Low Biocide Emission Antifouling Based on a Novel Route of Barnacle Intoxication

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AKADEMISK AVHANDLING

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Cover picture: “Panel painted with ‘Low Biocide Emission Antifouling’; treatment (down) compared to the control side (up)”. - *Photo by Mats Hulander*

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Dedicato ai miei piccoli '*juveniles'* Luca e Max

e alla mia *'megabalanus rosa'* Pernilla

Abstract

Marine biofouling can be defined as the colonization of man-made surfaces in seawater by microscopic and macroscopic organisms. This phenomenon can result in great loss of function and effectiveness both for cruising ships and for static constructions. Of special concern are the negative effects of hard fouler such as barnacles, which cause increased drag resistance resulting in increases in fuel consumption, and disruption of the corrosion protective layer of marine vessels and constructions. Present biocide-based antifouling strategies are based on a continuous exposure of biocides at the film/water interface and consequently release into the environment if the antifouling efficacy is to be maintained. Such biocide-based solutions can therefore not be regarded as sustainable.

The aim of this thesis is to describe the possibility to design biocide antifouling coatings based on a new strategy. Instead of releasing the bioactive molecule to the bulk water the biocide will be “entrapped” in the paint matrix and only after stimuli by organism interaction with the paint surface intoxication will take place. It was shown (Paper I) that using an experimental formulation, containing ivermectin, both in static panels and on boats, long lasting protection against barnacles was obtained. Moreover, using two model surfaces (Paper II), it was possible to separate and study the different contributions to the antifouling efficacy, finding that the low leaching of ivermectin had no contribution at all while surface’s modulus of the coating was the key factor. This supports the validity of the contact active antifouling hypothesis, rather than emission based. In (Paper III) we could follow the fate of barnacle growing on ivermectin containing coatings, and both field and laboratory tests could demonstrate that the intoxication of barnacles start when the juvenile organism reach ca. 0.6-0.7mm in diameter. Electronic microscopy images on the panels after the test, demonstrate that on control paint (no biocide) the juvenile barnacles (0.6-0.7mm diameter) already leaves imprint or penetration marks on the rosin based coatings. The distribution of ivermectin in the dry film seemed to be related with enhancement of barnacles contact intoxication. This was studied by fluorescence microscopy in (Paper I) and by the use ToF-SIMS in (Paper IV). This particular analytic method gives the possibility to follow organic biocides in paint film without the need of labelling or modify the biocide molecule in any extent.

The entrapped antifouling strategy opens up the possibility to achieve long term antifouling (>10 years) as there is no need to use erosive binders. Moreover, this system might also find it uses in marine constructions and other fields where maintenance is difficult.

Populärvetenskaplig sammanfattning

Det mesta av allt vi äter, använder eller har runt oss har kommit till oss med fraktfartyg och ofta transporterats runt halva jorden. Långväga frakter blir allt vanligare och mer accepterade i den globala handeln då sjöfart är det bästa och billigaste sättet att transportera ur miljösynpunkt. Vad vi vanliga konsumenter inte tänker på är att denna flotta förbränner ca 400 miljoner ton fossilt bränsle per år och skulle förbränna omkring 40% mer om skroven lämnades obehandlade, vilket skulle innebära flera hundra miljoner ton mer CO_2 ut i atmosfären varje år. Förutom miljöperspektivet inser vi kostnadsökningen. Tänker man sig att alla bilar och långtradare skulle köra på musselskal och havstulpaner i stället för på släta asfaltvägar kan man förstå vad det innebär för bränsleåtgången. Det framstår klart att problemet med påväxt behöver lösas och varför det är viktigt att oberoende forskare är involverade, både för att hålla ett öga på den viktiga bränslebesparingen men också för att vara medvetna om de möjliga konsekvenserna för miljön vid olika åtgärder som vidtas.
Effekten av nuvarande biocidbaserade bottenfärger baseras på ett ständigt utsläpp av biociden till färgens yta och sedan ut i det omgivande bulkvattnet för att upprätthålla effekten. Sådan miljöpåverkan på havsvattnet kan av miljöskäl i längden inte accepteras.

I min avhandling beskriver jag hur man kan bli av med en av de mest arbets- och kostnadskrävande organismerna, havstulpan, utan att behöva frisätta gifter till havet. I stället för att frisätta det bioaktiva ämnet till bulkvattnet är vår biocid infångad i färgstoffet och frigörs först när en organism försöker ta sig igenom ytan. Vi har satt ut plattor med olika bottenfärg i olika slags havsvatten och undersökt påväxten vid olika tidpunkter. Redan i ett första försök kunde man uppnå full och långvarig effekt mot havstulpaner med färg som byggts upp efter detta nya koncept (Paper I). I en annan studie (Paper II) visade det sig att ett lågt läckage av biociden inte bidrog till att hindra snäckpåväxt, men att ytans egenskaper (hårdhet) var nyckelfaktorn för att hindra denna. I ett senare arbete (Paper III) tittade vi närmare på vad som händer med havstulpaner när de försöker etablera sig på försöksytorna. Då kunde vi visa att havstulpaner kan sätta sig och klistra sig fast på ytor tills de blir 0.6 - 0.7 mm i diameter. Då börjar de påverka färgen och frisätter därmed biociden. De gräver alltså sina egna gravar.

Riassunto a carattere scientifico divulgativo

Il ‘fouling marino’ puó essere definito come colonizzazione di superfici immerse in acqua di mare, da parte di organismi microscopici e macroscopici. Questo fenomeno può causare grande perdita di funzionalitá e di efficacia sia per navi che per costruzioni statiche. Di particolare interesse sono gli effetti negativi di organismi calcarei come i balani, meglio conosciuti con il termine comune di ‘denti di cane’, che causano un aumento della frizione e conseguente aumento del consumo di carburante fino al 40%. Inoltre, una caratteristica qui molto importante dei ‘denti di cane’, é quella di penetrare lo strato di vernice protettiva anticorrosione di navi e costruzioni marine. Le vernici antivegetative presenti sul mercato si basano sull continuo rilascio di biocidi dalla superfice e questo comporta accumulo di sostanze nocive nel sedimento e nella biosfera. Di conseguenza, per loro natura, tali soluzioni non possono essere considerate ecosostenibili. Lo scopo di questa tesi è quello di dimostrare la possibilità di progettare vernici antivegetative basate su una nuova strategia. Invece di liberare il biocida all´interfaccia e quindi in mare, questo viene trattenuto nella vernice e solo dopo uno stimolo meccanico, attuato dall’interazione tra l'organismo e la vernice stessa, l´intossicazione del organismo incrostante ha luogo. E 'stato dimostrato, sia in test svolti su pannelli che su barche, che una vernice progettata su questo principio garantisce risultati duraturi contro i balani con un rilascio di biocida vicino allo zero e con quantitá utilizzate pari allo 0.1% del peso della vercice (1g/Litro). Utilizzando poi due superfici modello, è stato possibile separare e studiare i diversi fattori che concorrono all´efficacia. É stato dimostrato che il basso rilascio del biocida non aveva alcun contributo sulla efficacia antibalano, mentre la durezza della vernice ne era il fattore chiave, dimostrando ulteriormente l´ipotesi avanzata. Al fine di ottimizzare la futura progettazione e l'ottimizzazione delle vernici a zero emissioni di biocidi, uno studio piú approfondito del meccanismo era necesario. Quindi, in un più recente lavoro, abbiamo potuto seguire i balani durante il loro attacco e sviluppo, e siamo stati in grado di dimostrare che l'intossicazione dei balani utilizzando il biocida “intrappolato” inizia non prima dello stadio *juvenile* (circa 4-7 giorni dopo l'insediamento), quando i balani sono ca. 0.6-0.7 millimetri di diametro. Immagini di microscopia elettronica dimostrano che a questo punto i balani lasciano già un'impronta di penetrazione sulla vernice. Questo nuovo approccio apre la possibilità di raggiungere effetti senza bisogno di rilasciare i biocidi in mare. Si puó pensare ad un effetto a lungo termine (> 10 anni) in quanto non vi è alcuna necessità di usare vernici erosive. Inoltre, questo sistema potrebbe anche trovare applicazione nelle costruzioni marine e altri campi in cui la manutenzione è difficile e l´erosione da parte del flusso dell´acqua é minima.

