GÖTEBORGS UNIVERSITET

On the Efficacy and Ecotoxicity of Antifouling Biocides

Lethal and Sublethal Effects on Target and Non-target Organisms

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Abstract

From an environmental perspective, there is a need to reduce the amount of biocides from antifouling paints in the marine ecosystem as these biocides can exert a negative effect on the marine life. One way to do this is to optimize the use of biocides in antifouling paints, and thereby avoid unnecessary overdosing. This thesis has been produced within the research program Marine Paint which has the overall aim to produce an antifouling paint with a lower environmental impact than the paints existing on the market today. The aim of the studies presented in this thesis has been to evaluate the efficacy and ecotoxicity of eight antifouling biocides to both target and non-target organisms. The biocides investigated were: medetomidine, triphenylborane pyridine (TPBP), tolylfluanid, copper, irgarol, zinc pyrithione, copper pyrithione and 4,5-dichloro-2-*n*-octyl 3(2H)-isothiazolone (DCOIT). The target organisms investigated were the macroalga *Ulva lactuca* and periphyton (i.e. microbial communities). It is important to keep in mind that all target organisms that antifouling biocides are meant to affect, are also non-target organisms when they grow on natural substrates in the marine ecosystem. Therefore, effects on target organisms are not only of interest for efficacy evaluations, but also for ecotoxicological assessments of the biocides. Both the efficacy and ecotoxicity of the eight biocides has been evaluated for the target organisms in settlement assays in which the organisms were allowed to settle and grow in the presence of the biocides. Full concentration-response curves from 0 to 100 % effect were produced to enable future mixture predictions. Such mixture predictions can be used for paint optimization, but also in environmental applications such as hazard assessments.

Copper pyrithione was the biocide that most efficiently prevented growth of both *Ulva lactuca* and periphyton communities, and for *Ulva lactuca* is was also the biocide with the highest ecotoxicity. Due to different shapes of the concentration-response curves, the toxicity ranking was not consistent at all effect levels (from EC_{10} to EC_{98}), and irgarol was found to be more toxic to periphyton at lower concentrations than copper pyrithione.

In order to extend the ecotoxicological evaluations of the biocides beyond target organisms, effects on the non-target organism *Acartia tonsa* was investigated. *Acartia tonsa* is one of the most commonly occurring pelagic calanoid copepods in coastal waters world-wide. Effects on mortality and egg production were studied for three of the eight biocides, namely DCOIT, TPBP and medetomidine. It was shown that neither DCOIT nor medetomidine affected the egg production specifically, but inhibition of egg production occurred at the same concentration as mortality. TPBP was on the other hand shown to affect the egg production at concentrations lower than lethal concentrations.

Antifouling biocides present in the marine environment can exert selection pressure on marine life and through the process of natural selection induce tolerance development. An extreme tolerance to the antifouling biocide irgarol in a population of *Ulva lactuca* from the mouth of the Gullmar fjord has been described. This indicates that the use of antifouling paints has made its imprint on the marine ecosystem.

The results from this thesis have deepened the understanding of the biological effects of antifouling biocides. The well-defined concentration-response curves gives information on both efficacy and ecotoxicity, and the information can be used in a number of applications where either biocidal efficacy or ecotoxicity is of interest, such as hazard assessments and in the design of antifouling paints.

