violaceum* CV017 reporter strain assay from experiment 1a.

Collection day #9	Collection day #14
	

Polymerase chain reactions (PCR) - experiments 1 and 2

DNA was extracted with three methods to decide which one gave the best quality. The methods were Zhou *et al*, (1996), Lueders *et al*, (2004) and DNeasy Tissue Kit (Qiagen cat. No 69504). The DNeasy Tissue Kit (Qiagen cat No 69504) extraction method was selected for DNA extraction. Even if the DNeasy method from Qiagen (Qiagen cat No 69504) was the most efficient of the methods tested the DNA extracts were still rather poor both in quantity and quality.

The content and purity of the extracted DNA differed between samples. The first samples run were from experiment 1 collection day #9 and #22 and were from the Control and MUZ treatments since these were the only treatments from which DNA was purified successfully.

The first 35 PCR was made with a PCR system from Promega (cat. no M2861) which, even under different manipulations (se the method), only yielded poor results. The only treatment a PCR band was obtained in was the Control (day #9).

With the standard Fermentas PCR system the only band was also the only obtained in treatment MUZ from collection day #22. When diluting the template DNA to minimize the concentration of possible pollutants or polymerase inhibitors the only band was in treatment MUZ collection day #22, template dilution ×10.
In general there was almost generated a PCR product from treatment MUZ in every run. From this can be concluded that the MUZ paint did not have an inhibitory effect, which had been predicted in the beginning of the PCR because of the release of paint to the cotton bud when collecting biofilm, and that the biofilm from these treatments must lack what inhibits the PCR in all other treatments. The main difference between the collected experimental slides was the lack of algae on the MUZ slides.

Addition of additional $MgCl_2$ (from 2 µl to 4 µl) to treatments Control, T40, T43 and T44 (experiment 1), all from collection day #22, did not produce any PCR products.

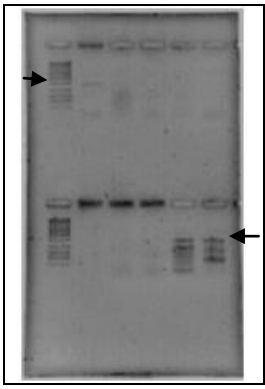


Figure 33: Gel with PCR products from experiment 1 and 2, sample days #22. Top: 1 kb ladder (Fermentas), treatments Control, T40, T43, T44 (experiment 1) and Control (experiment 2). Bottom: 1 kb ladder (Promega), T46, T49, T53, MU and MUZ (experiment 2). The arrows indicate where 1500 kb is placed.

Addition of BSA (0.8 µg / µl) to the PCR mix for treatments Control, T40, T43, T44 and Control (experiment 1), T46, T49, T53, MU and MUZ (experiment 2), all samples from collection day #22, produced PCR products in the Control (experiment 1) and in treatments MU and MUZ (experiment 2). The band (from the gel) from the Control was weak but the bands from the MU and MUZ treatments were clear but the lanes also contained other bands shorter than 1500 kb (figure 33).

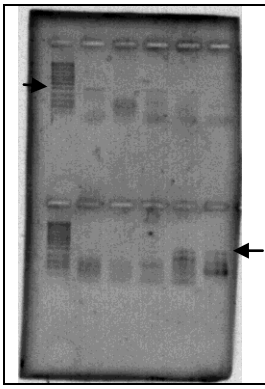


Figure 34: Gel with PCR products from experiment 1 and 2, sample days #22. Top: 1 kb ladder (Fermentas), treatments Control, T40, T43, T44 (experiment 1) and Control (experiment 2). Bottom: 1 kb ladder (Promega), T46, T49, T53, MU and MUZ (experiment 2). The arrows indicate where 1500 kb is placed.

When 5 % DMSO was added to the PCR mix which also contained BSA to the PCR mix used for treatments Control, T40, T43, T44 and Control (experiment 1), T46, T49, T53, MU and MUZ (experiment 2), all samples from collection day #22. There were PCR products in the Control and treatment T43, collection day #22 (experiment 1) and in treatments MU and MUZ, collection day #22 (experiment 2) (figure 34). There were also several smaller bands present in the all lanes and primer dimers in the bottom of the lanes.

Changing the denaturation (from 94°C to 95°C) or elongation (from 72°C to 74°C) temperatures did not improve the PCR products.

The PCR products obtained from the Control, T43 and MUZ treatments (experiment 1) and the Control, MU and MUZ treatments (experiment 2) were amplified and purified but without success. This was done in preparation for restriction fragment length polymorphism (RFLP) (this step was not a part of the original plan) and Denaturing Gradient Gel Elecrophoresis (DGGE).

Touch-down PCR with Master Mix (Fermentas) did not produce any PCR products at all and are therefore not included in table 10.

Table 10: PCR running plan for samples from experiment 1 and 2 collection day #22. The + in the table indicates a positive outcome of the PCR with a visible DNA band where the - indicates no visible band. There was not run any PCR in the blank cells. If nothing else is noted then the reaction was run at standard temperatures and cycles (table 6).

Sample D22	Sample no	Basic PCR mix	BSA	BSA + DMSO	BSA + DMSO + 40 cycles	Denaturation 95° C Elongation 74° C BSA + DMSO
C-1-22-1b	1	-	+	+	+	-
40-1-22-1b	2	-	-	-	-	-
43-1-22-1b	3	-	-	+	+	-
44-1-22-1b	4	-	-	-	-	-
MUZ-1-22-1b	5	+	-	+	+	-
C-2-22-1b	6			-	-	-
40-2-22-1b	7			-	-	-
43-2-22-1b	8			-	-	-
44-2-22-1b	9			-	-	-
MUZ-2-22-1b	10			-	-	-
C-3-22-1b	11			-	-	-
40-3-22-1b	12			-	-	-
43-3-22-1b	13			-	-	-
44-3-22-1b	14			-	-	-
MUZ-3-22-1b	15			-	-	-
C-1-22-2	16		-	-	-	-
46-1-22-2	17		-	-	-	-
49-1-22-2	18		-	-	-	-
53-1-22-2	19		-	-	-	-
MU-1-22-2	20		-	-	-	-
MUZ-1-22-2	21		-	-	-	-
C-2-22-2	22		-	-	-	-
46-2-22-2	23		-	-	-	-
49-2-22-2	24		-	-	-	-
53-2-22-2	25		-	-	-	-
MU-2-22-2	26		+	+	+	+
MUZ-2-22-2	27		+	+	+	+
C-3-22-2	28			-	-	-
46-3-22-2	29			-	-	-
49-3-22-2	30			-	-	-
53-3-22-2	31			+	+	-
MU-3-22-2	32			+	+	+
MUZ-3-22-2	33			+	+	-

Fatty acid results

Fatty acid extraction

There were screened for 26 different fatty acids (table 11) by fatty acid methylation extraction (FAME). The fatty acids are divided in several groups after their structure. All the screened fatty acids were also somewhat present in all treatments (tables 12, 26 and 27) however in very fluctuating concentrations, also internal in treatments.

Table 11: Fatty acids screened for FAME analysis. The fatty acids are divided in 5 groups under type. Saturated fatty acid (SFA), mono unsaturated fatty acids (MUFA), poly unsaturated fatty acids (PUFA), cyclopropane, hydroxy- and branched chains.

Fatty acids	Type
Methyl 2-hydroxydecanoate (2-OH-C10:0)	Hydroxy
Methyl undecanoate (C11:0)	SFA
Methyl dodecanoate (C12:0)	SFA
Methyl 2-hydroxydodecanoate (2-OH-	Hydroxy
Methyl 3-hydroxydodecanoate (3-OH-	Hydroxy
Methyl tridecanoate (C13:0)	SFA
Methyl tetradecanoate (C14:0)	SFA
Methyl 2-hydroxytetradecanoate (2-OH-	Hydroxy
Methyl 3-hydroxytetradecanoate (3-OH-	Hydroxy
Methyl pentadecanoate (C15:0)	SFA
Methyl 13-methyltetradecanoate (i-C15:0)	Branched
Methyl 12-methyltetradecanoate (α-C15:0)	Branched
Methyl hexadecanoate (C16:0)	SFA
Methyl 14-methylpentadecanoate (i-C16:0)	Branched
Methyl-2-hydroxyhexadeanoate (2-OH-	Hydroxy
Methyl cis-9-hexadecenoate (C16:1^{9})	MUFA
Methyl heptadecanoate (C17:0)	SFA
Methyl 15-methylhexadecanoate (i-C17:0)	Branched
Methyl cis-9,10-methylenehexadecanoate	Cyclopropane
Methyl octadecanoate (C18:0)	SFA
Methyl cis-9-octadecenoate (C18:1^{9})	MUFA
Methyl trans-9-octadecenoate (C18:1^{9})	MUFA
Methyl cis-9,12-octadecadienoate	PUFA
Methyl nonadecanoate (C19:0)	SFA
Methyl cis-9,10-methyleneoctadecanoate	Cyclopropane
Methyl eicosanoate (C20:0)	SFA

Fatty acid extraction – Community analysis experiment 1a

In table 12 is listed an overview of the fatty acids present in experiment 1a. There were screened for 26 fatty acids where 6 were hydroxy acids, 10 were saturated fatty acids (SFA), 3 were mono unsaturated fatty acids (MUFA), 1 was a poly unsaturated fatty acids (PUFA), 4 were branched fatty acids and 2 were cyclopropane fatty acids. There were some fatty acids which were present in all treatments. The saturated fatty acids C14:0, C15:0, C16:0, C17:0, C18:0 were present in all treatments at least on one collection day in the experiment. Another fatty acid which all treatments had in common was the mono unsaturated fatty acid (MUFA) C18:1ω9 and both the *cis* and *trans* version was present in all treatments. Treatment MUZ was the only treatment where the hydroxyl acid 2-OH-C12:0 was detected and the hydroxyl acid 3-OH-C14:0 was only found in treatment T40. T40 was also the only treatment where the

Table 12: Fatty acids screened for FAME analysis. The fatty acids present in experiment 1a.

Fatty acids	Type	Treatments				
		Control	T40	T43	T44	MUZ
Methyl 2-hydroxydecanoate (2-OH-C10:0)	Hydroxy		*	*	*	*
Methyl undecanoate (C11:0)	SFA		*			*
Methyl dodecanoate (C12:0)	SFA				*	*
Methyl 2-hydroxydodecanoate (2-OH-C12:0)	Hydroxy					*
Methyl 3-hydroxydodecanoate (3-OH-C12:0)	Hydroxy					
Methyl tridecanoate (C13:0)	SFA		*			*
Methyl tetradecanoate (C14:0)	SFA	*	*	*	*	*
Methyl 2-hydroxytetradecanoate (2-OH-C14:0)	Hydroxy		*			
Methyl 3-hydroxytetradecanoate (3-OH-C14:0)	Hydroxy					
Methyl pentadecanoate (C15:0)	SFA	*	*	*	*	*
Methyl 13-methyltetradecanoate (i-C15:0)	Branched			*		*
Methyl 12-methyltetradecanoate (α-C15:0)	Branched	*		*		
Methyl hexadecanoate (C16:0)	SFA	*	*	*	*	*
Methyl 14-methylpentadecanoate (i-C16:0)	Branched		*			
Methyl-2-hydroxyhexadeanoate (2-OH-C16:0)	Hydroxy					
Methyl cis-9-hexadecenoate (C16:1ω9)	MUFA		*	*	*	*
Methyl heptadecanoate (C17:0)	SFA	*	*	*	*	*
Methyl 15-methylhexadecanoate (i-C17:0)	Branched	*	*	*	*	
Methyl cis-9,10-methylenehexadecanoate (C17:0Δ)	Cyclopropa	*		*	*	*
Methyl octadecanoate (C18:0)	SFA	*	*	*	*	*
Methyl cis-9-octadecenoate (C18:1ω9)	MUFA	*	*	*	*	*
Methyl trans-9-octadecenoate (C18:1ω9)	MUFA	*	*	*	*	*
Methyl cis-9,12-octadecadienoate (C18:2ω9,12)	PUFA	*		*	*	*
Methyl nonadecanoate (C19:0)	SFA		*	*	*	*
Methyl cis-9,10-methyleneoctadecanoate (C19:0Δ)	Cyclopropa					
Methyl eicosanoate (C20:0)	SFA			*	*	*

branched fatty acid *i*-C16:0 was detected. The number of fatty acids detected on the treatments also differ (table 12). The Control had the lowest number of fatty acids detected (11) where the MUZ treatment had 18 out of 26. Treatments T40 (14), T43 (16) and T44 (15) number of fatty acids detected were in between the Control and the MUZ treatment.

The distribution between the different structural types of fatty acids in each treatment from experiment 1a is presented in figure 35.

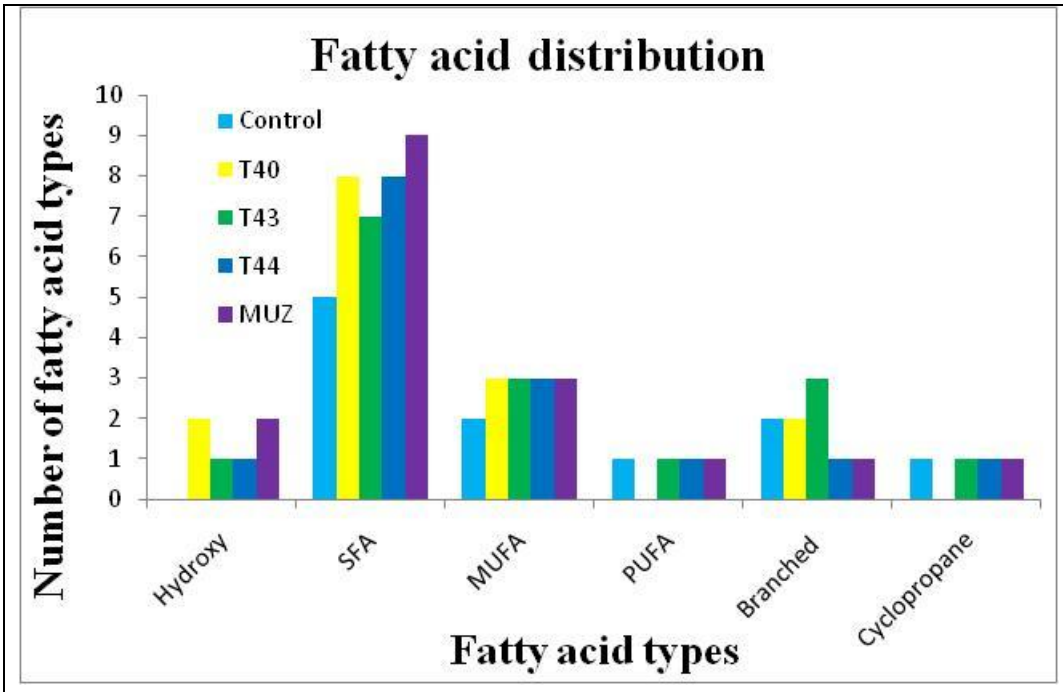


Figure 35: Distribution of the types of fatty acids detected in the FAME analysis for experiment 1a.

The dominating fatty acids were the saturated fatty acids (SFA) which also were the fatty acid type which there mostly was screened for. The treatment with most saturated fatty acids was the treatment MUZ and treatment T44 was the one with the least.

The fatty acids with the lowest density were poly unsaturated fatty acids and cyclopropanes. These two types were also the fatty acids screened the least for. There was only one fatty acid from each treatment in the fatty acid groups of poly unsaturated fatty acids and cyclopropane fatty acide except for treatment T40 which had no fatty acids from these two groups.

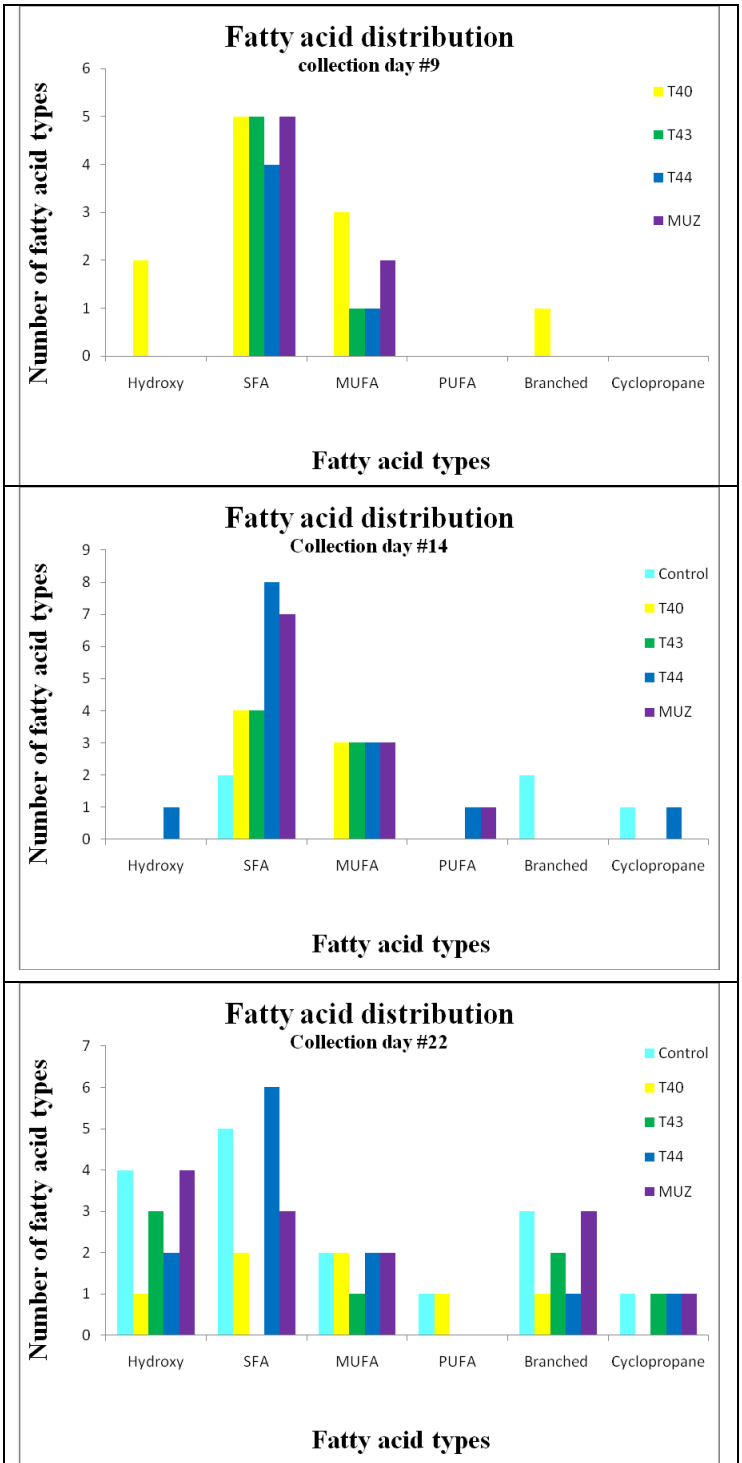


Figure 35: Distribution of the fatty acids detected in the FAME analysis in experiment 1a presented for each collection day.

There were not many hydroxy acids detected either. The maximum was two in the treatment T40 and in the MUZ treatment and all other treatments only had a single fatty acid of this type. The treatments almost had an equal amount of mono unsaturated (MUFA) fatty acids, three in each treatment, except from the Control which only had two fatty acids of this type detected. Treatments T40, T43 and the Control had two and three branched fatty acids where treatments T44 and MUZ only had one.

ANOVA – fatty acid extraction – experiment 1a

In figure 35, the number of fatty acids in each group is presented for each collection day. There was not observed any fatty acid succession over time (ANOVA, p>0.280, table 3) or differences between treatments (ANOVA, p>0.984, table 13) but there were seen differences between fatty acid groups (ANOVA, p>0.000, table 13).

Table 13: ANOVA test results from fatty acids groups in experiment 1a on collection days 9, 14 and 22. The significance limit was p=0.05.

ANOVA	df	MS	F-ratio	p
Treatment	4	0.166	0.095	0.984
Fatty acid group	5	8.955	5.131	<0.000
Time	2	2.258	1.294	0.280
Error	72	1.745		

The fatty acid groups which differed from each other were the saturated fatty acids (SFA) which were larger than the branched fatty acids (Tukey, p=0.019, A4), the cyclopropane fatty acids (Tukey, p=0.002, A4) and poly unsaturated fatty acids (PUFA) (Tukey, p=0.001, A4).