List of publications

This thesis is based on the following papers, referred to in the text by their Roman numerals (I-IV)

I. E. Pinori, M. Berglin, L.M. Brive, M. Hulander, M. Dahlström, H. Elwing
Multi-seasonal barnacle (*Balanus improvisus*) protection achieved by trace amounts of a macrocyclic lactone (ivermectin) included in rosin-based coatings.
Biofouling Vol. 27, No. 9, **2011**, 941–953

II. E. Pinori, H. Elwing, M. Berglin.
Impact of coating hardness on anti-barnacle efficacy in embedded biocide antifouling.
Biofouling (Accepted for publication)

III. E. Pinori; A. Holmqvist; M. Berglin; M. Dahlström; H. Elwing
The fate of cyprid and juvenile barnacles on rosin paint with 0.1% ivermectin.
In Manuscript

IV. M. Berglin, E. Pinori, H. Elwing, P. Sjövall
Distribution of Organic Biocide in Antifouling Paint as Determined using Time of Flight Secondary Ion Mass Spectroscopy (ToF-SIMS).
In Manuscript

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Preface and personal reflections

Usually when someone introduces itself as “researcher” working on “boat paints” the reaction is often a mix of curiosity and surprise when the words “boats” and “paints” arrive to the ear and are reflected on the face of the listener. Why should the researchers demonstrate interest in fighting marine organism from attaching on ship hulls? Is this not more a product development matter? Why should independent research address such questions?

If you will continue reading this book despite this statements this could be because you are involved in marine fouling yourself or are a member of the committee at my dissertation, either one of my friends/relatives or most hopefully, because you are a person moved by genuine curiosity. So let’s try to put the question in another perspective and demonstrate why these matters are interesting and touch our lives every day.

The majority of the goods we consume or use or just have around us every day have been transported by a cargo ship. These goods have travelled the half of the globe circumference to arrive in our hands. This is more and more common and accepted in the global market society and at the same time, is recognised as the best way to transport things in term of environmental impact. What the usual consumers ignore is that this fleet of ships, moving goods around the world, consume ca. **400 million tonnes fossil fuel** per year and would consume 40% more if their hulls were leaved untreated; this would mean **450 million tonnes more CO_2** emitted into the atmosphere every year. Beside the environmental perspective we can try to think about costs involved. Try to imagine if all the cars and trucks of the world should travel on mussels and barnacles instead for on smooth asphalt roads; then it should become more evident what this mean in term of fuel consumption. It will result clear why this problem needs to be solved, and why it is important to do that with independent researchers involved, keeping an eye on fuel saving aspects but also keeping in mind the possible negatives consequences to the marine environment due the countermeasures taken.

How do we control marine fouling today? It is done in a sustainable way? What could be done tomorrow to do lower the environmental impact? These are the questions discussed in this thesis.

1 BACKGROUND AND STATE OF THE ART

1.1 Brief history of antifouling development

Marine biofouling is a natural process with unwanted consequences on man-made surfaces. Micro- and macro- organisms find manmade surfaces immersed into the seawater as a very interesting place to settle on and grow. The fight for clean surfaces to colonize is hard between these organisms and novel immersed and free surfaces are always welcomed and fast covered by colony of organisms. A ship hull covered by soft fouling (bacterial and microalgae based film) face an increment in drag force up to 3-10% [1]. A ship hull covered by hard fouler (macro algae or calcareous organism such as barnacles) face an increment in drag and calculated increase in fuel consumption up to 40% compared with a cleaned and smooth hull surface [1-4]. Men have sailed the sea starting ca. 700 BC (Phoenicians and Carthaginians). Since then they have faced and combated marine fouling. This means that antifouling techniques have been developing in the last 2700 years starting with tar and wax containing different heavy metals and toxins known at the time.

In more recent times, copper sheathing was regarded as the best performing antifouling system. One proverbial and often cited example of the efficacy given by copper sheathing is Nelson’s victory at Trafalgar (1805). The fleet of the Royal Navy was copper bottomed while the French fleet was not. The faster sailing speed and the increased manoeuvrability could be one of the explanations for English victory according to many historical commenters.

In the second half of 19th century the need for larger ship and the industrial revolution give birth to the era of iron hulled ships. The wood made ships were over. This solved many problems such as mechanical resistance and operational lifetime, but unfortunately the effect of galvanic corrosion excluded the usage of copper sheathing for iron hulls. New solutions were sought for and paints containing toxins made the first debut. This can be considered the start of antifouling coating system as we know it today. Actually a lot of improvement has been done since that debut [5].

In the 1960 the fight against marine fouling was won with the introduction of Tributyltin (TBT). This very effective solution for iron hulls have shown unwanted environmental consequences [6]. TBT containing coatings have been globally banned since 2008 [7] after a long debate [1, 8, 9]. Nowadays the most adopted solutions goes under the name of tin-free antifouling paints , which are paints containing copper oxide and other co-biocides in soluble paint matrix [5]. These paints are still now the market leader with ca. 90% of the global fleet painted by copper containing antifouling paints.

1.2 The impact of fouling and antifouling

Some estimation made by International Maritime Organization (IMO), reveals that 90% of the global trading is based on ship transportation. The total fuel consumption for ship transportation, according to IMO (MARPOL, Annex VI) and EPA (Global Trade and Fuels Assessment, 2008) was estimated to be ca.370 million tonnes per years in the 2007 (with corresponding 1120 million tonnes CO_2 emitted) and will reach by 2020 ca.500 million tonnes (1475 million tonnes CO_2 emitted). A ship hull fouled with calcareous fouling compared to a smooth hull would show a rise in drag force and powering penalties up to 86% at cruising speed [3]. Efficient antifouling systems save nowadays ca.150 million tonnes fuel and ca.450 million tonnes CO_2 from being emitted in the atmosphere every year.

The strongest driving force in antifouling research is obviously fuel saving, but reaching this goal without impacting on the marine environment is the scope of the synergy between academy and industry. The hard equation to solve has on the left side, feasibility and good economical profile, and to the right side, the long lasting efficacy and being environmentally benign. Solving only one of these two terms of this equation is not enough for the future of sustainable antifouling.

Together with fuel consumption and problem in manoeuvrability [9], fouled ship's hulls and oil rigs [10-13] are known to be a vector for non-indigenous species transportation. Fouling organisms growing on man-made surfaces can also trigger corrosion [14-16], cause cooling system malfunction [17], mechanical failure and gain of weight of static structures in aquaculture [18, 19]. Another cost related to antifouling techniques is that, no matter which antifouling system the ship owner will choose, at regular intervals (3-5 years) depending on the operating conditions, the ship will need to be dry-docked in order to clean and repaint. This operation comport a down time for the ship owner that is very expensive itself. This is why long service life time of the coating is demanded by the market.

To make an impact on the environment a key factor is to penetrate the market, other ways the innovative solution will remain a very good idea with very little applicability, not making much of an impact in real life. After 30 years of scientific contributions on this subject still 90% of the global fleet is painted with copper containing paints, something have to be reconsidered, maybe we need to shift focus from the Petri dishes to ship hulls scale, losing a little bit of scientific elegance but risking to make a stronger impact into real life antifouling.

2 INTRODUCTION

In this thesis I discuss an alternative approach to the classical biocide release systems - an approach based on the ‘chemically enhanced post settlement mortality of barnacles’. This enhanced post settlement mortality is achieved by trace amount of biocide embedded in the paint, playing with physiochemical aspects of both the biocide and the paint components, which result in low release rate of the biocide (possible no-release if optimized further), long lasting antifouling efficacy, affordable cost of matrix materials and biocide, and high flexibility of applications, both on cruising or static surfaces. A unique aspect of this thesis is that, not only the scientific aspects (i.e. pharmacology aspects of antifouling; marine biology; material science; chemistry; biomimetic aspects etc.) but also feasibility and up-scalability aspects are taken into great consideration. This means that from the very beginning of idea evaluation and development, the both terms of the hard to solve equation have been taken into adequate consideration in order to present a sustainable and feasible antifouling alternative.

2.1 Marine fouling

For a didactic and generalized description, we could summarize the marine fouling as a regular succession of absorption and settlement of different organic molecules and organisms on an immersed surface (figure 1).

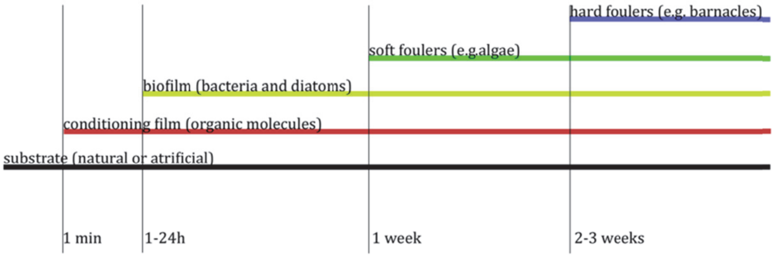

Figure 1. *Simplified temporal succession of biofouling process (adapted from [5])*

As soon as a new surface is immersed in the seawater, a cascade reaction starts. Immediately a ‘conditioning film’ involving organic compounds (e.g. proteins, lipids and polysaccharides) is absorbed on the fresh surface [20]. On this conditioning film the biofouling process can start. Already after some hours from the immersion mono-cellular organisms like bacteria, yeasts and micro-algae or diatoms absorb on the conditioning film. The bacteria produce and secrete extracellular matrix, made essentially of polymeric compounds (i.e. exopolysaccharide). This results in the formation of a ‘biofilm’. The biofilm support bacteria with channels for nutrient and other chemical communication and mechanical protection. The biofilm on a ship hull is even called ‘slime’ and can trigger corrosion and increase the drag force [1-4]. After one week from immersion spores of macro algae and other soft colonizers present into the water tend to be recruited and settle on this biofilm. After 2-3 weeks from the first immersion the larvae of benthic organism like crustacean, bryozoan, molluscs etc. called ‘hard fouler’ have attached and are now growing on the surface. Now the drag force can rise up with a calculated increase in fuel consumption up to 40% [1-4] compared to a smooth hull. Moreover this usually implicates faster corrosion, loss of function, mechanical failure and gain of weight.

