UPTEC X 09 028
Examensarbete 20 p
Juni 2009

Distribution and binding of a biocide in marine antifouling coating

David Wessling

UPPSALA UNIVERSITET

Molecular Biotechnology Programme

Uppsala University School of Engineering

UPTEC X 09 028	**Date of issue 2009-05**

Author

David Wessling

Title (English)

Distribution and binding of a biocide in marine antifouling coating

Title (Swedish)

Abstract

In this report we show the impact of certain solvents on the microscopic distribution of a fluorescent Biocide (Ivermectin) within an anti-fouling coatings layer. Furthermore, the degree of emission of Ivermectin from the surface was determined with the use of fluorescent derivatized Ivermectin. The results of such emission experiments were uncertain probably due to readsorption of Ivermectin to the coatings layer or to the surface of the equipments. The elutable (water-soluble) toxicity of the Ivemectin-containing coatings was also investigated with a Bioassay based on Brine shrimp (*Artemia salina*). We found that the apparent low toxicity of the elution water could partly be explained by toxic inactivation of Ivermectin with Abietic acid, a binder component in the coating.

Keywords

Marine fouling, Biocide, Toxicity, Coating

Supervisors

Hans Elwing
Göteborgs Universitet

Scientific reviewer

Karin Caldwell
Uppsala Universitet, Surface Biotechnology

Project name	Sponsors
Language **English**	Security
ISSN 1401-2138	Classification
Supplementary bibliographical information	Pages **24**

Biology Education Centre Biomedical Center Husargatan 3 Uppsala
Box 592 S-75124 Uppsala Tel +46 (0)18 4710000 Fax +46 (0)18 555217

Distribution and binding of a biocide in marine antifouling coating

David Wessling

Sammanfattning

Påväxt av alger och djur, särskilt havstulpan, är ett vanligt förekommande problem på artificiella ytor i havsmiljö. Så fort en yta sätts ner i vatten börjar adsorption av molekyler och föreningar att bilda en tunn hinna där sedan större organismer som havstulpan, musslor och havsborstmask kan få fäste. När större organismer fäster orsakar de stora problem för båtägare i termer av ökad bränsleförbrukning och tid och pengar som det kostar att avlägsna organismerna. För att minska problemen har båtfärger utvecklats som är mer eller mindre giftiga för organismer i pelagen och sedimentet. Idag är färgerna noggrant reglerade för att inte orsaka skador på miljön.

I detta arbete har jag undersökt vissa antifouling aspekter av Ivermectin, en typ av Biocid som ännu ej prövats i biofouling-sammanhang. Ett coating-skikt som innehåller Ivermectin är särskilt aktivt mot tillväxt av havstulpan men påverkar inte tidig kolonisering av havstulpanlarver. Jag har undersökt molekylens spridningsgrad i coating-skikt med hjälp av fluorescence märkt Ivermectin och fluorescence mikroskopi. Jag fann att distributionsmönstret av Ivermectin var beroende av vilket lösningsmedel som användes vid preparation av anti-fouling skiktten. Vidare har jag använt fluorescence märkt Ivermectin för att kvantifiera läckage av Ivermectin från färgskikten. Resultaten av dessa kvantifieringsförsök var osäkra, sannolikt beroende på återadsorption till färgskikten och till annan utrustning.

En biologisk metod (Bioassay) har även utvärderat hur mycket biologiskat aktivt Ivermectin som läcker ut från färgen för att avgöra hur farligt användande av just denna molekyl skulle vara. Resultaten från denna bioassay indikerar att Ivermectin har liten påverkan på Biologiskt liv i öppet vatten eller sediment.

Examensarbete 30 hp
Civilingenjörsprogrammet Molekylär Bioteknik

Uppsala universitet Maj 2009

Contents

Abbreviations & explanations

AVE	Avermectin
CDS	Controlled Depletion System
FRC	Foul Releasing Coating
GF	Glycerol Formal
HCA	Hydroxycoumarinyl acetic acid
IC	Immobilization Concentration
IVE	Ivermectin
LC50	Lethal Concentration for 50% of the individuals
SPC	Self Polishing Coating
TBT	Tributyltin

1. Introduction

1.1 Marine biofouling

Biofouling, especially of ships, has been a problem for many centuries and probably for as long as mankind has used marine transportation. Marine biofouling is caused by the attachment and settlement of marine organisms to manmade surfaces; it is a worldwide problem in marine systems. The settlements and accumulation of marine organisms on an inanimate substrate can cause large penalties to engineered structures [1]. Engineered structures are under constant attack from the marine environment. On ships hull biofouling results in an increase in roughness, which in turn increases the hydrodynamic drag as the boat moves thru water [2]. The attachment of organisms also reduces the maneuverability and efficiency. The increase in fuel consumption can be up to 40% [3], resulting in increase of the frequency of dry-docking on large ships, i.e. downtime for removal of fouling [3]. There is also an introduction of unwanted foreign species to seas where they are not naturally present [4].

As can be seen in Figure 1 the colonization is roughly composed of 4 phases with an overlapping time sequence; biochemical, bacterial, unicellular and finally multicellular eukaryote fouling [5]. Directly when a surface is put into contact with seawater an organic macro-molecular film is created. The film consists of proteins, polysaccharides and proteoglycans. The adsorption is purely physical. This adsorption is identical to what you can see in other environments such as human oral cavity and biomaterial materials [5]. Colonizing of Bacteria happens within hours, as many unicellular algae and cyanobacteria [2]. Bacteria adsorption is also controlled by physical laws with electrostatic interaction, gravity and van-der-Waals forces being the primary forces [6]. The first part of bacterial absorption is a reversible process where washing can remove the bacteria. Bacteria start with time to secrete extracellular bridge polymers that anchor to the substrate. The film now consists of dead material, cells and their slime. After several days of contact with seawater unicellular eukaryotes; yeast, protozoa and diatoms start to adhere. After weeks of immersion a 3D structure starts to form from all colonizing organisms and now multicellular larvae and algae spores start to settle in the film. After this phase the fouling community continually evolves depending on the environment.

The adhesion can be very diverse among different organisms and is often a two component process with both temporary and permanent adhesion. The attachment of organisms to a specific surface is an important aspect of biofouling. Adhesion and settlement is often a key stage in the lifecycle of marine organisms, so the pressure of colonizing a surface is great [1].

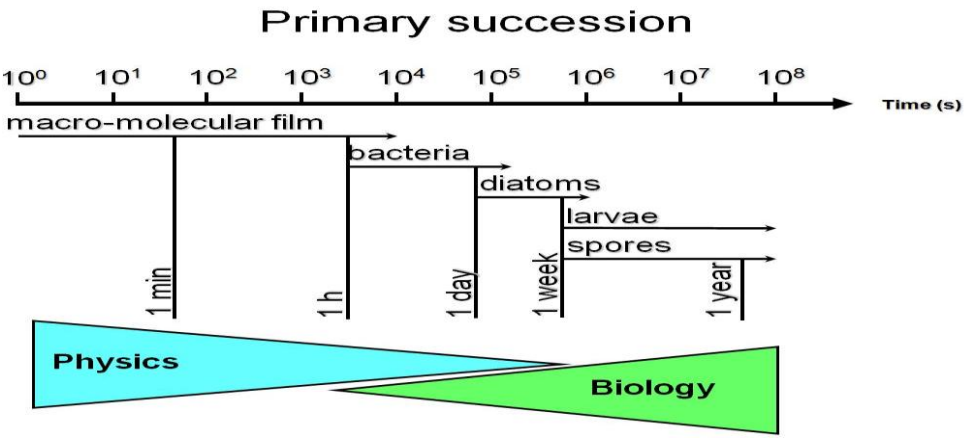


Fig 1. Highly schematized colonizing sequence leading to the establishment of a fouling community. The nearly instantaneous adsorption of macromolecules is followed several hours later by prokaryotic fouling. Diatoms and protozoa typically settle from the second day onward. Larvae and algal spores follow with a lag of one to several weeks. [3,4]

1.2 Barnacle

The marine biofouling consists of a multitude of species, both animal and plants; however, the barnacle is present in all marine environments and is considered to be one of the most serious biofouling organisms. They are arthropods, in the same group as insects. Barnacles are living only in marine environments and tend to live in shallow waters. The adult barnacle is usually fixed to a rocky surface, strongly attached in place and has 4, 6 or 8 hard calcareous plates to surround and protect their bodies. Most barnacles live on filtering the surrounding waters with their legs. The Barnacle has an excellent protection from water loss. Their plates are of calcite and impermeable. Barnacles do not release their gametes (eggs and sperms) into the sea but are able to fertilize each other. Most barnacles are hermaphrodites and can produce several thousands of napilus larvae. These larvae are released as plankton and undergo several moult stages until they reach cyprid stage. Cyprid uses a pair of attachment organs to "walk" the surface and search for a suitable surface to settle on. These organs have a chemical and touch detection and can recognize adults of its own species and suitable environments. Numerous surfaces may be explored and rejected before the cyprid becomes so desperate so that it selects a surface less favorable [7]. When it has found a good place it rotates its body and stands on its head while secreting proteins around itself making the attachment permanent. The now attached cyprid metamorphoses into an adult barnacle and from now on it is unable to move. The adult individual lives on filtering the water for nutrients. The life cycle of the barnacle is schematically shown in Figure 2.