On collection day #9 (upper graph in figure 35) saturated fatty acids (SFA) were the most dominating fatty acid group. There were few mono unsaturated fatty acids (MUFA) present in all treatments. Hydroxy acids and branched fatty acids were only present in treatment T40. There was significant difference between both the fatty acid groups (ANOVA, p<0.000, table 14) and the treatments (ANOVA, p=0.018, table 14) at collection day #9 in experiment 1a. There was a higher density of mono unsaturated fatty acids compared to the branched fatty acids (Tukey, p=0.008, A5).

Table 14: ANOVA test results from fatty acids groups in experiment 1a collection day 9. The significance limit was p=0.05.

ANOVA	df	MS	F-ratio	p
Fatty acid group	5	13.742	54.363	<0.000
Treatment	3	1.153	4.560	0.018
Error	15	0.253		

The saturated fatty acids group was the dominating and larger than all the other fatty acid groups (a21). The second largest group was the mono unsaturated fatty acids, which of cource was smaller than the saturated fatty acids (ANOVA, p>0.000, table 14) but larger than the other groups (A5). The branched-, poly unsaturated-, hydroxy-,

and cyclopropane fatty acids groups were all smaller than the groups of saturated fatty acids and mono unsaturated acids but all not different from each other (A5).
When comparing treatments only treatments T40 and T44 were different from each other where treatment T44 had a lower number of different fatty acids (Tukey, p=0.017, A6).

The fatty acid distribution in experiment 1a collection day #14 was different distributed than on collection day #9 (figure 35, middle). Only treatment T44 had a hydroxy acid detected. The saturated fatty acids were also the dominating fatty acid group on collection day #14 but different distributed than on collection day #9. There were also poly unsaturated fatty acids and cyclopropane fatty acids detected on collection day #14. There was no difference between the treatments on collection day #14 (ANOVA, p=0.232, table 15). There were differences between the different fatty acid groups (ANOVA, p<0.000, table 15).

Table 15: ANOVA test results from fatty acids groups in experiment 1a collection day #14. The significance limit was p=0.05.

ANOVA	df	MS	F-ratio	p
Fatty acid group	4	2.200	1.528	<0.000
Treatment	5	18.373	12.759	0.232
Error	20	1.440		

The saturated fatty acid group differed from all the other fatty acid groups (Tukey, A7) and there were no differences between the other groups.

On collection day #22 almost all fatty acid groups were presented in each treatment. There were no saturated fatty acid or cyclopropane fatty acids in T43 and in treatments T43, T44 and the MUZ treatment no poly unsaturated fatty acids were detected (figure 35 lower).
There was only difference between fatty acid groups (ANOVA, p=0.006, table 16) and not between the treatments (ANOVA, p=0,136, table 16).

Table 16: ANOVA test results from fatty acids groups in experiment 1a collection day #22. The significance limit was p=0.05.				
ANOVA	df	MS	F-ratio	p
Fatty acid group	4	2.583	1.982	0.006
Treatment	5	5.953	4.568	0.136
Error	20	1.303		

The saturated fatty acid group was larger than the groups of poly unsaturated- (Tukey, p=0.010, a22) and cyclopropane fatty acids (Tukey, p=0.034, A8). The hydroxy acid group was also larger than the poly unsaturated group (Tukey, p=0.034, A8).

Hydroxy fatty acids. The number of hydroxy fatty acids differed both over time (ANOVA, p=0.038, table 7) and between the treatments (ANOVA, p=0.005, table 17).

Table 17: ANOVA test results for the hydroxy fatty acids experiment 1a collection days #9, #14 and #22. The significance limit was p=0.05.				
ANOVA	df	MS	F-ratio	p
Treatments	4	3.235	4.662	0.038
Time	2	8.571	12.349	0.005
Error	7	0.694		

The treatments T40 and T44 differed from each other (Tukey, p=0.033, A9) properly due to treatment T40 was the only treatment with hydroxy acids present on collection day #9 where treatment T44 was the only treatment with hydroxy acids on collection day #14. Only one hydroxy acid differed among them on collection day #22.
The hydroxy acid number present on collection day #22 was much higher than on the two other collection days (Tukey, p=0.009 and p=0.010, table A10).

Saturated fatty acids (SFA). There was no difference between the treatments (ANOVA, p=0.338, table 18) when comparing their saturated fatty acid numbers but there was a difference over time (ANOVA, p=0.007, table 18).

Table 18: ANOVA test results for the saturated fatty acids experiment 1a collection days #9, #14 and #22. The significance limit was p=0.05.

ANOVA	df	MS	F-ratio	p
Treatments	4	1.656	1.360	0.338
Time	2	13.071	10.733	0.007
Error	7	1.218		

The number of fatty acids was larger on collection day #9 than in collection day #14 (Tukey, p=0.013, table A11) and #22 (Tukey, p=0.009, table A11).

Mono unsaturated fatty acids (MUFA). There was no difference between the number of mono unsaturated fatty acids in the treatments (ANOVA, p=0.907, table 19) or over time (ANOVA, p=0.881, table 19).

Table 19: ANOVA test results for the mono unsaturated fatty acids experiment 1a collection days #9, #14 and #22. The significance limit was p=0.05.

ANOVA	df	MS	F-ratio	p
Treatments	4	0.723	0.240	0.907
Time	2	0.387	0.129	0.881
Error	7	3.008		

Poly unsaturated fatty acids (PUFA). The poly unsaturated fatty acids did not differ from each other in treatments. (ANOVA, p=0.069, table 20) or over time (ANOVA, p=0.143, table 20).

Table 20: ANOVA test results for the poly unsaturated fatty acids experiment 1a collection days #9, #14 and #22. The significance limit was p=0.05.

ANOVA	df	MS	F-ratio	p
Treatments	4	11.667	3.561	0.069
Time	2	8.533	2.605	0.143
Error	7	3.276		

Branched fatty acids. There was no statistical difference between the branched fatty acids detected in treatments (ANOVA, p=0.472, table 21) or on the collection days (ANOVA, p=0.237, table 21).

Table 21: ANOVA test results for the branched fatty acids experiment 1a collection days #9, #14 and #22. The significance limit was p=0.05.

ANOVA	df	MS	F-ratio	p
Treatments	4	1.187	0.990	0.472
Time	2	2.133	1.778	0.237
Error	7	1.200		

Cyclopropane fatty acids. The cyclopropane number did not differ among the treatments (ANOVA, p=0.199, table 22) or between the collection days (ANOVA, p=0.069, table 22).

Table 22: ANOVA test results for the cyclopropane fatty acids experiment 1a collection days #9, #14 and #22. The significance limit was p=0.05.

ANOVA	df	MS	F-ratio	p
Treatments	4	0.267	2.000	0.199
Time	2	0.533	4.000	0.069
Error	7	0.133		

In figure 36 and 37 the treatments from experiment 1a are visualized in a MDS (multidimensional scaling) plot. The treatments are placed relative to each other.

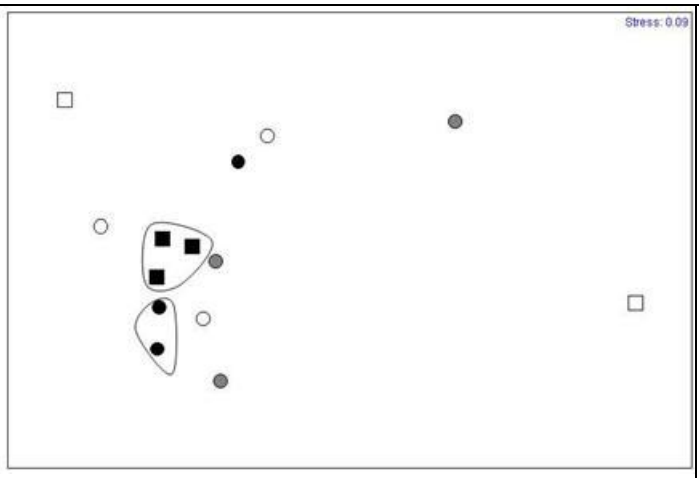


Figure 36: MDS plot of averages of fatty acids extracted from collected biofilm on experimental slides from experiment 1a (sampling days #9, #14 and #22). The following symbols illustrate treatments; Control (□), T40 (○), T43 (●), T44 (●) and MUZ (■).

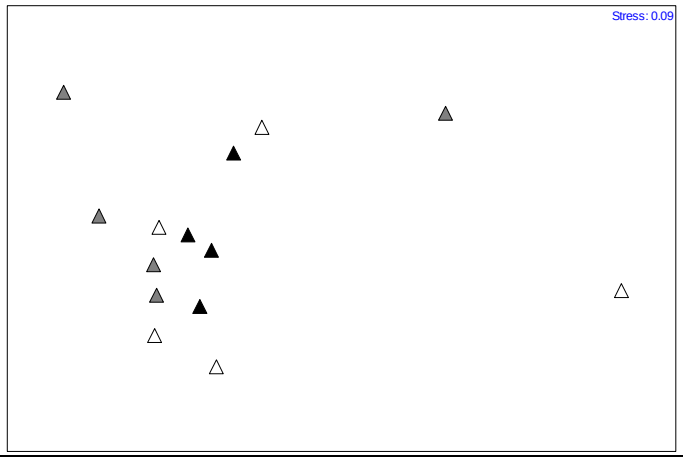


Figure 37: MDS plot of averages of fatty acids extracted from collected biofilm on experimental slides from experiment 1a (sampling days #9, #14 and #22). The following symbols display collection days; #9 (▲), #14 (Δ) and #22 (▲).

The MUZ treatments (full squares) from collection days #9, #14 and #22 are grouped in the middle of the plot (figure 36). Control treatments (open squares) from collection day #14 and collection day #22 are in the opposite ends of the plot and can therefore not have large similarities. The Control from collection day #9 is not visible and is therefore similar with another collection day or treatment. Treatments T43 (grey circles) are also spread across the plot and have therefore also relative low similarities. Treatments T44 (full circles) from collection days #14 and 22 clusters below the cluster of treatment MUZ where treatment T44 from collection day #9 is placed further away close to treatment T40 (open circles) collection day #14. Treatments T40 are not clustered but spread out between the other treatments.

In figure 37 the time/the collection days are visualized in a MDS plot. There is no clustering between collection days which suggests that time has no influence on the treatments fatty acid composition.

ANOSIM – experiment 1a

An analysis of similarities (ANOSIM) was made for comparing the treatments. Table 23 shows the results of the analysis where 100 indicates 100% similarity and 0 indicates that there is nothing in commen between the two data sets. The Control was different from treatments T43, T44 and MUZ and the MUZ treatment was also different from treatment T43 (ANOSIM, table 23).

There was no difference between the collection days (ANOSIM, p=52.4 %, A12).

Table 23: ANOSIM, Global R: 0.212, p= 0.2 %, all p values are given in percent.

Treatment	Control	T40	T43	T44	MUZ
Control	100				
T40	5.1	100			
T43	0.3	16.2	100		
T44	0.1	13.8	29.7	100	
MUZ	0.1	5.3	3.9	28.0	100

SIMPER – fatty acid extraction experiment 1a

A similarity of percentage (SIMPER) analysis was made to explain the differences in similaritites from the ANOSIM analysis. According to the SIMPER analysis the fatty acids dominating the experimental slides in general were methyl-hexadecanoate and methyl-octadecanoate. When running the SIMPER analysis more than 50 % of the analysis results (data not shown) were composed of these previous mentioned fatty acids. It was decided to remove these two dominant fatty acids from the further SIMPER analysis since they did not affect the outcome, other than largely affect the contribution percentage, but did cover the effect of the other fatty acids present. The SIMPER analysis explains which fatty acid that drives the differences between groups in the ANOSIM (table 23).

The average dissimilarities (table 24) are all quite high since all is above 50 %. The highest dissimilarities are between the Control and treatments T40, T43, T44 and the MUZ treatment.

Table 24: SIMPER analysis, average dissimilarities between treatments in percent.					
Treatment	Control	T40	T43	T44	MUZ
Control	100				
T40	81.12	100			
T43	82.33	68.38	100		
T44	81.29	64.82	67.04	100	
MUZ	79.37	58.65	63.37	58.34	100

In table 25 the SIMPER analysis result data are presented. In these tables an overview is seen of specific fatty acids which contribute to the dissimilarities (SIMPER, table 24) determined by the ANOSIM (table 23).

When comparing treatments T43 with the Control (table 25-A) and T44 with the Control (table 25-B) it is the same four fatty acids which contribute to approximately 50 % of the dissimilarity; methyl-cis-9,12-octadecadienoate (PUFA), methyl-*trans*-9-octadecenoate (MUFA), methyl-heptadecanoate (SFA) and methyl-tetradecanoate (SFA). The contributions from each fatty acid differ from the comparisons but the specific fatty acids are the same. When comparing the Control and the MUZ treatment (table 25-B) it is the methyl-heptadecanoate (SFA), methyl-cis-9,12-octadecadienoate (PUFA), methyl-pentadecanoate (SFA) and methyl-tetradecanoate (SFA) which contributes with more than 50 % of the determined dissimilarity. Comparing treatment T43 and the MUZ (table 25-C) treatment reveal that the fatty acids are responsible for approximately half the dissimilarity of methyl-heptadecanoate (SFA), methyl-pentadecanoate (SFA), methyl-tetradecanoate (SFA) and methyl-*trans*-9-octadecenoate (MUFA).

In general it is the same five fatty acids which contribute to the dissimilarities in all the treatment comparisons. Since several of the treatments are different from each other thew it must be the amount of the specific fatty acid which has influence on the dissimilarity.

Table 25: Presentation of the SIMPER analysis data for the fatty acid extraction in experiment 1a. All treatment comparisons which were significant different from each other due to the ANOSIM analysis are listed below in tables A-D. All average abundances are rounded off.

Table A: SIMPER analysis results of The Control and treatment T43. The dissimilarity between the treatments was 82.33 %.

Fatty acids	Average abundance T43	Average abundance Control	Average dissimilarity	Dissimilarity SD	Contribution in dissimilarity in %	Accumulation in %
Methyl-cis-9,12-octadecadienoate	69	2492	10.89	1.02	13.23	13.23
Methyl-trans-9-octadecenoate	4788	239	10.09	1.34	12.26	25.49
Methyl-heptadecanoate	786	73	9.43	1.07	11.45	36.94
Methyl-tetradecanoate	981	1400	9.25	0.68	11.23	48.17
Methyl-13-methyltetradecanoate	10	37	6.65	0.50	8.08	56.25
Methyl-15-methylhexadecanoate	8	3293	5.84	0.55	7.10	63.35
Methyl-pentadecanoate	67	367	5.45	1.08	6.62	69.97
Methyl-12-methyltetradecanoate	3126	5933	5.19	0.60	6.31	76.28
Methyl-cis-9-octadecenoate	91	69	4.72	0.88	5.73	82.00
Methyl-cis-9,10-methylenehexadecanoate	790	1116	2.80	0.74	3.40	85.40
Methyl-cis-9-hexadecenoate	66	0	2.66	0.57	3.23	88.63
Methyl-undecanoate	16	0	1.53	0.28	1.86	90.49

Table B: The results from the SIMPER analysis between the Control and treatment T44. The dissimilarity between the treatments was 81.29 %.

Fatty acids	Average abundance T44	Average abundance Control	Average dissimilarity	Dissimilarity SD	Contribution in dissimilarity in %	Accumulation in %
Methyl-cis-9,12-octadecadienoate	54	2492	10.28	1.09	12.65	12.65
Methyl-heptadecanoate	327	73	9.97	1.5	12.27	24.92
Methyl-tetradecanoate	640	1400	8.86	0.77	10.90	35.82
Methyl-trans-9-octadecenoate	217	239	7.21	1.24	8.86	44.68
Methyl-pentadecanoate	172	367	7.19	0.96	8.85	53.53
Methyl-13-methyltetradecanoate	0	37	6.02	0.48	7.41	60.94
Methyl-cis-9-hexadecenoate	69	0	4.46	.066	5.49	66.43
Methyl-eicosanoate	87	36	4.14	0.94	5.10	71.53
Methyl-12-methyltetradecanoate	0	5933	4.02	0.52	4.94	76.47
Methyl-15-methylhexadecanoate	19	3293	3.74	0.68	4.60	81.07
Methyl-cis-9-octadecenoate	68	69	3.69	0.89	4.53	85.61
Methyl-2-hydroxycanoate	46	0	3.62	0.52	4.45	90.06

Table C: Comparison of treatments Control and MUZ with SIMPER. The dissimilarity between the treatments was

79.37 %.

Fatty acids	Average abundance MUZ	Average abundance Control	Average dissimilarity	Dissimilarity SD	Contribution in %	Accumulation in %
Methyl-heptadecanoate	8719	73	18.45	2.11	23.16	23.16
Methyl-cis-9,12-octadecadienoate	561	2492	10.47	1.23	13.15	36.31
Methyl-pentadecanoate	3314	367	9.03	1.02	11.34	47.65
Methyl-tetradecanoate	1477	1400	8.10	0.83	10.18	57.82
Methyl-13-methyltetradecanoate	31	37	6.31	0.52	7.92	65.75
Methyl-trans-9-octadecenoate	946	239	4.75	1.44	5.96	71.71
Methyl-12-methyltetradecanoate	0	5933	4.08	0.52	5.12	76.83
Methyl-cis-9-hexadecenoate	560	0	3.37	1.04	4.23	81.06
Methyl-15-methylhexadecanoate	0	3293	3.36	0.58	4.22	85.28
Methyl-cis-9-octadecenoate	299	69	2.95	1.32	3.71	88.99
Methyl-cis-9,10-methylenehexadecanoate	62	1116	2.66	0.91	3.34	92.34

Table D: SIMPER analysis results from comparison between treatments T43 and MUZ. The dissimilarity between the treatments was 63.37 %.

Fatty acids	Average abundance MUZ	Average abundance Control	Average dissimilarity	Dissimilarity SD	Contribution in %	Accumulation in %
Methyl-heptadecanoate	786	8719	12.52	1.31	19.76	19.76
Methyl-pentadecanoate	67	3314	8.59	1.06	13.56	33.31
Methyl-tetradecanoate	981	1477	7.81	0.97	12.32	45.63
Methyl-trans-9-octadecenoate	4788	946	7.32	1.59	11.55	57.18
Methyl-cis-9-octadecenoate	91	299	4.33	1.06	6.83	64.01
Methyl-cis-9-hexadecenoate	66	560	3.91	1.07	6.17	70.18
Methyl-cis-9,12-octadecadienoate	69	561	3.76	1.29	5.93	76.11
Methyl-15-methylhexadecanoate	8	0	2.60	0.29	4.11	80.22
Methyl-cis-9,10-methylenehexadecanoate	790	62	1.93	0.81	3.04	83.26
Methyl-2-hydroxycanoate	18	70	1.67	0.69	2.64	85.90
Methyl-undecanoate	16	35	1.57	0.33	2.48	88.38
Methyl-12-methyltetradecanoate	3126	0	1.31	0.29	2.06	90.44

Summary

Even though the actual number of fatty acids detected rose from collection day #9 to #22, in experiment 1a, there was no overall difference over time or between treatments but only between the numbers of fatty acids present in each fatty acid group.

The group of saturated fatty acids (SFA) was larger than the other fatty acid groups on collection days #9 and #14. On collection day #22 the saturated fatty acids were only larger than the poly unsaturated- and the cyclopropane fatty acids and rather similar to the other fatty acid groups.

Collection day #9 was the only sampling day where there was a difference between treatments. Treatments T40 had a higher number of fatty acids than treatment T44.

Comparing the individual fatty acid groups treatment wise and over time revealed that there was a larger density of saturated fatty acids detected on collection day #9 compared to collection days #14 and #22.

The MDS plots revealed that there was no clustering between treatments on the three collection days except for in treatment MUZ which suggest that time have no or very little influence on the fatty acid composition.

The ANOSIM in experiment 1a showed that the Control treatment was different from the treatments T43, T44 and MUZ and the MUZ was also different from treatment T43.

The SIMPER revealed that it was the same five fatty acids which determined the differences between treatments; Methyl-pentadecanoate, Methyl-tetradecanoate and Methyl-heptadecanoate (SFA), Methyl-*trans*-9-octadecenoate (MUFA) and Methyl-cis-9,12-octadecadienoate (PUFA).