This sequence is a generalisation of the different patterns with several level of complexity that we can find in a real life situation. The marine fouling phenomenon varies in intensity and species involved with latitude and longitude. The abundance of larvae is dependent from the temperature and the light period, thus the fouling pressure result more constant over the year in tropical waters and more seasonal in the temperate regions. The succession also is not necessary as we described here for generalization and description purpose. In some case the macrofouler can settle without the presence of a biofilm or in other cases the presence of certain strain of bacterial biofilm can promote or reduce the settlement and recruitment of other macro species [21, 22]. Many studies have been published regarding the influence of bacterial biofilm to the subsequent recruitment in marine biofouling [23-27]. Moreover, it was only in recent times, with the expansion of the human activities off-shore and in deep water conditions (i.e. drilling structures exploiting off-shore oil reservoir, or deep water pipelines) that we had the opportunity to investigate and discover biofouling even at those unexpected depth [12]. Specialized fouling organisms can survive the low light and cold temperature. The fouling community is very heterogeneous, composed by ubiquitous and adaptive represents such as barnacles, together with very specialized ones.

In general we can conclude that attaching on solid surfaces is crucial for many aquatic organisms, both sessile and vagile. There is in fact a lack of free solid surfaces in nature. So the succession of the biofouling and the final result will be dependent of which organism is more favoured in a certain environment, because biofouling is a competition of the different organisms for the surface. The question is not if a surface will or will not be fouled by these organisms, but how long we can retard this phenomenon by employing fouling control system.

2.2 Biocide based antifouling

The antifouling technology as we know it today is mainly represented by biocide based antifouling paints. This started after the introduction of the iron hulled ships during the first half of the 19th century. At that point of the evolution of the shipping industry, wood ships were no longer sufficient to cover the demand of the naval transportation of goods and passengers. Copper sheathing was no longer possible due to galvanic corrosion problems. The introduction of paint containing copper oxide was the logic solution to this problem. Other toxins became the most popular solution for controlling fouling. The toxins or active ingredients used in antifouling paints will hereby called biocides to harmonize our terminology to the newly introduced regulations. Some regulatory aspects for the modern antifouling will be described later in this chapter.

The general principle of antifouling paints is to create a protective layer around the ship hull, working as control delivery system for the biocides. In order to achieve this goal, several components are necessary in the paint formulation, in order to control and maintain the release rate of the biocides. The released biocides have to be bioavailable to the target organisms at the surface. The release rate of the biocides from the paint matrix has to be kept above a limit threshold in order to reach and maintain a minimum inhibition concentration (MIC) of the biocide at the exposed surface [28, 29]. Because of the high cost bounded with the dry-docking for repainting, one of the market demands for antifouling system is a long in-between docking interval, typically now 3-5 years, but in the past even 7 years when TBT containing paints was allowed. For the above mentioned reasons the design of biocide based antifouling paint systems have been developing and changing during the last years [5]. In the past the insoluble paint matrix were used and the biocide was released by diffusion solely. This means a constant lowering of the release rate with time, as the distance from the biocide containing part of the matrix and the surface increase and the release rate become insufficient to reach MIC. Usually with insoluble matrix systems the ship owner was forced to clean and repaint the hull when much of the initially loaded biocide still was present into the coating, with logical negative economic and environmental consequences. More recently the design shifted into systems employing soluble paint matrix. These systems are called, depending of the mode of erosion employed, “self-depletion coating”, “ablative coating” or “self-polishing coating”. Taking advantage from the hydrolysis and the erosion of the active polymer constituting the paint the matrix, the dissolution of soluble pigments, the mass transport of other component of the coating [30] it is possible to keep similar relation between depletion of the biocide and the depletion of the matrix itself keeping the release rate above the threshold for the (MIC) minimum inhibition concentration of biocide. This gives longer lifetime of the coating and a better economical profile [29, 31-34] (figure 2).

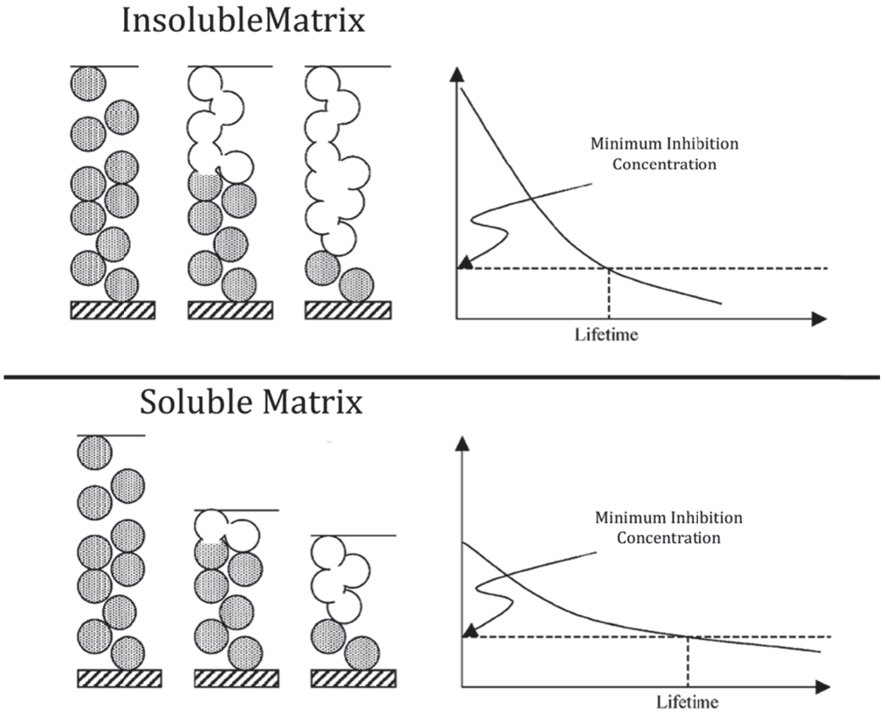

Figure 2. *Insoluble and soluble matrix, different mechanisms for sustained release of biocide. (Adapted from [5])*

The biocide release rates of the most common antifouling systems are usually expressed in micrograms per square centimetre per day [35]. The risks of unwanted impact on non-target organisms and bioaccumulation of biocides into the biosphere has always been considered as the main ecological drawback of this method. Looking deeper into this approach it become clear that another important technical drawback is the hard solved equation between the research of longer between-dry-dock interval and the constant erosion of the paint-matrix needed in order to maintain the sustained release of the biocides [29]. The paint industry must fine tune the different factors influencing this equation like loading amount of biocides in the coating, erosion rate of the matrix, water condition during the employment of the painted surface and like pH temperature and water flow on the surface [28, 36]. Unfortunately the fouling pressure is often higher when the erosion rate is lower, i.e. inside the marina, when the ship is not operating at cruising speed. The biocide based paint tends to release less biocide when most is needed and more biocide when less is needed. Thus optimisation of the release rate is important both for minimizing the environmental aspect, but even for the lifetime of the antifouling system itself.

In order to optimize the release profile of the biocide much effort has been done, i.e. encapsulation of the biocide into micro- or nano-carrier as i.e. in [37]. This reduces the burst effect of the initial immersion of a pristine antifouling coating, and can regulate the release of the biocide in difficult operational conditions during service life of the coating.

To tackle the intrinsic problem with the release of biocide into the surrounding water and the unwanted side effect on non-target organisms, a continuous search for alternative biocides is undergoing. The identikit of the optimum biocide presents some key features. The biocide need to have a rapid degradation into non harmful compounds once released into the water column. Another key quality sought for, is the high therapeutic ratio (ratio between the concentration needed to inhibit fouling and the concentration needed to kill organisms). The therapeutic ratio or LD_{50}/ED_{50} is the effectiveness of the compound in relation to its toxicity [38]. This means that the compounds to be favoured during the screening phase are the one having a large span between the concentration at which they are effective against the target organism (ED Effect Dose) and the concentration expected to kill the organism (LD Lethal Dose). This would guarantee settlement inhibition rather than lethality. Obviously, for the reason presented in the introduction, the alternative biocide still need to have a good economic and feasibility profile or no alternative biocides will be taken into consideration for the up-scale production. The search for copper alternatives produced the growing interest for (NAP) ‘natural antifouling products’ [38, 39]. Marine biologists interested in the biomolecular aspects of the marine life, have extract and tested for antifouling properties, many biomolecules. The idea is to mimic the nature's solution to the fouling problem. Algae, sponges and other marine organisms in fact have to face the problem of fouling too; this particular fouling acting upon living organisms, is called epibiosis. Especially algae and sponges, trying to protect their body from fouling organism, have been a source of inspiration [40]. One of the typical bottlenecks of this approach has been the up-scalability of the extraction or of the synthesis process of these rather complex molecules, in order to be incorporated in paints.