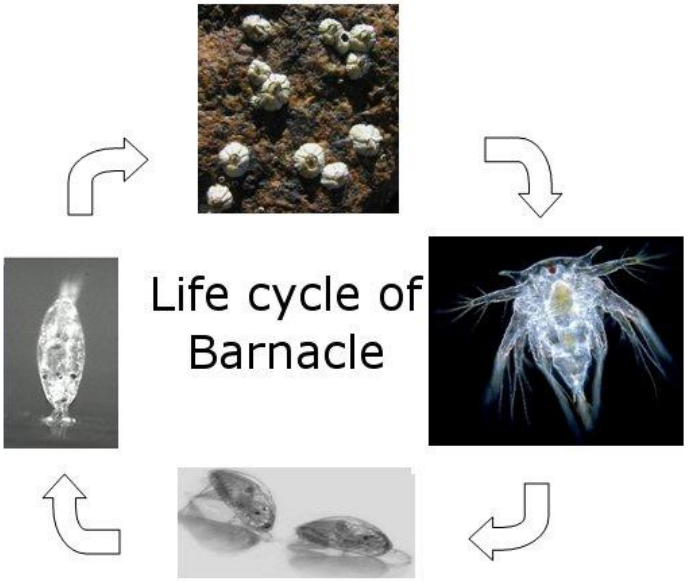


Figure 2. Barnacle life cycle starting at the top with adult animal that releases naupilus picture to the right. The naupilus swims around and after several moult stages it becomes a cyprid, picture at the bottom. The cyprid walks on surfaces and finds a suitable spot for colonizing. When the cyprid has found a good place to settle it turns its body up from the surface and attaches itself, picture to the left. It now starts to metamorphpose and evolve in to the adult barnacle.

1.3 Marine antifouling coatings

In order to minimize the impacts of foulers, many underwater structures are protected by antifouling coatings. Many types of coatings have however been found to be toxic to marine organisms. Today marine paint consists of two major types of coatings, foul release coating (FRC) or biocide containing coatings.

FRC functions due to its low surface energy which makes it a lot harder for organism to attach to the surface i.e. provide weak adhesion to the hull. When the vessel is moving at a critical speed any attached organisms release. Most of the FCR systems are based on silicone coatings. Silicone elastomers are expensive and sensitive to tearing; this makes the coating only applicable to specific vessels that can achieve the critical speed.

Biocide containing coatings have been commonly used on boats. Initially heavy metals such as lead, arsenic, mercury and their organic derivates where used. Today few heavy metals are allowed in paints and the environmental aspects of any paint is investigated. In a biocide containing paint the biggest problem is the release rate. Insoluble matrixes rely completely on the diffusion of the biocide for making it available to organisms. They suffer many problems with release and are often filled with high concentrations of biocide to counter the problem (fig 3).

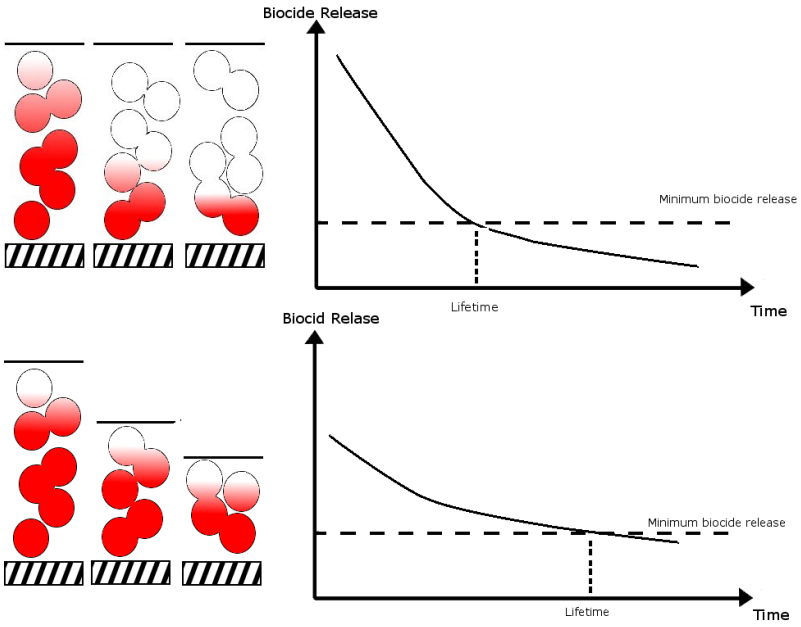


Figure 3. Illustration showing how an eroding system helps the release of biocide. Upper picture show a coating where the red is biocide and the balls are the paint. The biocide releases from the paint rapidly in the start but decreases fast as the biocide gets longer and longer to travel within the paint to reach the water.

Eroding paint has a high release in the start and with the eroding paint you get a continuing release of biocide until the paint has eroded away. With this system you can keep the biocide level closer to minimum release rate needed for antifouling thus saving both money and environment.

Controlled Depletion System (CDS) combined the property of the paint to erode and simultaneously release the included Biocide. The erosion is accomplished with a paint matrix (often rosin) and creates a thin layer where the sea water and coatings are mixed together. With the erosion the water can penetrate further into the paint and the diffusion of the biocide becomes stable.

Self Polishing Coating is another biocide containing paint that also erodes but the matrix here is not water-soluble. The self-polishing copolymer (SPC) technique uses both hydrolysis and erosion to control the antifouling activity [1]. The SPC paint "polishes" itself, making the surface smoother as the toxic compound is released from the paint. The paint works through hydrolysis of copper, zinc or silyl acrylate. The biocide in this paint is released at the hydrolysis reaction and hence very controlled by how fast the hydrolysis reaction in the paint is. The common reaction is based on an acrylate which hydrolyses and becomes soluble.

$$\underset{\text{acrylate (insoluble)}}{\text{polymer–COO–X (s)} + \text{Na}^+} \rightleftarrows \underset{\text{acidic polymer (soluble)}}{\text{polymer–COO}^-\text{Na}^+ \text{ (s)}} + \text{X}^+ \text{(aq)}$$

One commonly known SPC-paint was based on copolymerization of Tributyltin and an acrylate (TBT) which was heavily used on many boats in the 80s and early 90s but was later banned from all usage in the sea despite the excellent antifouling properties. TBT –SPC systems has today no good replacement. This has lead to an increased use of copper as anti fouling component. The copper performs very well in protecting the hull from invasion by most organisms, but with less effect when it comes to preventing the growth of algae and biofilms. However, the copper paint also comes with some drawbacks which make other approaches interesting. Copper is toxic in high concentration in water; this makes many skeptical to how good the use is for the marine environment.

Paints on boats are however confronted with the problem that sea-water differs in chemistry in different seas . Temperature, salinity and pH can render different paint very inefficient under certain conditions. Temperature is commonly known to increase the rate of chemical reactions. The salinity influences the dissolution of the paint as can be seen in the reaction of SPC. For paints containing rosin the pH is very important since an increase in pH increases the erosion drastically.

Today many are approaching the fouling problem by looking at natural defenses that repel or inhibit adhesion of fouling organisms. Any interesting biocide here can be tested in bioassays and here the the barnacle is a key organism. Many of the biocides found this way are water soluble and have problems with both of hydrophobic environment in the coating and with rapid release. Some ideas of encapsulation the active substance with microcapsules have been tested. Enzymes bound to the coating with different polymers such as PEG are also under testing. Many of the biological found substances are facing the problem to be active in or on the surface of a hydrophobic coat.

1.4 Biocides

After the ban of TBT many companies were in need of replacement biocides. Copper is the one commonly used but it has limitations such as environmental effects and price. To complement copper many different biocides primarily active against algae have been used [4].

In this work we will use a large hydrophobic class of molecule collectively named as Avermectins which was characterized in 1974. Avermectin is produced by a bacterium named *Streptomyces avermitilis.* This bacterium was isolated in the 70's and a strong antibiotic effect was observed. *S. avermitilis* (or, *S. avermectinius*) is the producer of anthelmintic macrolide "avermectin". The interesting and important property of *Streptomycetes* bacteria is their ability of producing a variety of antibiotics such as Streptomycin, Erythromycin and Tetracycline etc. through complex secondary metabolic pathways. The antibiotics produced by them are used as antibacterial, antifungal, antiviral, anitiparasitic, immunosuppressant and antitumor medicines. The Avermectins is a 16-membered lactone and and one of the Avermectin has been refined into a product today called Ivermectin that was used in this investigation. It has very good antiparasitic effects against helminthes, arachnids and insects but not against flatworms, protozoa, bacteria or fungi [8]. The general action of avermectin is that it blocks neurotransmission, interrupting the transmission of signals in the nervous system. Avermectin interacts with glutamate-gated chloride channels which are common in nematodes, insects and ticks. This increases the permeability of the cell membranes in the nervous system to chloride, resulting in a dysfunction of the nervous system [9].

In vertebrates avermectin affects the release of gamma-amino butyric acid (GABA). Most of the GABA receptors can be found in the brain and therefore the receptor is protected since avermectin cannot pass the blood/brain barrier. There are no findings of glutamate-gated chloride channels in mammals so the molecule is very useful in treating parasitic infections [10].

Ivermectin (Fig 4) is a hydrophobic molecule binding to soil, lipid and organic material; this results in a lower risk for pelagic water living organisms compared to sediment living .Ivermectin is therefore very likely associated with the sediment and particles within the marine environment and the fate of the compound is therefore linked to that of the particles [9]. Degradation studies in soil show degradation halflife of 90-240 days in winter, 7-14 day in summer and 3h when exposed to sunlight [10, 11]. In water sediment the half-life may be of the order of 100 days and in salmon farms it is debated whether or not it is dangerous [12].