Fatty acid extraction – Community analysis experiment 1b

The fatty acids detected through experiment 1b are listed in table 26. Almost all fatty acids detected for are present. All fatty acids are detected in treatment MUZ where the saturated fatty acid C11:0 only is present. All fatty acids are also present in the Control except from the C11:0 fatty acid which only is found in the MUZ treatment. Treatment T44 only has one hydroxy acid (2-OH-C10:0) and one branched (i-C17:0) fatty acid. T44 treatment is also the treatment with the lowest number of fatty acids detected (15). The treatments have 8 fatty acids in common, the saturated fatty acids C12:0, C14:0, C16:0, C17:0, C18:0, the mono unsaturated fatty acids C16:1ω9, C18:1ω9and the cyclopropane cyC17:0Δ.

Table 26: Fatty acids screened for FAME analysis. The fatty acids present in experiment 1b.

Fatty acids	Type	Treatments				
		Control	T40	T43	T44	MUZ
Methyl 2-hydroxydecanoate (2-OH-C10:0)	Hydroxy	*	*	*	*	*
Methyl undecanoate (C11:0)	SFA					*
Methyl dodecanoate (C12:0)	SFA	*	*	*	*	*
Methyl 2-hydroxydodecanoate (2-OH-C12:0)	Hydroxy	*	*	*		*
Methyl 3-hydroxydodecanoate (3-OH-C12:0)	Hydroxy	*	*	*		*
Methyl tridecanoate (C13:0)	SFA	*	*			*
Methyl tetradecanoate (C14:0)	SFA	*	*	*	*	*
Methyl 2-hydroxytetradecanoate (2-OH-C14:0)	Hydroxy	*	*	*		*
Methyl 3-hydroxytetradecanoate (3-OH-C14:0)	Hydroxy	*	*	*		*
Methyl pentadecanoate (C15:0)	SFA	*	*		*	*
Methyl 13-methyltetradecanoate (i-C15:0)	Branched	*	*	*		*
Methyl 12-methyltetradecanoate (α-C15:0)	Branched	*	*	*		*
Methyl hexadecanoate (C16:0)	SFA	*	*	*	*	*
Methyl 14-methylpentadecanoate (i-C16:0)	Branched	*	*	*		*
Methyl-2-hydroxyhexadeanoate (2-OH-C16:0)	Hydroxy	*		*		*
Methyl cis-9-hexadecenoate (C16:1ω9)	MUFA	*	*	*	*	*
Methyl heptadecanoate (C17:0)	SFA	*	*	*	*	*
Methyl 15-methylhexadecanoate (i-C17:0)	Branched	*	*		*	*
Methyl cis-9,10-methylenehexadecanoate (C17:0Δ)	Cyclopropa	*	*	*	*	*
Methyl octadecanoate (C18:0)	SFA	*	*	*	*	*
Methyl cis-9-octadecenoate (C18:1ω9)	MUFA	*	*	*	*	*
Methyl trans-9-octadecenoate (C18:1ω9)	MUFA	*	*		*	*
Methyl cis-9,12-octadecadienoate (C18:2ω 9,12)	PUFA	*	*	*	*	*
Methyl nonadecanoate (C19:0)	SFA	*		*	*	*
Methyl cis-9,10-methyleneoctadecanoate (C19:0Δ)	Cyclopropa	*		*		*
Methyl eicosanoate (C20:0)	SFA	*		*	*	*

The MDS plot is a visual representation of the communal between the treatments (figure 38 and 39). The treatments are placed relative to each other.

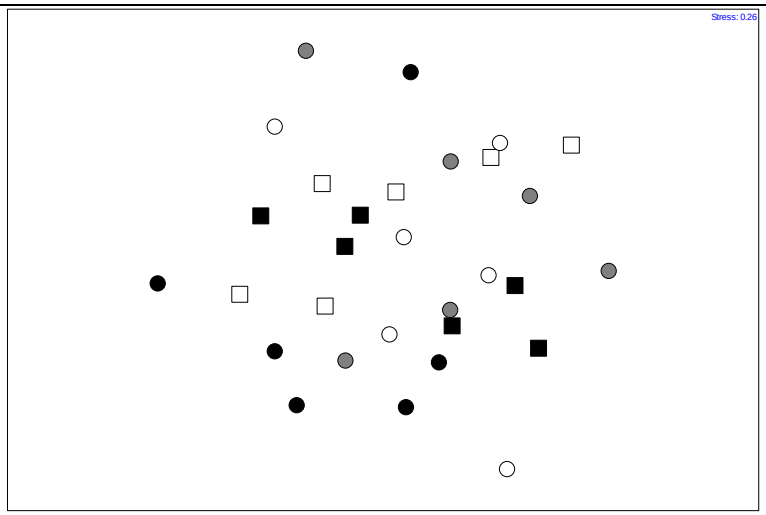


Figure 38: MDS plot of averages of fatty acids extracted from collected biofilm on experimental slides from experiment 1b (sampling days #1, #3, #6, #9, #14 and #22). The symbols illustrate treatments; Control (□), T40 (○), T43 (●), T44 (●) and MUZ (■).

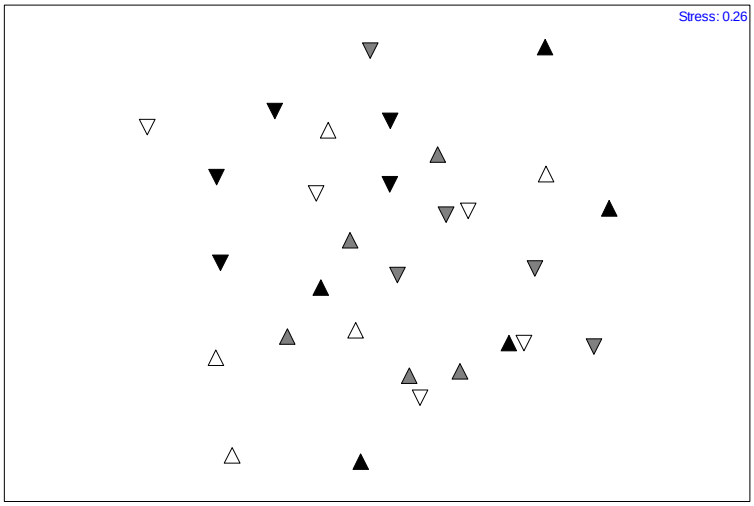


Figure 39: MDS plot of averages of fatty acids extracted from collected biofilm on experimental slides from experiment 1b (sampling days #1, #3, #6, #9, #14 and #22). The following symbols display collection days. #1 (▼), #3 (▼), #6 (▼), #9 (▲), #14 (Δ) and #22 (▲).

There is no clear clustering in any of the treatments (figure 38) or in any of the collection days (figure 39) which indicates that all treatments have low similarities between them but also internal in each treatment group.

ANOSIM – experiment 1b

There was no difference between the treatments or the collection days (ANOSIM, p=72.5 %, ANOSIM, p=41.9 %, A13 and A14).

Fatty acid extraction – Community analysis experiment 2

Numerous fatty acids were detected in the treatments from experiment 2. Six are present in all treatments in the experiment 2 (table 27). The saturated fatty acids C14:0, C17:0, C18:0, the hydroxy acid 2-OH-C10:0, the branched fatty acid α-C15:0 and the cyclopropane cyC17:0Δ. The treatment with the least fatty acids detected is treatment T49 and the one with the most is the MU treatment. The other treatments fatty acid counts are in between.

Table 27: Fatty acids detected in the FAME analysis in experiment 2.

Fatty acids	Type	Treatments					
		Control	T46	T49	T53	MU	MUZ
Methyl 2-hydroxydecanoate (2-OH-C10:0)	Hydroxy	*	*	*	*	*	*
Methyl undecanoate (C11:0)	SFA		*	*	*	*	
Methyl dodecanoate (C12:0)	SFA	*	*		*		*
Methyl 2-hydroxydodecanoate (2-OH-C12:0)	Hydroxy	*	*		*	*	
Methyl 3-hydroxydodecanoate (3-OH-C12:0)	Hydroxy	*	*	*	*		
Methyl tridecanoate (C13:0)	SFA			*	*	*	*
Methyl tetradecanoate (C14:0)	SFA	*	*	*	*	*	*
Methyl 2-hydroxytetradecanoate (2-OH-C14:0)	Hydroxy	*	*		*	*	*
Methyl 3-hydroxytetradecanoate (3-OH-C14:0)	Hydroxy	*		*			
Methyl pentadecanoate (C15:0)	SFA	*	*		*	*	
Methyl 13-methyltetradecanoate (i-C15:0)	Branched		*		*	*	*
Methyl 12-methyltetradecanoate (α-C15:0)	Branched	*	*	*	*	*	*
Methyl hexadecanoate (C16:0)	SFA	*	*	*		*	*
Methyl 14-methylpentadecanoate (i-C16:0)	Branched		*		*	*	*
Methyl-2-hydroxyhexadeanoate (2-OH-C16:0)	Hydroxy	*				*	
Methyl cis-9-hexadecenoate ($C16:1^{9}$)	MUFA	*				*	*
Methyl heptadecanoate (C17:0)	SFA	*	*	*	*	*	*
Methyl 15-methylhexadecanoate (i-C17:0)	Branched	*				*	*
Methyl cis-9,10-methylenehexadecanoate ($C17:0^{\Delta}$)	Cyclopropane	*	*	*	*	*	*
Methyl octadecanoate (C18:0)	SFA	*	*	*	*	*	*
Methyl cis-9-octadecenoate ($C18:1^{9}$)	MUFA	*	*			*	*
Methyl trans-9-octadecenoate ($C18:1^{9}$)	MUFA	*	*		*	*	
Methyl cis-9,12-octadecadienoate ($C18:2^{9,12}$)	PUFA	*	*		*		
Methyl nonadecanoate (C19:0)	SFA	*	*		*	*	*
Methyl cis-9,10-methyleneoctadecanoate ($C19:0^{\Delta}$)	Cyclopropane				*	*	
Methyl eicosanoate (C20:0)	SFA			*		*	*

The community based on fatty acid extraction is visualized in a MDS plot (figure 40 and 41). The treatments are placed relative to each other.

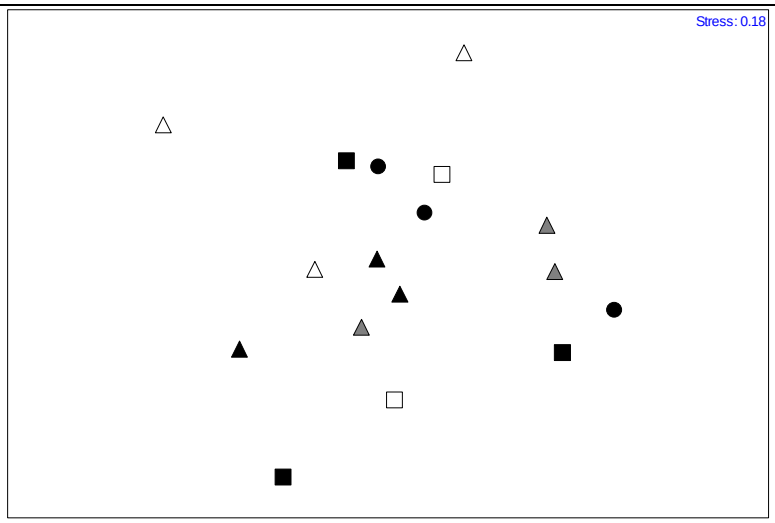


Figure 40: MDS plot of averages of fatty acids extracted from collected biofilm on experimental slides from experiment 2 (sampling days #3, #9 and #22). The symbols illustrate the following treatments; Control (□), T46 (▲), T49 (Δ), T53 (▲), MU (♦) and MUZ (■).

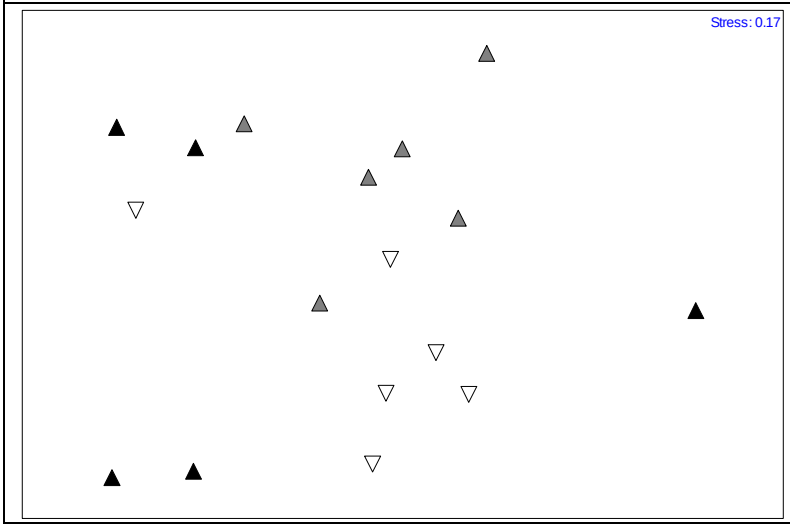


Figure 41: MDS plot of averages of fatty acids extracted from collected biofilm on experimental slides from experiment 2 (sampling days #3, #9 and #22). The symbols display collection days; #9 (▼), #14 (▲) and #22 (▲).

In figure 40 sample averages from experiment 2 are visualized. It can be noted that no treatments are grouping. The only pattern which can be observed is that in each treatment group two out of three are placed close to each other and the third in the opposite of the plot.

Figure 41 displays collection days and also here cannot be observed any clusterings.

ANOSIM – experiment 2

There was no difference between the treatments or the collection days (ANOSIM, p=74.6%, ANOSIM, p=56.2 %, A15 and A16).

Macrofouling results

Macrofouling – experiment 1a

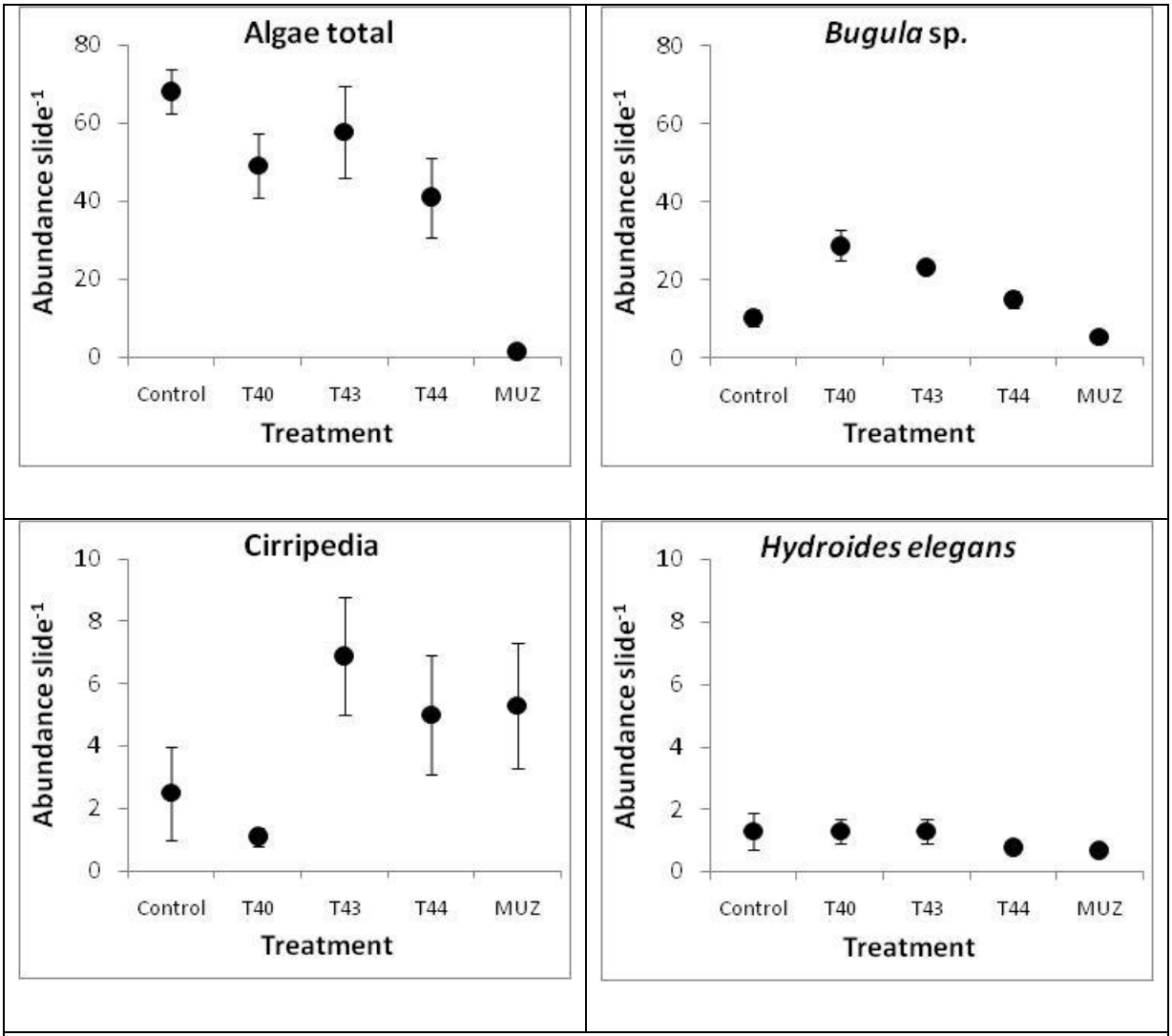


Figure 42: Abundance of algae and macro-invertebrates (macrofouling) on experimental slides from experiment 1a (sampling day #22). Numbers are mean values ± SE ($n_{Control}$= 6, $n_{40, 43, 44 \text{ and } MUZ}$= 7).

The density of algae and invertebrates (macrofouling) on slides from the various treatments from experiments in experiment 1a are presented in figure 42. No visible algae and invertebrates were observed at sampling events before day #22. At the sampling on day #22 there were found *Bugula* sp., *Hydroides elegans*, Cirripedia (barnacles), Infusorians and different algae.

ANOVA – Macrofouling experiment 1a

Algae. The Control had the highest abundance of algae whereas the MUZ treatment had the lowest algae density (figure 28). All treatments had a higher algal density than the MUZ treatment (Kruskal-Wallis, p=0.001) and the density on Control slides was also larger than the density on slides from treatment T44 (Conover-Inman, p=0.047, table A17). The algal density in treatments T40, T43, T44 were not different from each other (Conover-Inman, p>0.05table A17).

***Bugula* sp.** The abundance of *Bugula* sp. on experimental slides from different treatments differed from each other (ANOVA, p<0.000, table 28, figure 42). Treatment T40 had the highest density of *Bugula* sp. Which was larger than the density of treatments Control, T44 and MUZ (Tukey, p<0.000, p=0.034, p<0.000, table XA18). The abundance of *Bugula* sp. in treatment T44 was not different from that in treatment T43 or the Control (Tukey, p=0.333, p=0.146, tableXA18), but had a higher density than the MUZ treatment.

Table 28: ANOVA test experiment 1a *Bugula* sp.

ANOVA	df	MS	F-ratio	p
Treatment	4	10.884	19.877	< 0.000
Error	29	0.548		

Cirripedia. The density of Cirripedia on slides from treatment T40 and the Control treatment tended to be lower than on all other treatments (figure 42). This difference was, however, not significant (ANOVA, p=0.074, table 29) due to the rather larger variation among replicate samples.

Table 29: ANOVA test experiment 1a Cirripidia.

ANOVA	df	MS	F-ratio	p
Treatment	4	2.717	2.402	0.074
Error	28	1.131		

***H. elegans*.** There was no significant difference between the treatments (Kruskal-Wallis, p=0.203).

Infusoria. The largest abundance of infusorians was observed on slides from treatment T40 (119 on one slide) and the lowest in treatment T44 (2.9 per slide on average) (figure 42, Kruskal-Wallis, p<0.000). No infusorians were observed on

slides from the Control and the MUZ treatments. The density of infusorians was similar on treatments T43 (14.4 in average) and T44 (2.9 in average) (Conover-Inman, $p=0.107$, table A19), but both had a lower abundance than treatment T40 (Conover-Inman, $p<0.000$, $p=0.012$, table A19).