The search for environmentally friendly alternatives has a precise start point in the history of the antifouling technologies development. In the second half of the 20th century an organo-tin compound, the tributyltin (TBT) was regarded as the best solution in term of antifouling efficacy and economic profile. The fight against marine fouling was over. Seven years of smooth surface could be guaranteed from shipyards to the ship owners by applying TBT paints on the ship hull.

It was the dramatic effect on oyster aquaculture and the results from studies conducted in the Arcachon bay, France [41] and in the same period in the west of England [42] that underlined the economic and environmental negative impact of the use TBT in fouling protection systems. This discovering had two major consequences. First of all it triggered a global concern on TBT and the exponential increase of scientific literature about marine antifouling starting 1990 until today (Figure 3).

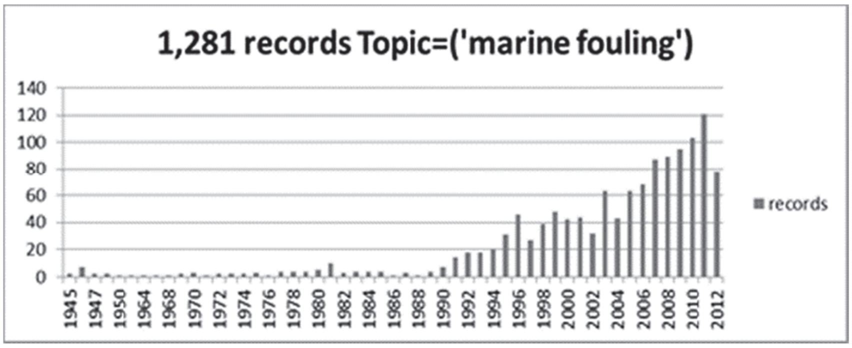

Figure 3. *Number of records per year from 1945 up to 2012 in the database 'ISI web of science' when the keyword used for the search is 'marine fouling'. (Updated in October 2012)*

Searching in the literature database for the term 'marine antifouling' and one will found that the number of records produced per year starting from 1945 as in a quite steady state ranging between 2-5 records per years and then, in correspondence with the TBT scandal increased exponentially from 1990 and forward. Today there are 1281 papers containing the term 'marine fouling' and 653 papers containing the term 'TBT Ban'. These have been produced in the last 22 years.

The second more practical consequence of the ban of TBT has been that, while waiting for future solutions, the market took a step back in the old fashioned copper oxide, which made its comeback as principal biocide in antifouling systems. This could be clearly observed in a study made ten years after the national banning of TBT containing paint in France 1982. This study revealed increased copper concentration in Arcachon Bay oyster [43].

After the ban of TBT a lot of improvement has been done and good result in term of effectiveness has been achieved by employing optimized biocides packages, copper oxide together with co-biocide mixed into eroding self-polishing or self-depleting paint systems. Nevertheless the level off efficacy and lifetime of coating achieved by TBT paints has not been replicated.

Despite all the research for alternative biocides, aspects such as the economical factor, the feasibility, the up-scalability, the strict regulation for the registration process of novel biocide, have built up a very difficult situation for the introduction of new compounds onto the market. This have considerable hindered the introduction of alternative biocide to replace copper, and thus the 90% of paints sold today still contain copper, which was the temporary solution after the banning of TBT (figure 4).

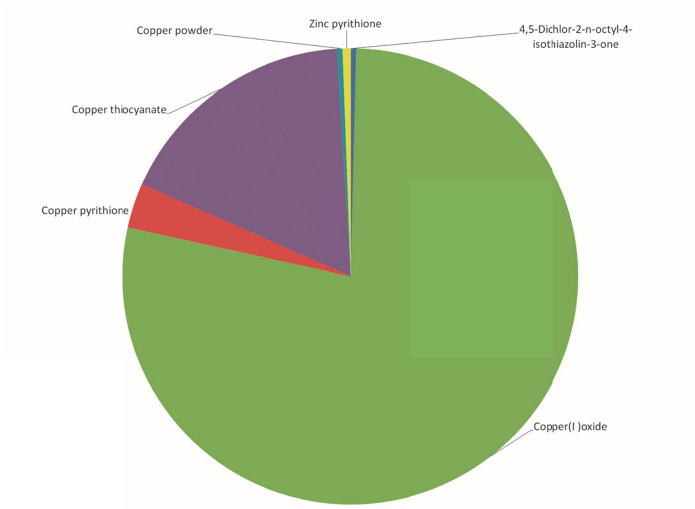

Figure 4. *Biocide product sold in Sweden in 2012 in proportion to the total mass. (The data for Zinc pyrithione are from 2011). (data source: Swedish Chemical Agency)*

The fate of the biocide is to be released and dispersed into the marine environment [44] thus compounds toxicity to non-target organism, 'Predicted Environmental Concentration' (PEC), 'Predicted No Effect Concentration' (PNEC) and bioaccumulation are always to be considered when dealing with risk assessment of biocides for antifouling. For the Swedish marine antifouling, every year ca. 140 tons of copper biocide are used.

Many signals arrived in the past few years in the direction of a change in the actual view on the usage of copper as antifouling biocide in marine environment. Some countries in EU, like Denmark, Sweden and Netherland, have or have had, beside the IMO regulations and the application of the

Biocidal Product Directive (BPR) guidelines, a special regulation on the import and selling of antifouling paints based on a maximum leeching rate of copper, especially for pleasure boat usage. Moreover a great push to copper replacement seems to come from the two separate senate bills that has been presented in USA; under the 2011 from State of California [45] and the same year from Washington state [46].

Other marine antifouling technologies, not based on biocides, are beyond the scope of this thesis. Nevertheless we would like to mention the nontoxic fouling release approach due to some interesting aspects and parallelisms with the approach discussed in this thesis.

The Fouling Release approach is based on the physical properties of the coating's surface [47-49]. Choose of material and design of the coating components produce low friction and low surface potential. While biocide based approach focuses on the chemical settlement inhibition, the fouling release approach focuses on reducing organism adhesion strength after settlement. The organisms can be released by the act of the water flow produced by cruising speed (i.e. typically for the barnacles the speed threshold is set to 13-15kn). Even if this approach is promising in terms of low environmental impact, the lack of usage flexibility (works better on fast cruising ships) and the cost of the matrix components (i.e. silicones and fluorinated polymers) make this solution still not favourable against the tin-free copper based paints, and this is reflected by the picture of the market share of biocide based coatings.

2.3 The Barnacle

Among the different organisms involved in marine fouling, one of the most resistant and thus ubiquitous is the Barnacle. Barnacles are a crustacean sub-class (Cirripedia), and among the various barnacles, *Balanus amphitrite* is considered one of the most problematic organisms in term of fouling [50]. They are present in almost all marine environments in the globe after millennia of transportation by ship hull. Being so resistant to different climate, salinity and chemical pollutant, barnacles are sometime dominating the polluted harbours and marinas around the globe. Moreover these areas are where boats and ships, painted by tin-free self-polishing antifouling, are most exposed to fouling, because of the nature itself of these antifouling systems, relying on the continuative erosion of depleted paint player in order to expose more biocide rich layer.

These problematic factors together with the property of barnacles cement to cure as strongly that once turned into adult removing them without damaging the underneath paint is very challenging... Copper sheathing had already demonstrated on the wood ship a very good efficacy to against barnacles, and thus copper oxide have been used in the plastic paints and after the ban of TBT in self-polishing paint. Being the scope of our research to find an alternative to copper oxide in paint, it has become natural for us to focus on this organism.

Barnacle life cycle involves six naupliar stages which can differ in size and morphology among Cirripedia [51]. The six naupliar stages are planktonic and or plantotrophic in the cirripedia and are considered being pelagic in order to give larvae both time to grow and disperse. After the six naupliar stages the larva turn into a final and non-feeding stage called cyprid. This is biologically conserved in the cirripedia. It is specialized in finding settlement on solid surface. The cyprid will explore the surface by ‘walking’ on it and testing with the antennules [50]. This surface could be a rock, a host organism, or a man-made surface. It has been demonstrated that the cyprid is very selective in this exploration and only if satisfied with the surface, both chemically and physically, and if stimulated to do so, it will settle [50]. After settlement the cypris larva will undergo metamorphosis. Several phase are involved, definable both by morphology and size changes. This process has been studied and described in a recent publication [52], using underwater video recording in laboratory. At those conditions the barnacles employed ca. 32 hours to undergo the transformation from cyprid to juvenile form. The success and speed of this process depend on environmental conditions, water flow and tidal phase. Species of pedunculated barnacles (*Lepas*) submerged during the entire metamorphose phase from cyprid to adult, can take several days to reach juvenile stadium [53]. Starting from the juvenile form the barnacle becomes a suspension feeder. The adult barnacle will produce and maintain a very interesting and well-studied cement [54-57] to attach firmly to the substratum.