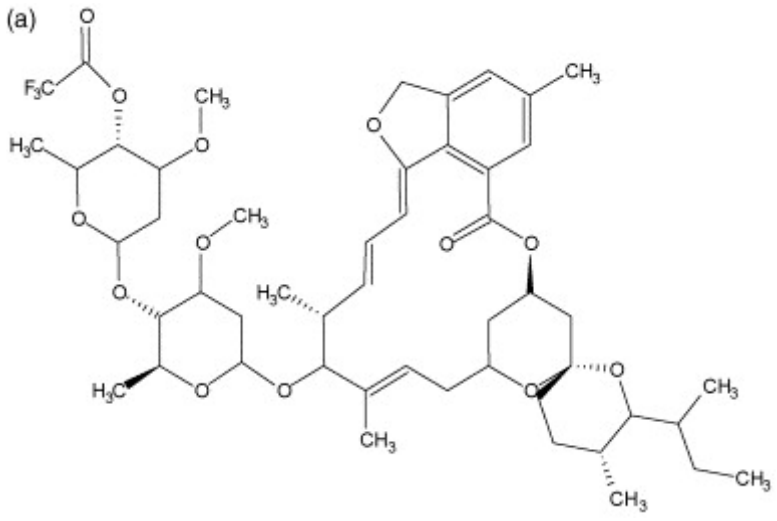


Figure 4. Ivermectin a large and hydrophobic molecule. Produced naturally by *S. avermitilis* a ground living bacterium. Ivermectin today has several uses as antiparasitic drug for cattle and is also used for medical purposes on humans, treating river blindness a disease cause by a parasitic worm.

The detection of avermectins is

frequently done in food products. Therefore several articles describing derivatization are available. The common for all molecules described is that they have an identical dihydroxylated tetrahydro benzofuran ring that upon derivatization with trifluoracetic acid anhydride and 1-methylimidazole can be converted into a chromophoric group [13].
The detection of Ivermectin in this investigation will be done by fluorescence microscopy. Fluorescence is superior in detection compared with LC-MS with respect to limit of detection. However as Ivermectin is not in itself a fluorescent molecule it requires an effective derivatization protocol and also stability testing of the derivatizated product, before it can be analyzed.

1.5 Previous Panel experiments

Previous research done by Prof. Hans Elwing and his research group at Gothenburg University has lead to the finding of Ivermectin as preventing colonization of the barnacle *B improvisus* in field-tests. Dr. Elwing made a screening of molecules with known effect on arthropods and with a large molecular size with the intention of not permitting it to diffuse away from the coating, as done by traditional biocides. They found Ivermectin as a working molecule but it proves to have been very lucky with the finding since Ivemectin seems to work only when combined with a co-solvent named Glycerol formal (GF).

The panels that have been used in previous experiments were Plexiglas with a size of 11x11 cm; They were painted in half with one paint containing Ivermectin where the other side was used as a control surface. These panels have been evaluated in water at Kristineberg Marine Research Station during the summer months and photographed carefully to monitor the growth of any fouling. One example of such a panel is shown in Figure 5.

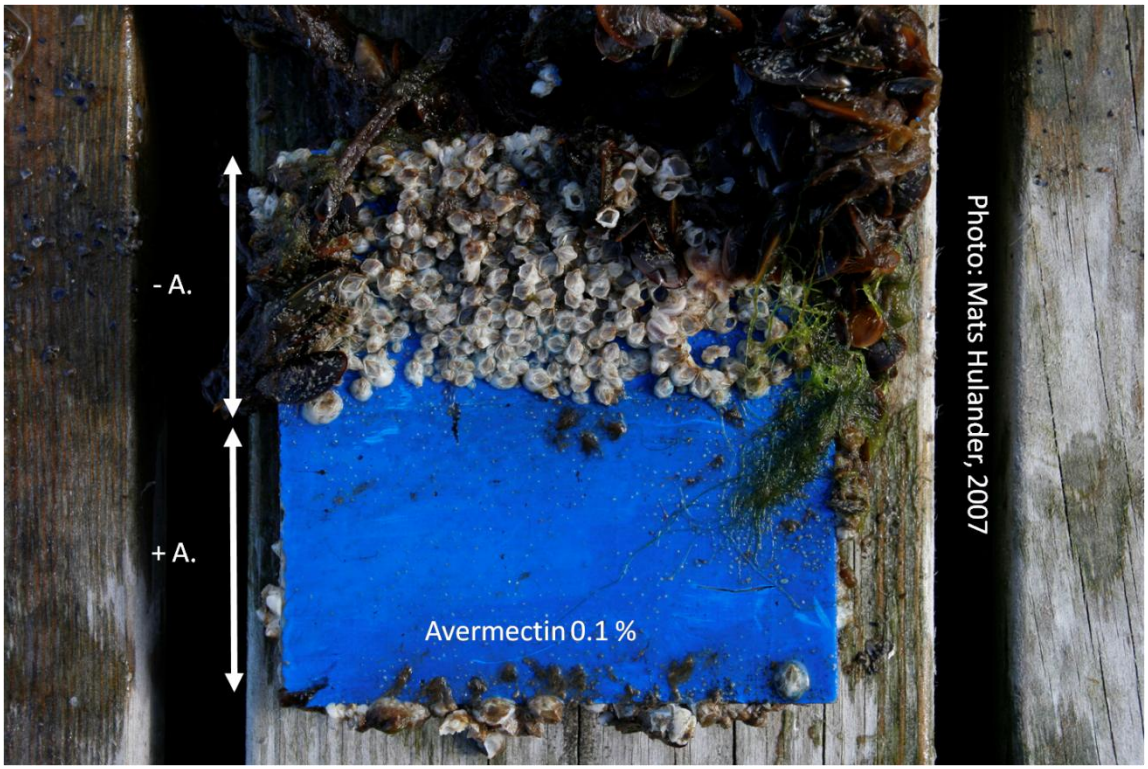


Figure 5.Photograph of a panel that has been incubated in marine environment at Kristineberg Marine Research Station during four summer month in 2007. Sides marked with –A does not contain any Ivermectin, +A have a 01% addition of Ivermectin in combination with GF. The anti barnacle effect is dramatic and on this panel it is close to 100% reduction in fouling.

Figure 6. Close-up on the same panel. Here we see the barnacle fully grown on the Control (A-) side wile you can see small white dots (barnacles) on the +A side. Barnacles are inhibited afteråpi settlement and unable to grow.

In the year 2008 a large test on boats was carried out. The test was done with two biocide free paints and a preparation of Ivermectin. The paint was applied by the owners as instructed, leaving a small band not containing Ivermectin on the hull. What came to be the general conclusion after this test was that Ivermectin effectively prevents barnacle colonization on ship hull. It also has a good effect on propellers, otherwise prone to barnacles settling. Boat owners claim it has equally or better effect than the copper based paints.

Figure 7. Picture of a boat that was in the field test during 2008. The string with lighter paint is a biocide free paint where barnacles have attached themselves. The surrounding area is painted with the same paint and added 0.1% Ivermectin and GF. The darker paint is because of different eroding and also because of different fouling from algae.

2. Aim

The complexity of marine biofouling makes it a very interesting area to work with. The many ways of foulers to attach themselves make researchers work with many different solutions. I will in this work concentrate on 3 aspects that have been discovered with the biocide molecule Ivermectin, but not completely understood yet. The Ivermectin preparation used so far is called "IVOMEC" (an injectable fluid) and consists of Ivermectin dissolved in glycerol formal (GF) and propylene glycol. The addition of GF in preparations has in field studies proven to be an important addition for the antifouling effect (unpublished results), how and why the effect is enhanced in the presence of GF are important questions to be answered.

How is the distribution of Ivermectin manifested within the coating and is it depending on solvent?
Pigment free paint matrices will be applied with and without GF, to see the effect of dispersion of Ivermectin in the coating. To do this a method for fluorescence - derivatization will be developed and optimized for microscopy.

How toxic is the Ivermectin system used as antifouling substance?
Biological effects on water living organisms will be studied by using a model organism i.e. Artemia Salina assay to determine toxic properties of the coatings against non-target marine organisms. This type of Artemia based assay is well known in the field of Avermectin research [16].

How much of the biocide is eluted into seawater from Ivermectin containing coatings?
Water immersion studies trying to evaluate how much of Ivermectin is released from the coating will be performed, which will be important for any registration purposes. A method aiming to quantify the released amount of Ivermectin will be developed with the fluorescence derivatization process, same as mentioned in the first question.

The molecular background of the antibarnacle effect is largely unknown but the goal is to shed more light on this question. I hope to find a general principle with the use of GF and Ivermectin and with that knowledge replace many other biocides that are currently being used.

3. Materials and methods

3.1. Reagents and equipment

The paint formulation that was used in all experiments was *Lotrec* LeFant SPF Mark 5 (Lidingö, Sweden), either as pigmented paint or as non pigmented varnish. The paint is a slowly eroding system which, according to the manufacturer, is supposed to give the barnacle a harder time attaching to it.

IVOMEC ® Merial is a compound used in veterinary medicine. It contains the active substance Ivermectin dissolved in GF and propyleneglycol. This Ivermectin based solution was used in previous field-tests . Pure Ivermectin was bought from a manufacturer in china as a freeze dried product and was used in most experiments. GF (99%) was obtained from Fluka. For the fluorescence reaction the following reagents where used; Acetonitile (99.9%) was obtained from Riedel-de Haën. 1-Methylimidazole (99%), Triethylamine (99.5%) and Trifluoroacetic acid anhydride (99%) were obtained from Fluka. Trifluoroacetic acid (99%) was obtained from Sigma-Aldrich. Chloroform (99%) for the fluorescence analysis was obtained from Merck. Colophony is an important binder substance in all marine anti-fouling paints and varnishes that are used today. Pure Colophony was obtained from Fluka and was used in some experiments to simulate the effect it might have on Ivermectin. Fluorescence pictures were taken on Leica microscope connected to a UV lamp with DAPI filter (EX: 345nm, EM: 455nm).

3.2. Methods

The methods used in this study for detecting Ivermectin come from the veterinary use of the compounds. It is regularly analyzed in serum, meat, milk or feces [14] where the animals have been digesting or been injected with the substance. The derivatization is specific to all of the avermectins because they contain an identical dihydroxylated tetrahydrobenzofuran ring. This ring can be derivatizated to an aromatic group with trifluororoacetic acid anhydride and 1-methylimidazole as shown in Fig 8. . TFAA and MI form an acylating reagent that reacts with the hydroxylic groups on the avermectin (fig 4). With addition of a nucleophilic base such as triethylamine the furan ring forms a strongly fluorescent aromatic group.