Photo documentation – macrofouling experiment 1a

The photos from sample day #22 are presented in figure 43 to give an overview of the slides just after collection. From a visual point the Control has the largest amount of algae and invertebrates where MUZ has the lowest amount and the treatments T40, T43 and T44 were in between.

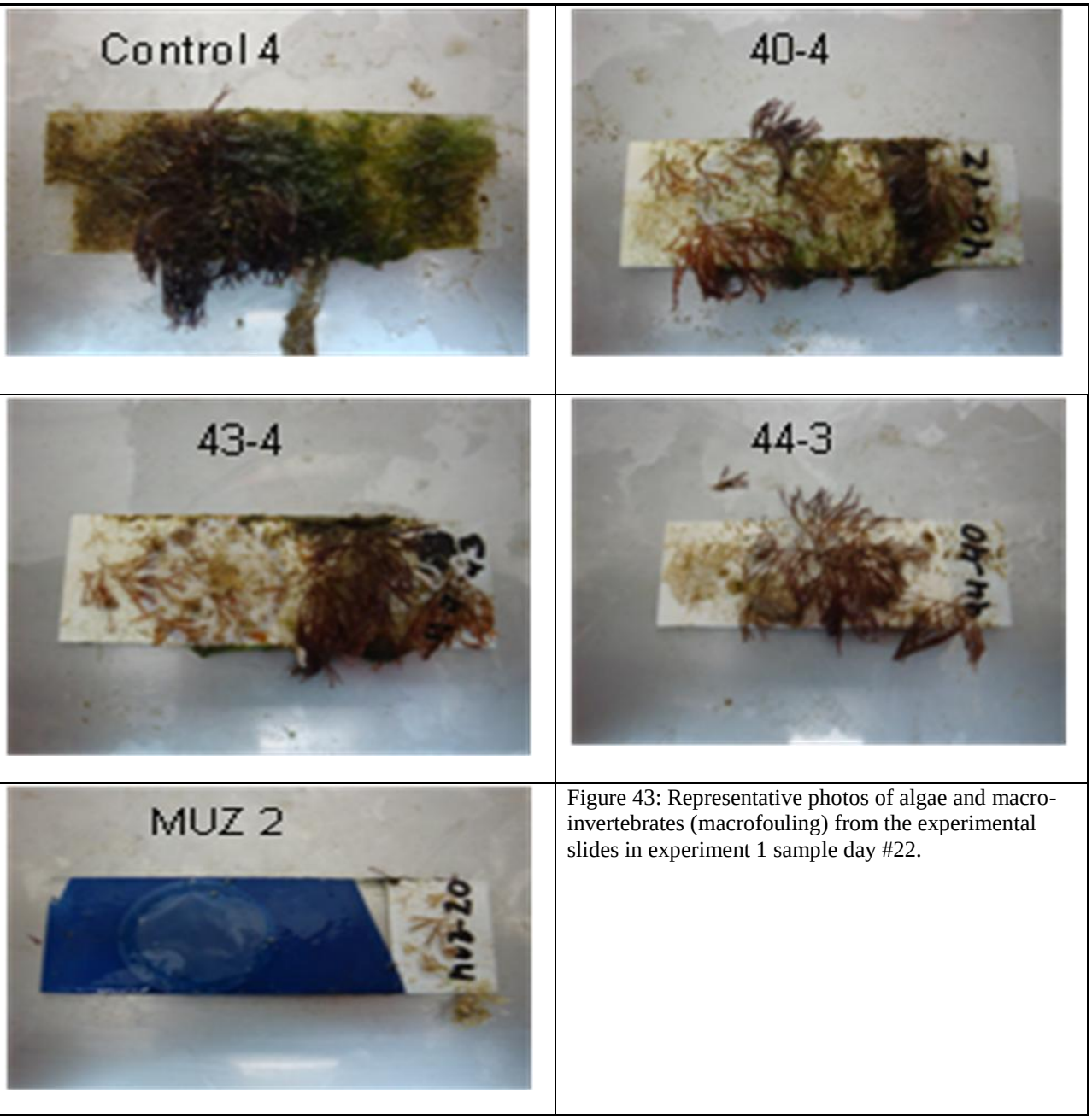


Figure 43: Representative photos of algae and macro-invertebrates (macrofouling) from the experimental slides in experiment 1 sample day #22.

Community analysis – macrofouling experiment 1a

The MDS plot is a visual representation of the treatments (figure 44). The treatments are placed relative to each other. Treatment MUZ (full squares) is spread out to the right, the Control treatment is grouped (open squares) to the upper left, except for a single outlier, and treatment T40 is grouped in between (open circles). Treatments T43 and T44 are mixed between the other groupings (full and grey circles).

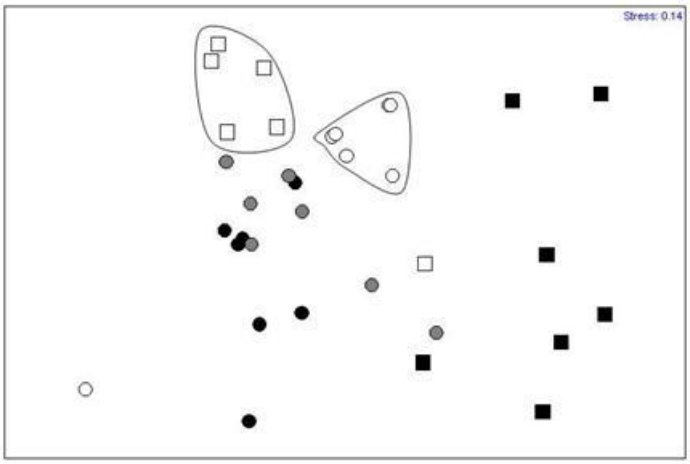


Figure 44: MDS plot of algae and invertebrates (macrofouling) on experimental slides from experiment 1a (sampling day #22). Control (□), T40 (○), T43 (●), T44 (●) and MUZ (■).

ANOSIM – macrofouling experiment 1a

The similarity analysis was performed to decifer how different the given data sets are from each other. All treatments were significant different from each other except for treatments T43 and T44 (ANOSIM, p=19 %) (table 30).

Table 30: ANOSIM, Global R: 0.453, p= 0.1 %, all p values are given in percent.

Treatment	Control	T40	T43	T44	MUZ
Control	100				
T40	0.2	100			
T43	0.2	0.1	100		
T44	4	0.1	19	100	
MUZ	0.1	0.3	0.3	0.2	100

SIMPER – macrofouling experiment 1a

The SIMPER analysis explains the differences between treatments from the ANOSIM analysis (table 30). The treatments with the lowest dissimilarity, treatments T43 and T44, were also the treatments which did not differ from each other in the ANOSIM. All other treatments were different from each other due to the ANISIM which in the SIMPER analysis all have dissimilarities above 20 % (table 31).

Table 31: SIMPER analysis, average dissimilarities between treatments in percent.

Treatment	Control	T40	T43	T44	MUZ
Control	0				
T40	24.54	0			
T43	28.06	26.22	0		
T44	24.92	24.29	19.27	0	
MUZ	43.44	37.48	41.37	37.66	0

When comparing the treatments pair wise with SIMPER (table 32) to investigate which species explain the differences between treatments deciphered by the ANOSIM only three species which in general had a major influence on the dissimilarities on all slides. These species were *Bugula* sp., algae and Cirripedia which were present on all experimental slides and were present in large abundances. Infusoria was only present on experimental slides from treatments T40, T43 and T44.

The Control had fewer *Bugula* sp. attached than any of the treatments T40, T43 and T44 but the *Bugula* sp. abundance only had dominating effect on the dissimilarity when compared with treatment T40 (table 32-A). When compared with treatment MUZ the Control had a larger number of species attached and a larger abundance of all settled species except for the Cirripedia (table 32-D).

Treatments T40, T43 and T44 had Infusoria settled on their surfaces. Since the Infusoria was only observed in these treatments they influence the dissimilarity notable when compared with the Control and the MUZ treatment. Treatment MUZ had very few algae attached and therefore the alga were dominating in the dissimilarity when treatment MUZ was compared with the other treatments.

Table 32: Presentation of the SIMPER analysis data for all treatment comparisons which were significant different from each other due to the ANOSIM analysis. The treatments are pair wise compared with the contributing species listed in tables A-I.

A: Comparison of treatments Control and T40' SIMPER analysis. The average dissimilarity between the treatments was 24.54 %.

Species	*Bugula* sp.	Algae	Cirripedia	Infusoria	*H. elegans*	Annilidae
Average Abundance C	13.33	50.83	2.50	0.00	1.00	0.33
Average Abundance T40	36.00	49.29	0.86	17.00	0.43	0.00
Average Dissimilarity	6.80	5.81	4.81	3.56	2.33	1.22
Dissimilarity SD	1.93	1.91	0.96	0.40	0.70	0.70
Contribution (%)	27.72	23.68	19.60	14.52	9.49	4.99
Accumulation (%)	27.72	51.40	71.00	85.52	95.01	0

B: SIMPER analysis data from comparison of treatments Control and T43. The average dissimilarity between the treatments was 28.06 %.

Species	Infusoria	Cirripedia	Algae	*Bugula* sp.	*H. elegans*	Annilidae
Average Abundance C	0.00	2.50	50.83	13.33	1.00	0.33
Average Abundance T43	14.43	6.86	57.86	26.43	0.43	0.00
Average Dissimilarity	10.51	6.07	5.43	2.83	2.12	1.11
Dissimilarity SD	1.84	1.89	1.31	1.50	0.89	0.70
Contribution (%)	37.44	21.65	19.33	10.07	7.56	3.95
Accumulation (%)	37.44	59.09	78.42	88.49	96.05	100.00

C: Treatments Control and T44' SIMPER analysis data. The average dissimilarity between the treatments was 24.92 %.

Species	Cirripedia	Infusoria	Algae	*Bugula* sp.	*H. elegans*	Annilidae
Average Abundance C	2.50	0.00	50.83	13.33	1.00	0.33
Average Abundance T44	4.71	2.71	40.71	19.29	0.29	0.00
Average Dissimilarity	6.79	5.43	4.98	4.41	2.17	1.14
Dissimilarity SD	1.46	2.17	1.20	1.31	0.91	0.70
Contribution (%)	27.24	21.77	19.99	17.71	8.70	4.58
Accumulation (%)	27.24	49.01	69.00	86.71	95.42	100.00

D: Comparison of treatment Control and MUZ. The SIMPER analysis dissimilarities between treatments Control and MUZ are 43.44 %.

Species	Algae	Cirripedia	*Bugula* sp.	*H. elegans*	Annilidae
Average Abundance C	50.83	2.50	13.33	1.00	0.33
Average Abundance MUZ	1.29	4.29	5.43	0.14	0.00
Average Dissimilarity	16.90	12.58	10.24	2.50	1.22
Dissimilarity SD	2.83	1.50	1.61	0.80	0.69
Contribution (%)	38.90	28.96	23.57	5.76	2.81
Accumulation (%)	38.90	67.86	91.43	97.19	100.00

E: SIMPER analysis data for treatments T40 and T43 and the average dissimilarity between them was 26.22 %.

Species	Infusoria	Cirripedia	*Bugula* sp.	Algae	*H. elegans*
Average Abundance T40	17.00	0.86	36.00	49.29	0.43
Average Abundance T43	14.43	6.86	26.43	57.86	0.43
Average Dissimilarity	10.83	5.21	4.81	3.74	1.63
Dissimilarity SD	1.87	1.79	2.40	1.20	0.72
Contribution (%)	41.31	19.85	18.35	14.28	6.21
Accumulation (%)	41.31	61.16	79.51	93.79	100.00

F: Species comparison of treatment T40 and T44 by SIMPER analysis. The average dissimilarity between the treatments was 24.29 %.

Species	Infusoria	Cirripedia	*Bugula* sp.	Algae	*H. elegans*
Average Abundance T40	17.00	0.86	36.00	49.29	0.43
Average Abundance T44	2.71	4.71	19.29	40.71	0.29
Average Dissimilarity	7.25	5.97	5.04	4.38	1.65
Dissimilarity SD	1.41	1.32	1.64	1.52	0.73
Contribution (%)	29.86	24.57	20.75	18.02	6.80
Accumulation (%)	29.86	54.44	75.18	93.20	100.00

G: Treatment comparison by SIMPER analysis between treatments T40 and MUZ. The average dissimilarity between the treatments was 37.48 %.

Species	Cirripedia	Algae	*Bugula* sp.	Infusoria	*H. elegans*
Average Abundance T40	0.86	49.29	36.00	17.00	0.43
Average Abundance MUZ	4.29	36.00	5.43	0.00	0.14
Average Dissimilarity	13.35	17.00	6.59	3.54	1.78
Dissimilarity SD	1.73	0.43	1.41	0.40	0.57
Contribution	35.62	0.00	17.59	9.45	4.75
Accumulation	35.62	0.00	85.79	95.25	100.00

H: Comparison of treatment MUZ and T43 SIMPER analysis. The average dissimilarity between the treatments was 41.37 %.

Species	Algae	Infusoria	Cirripedia	*Bugula* sp.	*H. elegans*
Average Abundance T43	57.86	14.43	6.86	26.43	0.43
Average Abundance MUZ	1.29	0.00	4.29	5.43	0.14
Average Dissimilarity	11.28	10.45	10.20	7.66	1.79
Dissimilarity SD	2.12	1.84	2.97	1.56	0.71
Contribution (%)	27.26	25.26	24.65	18.51	4.32
Accumulation (%)	27.26	52.52	77.17	95.68	100.00

I: SIMPER analysis of treatments T44 and MUZ The average dissimilarity between the treatments was 37.66 %.

Species	Algae	Cirripedia	*Bugula* sp.	Infusoria	*H. elegans*
Average Abundance T44	40.71	4.71	19.29	2.71	0.29
Average Abundance MUZ	1.29	4.29	5.43	0.00	0.14
Average Dissimilarity	12.49	10.43	7.53	5.40	1.81
Dissimilarity SD	2.10	1.99	1.41	2.17	0.72
Contribution (%)	33.15	27.70	20.00	14.33	4.82
Accumulation (%)	33.15	60.85	80.85	95.18	100.00

Macrofouling experiment 1b

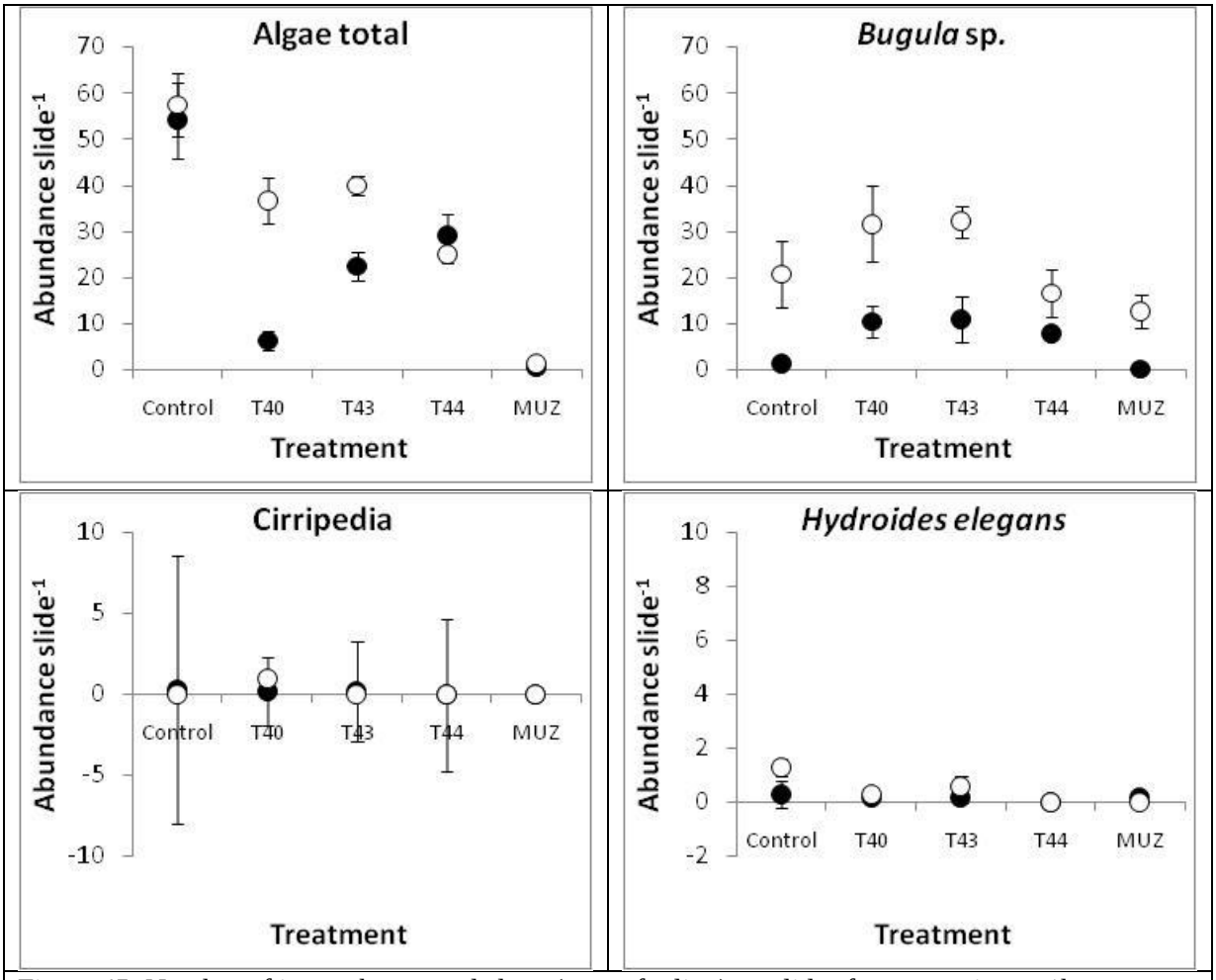


Figure 45: Number of invertebrates and algae (macrofouling) on slides from experiment 1b (sampling days 14, solid symbols ● and 22, open symbols ○). Mean values ± SE given per slide (n_{14}= 6, $n_{22, T43}$= 5, n_{22}= 6).

In experiment 1b were observed *Bugula* sp., *H. elegans*, Cirripedia and different algae on the experimental slides on sampling days #14 and #22, figure 45.

ANOVA – macrofouling experiment 1b

Algae. The abundance of algae increased over time (ANOVA, p=0.001, table 33). There was also difference in abundance between treatments (ANOVA, p=0.001, table 33) and an interaction between time and treatments (ANOVA, p=0.001, table 33). The density of algae was larger on Control slides than on slides from the other treatments (Tukey, p>0.000, table A20). Treatments T40, T43 and T44 had similar algal density and a higher abundance than slides from the MUZ treatment (Tukey, P<0.000, table A21).

Table 33: ANOVA test experiment algae.

ANOVA	df	MS	F-ratio	p
Treatment	4	1333.733	11.784	0.001
Time	1	4670.196	41.264	0.000
T × T	4	593.185	5.241	0.001
Error	49	113.180		

***Bugula* sp.** There was an increase in *Bugula* sp. density with time (ANOVA, p<0.000, table 34) and the density also differed between treatments (ANOVA, p=0.006, table 34). The density of *Bugula* sp. in treatments T40 and T43 was similar (Tukey, p=1.000, table A22), and higher than in the MUZ treatment (Tukey, p=0.020 and p=0.018, table A22). The density in treatment T44 and the Control treatment was similar and in between the two extremes (Tukey, p=0.998, table A22).

Table 34: ANOVA test experiment 1b *Bugula* sp.

ANOVA	df	MS	F-ratio	p
Treatment	4	515.741	4.080	0.006
Time	1	4088.236	32.342	0.000
T × T	4	97.719	0.773	0.548
Error	49	126.407		

Cirripedia. Very few Cirripedia had settled on the slides after 14 and 22 days (figure 35). No significance were detected due to increase in abundance over time (ANOVA, p=0.711, table 35). Also, there was no significant effect of treatments on settlement of Cirripedia (ANOVA, p=0.215, table 35).

Table 35: ANOVA test experiment 1b Cirripedia				
ANOVA	df	MS	F-ratio	p
Treatment	4	0.706	1.505	0.215
Time	1	0.065	0.139	0.711
T × T	4	0.605	1.288	0.288
Error	49	0.496		

H. elegans. Treatment had a significant effect on the abundance of *H. elegans.* (ANOVA, p<0.000, table 39), whereas time had no (ANOVA, p=0.583, table 39). The Control had a higher density of *H. elegans* than all the other treatments (Tukey, table A23), which were similar (Tukey, p>0.05table A23).