Populärvetenskaplig sammanfattning

På hårda ytor i havet pågår en ständig tävlan om utrymme. Många växter och djur behöver ett fast underlag att fästa vid för att överleva, och de ytor som finns tillgängliga blir snabbt koloniserade. När dessa ytor utgörs av båtskrov, fundament till bryggor, nätkorgar för fiskodling eller andra av människan skapade strukturer, blir plötsligt den marina mångfalden och dess förmåga att anpassa sig till nya miljöer problematisk. Framförallt inom sjöfarten orsakar påväxten av växter och djur stora problem genom att öka båtens vattenmotstånd och därmed öka bränsleförbrukning och följaktligen också utsläppet av avgaser. På engelska används ordet *biofouling* för att beskriva denna påväxt som i en grov direktöversättning till svenska blir *biologisk nedsmutsning*, vilket illustrerar hur illa betraktade dessa organismer i allmänhet är. Den vanligaste metoden för att motverka påväxt är användandet av biocidinnehållande båtbottenfärger. Genom att biociderna (gifter) långsamt läcker ut från färgen då den målade ytan kommer i kontakt med vatten skapas en giftig och ogästvänlig miljö närmast skrovet som håller borta påväxten. De växter och djur som biociderna är menade att påverka, dvs. de organismer som sätter sig på båtskrov, kallas för målorganismer. Eftersom biocider är framtagna för att ha en effekt på levande organismer så finns det alltid en risk för att de påverkar andra organismer i det marina ekosystemet, så kallade icke-målorganismer.

För att minska belastningen av biocider från båtbottenfärger på miljön kan man optimera användandet av biocider, dvs. använda den minsta möjliga mängd som krävs för att förhindra påväxt. Över 4000 olika arter har identifierats som förekommande på båtar och de inkluderar både mikroskopiska organismer såsom mikroalger och bakterier, samt makroskopiska organismer, exempelvis musslor och havstulpaner. Denna mångfald av liv innebär också en mångfald i tolerans för olika biocider och för att kunna förhindra **all** påväxt måste man dosera mängden biocid efter den mest toleranta arten. Att kombinera flera, olikverkande biocider i en färg kan därför göra att man kan minska mängden som behövs av de enskilda biociderna utan att minska effektiviteten hos färgen.

De studier som presenteras i denna avhandling har utförts inom forskningsprojektet *Marine Paint* som haft det övergripande målet att producera en mer miljövänlig båtbottenfärg. Studierna har mer specifikt syftat till att kartlägga olika biociders effektivitet och giftighet. För att kunna göra effektiva kombinationer av biocider behöver man ha en god förståelse för hur biociderna enskilt påverkar olika målorganismer, en kunskap som också behövs för att veta minsta möjliga mängd av en biocid som ger full hämmande effekt. Då syftet också varit att skapa en så miljövänlig båtbottenfärg som möjligt är det viktigt att även ta hänsyn till de olika biocidernas ”giftighet”, eller ekotoxikologiska egenskaper. Av den anledningen har även studier av effekter på icke-målorganismer inkluderats i denna avhandling.

Målorganismer som studerats är makroalgen *Ulva lactuca*, på svenska kallad *havssallat*, och mikrobiella samhällen bestående av främst mikroalger och bakterier. För dessa organismer har effektiviteten och giftigheten av åtta biocider utvärderats. De biocider som studerats är följande: medetomidine, triphenylborane pyridine (TPBP), tolylfluanid, koppar, irgarol, zincpyrithione, kopparpyrithione och 4,5-dichloro-2-n-octyl 3(2H)-isothiazolone (DCOIT). Genom att mäta hur väl målorganismerna kan fästa vid en yta och tillväxa då de exponeras för de olika biociderna kan man få svar på både vilken biocid som är mest effektiv för just den organismen och vilken koncentration av biociden som behövs för att helt förhindra påväxt. Relationen mellan den biologiska responsen och de olika biocidkoncentrationerna kan beskrivas matematiskt genom att beräkna koncentration-respons kurvan. Från denna kurva kan man få information om vilken koncentration som motsvarar en viss grad av respons, ofta angivet från 0 till 100 procent. Dessa koncentrationer benämns även effekt-koncentrationer. En biocids effektivitet beskrivs av de höga effekt-koncentrationerna (de koncentrationer som ger kraftig biologisk respons) medan de låga effekt-koncentrationerna (de koncentrationerna som ger en låg biologisk respons) beskriver biocidens ”miljögiftiga” (ekotoxikologiska) egenskaper. I utvärderingen av en biocid som ska användas för att förhindra påväxt är båda typer av effekt-koncentrationer viktiga.