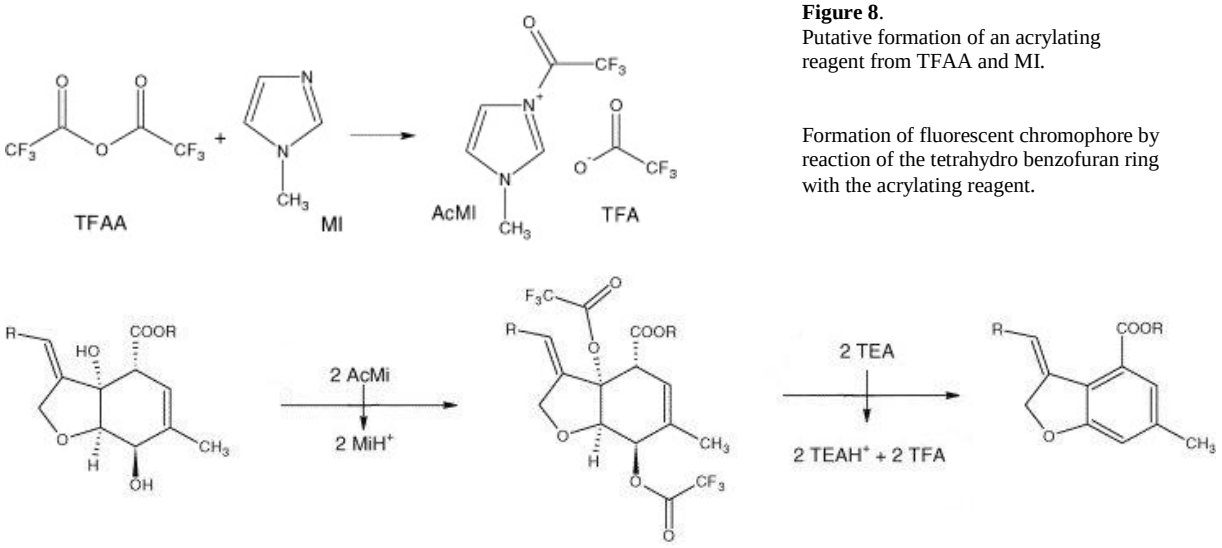


Figure 8.
Putative formation of an acrylating reagent from TFAA and MI.

Formation of fluorescent chromophore by reaction of the tetrahydro benzofuran ring with the acrylating reagent.

50mg IVE was diluted in 6,5ml acetonitrile (ACN). To the solution 1ml ACN:MI (1:1), 0,5ml TEA and 1,5ml TFAA:ACN(2:1) was added. The last reagents added make the solution warm and gets a yellow color. To end the derivatization 0,5ml TFA was added [13]. The final solution was transferred to a round flask for the rotary evaporator. As much liquid as possible was evaporated at 65^{o}C. To the evaporated solution water and chloroform was added and mixed 3 times to get rid of all water-soluble substances. Then the chloroform was evaporated and the yellow solid was saved in darkness. The final product has an excitation at 365nm and emission at 470nm [13, 15].

Microscopy pictures were taken on microscopy slides that were coated with an applicator manufactured to give the same thickness of the coating. Each slide was coated and left to dry for 24 hours in darkness before pictures were taken. The microscopy was adapted for pictures for stained cells, thus the filter used was a DAPI filter allowing at excitation 345nm and emission at 455nm.

Important to investigate was to see if GF was incorporated in the paint layer or evaporated during drying. Evaporation of GF was carried out with paint in petridishes where all compounds were weighed and thereafter left to dry. The weight difference was then observed after 24h.

For elution or release studies petridishes filled with 40ml of seawater were used. A Plexiglas surface of 5x5 cm^2 was painted. The coating was applied with a normal paintbrush 2 times with 15min between each application. The Plexiglas with coat was placed in the petridish and sealed with lid and parafilm. Elution was carried out on a shaking table with sealed petridishes all covered with aluminum foil. Dishes were placed in room temperature and aliquots of the water were sampled after different time intervals. Water sampled was stored at 4^oC and in darkness. Extraction of Ivermectin from the water samples was performed with Solid phase extraction (SPE) columns. Used in this study was Supelco Select HLB SPE 200MG/6ML tubes. Conditioning of the SPE tube was done with 5ml n-Hexane followed by 5ml acetonitrile. The water sample was then loaded. It was eluted with 5ml acetonitril. Samples were stored in darkness until measurements were carried out on the fluorescence spectrophotometer. Detection was carried out on DETECTOR X at 365nm excitation and 450nm emission. Excitation slit was set to 5nm and emission slit to 10nm.

Artemia assay was done according to known protocols [16]. Brine shrimp (*artemia salina*) germinating spores where obtained from a pet shop and put into water containing 3% NaCl. The water had a tube bubbling air to aerate the water. After 48h eggs hatched and the larvae were collected with a pipette. This procedure yields a higher concentration of larvae in saltwater. The assay was carried out in wells of a 96 well plate and each water-sample to be tested was stepwise diluted in each well 10 times with saltwater; each column thus contains a decreasing concentration of different water. Several brine shrimp larvae, (usually 7-30) were then added to the wells. The assay was evaluated using microscope and counting each larvae in all wells, determining the concentration of the sample to cause immobilization of the larva. Any larvae not moving in the well was counted as immobile, thus our values are immobilization concentration (IC). Control samples with no toxic component were present in each 96well plate. The reported IC100 for brine shrimp is around 165ng/ml. The lethal 50 concentration (LC_{50}) is reported to be 140-300ng ml^{-1} [17, 18].

4. Results

4.1 Microscopic Distribution of Ivermectin depending on solvent

Effects of solvents. GF appears to form small bubbles in the coat depending on concentration. From previous studies at the lab it has been shown that a GF concentration of 7% or higher is needed to get a good antifouling effect. From the pictures it appears that the coat differs drastically with the addition of GF. With small amounts of GF a smooth surface was seen and no difference from paint containing no GF. Addition of 5% GF gives areas indicating a phase separation. The coat now has two different areas of different color. The 5% seem to be a point where the isles form and with addition of more GF small "bubbles" form in the coat. With 10% I saw a grained surface and small bubbles 20-50um form in the coat. These bubbles are not clearly visible but are found inside the layer. The more GF added the larger the bubbles become; at 30% GF the size is up to 200um. Addition of GF to the paint gives it a macroscopic cloudy appearance, proportional to how much that was added in the coat. GF also makes the coating have a longer drying time and also makes it feel sticky. The bubbles are consistent in all areas of the coating and do not disappear even if the drying period is as long as a week. The paint itself was autofluorescent to some degree. When looking at the paint containing no fluorescent Ivermectin molecule it was seen that the bubbles contained faintly fluorescent material.

Distribution of Ivermectin. Fluorescence pictures show that Ivermectin was apparently not found inside the GF-bubbles. They get concentrated in the area around. With no fluorescent Ivermectin the bubbles get highlighted instead of the surroundings but with very small intensity. Without any GF added, a dispersed amount of Ivermectin with small cluster formations was noted. The size of clusters are hard to determine due to focus problems with such high fluorescence but approximate values of the clusters are areas of 30-100um in diameter. The pictures contain equal amounts of Ivermectin and the intensity should be the same with only a difference with respect to where the Ivermectin can be found. With calculations using an intensity threshold we can see that the intensity of Ivermectin is similar regardless of whether or not GF is present (fig 19). Difference is that the intensity is more disperse without GF. The derivat Ivermectin is sensitive to long exposure of UV light and after 2 minutes of exposure it is all bleached. This was used to see if Ivermectin behaved differently in different concentrations of GF. Pictures were taken in a series with normalization turned off. Each picture shows an intensity loss and for every picture a threshold was set and intensity was calculated. This shows that Ivermectin is degraded and bleached equally in both coatings independent of GF. Small clusters in the coat containing no GF can be seen even after long exposure with very low intensity. They are not being degraded, even after long exposure times and can therefore be viewed as molecules found in the paint normally. The coat containing no GF with Ivermectin looks very similar to other distribution pictures published before [19].

To compare different molecules to see if this was only a specific behavior of Ivermectin, different fluorescence probes where added to coatings containing 10% and 0% GF. All the different probes were slightly less hydrophobic than Ivermectin. The same phenomenon was seen where the bubbles are free from any of the probes. We see the same distribution of fluorescence without any addition of GF. This would imply that the derivatization of Ivermectin does not change how the biologically active Ivermectin would disperse. Thus, there appears to be a general dispersion pattern for molecules that are hydrophobic with the addition of GF.

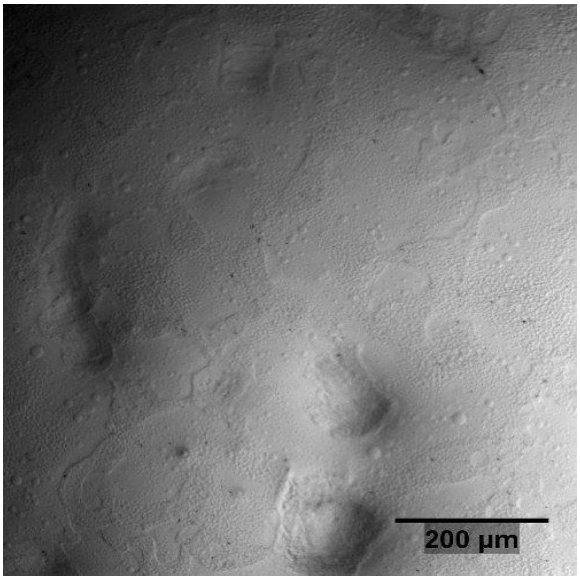


Figure 9. 3% glycerol formal concentration, a smoth surface similar to normal paint.