Table 36: ANOVA test experiment 1b *H. elegans.*				
ANOVA	df	MS	F-ratio	p
Treatment	4	3.461	9.581	0.000
Time	1	0.110	0.306	0.583
T × T	4	0.146	0.404	0.805
Error	49	0.361		

Community analysis – macrofouling experiment 1b

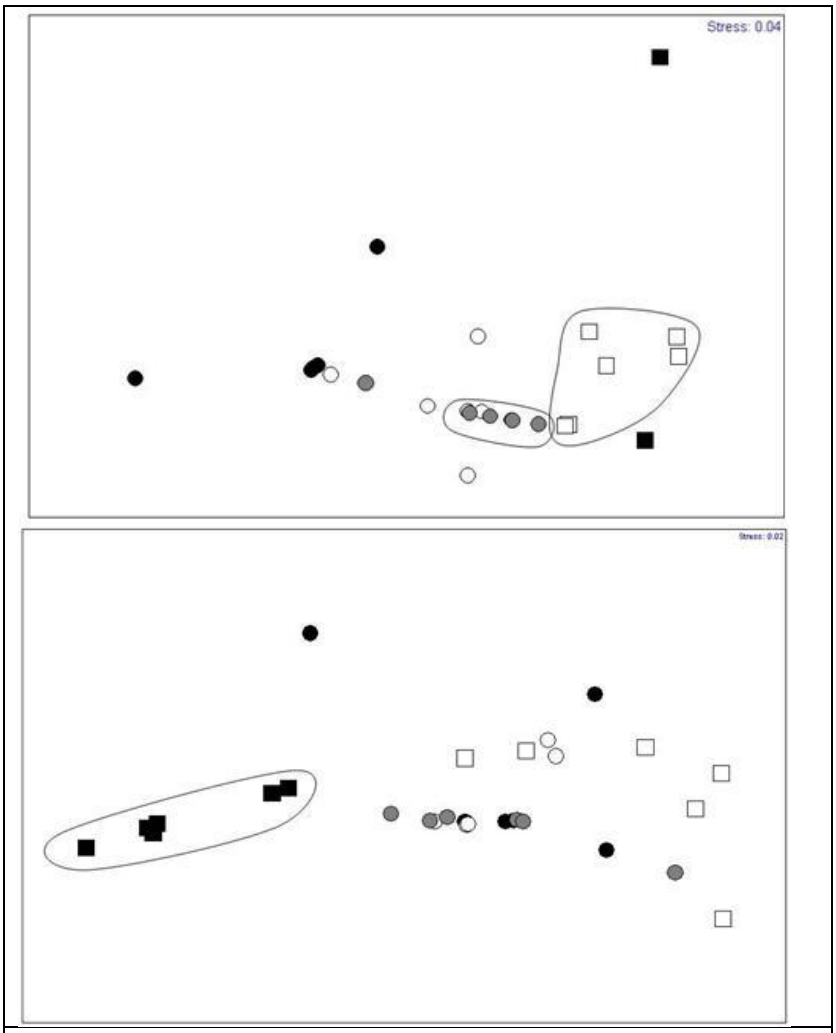


Figure 46: A: MDS plot of algae and invertebrates (macrofouling) on experimental slides from experiment 1b (sampling days #14 and #22). A; treatments from sampling day #14, B; treatments from sampling day #22. Control (□), T40 (○), T43 (●), T44 (●) and MUZ (■).

In figure 46 (upper) is presented a MDS plot of macrofouling found on sample day # 14. Treatment MUZ is split in two groups one in the right lower corner (full squares) and one in the upper right corner. Treatment 44 is grouped in the bottom of the plot next to the Control treatment and treatment MUZ with a single outlier (grey circles). The Control is also grouped in the left side of the plot (open squares). And treatments T40 and T43 are spread out in the left side and in the middle of the plot (full and open circles).

Figure 46 (lower); MDS plot of macrofouling found on the experimental slides on sample day # 22. Treatment MUZ (full squares) is grouped in the left side of the plot where the Control (open squares) treatment is spreas in the left side of the plot. Treatments T40, T43 and T44 (circles) are all found in the middle of the plot.

ANOSIM – macrofouling experiment 1b

The results from the similarity analysis in table 37 show that all treatments were significant different from each other except from treatments T43 and T44 (ANOSIM p=32 %, table 37).

Table 37: ANOSIM, Global R: 0.482, p= 0.1 %, all p values are given in percent.					
Treatment	Control	T40	T43	T44	MUZ
Control	100				
T40	0.1	100			
T43	0.3	2.5	100		
T44	0.2	0.9	32	100	
MUZ	0.1	0.1	0.1	0.1	100

SIMPER – macrofouling experiment 1b

The similarities from the ANOSIM are explained by the SIMPER analysis. This analysis explains the differences between treatments from the ANOSIM analysis by informing about how many percent two given treatments have in commen and also tells which species there is behind the observed differences (table 37). All treatments, except treatment MUZ, have a high similarity (>80 %) between replicates internally in the treatment. Treatments T43 and T44 are the only treatments in experiment 1b which are not different from each other. An overview of the average dissimilarities between treatments is presented in table 38.

Table X38: SIMPER analysis, average dissimilarities between treatments in percent.					
Treatment	Control	T40	T43	T44	MUZ
Control	0				
T40	30.46	0			
T43	22.13	15.92	0		
T44	24.92	18.23	13.33	0	
MUZ	41.68	38.06	37.46	36.69	0

In experiment 1b *Bugula* sp. and algae were the species dominating the dissimilarities between treatments determent in the SIMPER analysis (table 39). Approximately 50 % of the dissimilarities were accounted for by *Bugula* sp. except in the comparison between the Control and the MUZ treatment (table 39-D) and between treatments T40, T43 and T44 (table 39-E and F). Here the *Bugula* sp. contribution was lower, approximately 40 %, but still the dominating contributor.

There were few specimens of *H. elegans* and Cirripedia observed on the experimental slides in general and these species had very little influence on the dissimilarities. On slides from T44 no *H. elegans* were present and no Cirripedia was found on treatment MUZ. There was not found Infusoria on any of the slides.
The algae abundance was largest on the Control slides compared with all other treatments. On the other hand the *Bugula* sp. density was lower on the Control compared to the treatments T40, T43 and T44. Treatment MUZ had the lowest observed species density of al the treatments and had in particular a very low almost not present algal density. The highest dissimilarity was found between treatment MUZ and the Control treatment (table 39-D).

Table 39: Presentation of the SIMPER analysis data, experiment 1b, for all treatment comparisons which were significant different from each other due to the ANOSIM analysis. The treatments are pair wise compared with the contributing species listed in tables A-I.

A: SIMPER analysis of species collected from treatments Control and T40. The average dissimilarity between the treatments was 30.46 %.

Species	*Bugula* sp.	Algae	*H. elegans*	Cirripedia
Average Abundance Control	11.50	56.25	1.33	0.17
Average Abundance T40	21.83	21.54	0.25	0.58
Average Dissimilarity	15.37	9.30	3.81	1.98
Dissimilarity SD	1.72	1.39	1.44	0.71
Contribution (%)	50.47	30.52	12.52	6.49
Accumulation (%)	50.47	80.99	93.51	100.00

B: The average dissimilarity between treatments T43 and the Control was 22.13 %. The SIMPER analysis data are presented below.

Species	*Bugula* sp.	Algae	*H. elegans*	Cirripedia
Average Abundance Control	11.50	56.25	1.33	0.17
Average Abundance T43	21.27	30.45	0.36	0.09
Average Dissimilarity	11.83	0.36	3.69	1.23
Dissimilarity SD	1.59	1.57	1.38	0.54
Contribution (%)	53.46	24.33	16.66	5.56
Accumulation (%)	53.46	77.78	94.44	100.00

C: SIMPER analysis data from treatments Control and T44. The average dissimilarity between the treatments was calculated to 21.51 %.

Species	*Bugula* sp.	Algae	*H. elegans*	Cirripedia
Average Abundance C	11.50	56.25	1.33	0.17
Average Abundance T44	13.75	27.08	0.00	0.00
Average Dissimilarity	11.33	5.16	4.27	0.74
Dissimilarity SD	1.47	1.27	1.55	0.44
Contribution (%)	52.70	24.00	19.87	3.43
Accumulation (%)	52.70	76.69	96.57	100.00

D: Comparison of species settled and their density on treatment Control and MUZ. The dissimilarities between treatments Control and MUZ was determined to 41.68 % by SIMPER analysis.

Species	*Bugula* sp.	Algae	*H. elegans*	Cirripedia
Average Abundance C	11.50	56.25	1.33	0.17
Average Abundance MUZ	6.33	0.93	0.08	0.00
Average Dissimilarity	19.08	15.37	6.42	0.08
Dissimilarity SD	1.64	1.26	0.98	0.44
Contribution (%)	45.77	36.88	15.41	1.93
Accumulation (%)	45.77	82.66	98.07	100.00

E: The SIMPER analysis data for treatments T40 and T43. The average dissimilarity between them was 15.92 %.

Species	*Bugula* sp.	Algae	*H. elegans*	Cirripedia
Average Abundance T40	21.83	21.54	0.25	0.58
Average Abundance T43	21.27	30.45	0.36	0.09
Average Dissimilarity	6.19	5.77	2.05	1.91
Dissimilarity SD	1.30	1.02	0.81	0.64
Contribution (%)	38.90	36.21	12.87	12.02
Accumulation (%)	38.90	75.11	87.98	100.00

F: Species comparison of treatment T40 and T44 by SIMPER analysis. The average dissimilarity between the treatments was 18.23 %.

Species	*Bugula* sp.	Algae	Cirripedia	*H. elegans*

Average Abundance T40	21.83	21.54	0.58	0.25
Average Abundance T44	13.75	27.08	0.00	0.00
Average Dissimilarity	8.33	7.07	1.59	1.24
Dissimilarity SD	1.33	1.14	0.56	0.54
Contribution (%)	45.69	38.78	8.73	6.80
Accumulation (%)	45.69	84.47	93.20	100.00

G: Treatment comparison by SIMPER analysis between treatments T40 and MUZ. The average dissimilarity between the treatments was 38.06 %.

Species	*Bugula* sp.	Algae	*H. elegans*	Cirripedia
Average Abundance T40	21.83	21.54	0.25	0.58
Average Abundance MUZ	6.33	0.93	0.08	0.00
Average Dissimilarity	18.84	13.99	3.52	1.71
Dissimilarity SD	1.75	1.87	0.46	0.56
Contribution (%)	49.50	36.75	9.25	4.49
Accumulation (%)	49.50	86.26	95.51	100.00

H: SIMPER analysis of the comparison of treatments T43 and MUZ. The average dissimilarity between the treatments was 37.46 %.

Species	*Bugula* sp.	Algae	*H. elegans*	Cirripedia
Average Abundance T43	21.27	30.45	0.36	0.09
Average Abundance MUZ	6.33	0.93	0.08	0.00
Average Dissimilarity	18.77	14.29	3.72	0.69
Dissimilarity SD	2.73	1.78	0.48	0.31
Contribution (%)	50.10	38.14	9.92	1.85
Accumulation (%)	50.10	88.24	98.15	100.00

I: The SIMPER analysis determined the average dissimilarity between treatments T44 and MUZ to 36.69 % and the detailed species composition and contribution are listed below.

Species	*Bugula* sp.	Algae	*H. elegans*
Average Abundance T44	13.75	27.08	0.00
Average Abundance MUZ	6.33	0.93	0.08
Average Dissimilarity	19.32	14.89	2.48
Dissimilarity SD	2.47	1.53	0.30
Contribution (%)	52.66	40.57	6.77
Accumulation (%)	52.66	93.23	100.00

Macrofouling experiment 2

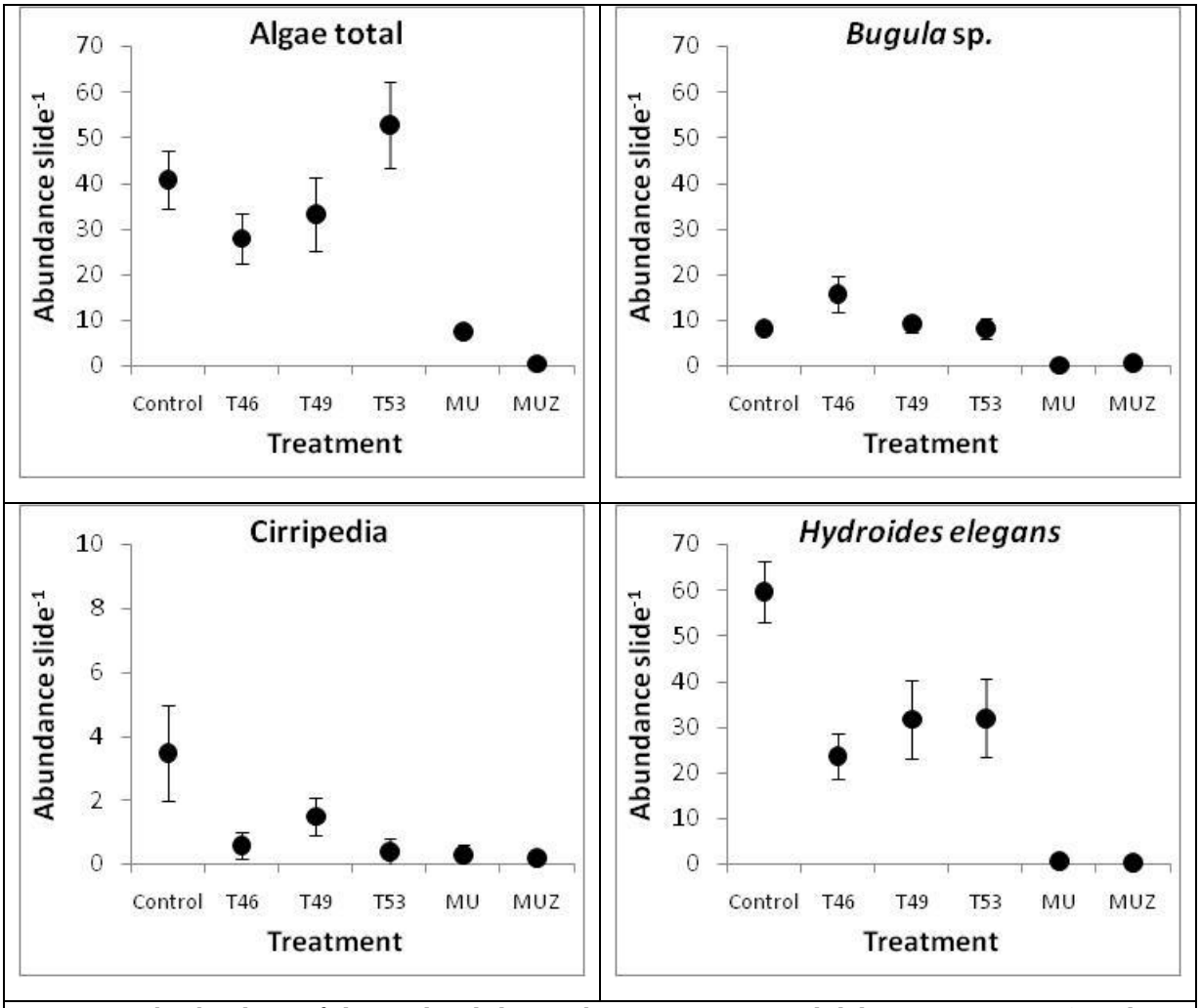


Figure 47: The abundance of algae and settled invertebrates on experimental slides in experiment 2 (sampling day 22). Mean values ± SE ($n_{46, 53}$= 5, $n_{Control, 49, MU, MUZ}$= 6).

In experiment 2 were observed *Bugula* sp., *H. elegans*, Cirripedia and various algae on the experimental slides. Further, one, mollusk was found on a slide from treatment T49 and a few Infusoria were observed on slides from treatment T53 (figure 47).

ANOVA – macrofouling experiment 2

Algae. The abundance of algae differed between treatments (Kruskal-Wallis, $p<0.000$). All treatments had a higher abundance of algae than the MU and MUZ treatments (Conover-Inman, $p<0.038$, table A24). Treatment T53 had further a higher abundance of algae than treatment T46 (Conover-Inman, $p=0.027$, table A24).

***Bugula* sp.** Treatments had a significant effect on *Bugula* sp. density (Kruskal-Wallis, $p<0.000$). Treatments T46, T49, T53 and the Control had all a higher density of *Bugula* sp. than the treatments MU and MUZ (Conover-Inman, $p<0.000$, table A25) which were similar (Conover-Inman, $p=0.632$, table table A25).

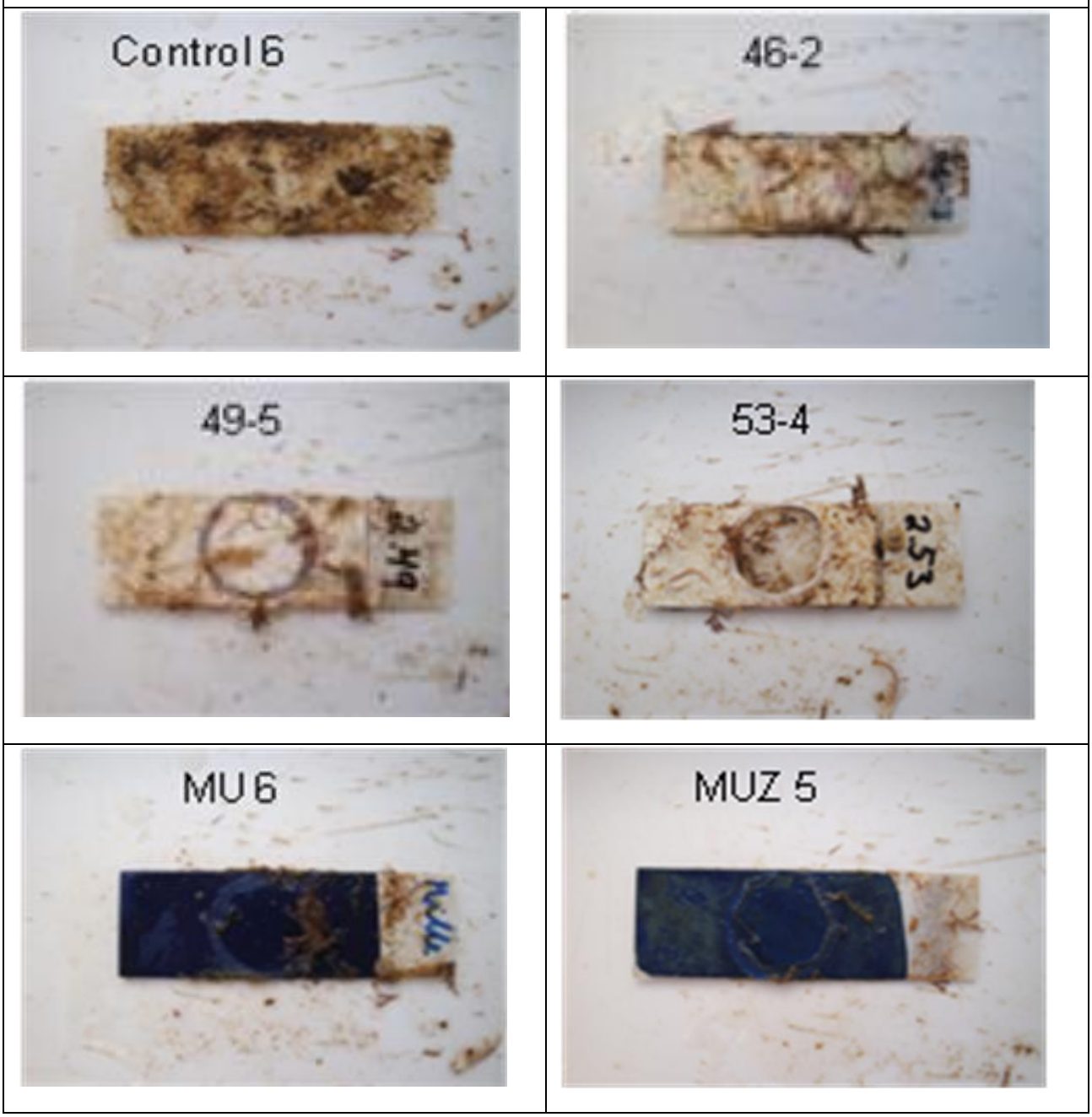
Cirripidia. The largest density of Cirripedia was observed in the Control treatment (Kruskal-Wallis, $p=0.012$, figure 47) and the lowest in MU and MUZ treatments, which were similar (Conover-Inman, $p=0.844$, table A26). The density of Cirripedia in treatment T49 was similar to that of the Control (Conover-Inman, $p=0.164$, table A26), which both had a higher Cirripedia abundance than the MU and MUZ treatments (Conover-Inman, table A26). Treatments T46, T53, MU and MUZ were not different from each other (Conover-Inman, table A26).