In the antifouling perspective the attachment phase is a crucial aspect to investigate and understand in order to take possible countermeasures in order to avoid firm attachment. This is especially the focus of many fouling release approaches [47-49]. The adult barnacle cement is very difficult to remove without damaging the underneath classical soluble matrix coating. This because the adhesion between barnacle and paint can becomes stronger than the adhesion between paint and the ship hull (or primer) itself.

Beside the production of adult cement, many species of barnacles adopt other strategy to improve their attachment on the substratum. Some species colonize and penetrate corals [58, 59], some species colonize and penetrate host animals skin (i.e. whales) and other penetrates algal leaves. It is however known, but not so emphasized in recent literature, the possibility that even more common barnacles (like *Balanus amphitrite* and *Balanus improvisus*) can penetrate the classical paint matrices used in biocide based antifouling when these fouling protection fails or the biocide is exhausted [60, 61] (Figure 5 and 6).

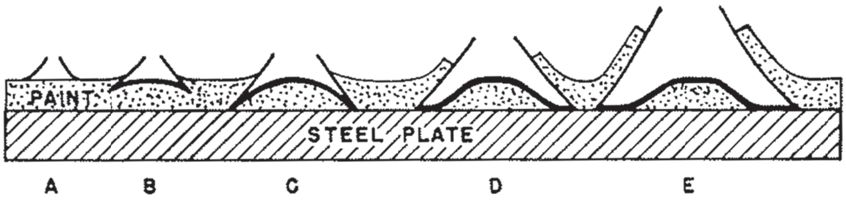

Figure 5. *Cartoon showing how barnacle "plows" into the surface of paint.* ***A*** *– Metamorphosed barnacle on paint surface.* ***B*** *and* ***C*** *–The edges of the shell grow downward until checked by the steel plate.* ***D*** *and* ***E*** *– Continued lateral growth forces the paint upward over the barnacle's shell. After [60]" figure caption text and diagram present in [61]chapter I page 17*

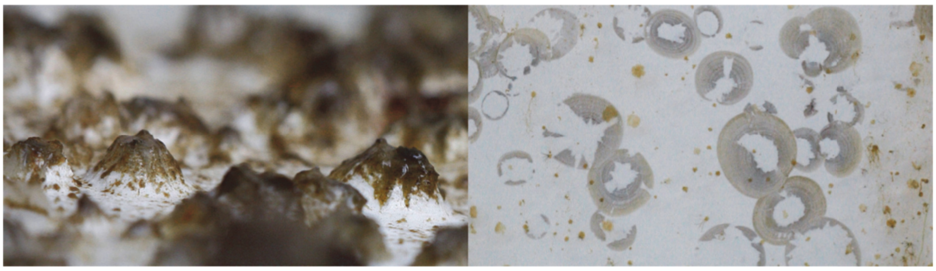

Figure 6*. Barnacle growing on polymeric waterborne paint (Paper II) based on polyvinyl Versatate immersed at the Swedish west coast water for one summer. The barnacles showed here are in the same growth phase as the phase E in (Figure 5). A skirt of paint is found on the shell and turning the panel upside-down one can notice the base plate checking the Plexiglas© plate (Paper II).*

This interaction, penetration and disruption of coating surface by growing barnacles, has not been under the focus of antifouling research as it is considered a consequence of a fail in antifouling protection. Biocide based antifouling approach focuses on settlement inhibition, while fouling release approach, even if it focuses on post-settlement aspects, does not take in consideration this phenomenon as a possible antifouling strategy, because this would mean a strong and firm barnacle adhesion and thus a fail.

The most famous and used bioassay for screening countermeasure against the acorn barnacle's attachment is the one developed by Dan Rittschof and co-workers [62, 63] using the barnacle cyprid. No antifouling bioassay [64] is design to follow barnacle growth on painted surfaces. Test on fully formulated paints and lasting for enough time to could accomplish for post settlement growth have always been done in field. The drawback of field test is that it is difficult to follow in detail all the metamorphosis and post metamorphosis changes, and the detail of the interaction barnacle/paint. Static panel's field tests are traditionally used for evaluating antifouling efficacy in season, or even year around.

This is why not so much was known in details on the process of coating penetration in the scientific literature. In this thesis we will present a study (Paper III) that individuate the initial phase of this process, on classical rosin paints, just after the juvenile form of Balanus is reached, this means when the barnacles are ca. 0.6-0.7mm in diameter. (Paper III).

The reason for this strong interaction between barnacles shell plaques and the paint coating could be found in the necessity of this sessile organism to test and find the best and more stable attachment point possible. If the substratum is too hard the penetration will not occur, this will mean a firm attachment point, as can be seen on the hard polymeric paint in (Paper II). On contrary, the soft surface in (Paper II) will get penetrated and the shell plaques will check the Plexiglas® plate, which may be a better attachment point than the soft paint coating, because a softer substratum could be a sign of instability in time, such as erosion of a biofilm under the shell plaques and cement. This is a speculation after years of observations on different substrata, such as common rosin based paint not containing any metal or biocide (Paper I and III) and on biocide-free polymer based paint (Paper II). It is important to remember that the coating penetration have been observed only on the negative control paints, this means on paint not containing any biocide.

3 LOW BIOCIDE EMISSION ANTI FOULING

3.1 Aim and inspiration

It is generally believed that the efficacy of a biocide based antifouling system is related to the amounts of leached biocide from the surface. Already in 2004 in the same group, a thesis on a pharmaceutical agent targeted against barnacles (medetomidine) [65] indicated that the release rate was not solely contributing to antifouling efficacy [66] . When I arrived in the project we noted in field experiments that the early colonization of cypris larvae on rosin paint containing 0.1% ivermectin was not affected, but the antibarnacle effect was in somehow time shifted in a later stage. This gave us the start point to think outside the box and start making hypothesis of how the penetration of the coating acted by barnacles could be turned in our advantage.

How can an observation about coating disruption be the inspiration for a protective coating system? The inspiration came when different observations were brought together: (i) the peculiarity shown by barnacles to penetrate, if possible, the substratum they have choose to live on; (ii) the observation both in our field test and in the literature that this penetration occurs not only on living organisms but even on antifouling paints [60]; (iii) a publication about an invasive species of brown alga *Fucus evanescens*, which could preserve its leaves from barnacles epibiosis better than a native congeneric *Fucus vesciculosus*, not by chemical settlement inhibition but acting on juvenile barnacles and enhancing the post settlement mortality [67].

Aiming in keeping both the feasibility and efficacy of the biocide approach with the low environmental impact of the fouling release approach, we formulated the idea of an enhanced post settlement mortality of the juvenile form of barnacles achieved not by biocide release but, as the *F. vesciculosus*, by waiting for the barnacles to push downward on the substratum, was evaluated. The result of this work is reported in this thesis, where I summarize in four papers five years of field and laboratory test in order to verify the hypothesis.

3.2 The model paint (Paper I)

The first step was to test if the idea could work in principle; this needed to be done in field due to the discussed difficulties to test antifouling paint in a laboratory bioassay. Putting together the observation in [60] with our field studies and the observation on *Fucus evanescens [67]*, we started by choosing the needed components in terms of paint matrix and active biocide in order to reproduce on manmade surfaces the observed enhanced post settlement mortality.

In order to replicate the possibility for barnacles to interact with coating surfaces and penetrate, we use the same paint matrix described under the observation of [60], the classical rosin based biocide free paint. This was a natural choice because the majority of the commercial antifouling paints are rosin based. We selected so-called 'biocide-free' rosin based paint, not containing any organic biocides and no other metal than zinc oxide. This paint is allowed for use on the east cost of Sweden (Baltic sea) where the limit for copper leaching are very low. In order to keep track of the possible antibarnacle effect of the 'biocide-free' paints itself, all the panels have been painted with base-paint/treated-paint side by side, in order to follow and compare the effect of the biocide versus a negative control.