Figure 10. 5% glycerol formal concentration, areas with different colors form and the surface has a sticky feeling to it.

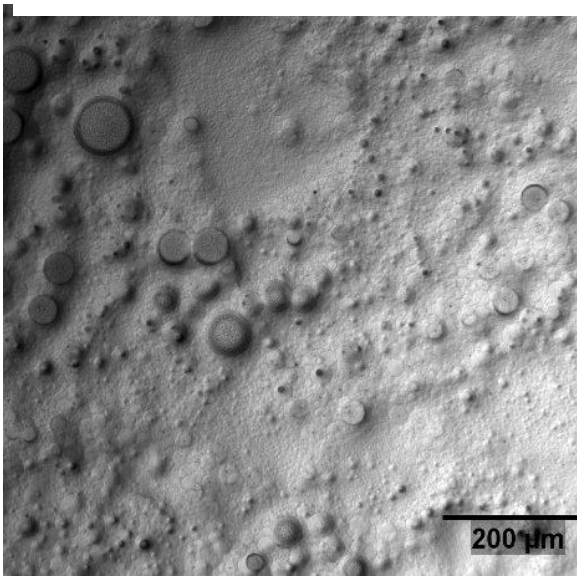


Figure 11. 10% glycerol formal concentration, bubbles form and the coat has a white appearance.

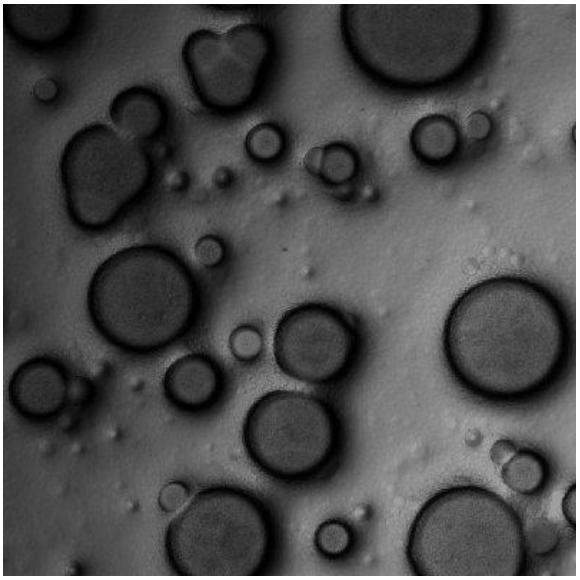


Figure 12. 30% glycerol formal concentration, large bubbles forming and making different phases within the coat.

Ordinary microscopic Pictures on coatings with different volumes of GF. The coat gets small bubbles; the scattering yielding a white foggy color with GF present. At 5% addition small areas form and with another addition of GF bubbles form. The bubbles increase in size the more GF you add.

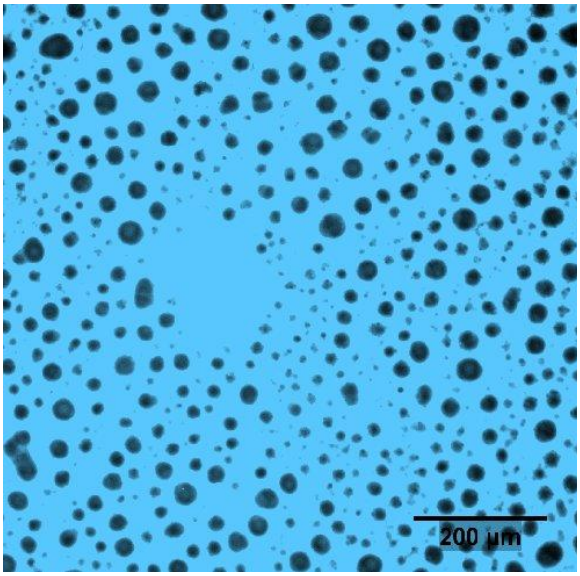


Figure 13. IVE with addition of glycerol formal, black bubbles contain no Ivermectin. The surrounding area have a very disperged Ivermectin

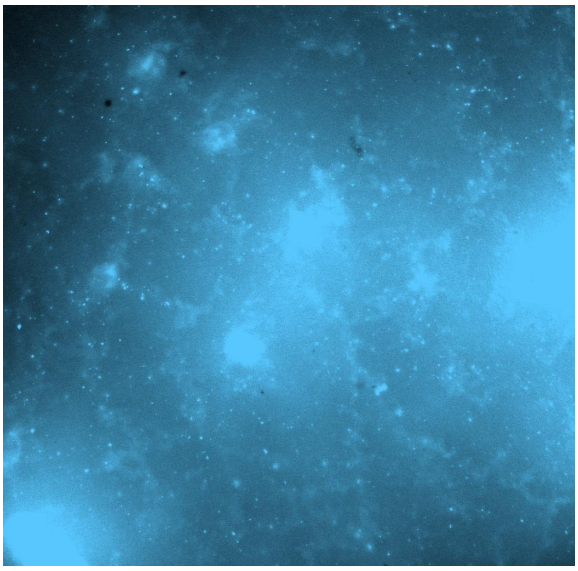


Figure 14. IVE with no addition of glycerol formal, Clusters of compact Ivermectin can be found.

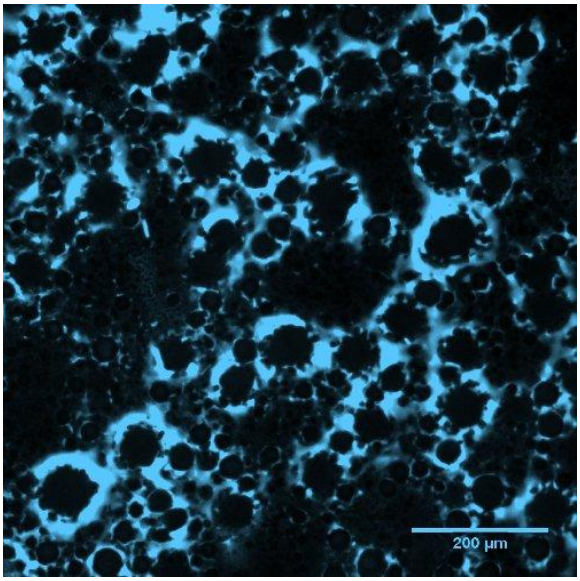


Figure 15. Hydroxy coumarin acetic acid mixed in pain with glycerol formal. Same disperge pattern as Ivermectin. The areas around the bubbles are not all fluorosent due to the molecule being less hydrophobic then Ivermectin.

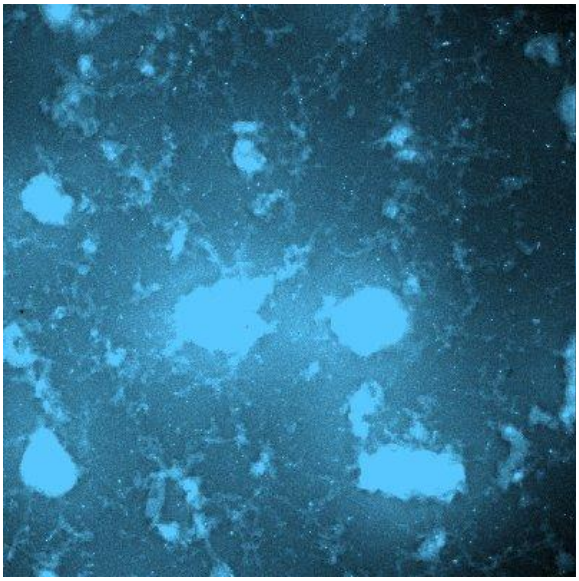


Figure 16. HCA with no addition of glycerol formal, very similar to Ivermectin. Clusters are more compact and more defined.

Pictures showing the behavior of Ivermectin and hydroxycoumarinyl acetic acid. The dispersion is similar for both GF containing coat and no GF. GF bubbles form in both coatings and the fluorescent molecule are in the surrounding area. With HCA the molecules are closer to the bubbles and not as dispersed, due to it being a more hydrophilic molecule than IVE and the coating is very hydrophobic. In coatings containing no GF the clusters can be seen in both coatings, more clear clusters for HCA again for being more hydrophilic.