***H. elegans*.** Treatments had a significant effect on the abundance of *H. elegans* (Kruskal-Wallis, $p<0.000$, table 47). The treatment T46 had the highest abundance of *H. elegans* whereas the MU and MUZ treatments had the lowest (Conover-Inman, $p<0.000$, table A27). The density in treatments T46, T49 and T53 was similar (Conover-Inman, table A27) and were all higher than in the MU and MUZ treatments (Conover-Inman, table A27), which were similar (Conover-Inman, $p=0.204$, table A27). The Control treatments *H. elegans* density was lower than treatments T46, T49 and T53 (Conover-Inman, $p=0.001$, $p=0.004$, $p=0.009$, table A27) but higher than that of the MU and MUZ treatments (Conover-Inman, $p<0.000$, table A27).

Photo documentation – experiment 2

The photos from sample day #22 are presented in figure 48 to give an overview of the slides just after collection. From a visual point the Control has the largest amount of algae and invertebrates where MU and MUZ has the lowest amount and the treatments T46, T49 and T53 were in between.

Figure 48: Representative photos of algae and macro-invertebrates (macrofouling) from the experimental slides in experiment 2 sample day #22.



Community analysis – macrofouling experiment 2

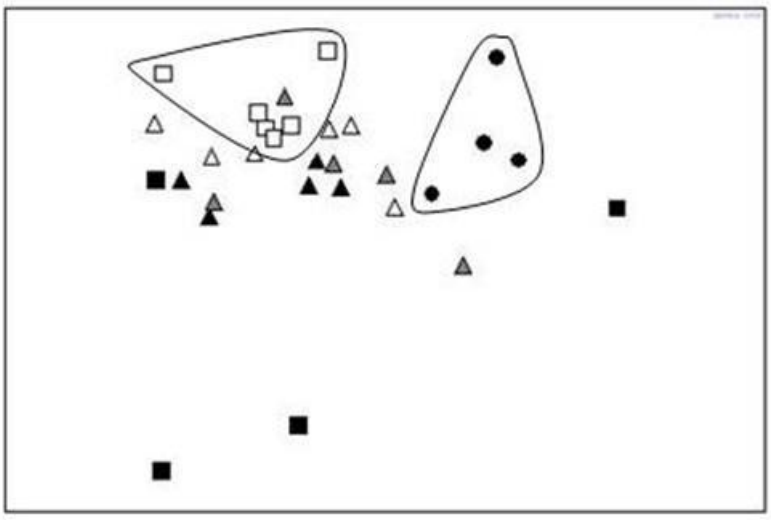


Figure 49: MDS plot of algae and invertebrates (macrofouling) on experimental slides from experiment 2 (sampling day #22). The following symbols illustrate treatments; Control (□), T46 (▲), T49 (Δ), T53 (▲), MU (●) and MUZ (■).

MDS plot of macrofouling found on sample day # 22 (figure 49). The Control treatment (open squares) is grouped nicely in the upper left side of the plot. Treatment MU is also grouped nicely and is found in the upper right side of the plot (full circle). Treatment MUZ is spread out through most of the plot. Treatments T46, T49 and T53 are somewhat grouped but all placed close together in the upper part of the plot.

ANOSIM – macrofouling experiment 2

The results from the analysis of similarities show that most treatments were different from each other but there were a few exceptions. Treatment T49 was not different from the Control treatment and treatment T53. Treatment MUZ was not different from treatments T46 and T53 and treatments T46 and T49 were also not different from each other (table 40).

Table 40: ANOSIM, Global R: 0.398, p= 0.1 %, all p values are given in percent.

Treatment	C	T46	T49	T53	MU	MUZ
C	100					
T46	0.6	100				
T49	24.2	78.4	100			
T53	0.2	2.4	16	100		
MU	0.2	0.2	0.2	0.2	100	
MUZ	0.4	12.3	3	8.7	3.9	100

SIMPER – macrofouling experiment 2

The SIMPER analysis explains the differences between treatments from the ANOSIM analysis. In table 41 is listed the percentages the given treatments have in commen and in table 42 is the species and their abundances which accounts for the observed differences listed.

Table 41: SIMPER analysis, average dissimilarities between treatments in percent.

Treatment	C	T46	T49	T53	MU	MUZ
C	0					
T46	18.68	0				
T49	17.60	16.90	0			
T53	23.65	23.02	12.34	0		
MU	39.08	37.13	36.14	33.10	0	
MUZ	50.00	44.36	46.69	47.74	39.49	0

The following treatments were not different from each other according to the ANOSIM; treatment T49 and the Control, treatments T46 and MUZ, treatments MUZ and T53 and treatments T49 and T53. All other treatments were different from each other (ANOSIM, table 40). The dissimilarity between treatments T49 and the Control and treatments T49 and T53 were low compared to the dissimilarities between the other treatments (table 41). The dissimilarities between treatment MUZ and treatments T46 and T53 were among the highest dissimilarities in the SIMPER (table

41) but according to the ANOSIM (table 40) there were no difference between the MUZ treatment and treatments T46 and T53.

The SIMPER analysis performed on data from experiment revealing large variations in which species contributed mostly to the dissimilarities between treatments observed in the ANOSIM test (table 42). The SIMPER analysis revealed that the dominating organisms with the highest average abundances on the experimental slides in experiment 2 were *H. elegans* and algae. Treatments MU and MUZ were excluded in this generalization since they had very low abundance of all species. In treatment MU the highest abundances were made by algae and *Bugula* sp. growth (table 42-I) where treatment MUZ followed the same pattern as all other treatments in experiment 2. There was found a specimen from Mollusca on slides from treatment T49 which barely had any influence when treatment T49 was compared to other treatments (table 42-D and G). Infusoria was only observed on slides from treatment T53 and was the largest contributor to the dissimilarity when compared with the Control treatment and treatment T46 (table 42-B and E) where there was low contribution when compared with treatment MU (table 42-H).
The *Bugula* sp. and Cirripedia average abundances were low compared to the other species abundances but still had large influence on the dissimilarities in some treatment comparisons. Comparisons between treatments MU and MUZ, T46 and MU, C and T46 all had *Bugula* sp. as the largest contributor to their dissimilarity. All other treatments comparisons had *Bugula* sp. as the second largest contributor except from comparison between treatments Control and T53 where it gave the lowest contribution. The algae had large average densities but had only a medium contribution the dissimilarities.

Table 42: Presentation of the SIMPER analysis data from experiment 2. All treatment comparisons which were significant different from each other due to the ANOSIM analysis is presented pair wise in tables A-I.

A: Treatments Control and T46' SIMPER analysis results and average dissimilarity which was 18.86 %.

Species	*Bugula* sp.	Algae	*H. elegans*	Cirripedia
Average Abundance C	7.50	40.83	59.67	3.50
Average Abundance T46	16.20	28.00	23.80	1.20
Average Dissimilarity	6.34	4.82	4.62	3.08
Dissimilarity SD	1.89	1.56	1.42	1.24
Contribution (%)	33.60	25.56	24.50	16.34
Accumulation (%)	33.60	59.16	83.66	100.00

B: Data from comparison of treatments Control and T53 by SIMPER analysis. The average dissimilarity between the treatments was 23.65 %.

Species	Infusoria	*H. elegans*	Algae	Cirripedia	*Bugula* sp.
Average Abundance C	0.00	59.67	40.83	3.50	7.50
Average Abundance T53	6.20	32.00	53.00	0.40	8.20
Average Dissimilarity	6.29	5.76	5.14	4.13	2.33
Dissimilarity SD	1.68	1.25	1.32	1.59	1.26
Contribution (%)	26.61	24.35	21.73	17.47	9.84
Accumulation (%)	26.61	50.97	72.69	90.16	100.00

C: The Control treatment and treatment MUZ's SIMPER analysis data. The average dissimilarity between the two treatments was determined to 50.00 %.

Species	*H. elegans*	*Bugula* sp.	Algae	Cirripedia
Average Abundance C	59.67	7.50	40.83	3.50
Average Abundance MUZ	0.33	1.00	0.58	0.17
Average Dissimilarity	21.30	11.39	10.58	6.72
Dissimilarity SD	2.26	1.94	1.66	1.81
Contribution (%)	42.61	22.78	21.17	13.45
Accumulation (%)	42.61	65.38	86.55	100.00

D: Comparison of treatment T49 and MUZ by SIMPER analysis. The SIMPER analysis dissimilarity between treatments Control and MUZ was 46.69 %.

Species	*H. elegans*	*Bugula* sp.	Algae	Cirripedia	Mollusca
Average Abundance T49	31.83	9.50	33.33	1.17	0.17
Average Abundance MUZ	0.33	1.00	0.58	0.17	0.00
Average Dissimilarity	18.78	11.75	10.61	4.98	0.58

Dissimilarity SD	2.19	2.19	1.55	1.03	0.44
Contribution (%)	40.21	25.16	22.73	10.66	1.24
Accumulation (%)	40.21	65.37	88.10	98.76	100.00

E: SIMPER analysis results for treatments T46 and T53 and their average dissimilarity between them which was determined to 23.02 %.

Species	Infusoria	*Bugula* sp.	Algae	*H. elegans*	Cirripedia
Average Abundance T46	0.00	16.20	28.00	23.80	1.20
Average Abundance T53	6.20	8.20	53.00	32.00	0.40
Average Dissimilarity	6.23	5.56	4.82	3.85	2.56
Dissimilarity SD	1.67	1.65	1.30	1.32	1.15
Contribution (%)	27.06	24.17	20.93	16.71	11.14
Accumulation (%)	27.06	51.23	72.16	88.86	100.00

F: Species comparison of treatment T46 and MU by SIMPER analysis. The average dissimilarity between the treatments was 37.13 %.

Species	*Bugula* sp.	*H. elegans*	Algae	Cirripedia
Average Abundance T46	16.20	23.80	28.00	1.20
Average Abundance MU	0.17	0.83	7.50	0.33
Average Dissimilarity	13.74	9.69	9.76	4.03
Dissimilarity SD	2.84	1.65	1.87	1.15
Contribution (%)	37.00	26.10	26.05	10.85
Accumulation (%)	37.00	63.10	89.15	100.00

G: Comparison of treatments T49 and MU by SIMPER analysis. The average dissimilarity between the treatments was 36.14 %.

Species	*H. elegans*	*Bugula* sp.	Algae	Cirripedia	Mollusca
Average Abundance T49	31.83	9.50	33.33	1.17	0.17
Average Abundance MU	0.83	0.17	7.50	0.33	0.00
Average Dissimilarity	11.70	10.13	9.66	4.08	0.57
Dissimilarity SD	1.56	2.43	1.44	1.22	0.44
Contribution (%)	32.38	28.03	26.73	11.29	1.57
Accumulation (%)	32.38	60.41	87.14	98.43	100.00

H: Comparison of treatment T53 and MU by SIMPER analysis. The average dissimilarity between the treatments was 33.01 %.

Species	*H. elegans*	*Bugula* sp.	Infusoria	Algae	Cirripedia

Average Abundance T53	32.00	8.20	6.20	53.00	0.40
Average Abundance MU	0.83	0.17	0.00	7.50	0.33
Average Dissimilarity	8.97	7.66	7.13	6.67	2.66
Dissimilarity SD	1.43	2.20	1.66	1.57	0.62
Contribution (%)	27.11	23.14	21.55	20.16	8.04
Accumulation (%)	27.11	50.24	71.80	91.96	100.00

I: SIMPER analysis of treatments MU and MUZ. The average dissimilarity between the treatments was 39.49 %.

Species	*Bugula* sp.	*H. elegans*	Algae	Cirripedia
Average Abundance MUZ	1.00	0.33	0.58	0.17
Average Abundance MU	0.17	0.83	7.50	0.33
Average Dissimilarity	12.40	11.88	10.37	4.48
Dissimilarity SD	0.95	1.97	1.22	0.62
Contribution (%)	31.41	30.09	26.25	12.25
Accumulation (%)	31.41	61.50	87.75	100.00

Summary

In general all treatments from the three experiments (1a, 1b and 2) had a higher algae density than the MU and MUZ treatments. In experiment 1a and 1b the Control treatments were higher than all other treatments and the treatments T40, T43 and T44 were similar and had a lower density than the Control treatment but a higher density than the MUZ treatment. In experiment 2 all treatments had a higher algae density than treatments MU and MUZ. Treatments T53, T49 and the Control treatment were similar and treatments T46, T49 and the Control treatment were similar.

Treatment T40 was the treatment with the highest *Bugula* sp. density in both experiment 1a and, with treatment T43 in, 1b where treatment MUZ had the lowest density. The other treatments were similar to treatment MUZ (experiment 1a) or lay in between (experiment 1b). In experiment 2 all treatments had a higher *Bugula* sp. abundance that treatments MU and MUZ.

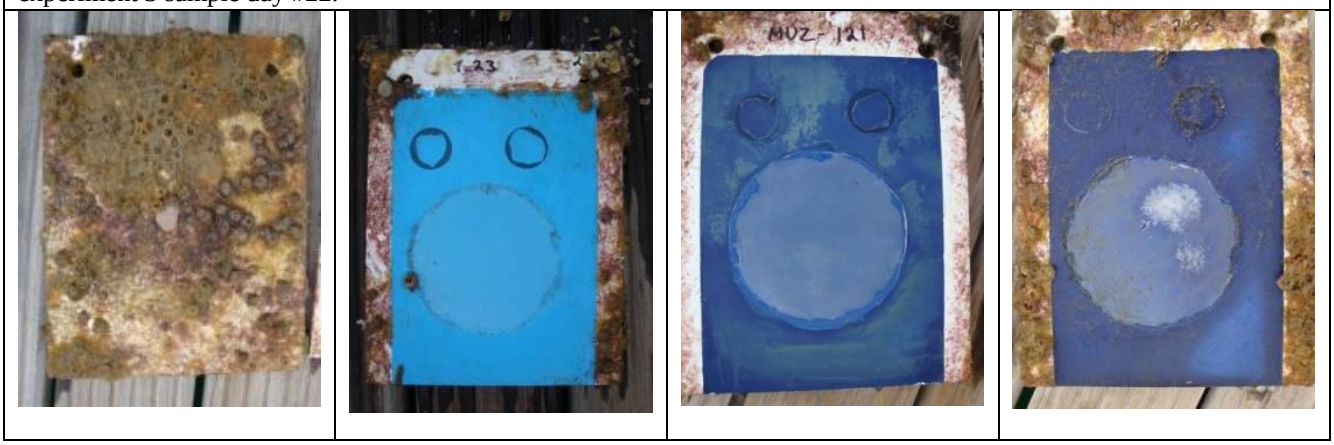
The Cirripedia density was similar for all treatments in experiment 1a and 1b and in experiment 2 the treatment T49 and the Control treatment had higher densities than treatments T46, T49, MU and MUZ.

The *H. elegans* density was in general very low in experiments 1a and b where it increased in experiment 2. In experiment 1a all treatments were similar where the Control treatment had a higher density than the other treatment in experiment 1b. In experiment 2 treatments T46, T49 and T53 were similar and had the highest abundance, where treatments MU and MUZ had the lowest and the Control treatment was in between.

Photo documentation – experiment 3

The photos from experiment 3 collection day #22, where there are used large plates, are presented in figure 50. The Control has without doubt the largest amount of algae and invertebrates namely Cirripedia which has a very large bloom after a red tide in the beginning of the experiment. There are almost no algae or macrofouling on the other treatments. A few Cirripedia settled on the large filter or on the rim off the filters. Treatment UV has the least amount of fouling and treatments MU and MUZ have very little but more than treatment UV.

Figure 50: Representative photos of algae and macro-invertebrates (macrofouling) from the experimental slides in experiment 3 sample day #22.



Larval settlement assays

No larvae settlement assays were performed before on day #15, 16 and 17. The reason for the late filming was a red tide in the beginning of the experiment which eliminated a large part of the marine organisms. When the organisms started to recover there was a large bloom in Cirripedia, which in few days covered all surfaces, not treated with antifouling coatings, which made the larva settlement almost impossible to accomplish.

Photo documentation – experiment 4

The photos from experiment 4 collection day #22 are presented in figure 51. There were used large plates. When the experiment 4 was run the temperature was so high that there was not much fouling activity (figure 24). The Control treatment was also in experiments 4 the treatment with the highest density of fouling. Treatment UV was the treatment with the lowest amount of fouling. The MU and MUZ treatments only had a small amount of algae growing on them.

Figure 51: Representative photos of algae and macro-invertebrates (macrofouling) from the experimental slides in experiment 4. The photos were collected on sample day #22.

Larval settlement assays

Larvae settlement was observed both *in situ* and *in lab*. The highest density of larvae was at dawn and up to an hour after. This was also the only time at day settlement was observed. The larval settlement was very low, 1 - 2 per hour and in most cases no settlement was observed at all, neither *in situ* nor *in lab*. The low larvae abundance could be due to the high temperature of the summer season in Oman.

The larvae observed were cirripedia cyprids. There was not observed any specific settlement patterns at all. The larvae which settled on the filters swam by the filter, often in a straight line/path, and suddenly just settled on the clean surface.

Discussion

Because of the large economical burden connected with biological marine fouling a large industry has grown around developing and producing marine coatings which inhibits growth on for example ship hulls but also on harbor pillars, in pipe lines and other manmade marine constructions. After the ban of organotin in coatings, as tri-butyl-tin (TBT) paints, a large interest has grown in development of antifouling coatings. Paints containing TBT were the most efficient paints against biological fouling but had also grave effects on the surrounding non target biological life. A demand for an efficient environmental friendly antifouling coating has arisen and still no permanent solutions are in reach.
The questions of interest investigated in this thesis were how the succession of bacterial, algae and invertebrate fouling performed over time. Another interesting question was how commercial and experimental antifouling coatings would influence on the succession and community distribution of the bacteria and macrofouling over time.

Bacterial succession

Formation of a marine biofilm environment is driven by recruitment of organisms from the bulk liquid. Organisms, from the water, arrive by diffusion (Whitehead & Verran, 2008). The settlements are influenced by surface tension (Davies *et al*, 1993) and the bacteria settling ability. As the biofilm grows the cell number becomes more abundant and the competition for resources increases. This leads to a decrease in diversity which will increase again when the biofilm matures because of niche differentiation and internal recycling of resources in the biofilm (figure 52) (Jackson *et al*, 2001; Jackson, 2003; Lyautey *et al*, 2005).

Increase in bacterial density over time and with temperature was expected in the present study and have been reported and described numerous times in the literature (Bourne *et al*, 2006; Chet *et al*, 1975; Dempsey, 1981; Dryden *et al*, 2004; Jackson & Jones, 1988; Molino *et al*, 2009; Mueller *et al*, 2006) and fitted approximately with

the bacterial densities in this experiment (Bourne *et al*, 2006; Rao, 2010). The biofilm development was slightly faster (approximately a day) in Muscat Oman compared to Williamstown Australia, a harbor with copper pollution (Molino *et al*, 2009).

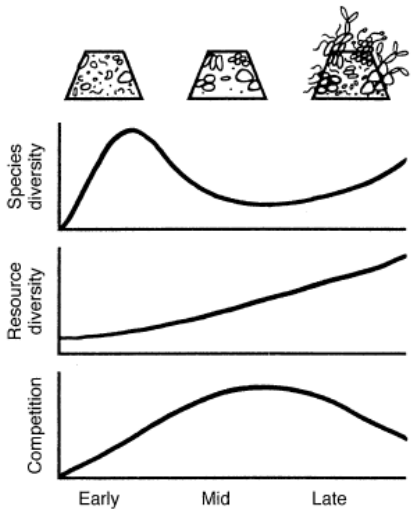


Figure 52: Model of biofilm development by Jackson *et al* (2003). The collection days were 2, 7 (early), 15, 30 (mid), 60, and 90 (late).