3.3 The probe biocide (Paper I)

The probe biocide was a key factor for the 'proof of concept'. Aiming to a very low release but high effect for the target organism, we started screening for possible candidates. For the low release part of this question we assumed the model paint as a control delivery system. Thus we followed the old Higuchi [68, 69] equation to have an overlook at the controlling factors of the diffusion of a solid drug from a solid matrix:

$$\mathrm{RR}=\sqrt{2\mathrm{DS}\varepsilon\left(\mathrm{A}-\frac{1}{2}\mathrm{S}\varepsilon\right)}\times\sqrt{\mathrm{t}}=\mathrm{Kh}\sqrt{\mathrm{t}} \qquad \text{[Eq.1]}$$

The RR 'release rate' of the biocide depends on the physiochemical properties of both the biocide and the matrix. Biocide concentration (A) and water solubility (S) are obviously important factors when controlling the release rate. Affinity between the biocide and the matrix (or some other components of the matrix) [70] influences the diffusion coefficient (D). Another important property is the molecular volume of the biocide, affecting the diffusion of the molecule trough the matrix porosity (ε) [71, 72]. Matrix porosity can have a large effect on diffusion, especially in crystalline or dense matrix. One can conclude that a biocide at low concentration, with low water solubility, high affinity to the matrix and high molecular volume will have a very low release rate as discussed in (Paper I).

Screening the literature for molecule showing toxicity to crustacean and fitting the sought physiochemical characteristics, we found the avermectins as good source of candidates. Avermectins are produced by the soil living bacterium *Streptomyces avermitilis* and among the different forms and derivatives used in the market of avermectin, ivermectin (22, 23–Dihydroavermectin) was selected as the model biocide for this investigation (Figure 7).

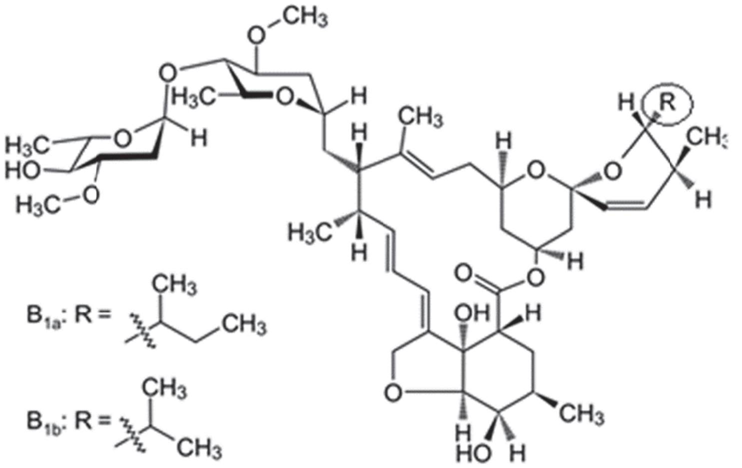

Figure 7. *The structure of ivermectin, a derivative (22,23-dihydrated avermectin) of avermectin produced by the soil and sediment bacterium Streptomyces avermitilis as a racemic mix (80%* B_{1a} *+ 20%* B_{1b}*).*

Ivermectin presents low water solubility (4 mg L^{-1}), high K_{oc} (12660-15700) [73], indicating a high affinity to organic molecules such as rosin and high K_{ow} (1651) [74]. All these properties suggest a low release rate from the paint film considering the Higuchi model. Moreover Ivermectin demonstrate an IC_{100} (100% immobilization concentration) of 430ng ml^{-1} for the Brine shrimp *Artemia salina* [75]. This gave us indication of possible activity against other crustacean as barnacles even when used in low concentration in the paint film.

3.4 Multi-seasonal efficacy (Paper I)

In order to prove this hypothesis we needed to do field tests because of the discussed difficulties in making bioassay on paint formulation. Another important aspect and not less important was the price of the biocide. Ivermectin is a well-known anthelmintic agent used in agriculture and in veterinary and human medicine and is relatively cheap. A static panel field test using rosin paint containing 0.1% ivermectin demonstrated the possibility of protecting surfaces from barnacles, for several season (Paper I). At the same time tests in laboratory confirmed the predicted low release rate at nanogram level per square centimetre per day. Even more promising was the fact that at this point we did not optimized the matrix properties according to Higuchi, but the choose was on a regular rosin based paint because of the observation reported in literature of the barnacle penetrating these kind of paint matrix [60]. All the details are present in (Paper I) of this thesis.

Another important aspect of this concept is that the biocide distribution in the matrix after film formation will most likely play an important role for the contact-efficacy. This was studied and discussed in Paper I and the effect of dispersion enhancers called “co-solvent” is studied by fluorescence microscopy. This was possible due to a dihydroxylated tetrahydro benzofuran ring in the molecule that could be made cromophoric upon derivatisation [76]. Moreover the distribution of this organic biocide could be followed even without modifying the molecule at all. This is discussed in (Paper IV) using a novel techniques in this field, i.e. ToF-SIMS (time of flight secondary ion mass spectroscopy).

3.5 Contact or chronic intoxication? (Paper II)

After the initial results, some doubts regarding a possible chronic intoxication effect arising from the low, but present, leaching of biocide were considered. In order to demonstrate the total independency between the low release rate and the efficacy, another study was designed and is presented in this thesis (Paper II). The demonstration of this independency is not important in order to registration or commercialize this system, but is important for the ‘proof of concept’ itself. In fact, in my opinion, no improvement in the state of the art is achieved if a biocide with very high activity against the target organism is proven to show efficacy at very low release rate, this is elementary logic. The level of the release rate is not an absolute value, but has to be relative to the toxicity of the biocide for the target organisms, it is a question on MIC (minimum inhibition concentration) as was discussed in the precedents chapters.

The real novelty is achieved in this thesis when the complete independence between the release of ivermectin and the anti-barnacle efficacy was demonstrated. That is the key result of the work done in the last five years.

3.6 The model paints (Paper II)

In order to test and demonstrate the contact upon penetration or the “post settlement mortality” hypothesis, two model coatings were designed (by ‘Finndisp’, Finland) with different elastic modulus. One based mainly on a polyvinyl versatate (PV), and the other mainly based on polystyrene (PS). Both polymers are insoluble in waters, have a low erosion rate and should be resistant to the sea water exposure. The two model paint demonstrated same low erosion, same affinity for the biocide studied by surface plasmon resonance (SPR), same Release Rate of the biocide (nanogram level per cm^2 per day) and, as predicted, enough different mechanical properties to permit barnacle penetration only on the soft one.

3.7 Field test results (Paper II)

An 80 days field test exposure of triplicates panels was made. On the hard model paint system cyprid larvae could establish both on the control and the treated side of the panels. On the soft paint no barnacles where found on the treated side. This indicates a strong correlation between the modulus, the barnacle’s penetration and the antibarnacle efficacy of such antifouling paint system. Moreover the release rate being the same for the polyvinyl versatate (PV) and for the polystyrene (PS) system demonstrates that the low emission of ivermectin at nanogram level is not enough to demonstrate chronic intoxication.

3.8 Fate of juvenile barnacles (Paper III)

In order to study the cyprid fate on the embedded biocide paint systems, a new bioassay was developed. As discussed in the previous chapters the difficulties of reproducing settlement conditions in laboratory conditions on fully formulated paints (with cypris larvae reared at ‘Sven Loven Centre for Marine Science’ in Tjärnö (Sweden)), were not so easy to overcome. After several attempts a compromise was adopted. The settlement was done in field and then, after 3 days exposure, the panels were brought laboratory. A basin with ‘water open system’ (i.e. superficial sea water inlet regulated at the same rate as the water outlet) was prepared in order to keep the barnacles in best condition possible and minimize the accumulation of toxins from the paints. The barnacles were maintained with diatoms algae cultured in the laboratory, and a net with mesh of ca. 200µm was used in order to keep food inside the basin. The barnacles were studied by stereo microscopy every second day to follow the development and growth. The barnacles on the treated side of the panels could grow up to, and not more than the juvenile form, corresponding at stereo microscopy observation, to (Figure 8, C) circa 06-07mm.