Bleaching study of paint with glycerol formal

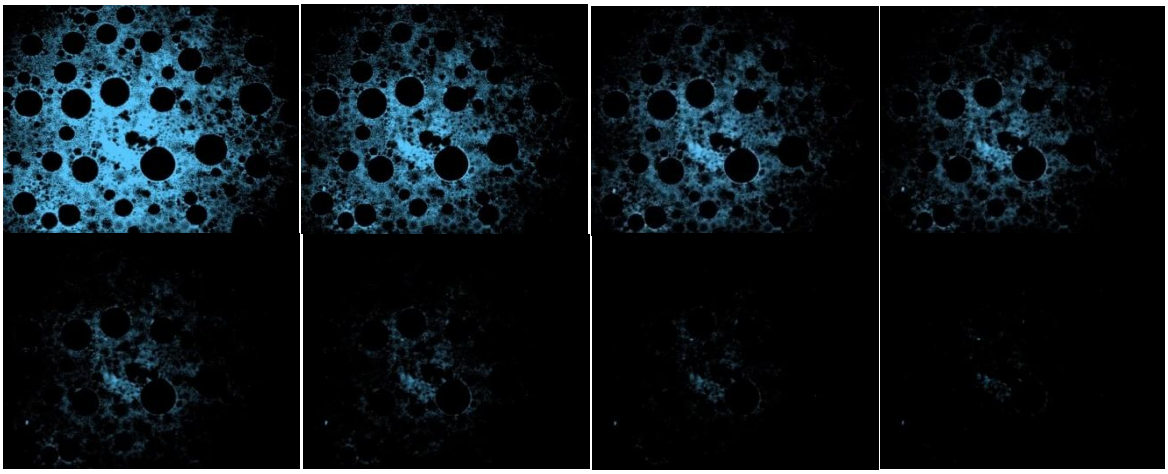


Figure 17

Bleaching of paint containing no glycerol formal

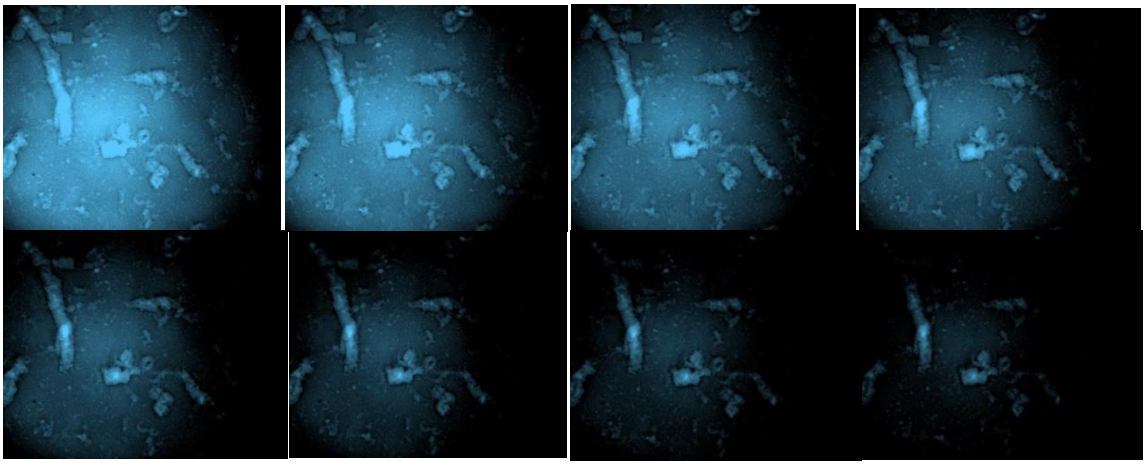
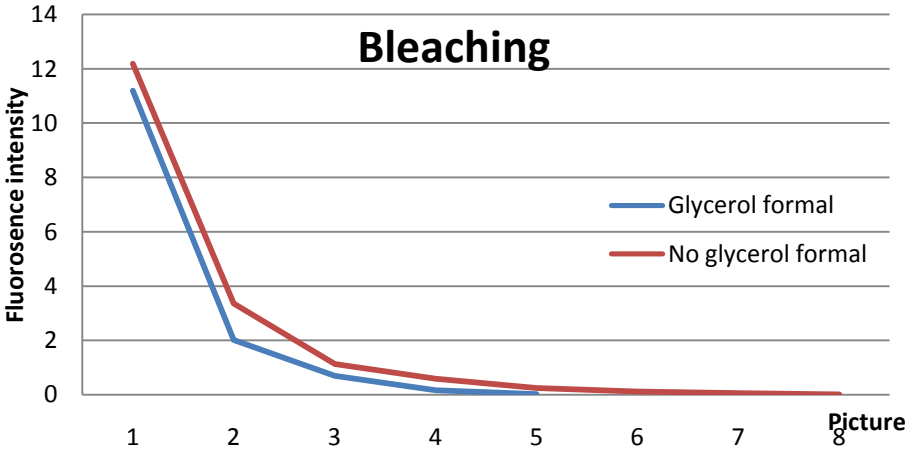


Figure 18

Series of pictures showing the bleaching of the fluorescent IVE. The intensity of each picture was calculated. The bleaching speed was determined to be very similar independent on GF. Small clusters can still be seen on the coat containing no IVE. The dispergered IVE that can be seen in later pictures for no GF is of such low intensity that calculations of intensity stop already at picture nr6. For GF there is no cluster that stays longer, and after about 4 pictures the intensity is nonexistent.



Picture	Intensity
GF - 1	11,2
GF - 2	2
GF - 3	0,7
No GF -1	12,2
No GF -2	3,4
No GF -3	1,1

Figure 19. Graphical representation of bleaching. Each picture was analyzed and fluorescence intensity (y-axis) was calculated for each picture. Each picture was taken in a rapid series (x-axis). The calculations reveal an equal bleaching of both surfaces. Paint containing no glycerol formal has clusters that take longer to bleach but the general bleaching process is the same regardless of glycerol formal concentration.

4.2 Drying of glycerol formal containing coatings

To answer the question if GF was evaporated or not from the coated layers, a simple coating-experiment was done in which all coatings were carefully weighed. Paint containing GF does not loose equally much of its solvent (table 1). The normal paint evaporates approximately 38-39% of its wet weight. With addition of GF the dried weight increases and thus the GF stays after the organic solvents have evaporated. From the pictures seen it is highly likely that it is GF that forms the small bubbles in the coat and that the bubbles are not empty. This is also supported by the pictures showing a small fluorescence in the bubbles. With calculations I confirmed that it is GF that stays in the coat. By removing the GF weight from both wet weight and dried weight the weight loss becomes 40% which is in line with the paints weight loss when not containing GF.

Glycerol formal	Wet weight	Dried weight	% Weight loss
0g (0%)	0.83g	0.51g	38.5%
0g (0%)	0.86g	0.53g	38.4%
0g (0%)	0.72g	0.44g	38.9%
0.11g (10% volume)	0.73g	0.48g	34.2%
0.09g (10% volume)	0.82g	0.59g	28.0%
0.10g (10% volume)	0.83g	0.52g	37.3%

Table 1 showing paints not containing and containing GF. The paints were applied on a surface and allowed to dry for 24h and thereafter I determined how much of the solvent that has evaporated. GF is still present after other solvents have evaporated.

4.3 Release fluorescent molecules in water

To give an exact concentration of how much Ivermectin that is eluted from the coatings, a chemical detection method was needed. Our method now was to take coatings containing Ivermectin, elute them and from there concentrate it in acetonitrile for derivatisation and detection. The calibration curve for this was done from water containing known amounts of Ivermectin. Tests were carried out with a Supelco Select HLB SPE 200MG/6ML column and eluted into acentonitril. The derivatization process was then carried out on the eluate from columns. After several tests it was concluded that it was very hard getting reproducible results and the calibration curve would be too inaccurate to rely on. Response values at 10-1200 for the same concentrations could be seen and any efforts to improve reproducibility did not help. This drawback made a change in method and instead of derivatization after elution; coatings were painted with already derivatized Ivermectin.

The calibration curve here was produced from known concentrations of derivatizated Ivermectin and the response from the detector is represented in Figure 21. It follows a linear pattern as expected and a calibration curve was calculated from it. We see an increased variation at responses for 0ng Ivermectin and 50ng per ml. The 0mg sample variations can be seen as the normal noise and that will be constant. Variations in 50mg per ml is not known why it appears and on the same sample concentration. All other samples were very consistent and no large variations could be seen. The linear approximation is calculated to [C] = 0.63*response -5.

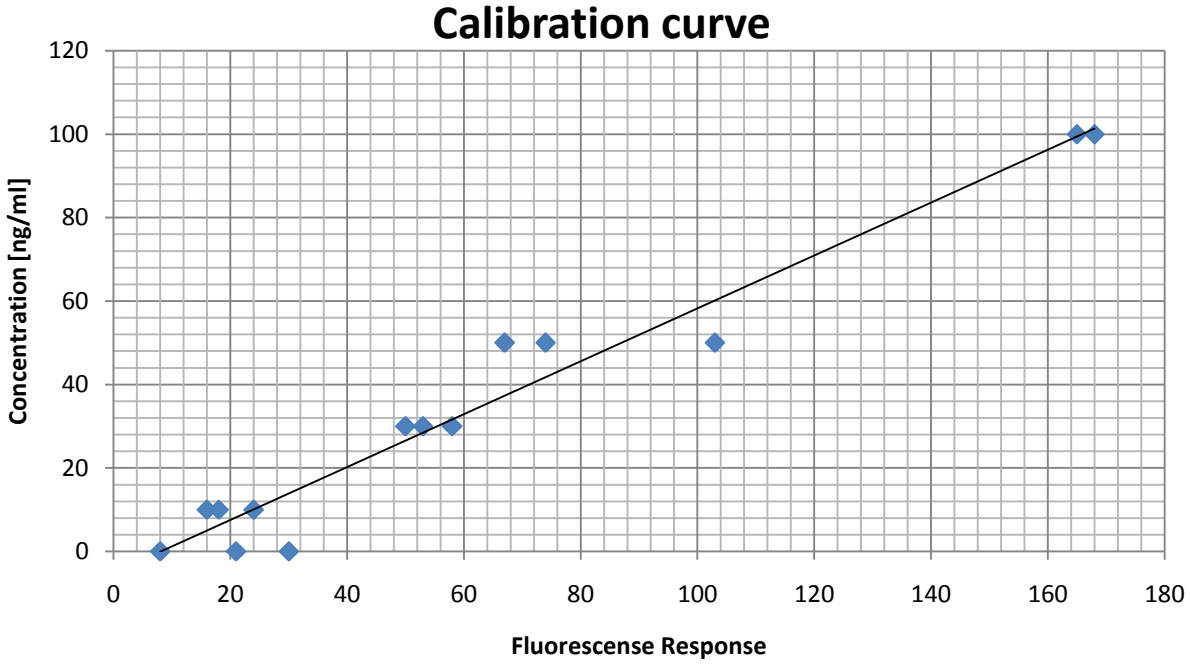


Figure 21. Calibration curve of fluorescent Ivermectin extracted from water. Calibration curve is linear with aberrations at concentrations of 0mg/ml and at 50mg/ml. 0mg/ml can be seen as detaction limit of the instrument simply regestring background. At 50mg/ml the aberrations have no good reason to show up. The linear approximation is calculated to [C] = 0.63*response -5.