The densities of bacteria in the present experiment were, unexpectedly, much higher on the filters attached to the antifouling treatments than compared to the control treatment, the non-treated glass surfaces (Chet, *et al*, 1975; Doğruöz *et al*, 2009, Faúndez *et al*, 2004).

Bacterial succession images documented from the foul-release (FR) antifouling paint Intersleek 700 was rather similar to the one observed on treatments T43 (figure 26) and T49 (figure 28) but shared no visual similarities with the other treatments (Molino *et al*, 2009). This could suggest species similarities between the biofilms on Intersleek 700, T43 and T49.

In literature it is reported that different substrates with the same antifouling coating have an influence on the biofilm density. When adding copper or tri-butyl-tin to the paint the initial development of biofilm was inhibited but after 3-4 days the bacterial density was similar to the paints without these two compounds (Tang & Cooney, 1998). This fact should be kept in mind when comparing results between experiments which have not followed completely the same procedures. The only paints with copper added in the present project were the MU and MUZ treatments. The experimental slides were not monitored from day 0-2, in experiment 2, but at day 3 where they had the same bacterial density as T53 (zinc sulphide with a commercial protease). Both treatment MU and MUZ had larger densities than the other treatments in the experiments. Dempsey (1981) did observe a somewhat similar biofilm development on copper based antifouling paints as for treatment MU in this thesis.

Zinc pyrithione resistance has been observed in *P. aeruginosa* PAO1 (Malek *et al*, 2002; Malek *et al*, 2009), copper resistance in several bacteria (Burton, 1987; He *et*

al, 2010; Riquelme *et al*, 1997) including *P. aeruginosa* PAO strains (Vargas *et al*, 1995) and this could be an explanation to the high bacteria count in the MUZ treatment (Petersen *et al*, 2004). In experiments with zinc acetate *P. aeruginosa* was less inhibited than *Staphylococcus auerus* and *Staphylococcus epidermidis* (Atmaca *et al*, 1997) which again could explain the large bacterial densities on the antifouling paints compared to the Control treatment due to favorable conditions to bacteria resistant to the zinc concentrations used in the antifouling paints. In experiments with zinc oxide, rosin and resin acids only gram positive facultative aerobic bacteria was inhibited by the inhibitory compounds, while there was not observed any inhibitory effect on the gram negative bacteria in the experiment where among others *P. aeruginosa* and *E. coli* was included (Söderberg *et al*, 1990).

Community analysis of bacteria by fatty acid composition

Fatty acid extractions revealed that temporal development was presence (figure 53). The temporal development of fatty acids between the collection days could be due to changes in biofilm community over time but could also be due to differences in temperature (Cullen *et al*, 1971; Nichols *et al*, 1997), biofilm conditions (Carvalho & Fernandes, 2010; Grogan & Cronan, 1997; Jacques, 1981; Nichols *et al*, 1997) or to changes in bacterial growth phases (Grogan & Cronan, 1997; Su *et al*, 1979).

In this experiment there was only screened for one type of poly unsaturated fatty acids (PUFA) C18:2ω9,12 and this fatty acid was only observed a few times (figure 53). On sample day 14 it was present in treatments T44 and the MUZ treatment where on sample day #22 it was detected in the Control and treatment T40. Referring to Su & Yang (2009) there was either found microeucaryotes on the experimental slides on the given treatments on the two sampling days or there were bacteria from Cytophagales or Proteobacteria present.

Gharaibeh & Voorhees, (1996) found iso and anteiso C15:0 and C17:0 along with C18:0 were predominant in the five gram positive bacteria they worked with where C16:1, C16:0, C18:1 and cyclopropane cyC17:0 and cyC19:0 were significant in the five strains of gram negative bacteria they tested. On collection day #9 there were no treatments with the fatty acid profile from the gram positive bacteria described by

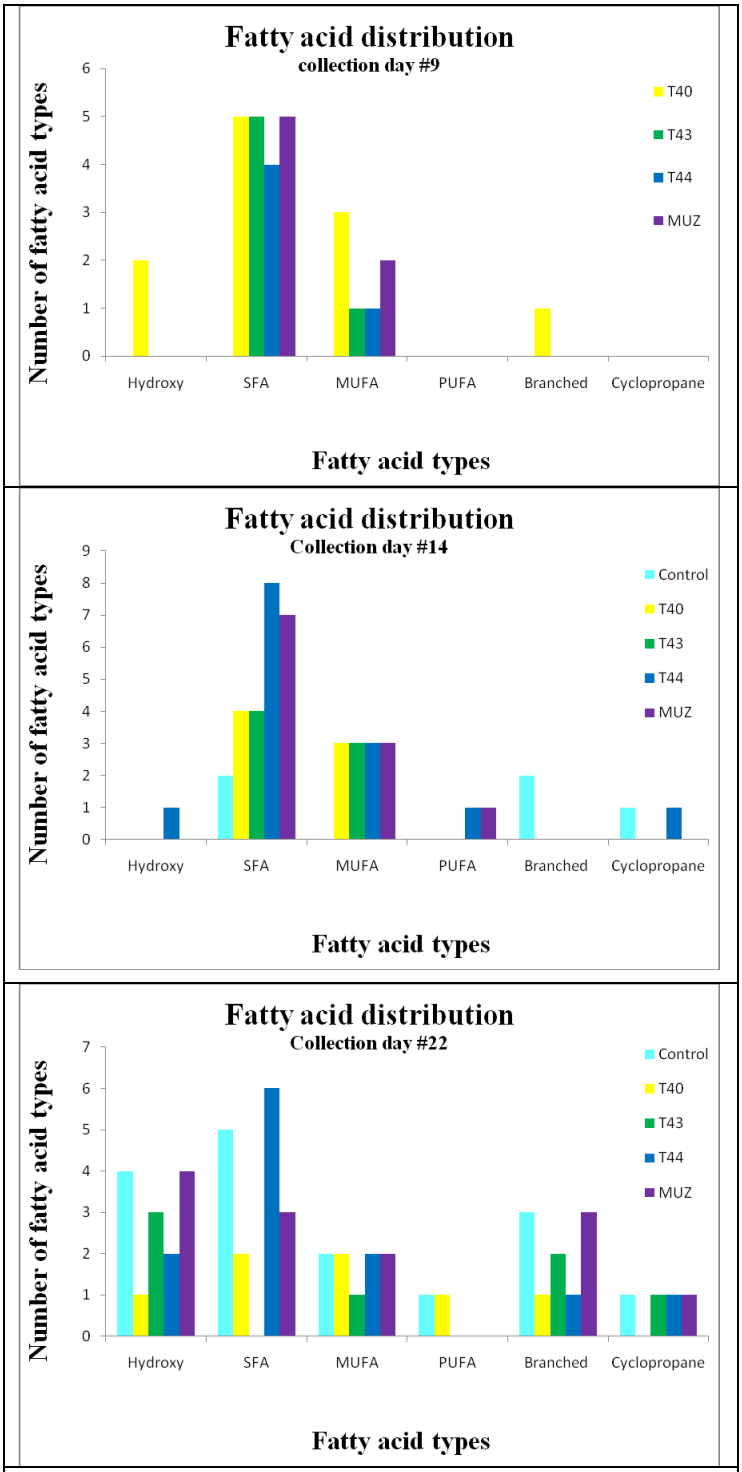


Figure 53: Temporal development of the fatty acids detected in the FAME analysis in experiment 1a presented for each collection day.

Gharaibeh & Voorhees, (1996) but on collection day #14 some of the samples in the Control treatment had a similar profile and a single sample from the Control treatment and the treatment MUZ also had similar fatty acid profiles on collection day #22. On collection day #9 all treatments had similar profiles to the gram negative bacteria in Gharaibeh & Voorhees, (1996) and the same was similar for treatments T43, T44 and MUZ on collection day #14 and treatments T44 and the Control on sample day 22. The only exception was that, there was no cyclopropyl cyC19:0 detected in the fatty acid extractions in this thesis. Fatty acid profiles from this thesis were rather similar to profiles from the following gram negative bacteria *Escherichia coli* strain K-12, *Enterobacter aerogenes* (ATCC 13048), *Pseudomonas aeruginosa* (ATCC 10145), *Pseudomonas fluorescens* (ATCC 13525) and *Serratia marcescens* (ATCC 13880) (Gharaibeh & Voorhees, 1996). All have been isolated from marine waters (Dhanasekaran *et al*, 2009; Høfle & Brettar, 1996; Vazquez *et al*, 2000; Verthé *et al*, 2004; Yu *et al*, 2004) but none are concidered specific marine species.

Algal succession

The algae observed and counted in this thesis were mainly macroalgae which first were observed from collection day #14. Among microalgae only a few diatoms were observed on the black filters in the Control treatments (data not shown). The commercial paints used in this experiment had very little algal growth. The MUZ treatment with both copper and zinc pyrithione had less algal growth than treatment MU only having copper as the active compound. These results are consistent with some results from the literature which also reports zinc pyrithione to be more toxic than copper (Bao *et al*, 2008; Koutsaftis & Aoyama, 2006). Faimali *et al*, (2003) found copper pyrithione to have a higher effect than zinc pyrithione and Myers *et al*, (2006) found that zinc pyrithione was more effective than antifouling coatings with other zinc compounds and coatings considered environmental friendly.

Macrofouling succession

The treatment which had the lowest inhibition ability on a macrofouling species varied between the experiments but there were still some overall tendencies. In general the treatments MU and MUZ were the least fouled treatments in the experiments due to the active compounds in these two commercial paints are zinc pyrithione and copper which in general have a higher toxicity than zinc sulfide, zinc oxide and titanium dioxide (Bao *et al*, 2008; Dafforn *et al*, 2008; Dafforn *et al*, 2009; Faimali *et al*, 2003; Garaventa *et al*, 2010; Piola & Johnston, 2005; Romano *et al*, 2010).

The most fouled treatments differed between experiments and treatments. Some fouling organisms were more prominent than others by being present in every experiment in large amounts. The algal density was high in all experiments. *Bugula* sp. had high densities in experiments 1a and 1b and low density in experiment 2. The Cirripedia abundance was high in experiment 1a but low in experiments 1b and 2 where the *H. elegans* was low in experiments 1a and 1b and high in experiment 2. The temporal variation observed between the fouling abundances in experiments 1a, 1b and experiment 2 could be due to a red tide in the beginning of experiment 2. This tide displaced the succession of macrofouling, which was followed regularly (data not shown), and not visible around for example day 14 (as seen in experiment 1b) but when settling started the macrofouling succession was fast. There was also a red tide in the beginning of experiment 3 which covered the slides totally in barnacles in a

very short period of time and is the reason that no macrofouling results was used from experiments 3 and 4 since the red tide ruined the possibility for comparison between the panels. The tide also killed almost all other larvae than barnacle cyprids which also were in wery few numbers after the tide that there was not the sufficient number of any larval to complete the larva settlement assays.

Treatments MU and MUZ were expected to have an effect against macrofouling and they performed as expected. Treatment T44 with zinc oxide was used due to the fact that it is used in anti–fouling today and has a classification (R53). Zinc oxide is not very soluble but the pigment promotes the coatings ability to self polish (Olsen, 2009). The activity against macrofouling was average and the slides were placed stationary which inhibited the polishing ability. Due to the missing polishing factor this coating performed below expectation. Treatments T40 and T43 had zinc sulphide as the active compound which is practically insoluble in water. This compound is approved by the Food and Drug Administration (FDA) which means that it is allowed in contact with food and is therefore not expected to have any antifouling effect. This compound was chosen because it is a soft pigment and the polishing effect can be achieved easier (pers. comm. Eva Wallström). These two treatments had the poorest antifouling activity against macrofouling in the experiment which was as expected. The aerogels polishing ability did not improve the performance of treatment T40 at all probably due to the fact that the experimental slides were placed stationary.

Experiments have shown that antifouling compounds can have different effects against different organisms (Dafforn *et al*, 2009; Han *et al*, 2008; Weiss, 1947). In some cases the compound has no effect and this can give the given fouling organism an advantage against other organisms (Dafforn *et al*, 2009; Johnston & Keough, 2003) especially if the local marine environment is exposed to heavy metal pollution (Johnston & Keough, 2003; Piola & Johnston, 2005). There has not been observed any heavy metal marine pollution in Bay of Oman, for example from long term use of antifouling coatings (de Mora *et al*, 2004; Fowler *et al*, 2007; Hassan *et al*, 1996; IAEA). No fouling organisms observed should have had any selective adaptations against the antifouling compounds used in the paints.

Future research

To complete this project further research is needed. Confirmation of the bacterial strains present on the experimental slides used in this project would support further development in antifouling paints.

In figures 10 and 11 is given a future research plan (in grey). The main aims of the plan is (1) further support the community analysis by MDS plots of the fatty acid extractions by DGGE. Since fatty acid composition in bacteria is strongly affected by factors as temperature (Aerts *et al*, 1985; Kamimura *et al*, 1992; Kamimura *et al*, 1993; Monteoliva-Sanchez & Ramos-Cormenzana, 1986; Monteoliva-Sanchez *et al*, 1988; Nordström, 1993), nutrients availability (Yano *et al*, 1998), salinity (Monteoliva-Sanchez & Ramos-Cormenzana, 1986; Monteoliva-Sanchez *et al*, 1988) and age of biofilm (Guckert *et al*, 1986; Oliver & Colwell, 1973; Werker & Hall, 2000) and DGGE are based on gel electrophoresis of PCR fragments DGGE can give a molecular fingerprint of the bacterial community. PCR are run with DGGE primers and the sequences generated, which are of the same size, are separated by the gel electrophoresis because the different PCR products which have different denaturation characteristics encounter increasing denaturation chemicals as the gel is run (Ercolini, 2004; Muyers & Smalla, 1998). (2) Sequencing of purified PCR products of 16S rDNA will give specific sequence profiles for each strain which could reveal what kind of bacterial strains or groups were present by comparison with existing genome profiles on available public databases.

This “old school” way of determine the bacteria strains in the biofilm are long, expensive and full of hard work but perfectly doable in small laboratories with small sample sizes. Large scale sequencing of mixed unknown environmental data can give immense amounts of data and the workload would be long and tedious.

Second generation of sequencing uses a whole genome shotgun (WGS) strategy. The genome is split into short sequences of between 20-50bp (Paszkiewicz & Studholme, 2010; Reinhart *et al*, 2009; Schloss & handelsman, 2005). The bp length was approximately 52bp when sequencing the genome of the giant panda (Li *et al*, 2010a). The short sequences are sequenced by for example Illumina with their Illumina Genome Analyzer sequencing technology (Illumina.com), and then assembled with

for example SOAPdenovo (http://soap.genomics.org.cn) which is a genome assembler specially developed for next-generation short-read sequences (Li *et al*, 2010a; Li *et al*, 2010b). There exist several other computer programs for assembling short sequence reads (Paszkiewicz & Studholme, 2010).
With this novel technology it is possible to sequence mixed biofilms directly from the assembly surface with no need for culturing (Allen & Banfield, 2005; Cassler *et al*, 2008; DeLong, 2005; Handelsman, 2004; Kimura, 2006; Reinhart *et al*, 2009; Schloss & Handelsman, 2005) which previously was an obstacle for understanding these unculturable microbes (Kellenberger, 2001; Liesack & Stackebrandt, 1992; Sharma *et al*, 2005; Xu *et al*, 1982) as for example many marine bacteria, several soil bacteria and many bacteria with parasitic behavior.
The down side of the WGS method in environmental data is that species in high abundance will dominate the sequences and thereby cover for sequences with lover abundance. An example of this problem was seen in the Sargasso Sea where there was produced 1.045 billion bp of sequence data distributed among approximately 1800 genomic species. Only a few near-complete genomes of the most abundant species were assembled due to this massive number of gene products (Venter *et al*, 2004).

Besides species determination of bacterial species in the biofilm collected further research in the larval settlement behaviour is nessesary. Further investigation in larval settlement cues and repellents are important to gain knowledge on how macrofouling is inhibited most efficient and environmental friendly.
To interrupt the fouling succession in its different phases, for a greater effect, it is nessesary to have a deeper understanding of how both biofilm and macrofouling develop and is controlled. A plausible solution is to create a cocktail of settlement repellents designed to control both bacterial growth, some bacteria inhibit settlement of other organisms, and the specific macrofouling organisms. Several organisms which inhibit or control growth of both bacterial and macrofouling have been described in the literature (Bhadury & Wrigth, 2004; Cabrefiga *et al*, 2007; da Gama *et al*, 2008; Dakhama *et al*, 1993; Gallagher & Manoil, 2001; Gram *et al*, 1996; Gram *et al*, 1999; Kim *et al*, 2007; Manjula *et al*, 2004; O'Conner & Richardson, 1996; Raveendran & Limna Mol, 2009; Steinberg *et al*, 1998). The areogel used as carrier of active compound in this experiment would be very able to carry this mixture of active compounds.

Perspectives in paint development

Since the ban of use of organotins in marine antifouling coatings a range of alternative biocides have been used instead. These coatings contain compounds as Irgarol 1051, diuron, Sea-Nine 211, chlorothalonil, dichlofluanid and zinc pyrithione (Konstantinou & Albanis 2004; Omae, 2003a). These compounds are the most used worldwide. Several of these have also been found to accumulate in the environment and are detectable both in coastal waters and in marine organisms (Bellas, 2006; Konstantinou & Albanis 2004; Omae, 2003a).

Because of the consequences for the marine organisms new antifouling strategies are needed. Development of more environmental friendly antifouling coatings are under construction. These coatings are based on using the same strategies as several marine organisms which are quite successful in keeping themselves free of macrofouling (Bhadury & Wrigth, 2004; Steinberg *et al*, 1998).

Coatings with incorporated natural marine compounds have been tested with varying effects. Min & Oh, (2009) tested the effect of catfish oil, origanum, and found it more efficient against *Salmonella* Typhimurium than *E. coli* strain O157:H7. A study with Brazilian macro algae revealed that more than 50 % of the red algae used in the study revealed strong inhibition of the mussel *Perna perna* when used in antifouling assays as extracts (da Gama *et al*, 2008).

Some paint which have natural product antifoulants (NPAs) incorporated are already in production and available on the market such as Sea Nine-211, Netsafe and Pearlsafe. The active compound in Sea Nine-211 is 4,5-dichloro-2-*n*-octyl-4-isothiazolin-3-one (DCOI) which is a member of the isothiazolone family (Raveendran & Limna Mol, 2009). The active compound in Netsafe and Pearlsafe is developed from analogues extracted from the Australian red seaweed *Delisea pulchra* (Gram *et al*, 1996; Steinberg *et al*, 1995). An antifouling paint based on an analogue of 2,5,6-tribromo-1-methylgramine isolated from *Z. pellucidum* has proven to be fouling free for up to two months in seawater but only from barnacle larvae (Omae, 2003b).

In general it seems as the future in antifouling coatings will lie in incorporation of natural antifouling compounds into paints. Since the natural compounds seem to have different effects on different organisms, let it be bacteria or larger foulants, a wise strategy would be to incorporate several compounds which target different foulants.

Method evaluation

When comparing the results from the bacterial counts and the macrofouling it is important to take in consideration that the bacteria were counted on black filters and the macrofouling was counted directly on the antifouling treated microscope slide surfaces. The black filter was used for an easy way to count bacteria by DAPI staining but also to even out surface differences between the treatments. The black filters gave an even surface with the same surface charge, structure and color and still allowed the antifouling compounds to affect the biofilm formation. The surface structure and charges differed between the attached filters and antifouling paints and there could therefore be differences in the biofilms on the black filters and on the biofilm covered slides which of course would have affected fatty acid composition and the macrofouling. There was in fact observed less macrofouling on the black filters compared to the surrounding antifouling coating (data not shown).

The black filter pore size of 0.2 μm should not influence the diffusion of antifouling compounds, since these molecules are rather small from the antifouling paints and there should therefore not be any differences between exposure to treatment on the filters and on the surface of the treated slides.

One other factor, which has not been taken into consideration, is the artificial condition, that there has been no efficient polishing of the paints which would normally occur on paints coating a ship hull since the racks were suspended from the pier in the marina. This factor could also affect the succession of micro and macrofouling.