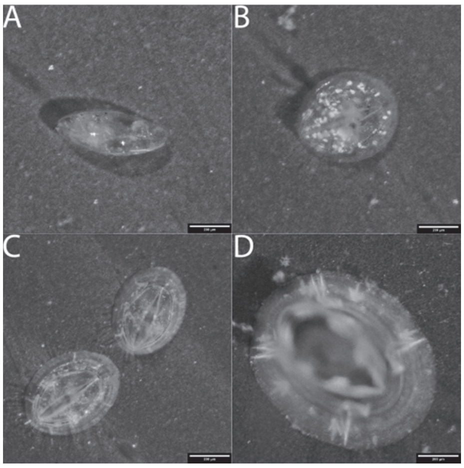

Figure 8 *Succession of barnacle’s metamorphosis and growth on rosin painted panels containing 0.1% ivermectin. The phase definition corresponding to the morphological difference between is as follow: A-Cipris Larva; B-Newly metamorphosed barnacle; C-Juvenile barnacle; D-Adult barnacle. This has been used as definition of the growth study in the laboratory bioassay presented in (Paper III)*

A parallel test was done in field. Rosin based paints with and without ivermectin 0.1% were applied on panels and deployed in sea water in order to study settlement recruitment and growth in natural conditions. The field test showed some initial recruitment inhibition by the ivermectin treated rosin paints. This inhibition disappears after the first week of exposure. After this period new recruitment is permitted, and at this point the barnacles could undergo metamorphosis and grow up to juvenile stadium to around 4-7 days old on field panels. In the laboratory experiment the results were similar. All the details of the experiments' design are present in (Paper III) of this thesis

3.9 The "imprints" of juvenile (Paper III)

In order to further develop a low or no release system based on embedded biocides it is of great importance to determine at which stages in the barnacle life cycle they interact strongly with the coating and can trigger the bioavailability of the embedded biocide. Biocide free control paints were exposed in field for different time intervals (See Paper III) and, once removed the juvenile barnacles on it, they were studied in laboratory by scanning electron microscopy (SEM). The eventual imprints left by barnacles growing were investigated. Indentation marks where found starting with diameters of ca. 0.6-0.7mm (Figure 9). This correlates quite exactly with the observation made both in the laboratory and field experiment, the intoxication takes place on juvenile form when they are about 7 days old and within the phase C in (Figure 8). At this stage their diameter is ca. 0.6-0.7mm. This could mean that the intoxication takes place immediately as the juvenile start pushing downwards. But before drawing final conclusion, future study should investigate the presence or absence of imprinting marks in a more detailed and at more time points than conducted in this experiment.

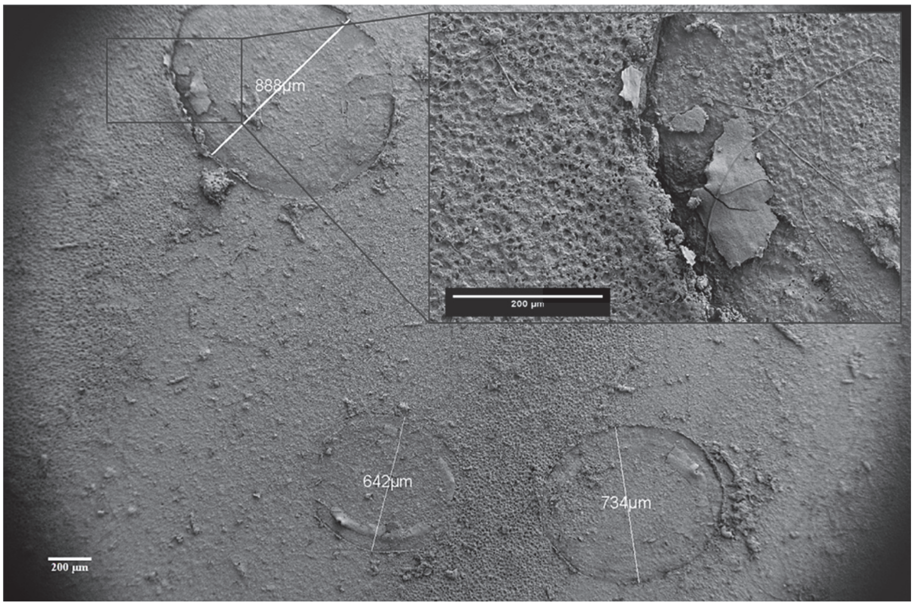

Figure 9 *imprints left by juvenile barnacles on the control rosin paint used in the test described in (Paper III). As can be seen in the picture, indentation marks (see the zoom insert) left by the shell plaques of the juveniles are present when the organism are 0.6-0.7mm in diameter.*

4 NEW TOOLS FOR NEW QUESTIONS (PAPER IV)

The distribution of a biocide and its distance from the surface of the coating, plays an important role in the regulation of diffusion and thus release in the classical biocide based coatings [77]. It has been showed in Paper I that even the aggregation of biocide could influence the bioavailability of the biocide for the target organism. Thus analytical techniques have been developed in order to get information of the distribution of the active ingredients inside the paint film after the formation of the solid film. In general the way to go is follow metal compounds such as copper or zinc by SEM and EDX. In the case of organic compound such as those studied in our group (medetomidine and ivermectin) it is impossible to follow them with these instruments. One needs to label or modify the biocide with the risk of changing the physiochemical characteristics that plays important role in the fate of these molecules during film formation, possibly altering the final results.

One possible solution to this problem is the use of a very interesting technique, called ‘ToF-SIMS’ ‘Time of Flight - Secondary Ion Mass Spectroscopy’. This technique is based on mass spectrometry profiles, which result after an electron beam has been directed onto the surface. The instruments can reconstruct the local mass spectra profile. Thus one can obtain mass spectra ‘pixel by pixel’ shooting by the ion and sweeping the area of interest. In this way one can obtain a chemical picture at microscopic level, both for the very first outmost layer of the surface or, employing a so called dynamic ToF-SIMS mode, one could sputter down trough few µm of the specimen at the same time as the analysis occur and thus reconstruct a 3D chemical picture at microscopic level of the specimen.

We were able to follow the fate and distribution of the ivermectin after film formation in rosin lacquer, and we could correlate pattern in distribution with solvent polarity used in the wet paint formulation (Paper IV). Of particular importance was the study of similar system as the ones reported in (Paper I) in order to confirm the results obtained in Fluorescence Microscopy with the one obtained by ToF-SIMS.

In the case of ivermectin and the ‘enhanced post settlement mortality’ obtained by contact between organism and biocide, it is of fundamental importance to have an evenly distributed biocide in the final film, otherwise the concentration organism may experience could be too low in some region of the coating if aggregates or precipitation takes place. Thus in the case of ivermectin, where the mechanism is not based on biocide release, but on direct contact, the study of active molecule distribution could be crucial.

Especially in (Paper IV) we could correlate the results obtain by ToF-SIMS with the preliminary results obtained by Fluorescence Microscopy in (Paper I). The addiction of glycerol formal to the formulation gave rise to a phase separation of the different component of the rosin with an emulsion like formation. The ivermectin seemed to be more prone to stay in the lipophilic phase creating a net-like structure rich in ivermectin around bubble of 20-50µm of aliphatic carbon. This compared with the more aggregated ivermectin when added without the co-solvent gave in field a slower efficacy, this means that the effect is in somehow postposed in time from the settlement to the intoxication, and bigger juvenile could be found at the beginning. Nevertheless the two formulations, with and without co-solvent, were free from barnacles at the end of the season and even after the second season of continuative exposure.

5 SUMMARY OF PAPERS

Paper I

E. Pinori, M. Berglin, L.M. Brive, M. Hulander, M. Dahlström, H. Elwing
Multi-seasonal barnacle (Balanus improvisus) protection achieved by trace amounts of a macrocyclic lactone (ivermectin) included in rosin-based coatings.
Biofouling Vol. 27, No. 9, **2011**, 941–953

Contribution

I participated in planning the field study with H.E. and M.B. I carried out experiments such as static panels' field test, fluorescence study of ivermectin distribution, chemical transformation and purification of ivermectin in order to obtain a cromophore moiety in the molecule. I carried out the release rate study, both extraction method and measurements of biocide together with L.M.B. I analyzed all the data, compiled an *ImageJ* based routine to automatize the barnacle counting on panels, and wrote the manuscript in draft.

Summary

In this study was performed a static panel field test (to verify and normalize several past boat test) in order to test the possibility to achieve antibarnacle efficacy by using molecule with high affinity to the paint matrix, high activity against the target organism and low emission from the paint into the water. The results from both panels and boats tests were outstanding and some experiment and characterization in laboratory confirmed the low biocide release 0.7-3 ng cm^{-2} day^{-1}and the importance of mechanical property as hardness and biocide distribution into the dry film of the paint.

The key finding of this study was that these kind of antifouling paints designed according to this hypothesis did not influenced as much the recruitment and settlement of cyprid larvae, while in a later observation on the same panels, no adult barnacles could be observe. This was speaking for the hypothesis of mechanical interaction barnacle/paint being the key factor for the intoxication and not the release of biocide from the surface of the paint as in the classical biocide antifouling paint.

Nevertheless a low but present leaching of biocide could cause a chronic intoxication effect to be confused with post settlement juvenile intoxication by contact. This aspect had to be investigated with a further work.

Paper II

E. Pinori, M. Berglin, H. Elwing
Impact of coating hardness on anti-barnacle efficacy in embedded biocide antifouling.
Biofouling (Accepted and in press, May 2013)

Contribution

I planed the study together with M.B. and carried out the preparation of field test. I carried out evaluation of efficacy together with M.B. and H.E. I planned and carried out the ISO standard method for the extraction and measurements of biocide release rate with help from Lena Brive at SP. I carried out the hardness and erosion study on the different matrices.