Samples to test for elution were treated the same way as in the artemia assay. There were fewer samples here for the limited amount of fluorescent Ivermectin hence no coating containing 10mg per ml could be done. Samples containing 0% 2% 7% 21% GF with 1mg/ml Ivermectin were painted and set in elution for 16days. All samples showed a relatively low concentration of Ivermectin in the water. An increase in release could however bee seen for paints containing 7% or more GF. The effect from GF is that it increases the release of fluorescence but not in a linear fashion. The limit of having 7% GF seems to do something with the release rate. Complete fluorescence spectra of those coats containing 7% or more GF had more noise than the others (figure 22). The bubbles that we know here increase the general release of fluorescent molecules. If the measured fluorescence is strictly due to Ivermectin is hard to tell knowing that GF attracts other fluorescent molecules in the paint also.

A test sample was also studied that consisted only of water containing 1000ng/ml Ivermectin, but that was otherwise treated the same way as the other samples. The result for that showed the exact same as paints with higher contents of GF. It should however have shown the correct concentration and not a low concentration. This makes concentrations calculated from the real paints uncertain and no good knowledge can be obtained how much Ivermectin that is measurable following release. What we can say is that Ivermectin is not present in the water as fluorescent detectible molecule. It has either been broken down or adsorbed to the surrounding hydrophobic walls in the petri dish.

Sample	Fluorescence Response	Calculated Concentration [ng/ml]
0% GF	26	11,3
2% GF	29	13,3
7% GF	41	20,9
21% GF	49	25,9
Standard paint	25	10,7
Controll 1000ng/ml	43	22,1

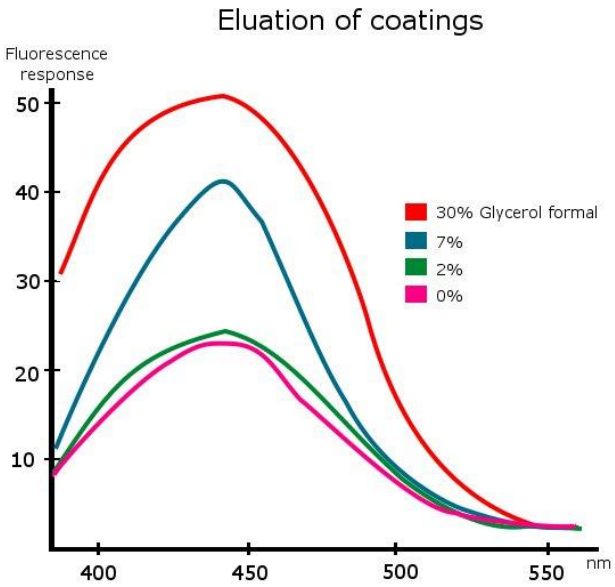


Figure 22. Results from elution of fluorescent Ivermectin. Samples were painted on 11x11cm Plexiglas and placed in water for 16days on a shaking table. Water was collected and extracted with column. The eluate was analyzed on fluoro spectrometer. The results can be seen in the graph and calculations from calibration curve can be seen above. Glycerol formal has the effect of increasing diffusion of fluorescent molecules. With 30% glycerol formal a wide peak can be seen. The wider peak implies more background than for Ivermectin alone. The calculations done for how high release of Ivermectin the different systems generate was done with bases on the peak value at 450nm. Calculations showed a low release between 10-25ng/ml. The control sample containing 1000ng/ml did however show a calculated concentration of 22ng/ml. This would mean that if we have a bigger release we cannot say that it shows with this method. Ivermectin is either degrades or adsorbed on the surrounding material. The results can therefore not be trusted and a new method for evaluating elution is needed.

4.4 Elution experiment with artemia toxicity

The artemia assay was carried out with different test solutions containing known concentrations of Ivermectin. Artemia was completely immobilized at a concentration of 100ng per ml after 1hour of exposure. After 24h complete immobilization could be seen at 10ng per ml of water. This is a higher *A. Salina* sensitivity than reported by others [17, 18]. Results were reproducible in several assays. The veterinary compound IVOMEC® containing 10mg Ivermectin per ml solution was tested as reference and showed similar toxicity as the pure preparation.

Ivermectin is a highly hydrophobic molecule and has strong affinity for soil and other particles [9-12]. An addition of colophony to the wells was added to see how Ivermectin toxicity was affected in water, and any change in toxic effect would be interesting. Colophony is a common component in many paints so Ivermectin should be in contact with it when added to paint. Test solutions with increasing colophony concentration reduced the acute toxicity the more colophony that was added dramatically. With colophony concentrations 40 times higher than Ivermectin a cut-off point in toxic effect was seen with no immobilization at all. 10-20 times higher concentrations showed a reduced effect when the Ivermectin concentrations were lower (fig 20).This shows that Ivermectin interacts with colophony and is not available to *A. Salina.* After 24h the toxic effect was however back to its normal values and no difference between colophony additions could be seen.

IVE : Colophony 1h exposure	100ug	10ug	1ug	100ng	10ng	1ng
1:0	100	100	100	100	0	0
1:10	100	100	50	0	0	0
1:20	100	100	70	70	0	0
1:40	100	0	10	10	0	0
1:60	100	0	0	0	0	0
IVOMEC®	100	100	100	100	0	0

Table 2 showing mortality of *A. Salina* after 1h exposure with different amounts of colophony added. The higher colophony concentration relative to IVE the less toxic effect is seen. IVOMEC® is a veterinary compound with 10mg IVE /ml. Both the pure IVE and IVOMEC® give the same toxic effect.

IVE : Colophony 24h exposure	100ug	10ug	1ug	100ng	10ng	1ng
1:0	100	100	100	100	100	0
1:10	100	100	100	100	50	0
1:20	100	100	100	100	100	50
1:40	100	100	100	100	100	50
1:60	100	100	100	100	100	50
IVOMEC®	100	100	100	100	100	0

Table 3 with mortality of *A. Salina* after 24h exposure. Different concentrations of colophony relative the Ivermectin concentration. There is no difference in mortality with different colophony concentration after 24h.

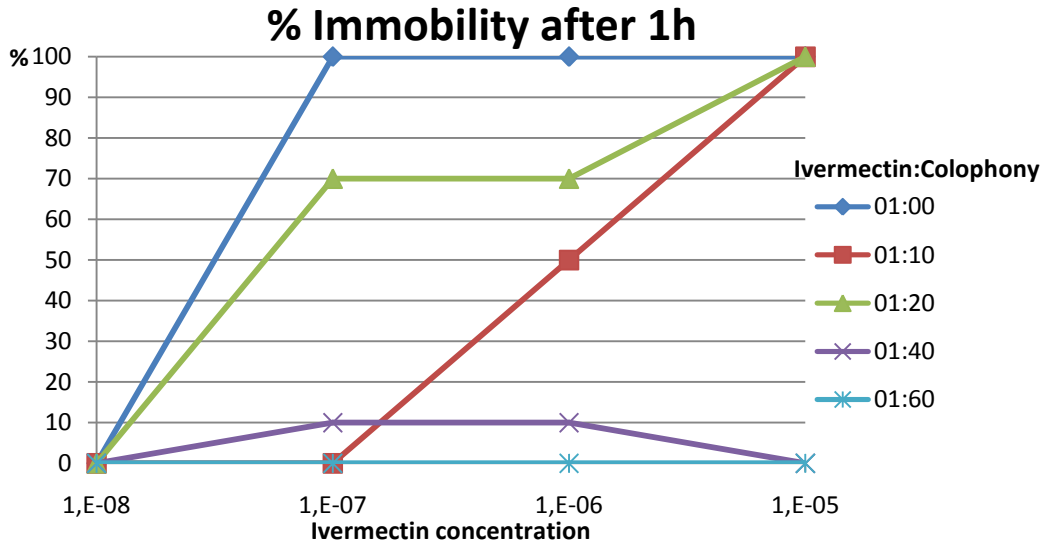


Figure 20. Graphical representation of table 2. showing how the immobilization is affected with different concentrations of colophony relative to Ivermectin. The higher concentration of colophony there is the less effect from Ivermectin. At 10 to 20 times more colophony a reduced effect is seen. With concentrations 40 to 60 times higher the toxic effect is removed. After 24h of exposure there is no difference in immobilization depending on colophony concentration. The colophony only affects the acute toxicity.

Varnish painted contained 3% 7% 21% GF and Ivermectin 1mg/ml and 10mg/ml extracted in water for 10days were tested. Samples were taken out at 1h 4h 24h and at last 10 days and saved for assay. The effect from Ivermectin on *A. Salina* was not noticeable and no difference could be seen in any of the samples. 1h, 4h and 24h showed no toxic effect at all with all artemia alive after 24h of exposure. Water collected after 10days of elution showed toxicity when not diluted, however the

water was toxic from all types of coatings including those with no Ivermectin and no GF. The paint itself does leak toxic substances into water after 10days of eluation that kills *A. Salina*. At a dilution of 1:10 the toxic effect could not be seen any more thus any concentration of toxic molecules was low and not related in any way to Ivermectin concentration in the paint.

5 Discussion

With all results given to us we can start thinking about how the coat works. What was known from previous studies was that 10% GF is needed to get the anti barnacle effect. The fluorescence microscopy shows how GF makes the coat contain bubbles. There is no increased distribution; you could talk about less area to be located on hence increasing the concentration in some parts of the coating. All in all there is a dramatic change in how the coat looks with addition of GF.

GF has an important role for the effect of Ivermectin. In what way it is unclear to say but that its presence enhances the effect is undisputed from field studies. GF not only changes how Ivermectin is dispersed in the layer but also makes the drying process slower and the paint feel stickier with addition of it. It has the property of working as a softener for the paint. So GF has 2 effects; to change the dispersion and to make the paint softer.