Conclusion and perspectives

Interestingly, the treatments MU and MUZ had the highest bacterial counts in all experiments which are contradicting all expectations and according to the literature (Chet *et al*, 1975; Doğruöz *et al*, 2009; Elguind *et al*, 2009; Faúndez *et al*, 2004). Bacterial resistance to both copper and zinc pyrithione are known but are normally documented from areas polluted with heavy metals (Burton, 1987; He *et al*, 2010; Malek *et al*, 2002; Malek *et al*, 2009; Riquelme *et al*, 1997). An explanation to this could be that the pier where the experiments in this thesis took place suffers from (1) a light pollution due to the coatings on the boats there and a rather low copper and zinc pyrithione concentration in the antifouling paints due to environmental restrictions or (2) that resistant bacteria have been transported from other polluted areas and give the bacteria an advantage in the area where the present experiments were performed.

The fatty acid community analysis was an easy and fast way to determine if the communities on the slides were similar or not due to fatty acid profiles. This method was on the other hand not suited for detection of specific bacterial domains, genera or species present in the biofilms since these demand pure culture profiles of the given bacteria for comparison. There were however indications suggesting bacteria from the groups of Cytophagales or Proteobacteria were presence (Su & Yang, 2009) and five other gram negative bacteria (Gharaibeh & Voorhees, 1996).

Based on the results in this thesis it can be hypothesized that bacteria which are resistance to copper and zinc pyrithione maybe also have an inhibitory effect on macrofouling settlement since almost no macrofouling was observed on the two commercial treatments MU and MUZ. The low macrofouling counts on treatments MU and MUZ could also simply be due to the copper and zinc pyrithione in the paints but still do not explain the high bacteria densities.

It must be hypothesized that bacteria which are resistant to copper and zinc pyrithione also inhibit macrofouling settlement since almost no was observed on the commercial treatments MU and MUZ.

Strains of *Pseudomonas aeruginosa* or *Pseudomonas fluorescens* could be present in the biofilms on the MU and MUZ treatments since both strains have proven to have

resistance against copper (Poirier *et al*, 2005; Poirier *et al*, 2009; Vargas *et al*, 1995; Yang *et al*, 1996; Yang *et al*, 1993). *Pseudomonas aeruginosa* is also resistant to zinc pyrithione (Malek *et al*, 2002; Malek *et al*, 2009) and *Pseudomonas fluorescens* can survive some low zinc pyrithione concentrations but do not thrive (Caicedo *et al*, 2010; Grossman, 2007). Grossman, (2007) found that *Pseudomonas fluorescens* lacked the ability to induce resistance against zinc pyrithione in three generations. *P. aeruginosa* biofilms in the growing phase have a higher resistance against heavy metals, including copper, compared to biofilms in a stationary phase or single planktonic cells. *P. aeruginosa* cells also adopt a copper resistant phenotype after exposure to copper (Teitzel & Parsek, 2003).
Both bacterial strains also show inhibitory abilities against micro- and macro fouling (Cabrefiga *et al*, 2007; Dakhama *et al*, 1993; Gallagher & Manoil, 2001; Gram *et al*, 1999; Kim *et al*, 2007; O'Conner & Richardson, 1996; Manjula *et al*, 2004).

It must also be hypothesized that these bacteria which colonize paints which inhibit AHLs quorum sensing must either use another quorum sensing compounds or system, since AHL production was inhibited in the CV017 assay in treatments MU and MUZ, or simply just is not affected by the paints inhibitory effect as the reporter strain.

In regard to this project it would be beneficial to determine which bacterial strains the biofilm on the MU and MUZ paints consisted of and work them or their active antifouling compound(s) into an antifouling coating. Addition of compounds with specific antifouling activity against the large foulers as algae, *Bugula* spp., barnacles, mussels and tube worms to create an antifouling mixture which works against the succession on several levels would be an optimal solution. Here the aerogel used for carrying the active compounds x in treatments T40 and T46 would be useful for this type of design.

The treatment with the poorest antifouling activity against macrofouling was clearly treatments T40 and T43 and of course the Control. The treatments with the best antifouling activity against the macrofouling were the commercial paints MU and MUZ. Both of these commercial paints demonstrated a consistant antifouling ability against all fouling organisms observed; algae, *Bugula* sp., *H. elegans* and Cirripedia.

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Appendix

Statistical test results from bacterial counts from experiments 1 and 2

Presentation of the statistical results from Tukey, Conover-Inmann tests from the bacterial counts in experiments 1 and 2.

A1: Tukey test for bacteria numbers from experiment 1.

Treatments	T40	T43	T44	Control	MUZ
T40	1.000				
T43	0.001	1.000			
T44	0.001	0.552	1.000		
Control	0.999	0.004	< 0.000	1.000	
MUZ	0.001	0.755	0.997	< 0.000	1.000

A2: Test results from Tukey test for the bacterial counts from experiment 2.

Treatments	Control	MU	MUZ
Control	1.000		
MU	0.001	1.000	
MUZ	0.003	0.038	1.000

A3: The p-values from the Conover-Inman test performed in experiment 2 for the bacterial counts.

Treatments	T46	T49	T53	Control	MU	MUZ
T46	1.000					
T49	0.925	1.000				
T53	0.274	0.256	1.000			
Control	< 0.000	0.001	< 0.000	1.000		
MU	0.045	0.045	0.448	< 0.000	1.000	
MUZ	0.001	0.003	< 0.000	< 0.215	< 0.000	1.000

Statistical test results from the fatty acid extractions from experiments 1a, 1b and 2

Presentation of the statistical results from the ANOSIM results from the fatty acid extraction in experiments 1a, 1b and 2.

A4: Tukey test for the for the ANOVA results from experiment 1a collection days #9, #14 and #22, p> 0.000.

Fatty acid groups	Hydroxy	SFA	MUFA	PUFA	Branched	Cyclopropane
Hydroxy	1.000					
SFA	0.435	1.000				
MUFA	1.000	0.276	1.000			
PUFA	0.159	0.001	0.276	1.000		
Branched	0.708	0.019	0.861	0.916	1.000	
Cyclopropane	0.276	0.002	0.435	1.000	0.980	1.000

A5: Tukey test for the for the ANOVA results from experiment 1a collection day #9, p> 0.000.

Fatty acid groups	Hydroxy	SFA	MUFA	PUFA	Branched	Cyclopropane
Hydroxy	1.000					
SFA	>0.000	1.000				
MUFA	0.030	>0.000	1.000			
PUFA	0.723	>0.000	0.002	1.000		
Branched	0.979	>0.000	0.008	0.979	1.000	
Cyclopropane	0.979	>0.000	0.002	1.000	0.979	1.000

A6: Tukey test for the for the ANOVA results from experiment 1a collection day #9, p= 0.018.

Treatments	T40	T43	T44	MUZ
T40	1.000			
T43	0.051	1.000		
T44	0.017	0.938	1.000	
MUZ	0.143	0.938	0.667	1.000

A7: Tukey test for the for the ANOVA results from experiment 1a collection day #14, p<0.000

Fatty acid groups	Hydroxy	SFA	MUFA	PUFA	Branched	Cyclopropane
Hydroxy	1.000					
SFA	<0.000	1.000				
MUFA	0.082	0.028	1.000			
PUFA	1.000	<0.000	0.134	1.000		
Branched	1.000	<0.000	0.134	1.000	1.000	
Cyclopropane	1.000	<0.000	0.134	1.000	1.000	1.000

A8: Tukey test for the for the ANOVA results from experiment 1a collection day #22, p=0.006

Fatty acid groups	Hydroxy	SFA	MUFA	PUFA	Branched	Cyclopropane
Hydroxy	1.000					
SFA	0.993	1.000				
MUFA	0.735	0.409	1.000			
PUFA	0.034	0.010	0.409	1.000		
Branched	0.872	0.570	1.000	0.274	1.000	
Cyclopropane	0.104	0.034	0.735	0.993	0.570	1.000

A9: Tukey test for the for the ANOVA results for the treatments in hydroxy acids from experiment 1a collection day #9, #14 and #22, p=0.038.

Treatments	T40	T43	T44	Control	MUZ
T40	1.000				
T43	0.060	1.000			
T44	0.033	0.986	1.000		

Control	0.409	0.791	0.566	1.000	
MUZ	0.370	0.609	0.370	1.000	1.000

A10: Tukey test for the for the ANOVA results for the time in hydroxy acids from experiment 1a collection day #9, #14 and #22, p=0.005.

Time	9	14	22
9	1.000		
14	0.883	1.000	
22	0.009	0.010	1.000

A11: Tukey test for the for the ANOVA results for the time in saturated fatty acids from experiment 1a collection day #9, #14 and #22, p=0.007.

Time	9	14	22
9	1.000		
14	0.013	1.000	
22	0.009	0.956	1.000

A12: ANOSIM for the time in experiment 1a, Global R: - 0.009, p= 52.4 %, all p values are given in percent.

Time	9	14	22
9	100		
14	72.6	100	
22	22.2	45.3	100

A13: ANOSIM for the treatments in experiment 1a, Global R: - 0.033, p= 72.5 %, all p values are given in percent.

Treatments	T40	T43	T44	Control	MUZ
T40	1.000				
T43	77	1.000			
T44	55.2	37.9	1.000		
Control	62.6	72.6	9.00	1.000	
MUZ	20.5	24.3	37.37	50.3	1.000

A14: ANOSIM for the time in experiment 1b, Global R: - 0.013, p= 41.9 %, all p values are given in percent.

Time	1	3	6	9	14	21
1	1.000					
3	43.4	1.000				
6	81.2	72.4	1.000			
9	89.5	62.0	97.6	1.000		
14	16.00	20.9	39.3	52.6	1.000	
22	25.0	5.1	23.3	30.2		1.000

A15: ANOSIM for the treatments in experiment 1b, Global R: - 0.054, p=7 4.6 %, all p values are given in percent.

Treatments	T46	T49	T53	Control	MU	MUZ
T46	1.000					
T49	14.0	1.000				
T53	55.0	60.3	1.000			
Control	24.0	99.0	99.0	1.000		
MU	27.0	96.0	90.0	86.0	1.000	
MUZ	24.0	32.0	26.0	54.0	52.0	1.000

A16: ANOSIM for the time in experiment 2, Global R: - 0.016, p= 56.2 %, all p values are given in percent.

Time	3	9	22
3	100		
9	48.2	100	
22	52.8	89.2	100

Statistical test results from macrofouling from experiments 1a, 1b and 2

Presentation of the statistical results from Tukey and Conover-Inmann tests.

A17: The p-values from the Conover-Inman test for experiment 1a – Algae'

Treatments	T40	T43	T44	Control	MUZ
T40	1.000				
T43	0.354	1.000			
T44	0.569	0.145	1.000		
Control	0.139	0.529	0.047	1.000	
MUZ	0.000	0.000	0.001	0.000	1.000

A18: p-values from Tukey test experiment 1a – *Bugula* sp.

Treatments	T40	T43	T44	Control	MUZ
T40	1.000				
T43	0.776	1.000			
T44	0.034	0.333	1.000		
Control	< 0.000	0.002	0.146	1.000	
MUZ	< 0.000	0.000	0.001	0.240	1.000

A19: The p-values from the Conover-Inman test performed for Infusoria in experiment 1a

Treatments	T40	T43	T44	Control	MUZ
T40	1.000				
T43	< 0.000	1.000			
T44	0.012	0.107	1.000		
Control	0.315	< 0.000	0.001	1.000	
MUZ	0.296	< 0.000	0.001	1.000	1.000

A20: The p-values from the Tukey test for the algae in experiment 1b

Treatments	T40	T43	T44	Control	MUZ
T40	1.000				
T43	0.204	1.000			
T44	0.707	0.881	1.000		
Control	< 0.000	< 0.000	< 0.000	1.000	
MUZ	< 0.000	< 0.000	< 0.001	< 0.000	1.000

A21: Tukey test p-values from algae in experiment 1b collection day #14 and #22

Treatments	T40-14	T43-14	T44-14	Control-14	MUZ-14	T40-22	T43-22	T44-22	Control-22	MUZ-22
T40-14	1.000									
T43-14	0.237	1.000								
T44-14	0.018	0.984	1.000							
Control-14	< 0.000	< 0.000	0.006	1.000						
MUZ-14	0.994	0.026	0.001	< 0.000	1.000					
T40-22	< 0.000	0.403	0.965	0.149	< 0.000	1.000				
T43-22	< 0.000	0.196	0.800	0.471	< 0.000	1.000	1.000			
T44-22	< 0.000	1.000	1.000	0.001	0.008	0.669	0.390	1.000		
Control-22	< 0.000	< 0.000	0.001	1.000	< 0.000	0.041	0.196	0.011	1.000	
MUZ-22	0.998	0.035	0.001	< 0.000	1.000	< 0.000	< 0.000	< 0.000	< 0.000	1.000

A22: Tukey test for *Bugula* sp. from experiment 1b.

Treatments	T40	T43	T44	Control	MUZ
T40	1.000				
T43	1.000	1.000			
T44	0.328	0.296	1.000		

Control	0.191	0.172	0.998	1.000	
MUZ	0.020	0.018	0.699	0.863	1.000

A23: p-values from the Tukey test for *Hydroides elegans* in experiment 1b

Treatments	T40	T43	T44	Control	MUZ
T40	1.000				
T43	0.984	1.000			
T44	0.845	0.552	1.000		
Control	0.001	0.004	< 0.000	1.000	
MUZ	0.960	0.755	0.997	< 0.000	1.000

A24: The Conover-Inman test results for algae collected in experiment 2

Treatments	T46	T49	T53	Control	MU	MUZ
T46	1.000					
T49	0.550	1.000				
T53	0.027	0.079	1.000			
Control	0.165	0.397	0.324	1.000		
MU	0.007	0.001	< 0.000	< 0.000	1.000	
MUZ	< 0.000	< 0.000	< 0.000	< 0.000	0.038	1.000

A25: *Bugula* sp. Conover-Inman test results from experiment 2

Treatments	T46	T49	T53	Control	MU	MUZ
T46	1.000					
T49	0.117	1.000				
T53	0.054	0.643	1.000			
Control	0.062	0.397	0.870	1.000		
MU	< 0.000	< 0.001	< 0.000	< 0.000	1.000	
MUZ	< 0.000	< 0.000	< 0.000	< 0.000	0.632	1.000

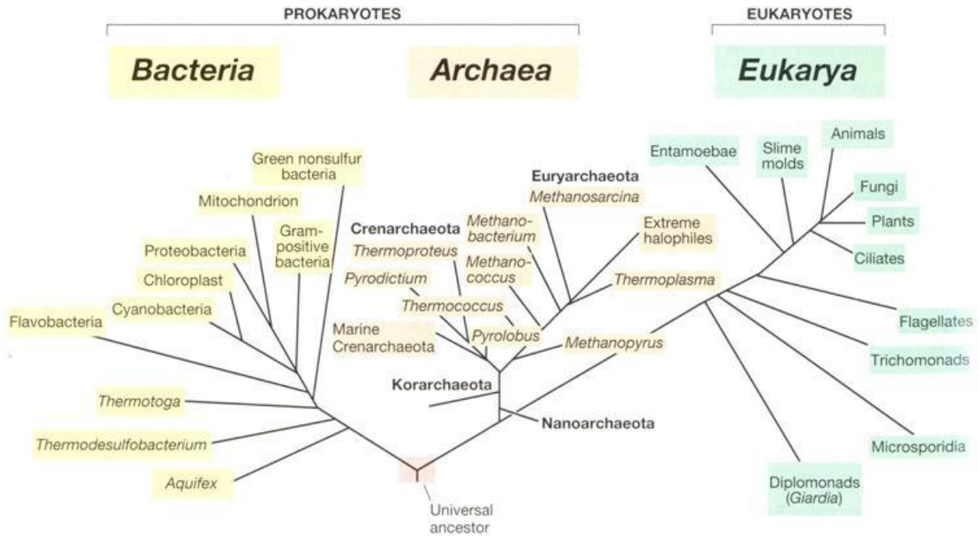
A 26: Conover-Inman test results for Cirripidia from experiment 2

Treatments	T46	T49	T53	Control	MU	MUZ
T46	1.000					
T49	0.171	1.000				
T53	0.606	0.061	1.000			
Control	0.010	0.164	0.003	1.000		
MU	0.506	0.038	0.899	0.001	1.000	
MUZ	0.396	0.024	0.753	0.001	0.844	1.000

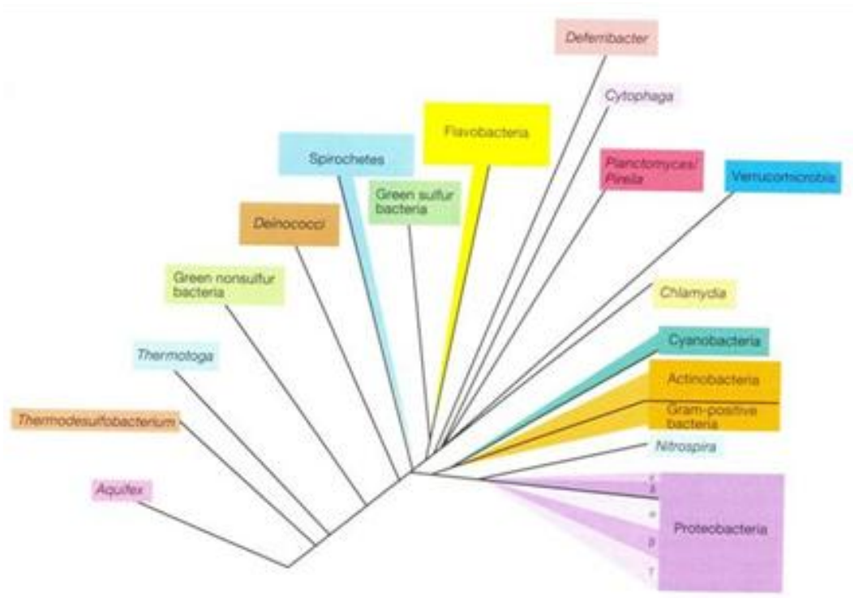
A27: p-values from the Conover-Inman test for *Hydroides elegans* collected in experiment 2

Treatments	T46	T49	T53	Control	MU	MUZ
T46	1.000					
T49	0.385	1.000				
T53	0.312	0.850	1.000			
Control	0.001	0.004	0.009	1.000		
MU	< 0.000	< 0.000	< 0.000	< 0.000	1.000	
MUZ	< 0.000	< 0.000	< 0.000	< 0.000	0.244	1.000

Bacterial maps



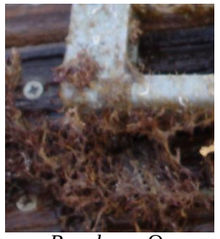
Phylogenetic three of prokaryotes and eukaryotes (Madigan & Martinko, 2006).



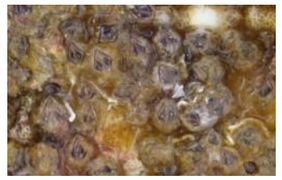
Phylogenetic three of bacteria (Madigan & Martinko, 2006).

Species and paints overview

Overview of the animals observed as fouling organisms on the experimental slides in this thesis.



Bugula sp. Oman



Barnacles, Oman

H. elegans, Oman

Overview of the paints used on the experimental slides and panels in this thesis.			
Treatment	Explanation surface	Type	Experiment
Control	Silane prep glass	Control	1a and 1b
T43	Water-borne paint with zinc sulphide without aerogel	Engineered	1a and 1b
T40	Water-borne paint with zinc sulphide and aerogel with enzyme x	Engineered	1a and 1b
T44	Water-borne paint with zinc oxide without aerogel	Engineered	1a and 1b
MUZ	Mille Ultimate as 2-component with copper and zinc pyrithione	Commercial	1a and 1b
Control	Silane prep glass	Control	2
T46	Water-borne paint with Zinc sulphide and aerogel with active compound x	Engineered	2
T49	Water-borne paint with titandioxide (anatas)	Engineered	2
T53	Water-borne paint with zinc sulphide with commercial protease	Engineered	2
MU	Mille Ultimate with copper but without zinc pyrithione	Commercial	2
MUZ	Mille Ultimate as 2-component with copper and zinc pyrithione	Commercial	2
Control	PVC plate	Control	3 and 4
MUZ	Mille Ultimate as 2-component with copper and zinc pyrithione	Commercial	3 and 4
MU	Mille Ultimate with copper but without zinc pyrithione	Commercial	3 and 4
UV	Zinc pyrithione and pigments x	Engineered	3 and 4