Summary

In this paper we investigated the question left suspended in the previous study. Actually that is the key question for the novel concept presented here. “Is the antibarnacle efficacy shown from the ‘Low Emission Antri-Fouling’ paints completely independent from the low released ivermectin?” In order to answer to this crucial question we designed two model surfaces, and were able to achieve the same ivermectin release rate (As studied this time with an international standard method ISO 1518-1), the same affinity biocide/matrix and the same erosion rate of the paint. The only parameter designed to be different between these two formulations was the hardness. Negative controls (no biocide present) of these two paints were deployed into the Swedish west coast. One resulted hard enough to inhibit barnacle penetration and coating disruption. The adult barnacle on this polystyrene based polymer paint, were able to grow staying just on the top layer of the coating. The other control paint was enough soft to be penetrated from the growing barnacles, which could reach the Plexiglas base plate underneath. Once these two model surfaces were added by the same amount of ivermectin as in the previous study (0.1% w/v) the effect of this difference in modulus was clear. Despite the same release rate of ivermectin for the two formulations, the barnacle growing on te top layer of the polystyrene based formulation did not suffered intoxication for the all duration of the field test (3 summer months). On contrary on the softer paint, the same release rate gave a full protection against barnacles. More precisely the cyprid larvae and the juvenile could be spotted as undisturbed at the first observation time point, and then no more trace of barnacles were registered in the future observation. Something bounded with the modulus of the formulation was triggering the intoxication mechanism. And the released ivermectin alone could not intoxicate the barnacles as clear from the hard paint results. This was the final demonstration that the mechanism act not by released biocide but from a triggered bioavailability depending on the interaction between the barnacles and the paint matrix, where the modulus is a key factor.

When in the barnacle's life cycle this interaction does trigger the intoxication was the natural question arising from this study. And then a further study (Paper III) was necessary in order to have a closer look at "my organisms" as Prof. Dan Rittschof advised me to do during the ICMCF conference in Newcastle 2012.

Paper III

E. Pinori, A. Holmqvist, M. Berglin, M. Dahlström, H. Elwing
The fate of cyprid and juvenile barnacles on rosin paint with 0.1% ivermectin.
In Manuscript

Contribution

I planned, realised and followed the mixed field/laboratory barnacle's bioassay. I analysed the results and wrote the manuscript in draft. I planned, prepared and carried out together with M.B. post-experimental study of imprints by SEM. The field test on panels in order to follow the recruitment and mortality in field during 32 days was carried out by M.D. and A.H.

Summary

Rosin paints not contain any heavy metal, neither zinc oxide nor copper were applied on Plexiglas. In one bioassay the panels were exposed for 3 days in field in order to recruit cypris larvae and then one week monitoring in laboratory was done. The same formulations were replicated in many panels and deployed in field taking them up at different intervals for observation from day 1 to 32.

In the laboratory bioassay the barnacles recruited in field were kept in a basin with open water system. The barnacles were maintained by diatoms cultured in laboratory. The panels with different formulation and control and treated paints were observed under 7 days in lab (total 10 days from first immersion). The barnacles growth was studied by stereomicroscopy as the panels were kept under water, and the morphological changes were recorded using a schematic 4 phases defined as A B C D in (Figure 8 in this thesis). For all the formulations when ivermectin 0.1% was added, the growth of barnacle could only reach phase C 0.6-0.7 mm diameter, defined as juvenile barnacle 4-7 days old. Thus the triggering of intoxication has to be individuated in this phase. SEM study of the control panels after the experiment, demonstrate that on the control paint the juveniles had left indentation marks (0.6-0.7mm diameter). This finally confirmed that this novel mechanism proposed in order to reach a emission free antifouling system is independent from the release of ivermectin, thus it is possible to work on the materials properties in order to reduce to the minimum possible the release rate maintaining antibarnacle-efficacy.

Paper IV

M. Berglin, E. Pinori, H. Elwing, P. Sjövall
Distribution of Organic Biocide in Antifouling Paint as Determined using Time of Flight Secondary Ion Mass Spectroscopy (ToF-SIMS)
(*In Manuscript)*

Contribution

I planned the study of the paint film by ToF-SIMS together with P.S. Measurements were carried out at SP by P.S and me. Me, M.B. and P.S., wrote the manuscript in draft.

Summary

The distribution of bioactive compounds in an antifouling paint affects the release which ultimately controls the efficacy of the paint. In this paper we demonstrate how ToF-SIMS can be used to determine the distribution of an organic biocide i.e. ivermectin in rosin based coatings. Ivermectin was added to rosin based matrix dissolved in ethyl acetate or dissolved in an amphiphilic solvent i.e. glycerol formal. After film formation the distribution of ivermectin, both at the surface and as a function of depth after sputtering, was visualized. Aggregates or particles of ivermectin ranging in size from about 5 µm up to 25 µm were found when using ethyl acetate as solvent. The aggregates where randomly distributed in the coating indicating a heterogeneous film structure which might affect the local paint efficacy. When using glycerol formal as solvent two distinct phases on the surface was detected. First phase were composed of circular domains ranging in size from around 50 up to 100 µm in diameter enriched in hydrocarbons but with no trace of ivermectin. The high hydrocarbon content indicates this phase to be enriched in the rosin binder. The other phase on the surface contained ivermectin, rather well distributed, with no signs of the aggregated structure as was found on the surface when ethyl acetate where used. The advantage of using Tof-SIMS was that no labeling or other surface preparation steps were needed prior to analysis. Also, ToF-SIMS was able to selectively determine the 3D distribution of an organic biocide in an organic binder of rather similar structure, a challenge using other techniques such as SEM-EDX or ATR-FT/IR

6 CONCLUSIONS AND FUTURE OUTLOOK

6.1 Conclusions

At the end of these challenging five years, we can finally introduce and describe a new perspective in antifouling. We have now demonstrated that the 'Low biocide Emission Anti-Fouling' approach is based on a novel route of intoxication for one of the most problematic organisms in marine fouling. This novel route of intoxication makes it possible to have barnacle free surface without any need to expose or release the biocide into the water column. Thus minimize the risk of accumulation of biocide into the biosphere. Beside the lowered risk for bioaccumulation, this improve the service lifetime of the coating, eliminate the necessity of matrix's erosion to control and keep the sustained release and thus permit a much broader applicability and flexibility in segment of use, working as well on statics as on dynamic surfaces, on cooling systems and on ship hulls, fast cruising as slow cruising, container ship as small motor boats or sailor boats. Moreover of particular importance is the heavy metal free aspect for the aquaculture application. Finally eliminating the necessity of continuous biocide release we could eliminate the necessity of loading large amounts of biocide in the paint, with a consequent better economical profile for the paint producers.

It has been a long journey even because hypothesis like this one, where the molecule-matrix-organism interaction needs to be studied, are impossible to be tested in laboratory bioassay. Thus starting from the idea evaluation part to the screening part, everything was forced to be done in field and it is quite time consuming to go by trial and error method in sea water field tests in Scandinavia. You cannot afford too many trials and too many errors in five years.

A particular aspect of this project is that, already from the beginning of idea evaluation, a low-end approach has been employed. The choosing of biocide and matrixes has been done having in mind at the same time the physicochemical aspects and the economic aspects to give the all project a character of feasibility. It is for this reason that we are confident that what we present here today is not only effective but even a feasible approach. Not so far from today this could make impact in real life, and this is the most exciting part when working on problem solving science, that the results can be used in real life.

6.2 Future outlook

It has been a great experience to start on a blank page in a completely new approach, starting from the concept and testing all the primary questions, but nevertheless, even more questions are open now that the concept is presented. The correlation between the modulus and the triggering of the intoxication process need to be studied more in details and on different matrices. A gradient of hardness is in project, in order to find out which is exactly the threshold value for rosin (respectively for other matrix) in order to get embedded biocide systems to work and have efficacy against the target organism.

Another aspect we would like to explore in the near future is the possibility to enhance the barrier property of the matrix and the affinity of the active molecule to the matrix component to test a ‘near zero emission’ system against fouling.

More biological questions are the study of the mode of action and the exact route for the bioavailability into the organism of the ivermectin. A closer look at the organism after exposure on paint containing labelled ivermectin in order to study in details which is the pathways from the matrix to the target receptor (Glutamate-gated chloride channels).

Another very important aspect to explore in the future is the possibility to employ this or a similar approach for fouling control of other important organisms (such as i.e. *Bugula neritina,or* ; or even if possible micro and macro algae). Some preliminary results from field test in Guanabara bay, Rio de Janeiro tells us that *B. neritina*, could be controlled by rosin based paint containing 0.1% ivermectin (data presented at ICMCF 2012, Seattle), but the mechanism of intoxication has not been studied yet in detail and we don´t know at this stage if the intoxication happens for chronic intoxication or per interaction triggered contact intoxication.

Fortunately this is still so interesting and hopefully many other scientists after me will jump into this approach and discover fundamental keys in order to realise a sustainable antifouling system for replacing heavy metals in the near future.

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Doing research is a luxury, and this luxury does not need only money but even a supporting and comprehensive wife, especially with three little child such as Luca, Max and me. *Grazie amore!*

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