Ivermectin strongly binds to soil and other particles and it was seen that it was a reversible binding. In coatings it is bound and does not diffuse from the layer since no toxic effect can be seen in water. It does not hinder barnacle settling but inhibits barnacle growth. The toxicity stays in the coat and does not affect sea living organisms, as shown from the artemia assay. This principle can be seen as post settlement inhibition and is something new for the antifouling industry. All available paint is now based on emission of the biocide and thus makes the enviroment contaminated and the new research is concentrated on reducing release and therby the danger of biocide in the environment. What have been shown here is that there is no release of Ivermectin that affects artemia, normally sensitive to 1ng/ml. The effect is however confusing that it exist; a substance with strong affinity for the coat should not give a effect on the barnacle. The barnacle larvae settle on the surface and have no problem walking and finding a good position, but they stop growing when they should go through metamorphis. Ivermectin is not known to only affect the metamorphic state of barnacle any Ivermectin should have effect on the barnacle to any stage. What hypothesis we here can find is that the barnacle larvae attach and dig on the surface. When they push themselves close and into the coating they get so close that the Ivermectin becomes available and the organism gets affected by it. We have proven that colophony delays the effect of Ivermectin, with the artemia study showing only effect after 24h exposure. Ivermectin binds strongly but it is reversible and when you get close enough for a period of time it will be toxic. This is a new principle based on contact inhibition that can be found on seaweeds [20].

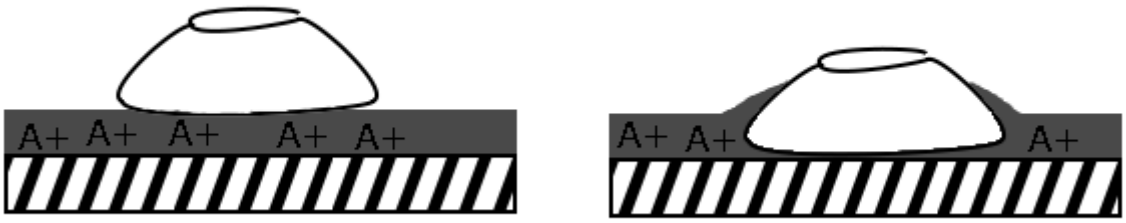


Figure 23. Hypothethis of how the antifouling effect is achieved. To the left we have a hard paint where the barnacle cannot dig. To the right a paint that is soft where the barnacle is digging into the paint and with that it gets very close to the biocide. Biocide is tightly adsorbed into the coating but can in close contact be exposed to the organism. Pictures showing how barnacle dig can be seen in figure 6. Results of exposure of Ivermectin adsorbed to colophony shows toxicity to artemia. This strengthens the hypothesis that we have a contact inhibition.

The release rate of Ivermectin could not be measured with available equipment. Irreproducible results when trying to derivate and detect from water. Studies done before were carried out with HPLC; here it was done with a fluorospectrometer. The measurements of fluorescent Ivermectin from water were done with good results. However the result with water at a known concentrtation

of 1000ng/ml showed did not show accurate calculated concentration. This could imply that Ivermectin is broken down or that it adsorbs to the petridish. The method used here for eluting and detecting is not enough to say if the Ivermectin can do harm to the environment. If the Ivermectin is degraded it will not cause any environmental problems, if it binds to particles then the possibility of it leaving the coating free is very unlikely. The concern for sediment living organism is an area where no answer can be given. The frequently used eroding paints will with time release all biocide in the water. Any particles containing the Ivermectin might now be dangerous to the environment, not acutely but during a long term exposure as seen with *A. Salina*. Ivermectin can be enriched in the sediment where sensitive worms are dwelling and cause damage if they are exposed in the same way as the barnacle when eating it.

How Ivermectin affects the barnacle is currently not exactly known. To get an exact and detailed knowledge there is necessary to have a biological study of how Ivermectin can reach the barnacle. The strange part is that the toxic molecule becomes available with no evidence of leaching out of the water. With a full biological study of the barnacle the exact mechanism of the paint would be solved. Further studies are needed to get an exact value of how much Ivermectin that is released, with an eroding paint it will always come out in the environment. This also gives the thought of having a non eroding paint. Thinking of erosion and leaching as artifacts in the system a new concept of non leaching paint could be brought out on the market.

A new environmental interest from boat owners and governments would find that contact inhibition is an interesting alternative. The enhanced effect can also be added to current eroding paints to get a dramatic effect reducing a lot of work for boat owners today.

6 Conclusion

GF changes how the coating surface looks by creating small bubbles. These bubbles are seen at 7% addition of GF, the same critical point where no barnacles adhere. The exact function of GF cannot be explained more than that it is needed for the antifouling effect. The analysis of coatings with Ivermectin as biocide showed that Ivermectin is not leaching from the coat in large quantities. There is no biological effect on *A. Salina* from any of the coatings. The chemical detection method did not yield good enough results and the exact value of how much Ivermectin that is leaching out cannot be said. It has to be redone and done with liquid chromatography in analogy as most published methods for detecting Ivermectin is done.

The combined new results have given a hypothesis of how the biocide gets in contact with the fouling organisms. A new principle is formulated based on inhibition on contact instead of the normal release of toxic material. The hypothesis needs to be further investigated and tested, especially with reproducible and sensitive detection methods for Ivermectin.

7 Acknowledgements

I have really enjoyed working at Goteborg's University with the Interface Biophysics group and I especially want to thank Hans Elwing for giving me the opportunity to do my degree project in his research group. I would also like to thank Emiliano Pinori for his great support and feed-back on everything on laboratory work. Furthermore, I would like to thank Matias Berglin for his many ideas during the project, Mats Hullander for all of his beautiful pictures on all the panels.

8 References

1. Chambers, L.D., et al., *Modern approaches to marine antifouling coatings.* Surface and Coatings Technology, 2006. **201**(6): p. 3642-3652.
2. Callow, M.E. and J.E. Callow, *Marine biofouling: a sticky problem.* Biologist (London), 2002. **49**(1): p. 10-4.
3. Champ, M.A., *A review of organotin regulatory strategies, pending actions, related costs and benefits.* The Science of The Total Environment, 2000. **258**(1-2): p. 21-71.
4. Yebra, D.M., S. Kiil, and K. Dam-Johansen, *Antifouling technology--past, present and future steps towards efficient and environmentally friendly antifouling coatings.* Progress in Organic Coatings, 2004. **50**(2): p. 75-104.
5. Wahl, M., *Marine Epibiosis .1. Fouling and Antifouling - Some Basic Aspects.* Marine Ecology-Progress Series, 1989. **58**(1-2): p. 175-189.
6. Abarzua, S. and S. Jakubowski, *Biotechnological Investigation for the Prevention of Biofouling .1. Biological and Biochemical Principles for the Prevention of Biofouling.* Marine Ecology-Progress Series, 1995. **123**(1-3): p. 301-312.
7. Aldred, N. and A.S. Clare, *The adhesive strategies of cyprids and development of barnacle-resistant marine coatings.* Biofouling, 2008. **24**(5): p. 351-363.
8. Õmura, S., *Ivermectin: 25 years and still going strong.* International Journal of Antimicrobial Agents, 2008. **31**(2): p. 91-98.
9. Davies, I.M. and G.K. Rodger, *A review of the use of Ivermectin as a treatment for sea lice [Lepeophtheirus salmonis (Kroyer) and Caligus elongatus Nordmann] infestation in farmed Atlantic salmon (Salmo salar L.).* Aquaculture Research, 2000. **31**(11): p. 869-883.
10. Fisher, M.H. and H. Mrozik, *The Chemistry and Pharmacology of Avermectins.* Annual Review of Pharmacology and Toxicology, 1992. **32**: p. 537-553.
11. McKellar, Q.A. and H.A. Benchaoui, *Avermectins and milbemycins.* Journal of Veterinary Pharmacology and Therapeutics, 1996. **19**(5): p. 331-351.
12. Davies, I.M., et al., *Environmental risk of Ivermectin to sediment dwelling organisms.* Aquaculture, 1998. **163**(1-2): p. 29-46.
13. Berendsen, B.J., P.P. Mulder, and H.J. van Rhijn, *The derivatisation of avermectins and milbemycins in milk: new insights and improvement of the procedure.* Anal Chim Acta, 2007. **585**(1): p. 126-33.
14. Jiang, H.Y., et al., *LC-fluorescence detection of abamectin, Ivermectin, doramectin, and eprinomectin in rabbit feces.* Chromatographia, 2008. **68**(3-4): p. 259-262.
15. Knold, L., et al., *Validation of a simple liquid chromatographic method for determination and quantitation of residual Ivermectin and doramectin in pig liver.* Journal of Aoac International, 2002. **85**(2): p. 365-368.
16. Blizzard, T.A., et al., *Brine Shrimp (Artemia-Salina) as a Convenient Bioassay for Avermectin Analogs.* Journal of Antibiotics, 1989. **42**(8): p. 1304-1307.
17. Grant, A. and A.D. Briggs, *Toxicity of Ivermectin to estuarine and marine invertebrates.* Marine Pollution Bulletin, 1998. **36**(7): p. 540-541.
18. Dharia, J.R., B.M. Gavande, and S.K. Gupta, *Ecotoxicity Studies of Some Organotin Monomers and Polymers.* Toxicological and Environmental Chemistry, 1989. **24**(3): p. 149-154.
19. Faÿ, F., et al., *Antifouling activity of marine paints: Study of erosion.* Progress in Organic Coatings, 2007. **60**(3): p. 194-206.
20. Wikstrom, S.A. and H. Pavia, *Chemical settlement inhibition versus post-settlement mortality as an explanation for differential fouling of two congeneric seaweeds.* Oecologia, 2004. **138**(2): p. 223-230.

All pictures have been approved of use by prof. Hans Elwing Gothenburg University