För att utvidga den miljömässiga utvärderingen av biociderna har även studier av effekter på hoppkräftan *Acartia tonsa*, som är en icke-målorganism, gjorts. *Acartia tonsa* är en av de vanligaste arterna hoppkräftor, och som djurplankton lever arten i den fria vattenmassan. Arter som lever i de övre lagren av den fria vattenmassan löper en hög risk att exponeras för biocider från båtbottenfärger, eftersom båtarna finns direkt i deras levnadsmiljö. Effekter av tre av de totalt åtta biociderna på både överlevnad och reproduktionsförmåga har studerats. Då man utvärderar en biocids giftighet av miljömässiga skäl är det önskvärt att känna till den mest känsliga parametern som påverkas av giftet. Inom ekotoxikologi testar man därför ofta subletala effekter, dvs. effekter som inträffar vid koncentrationer som är lägre än dödliga koncentraitoner. Innan studierna på *Acartia tonsa* påbörjades var hypotesen att reproduktionen, i form av antal producerade ägg, skulle vara en känsligare parameter än dödlighet, men detta visade sig stämma bara för en av de tre studerade biociderna. För de övriga två biociderna producerade honorna ägg tills de dog, dvs. äggproduktionen påverkades i samma grad som dödligheten.

Vi har också kunnat visa på att biocider från båtbottenfärger utgör ett selektionstryck i den marina miljön. I en population av makroalgen *Ulva lactuca* från mynningen till Gullmarsfjorden har vi kunnat påvisa en extrem tolerans för biociden irgarol. Sporer från vuxna individer från den undersökta populationen kunde både fästa vid en yta och därefter tillväxa i höga koncentrationer av biociden. Förutom att detta indikerar att irgarol finns i den marina miljön i tillräckligt höga koncentrationer för att skapa en hög tolerans hos alger, så belyser den toleranta populationen också problematiken

med toleranta målorganismer. Det räcker med **en** planta som är tolerant mot biociderna i en båtbottenfärg för att orsaka en kraftig påväxt på båtskrovet.

Resultaten som presenteras i denna avhandling har bidragit till att öka förståelsen av hur biocider som används i båtbottenfärger påverkar både målorganismer och icke-målorganismer. Dessa resultat kan användas i framtida optimeringar av båtbottenfärger, men också i andra sammanhang som exempelvis för att förutsäga biocidblandningars effekter på miljön.

”Det är bara att bryta ihop och komma igen”

- Per Elofsson efter loppet mot Johann Mühlegg, OS 2002

List of publications

This thesis is based on the following papers, which are referred to by the corresponding roman number given below.

Paper I

Wendt I, Arrhenius Å, Backhaus T, Hilvarsson A, Holm K, Langford K, Tunovic T, Blanck H. (2013) Effects of five antifouling biocides on settlement and growth of zoospores from the marine macroalga *Ulva lactuca* L. *Bulletin of Environmental Contamination and Toxicology*, 91:426-432 DOI: http://dx.doi.org/10.1007/s00128-013-1057-9

Paper II

Arrhenius Å, Backhaus T, Blanck H, Hilvarsson A, Wendt I, Zgrundo A. (2013) A new rapid antifouling efficacy assay using natural periphyton communities. Manuscript

Paper III

Wendt I, Arrhenius Å, Backhaus T, Blanck H. (2013) The toxicity of the three antifouling biocides DCOIT, TPBP and medetomidine to the marine pelagic copepod *Acartia tonsa*. Manuscript submitted to *Ecotoxicology and Environmental Safety*

Paper IV

Wendt I, Arrhenius Å, Backhaus T, Hilvarsson A, Holm K, Langford K, Tunovic T, Blanck H. (2013) Extreme irgarol tolerance in an *Ulva lactuca* L. population on the Swedish west coast. *Marine Pollution Bulletin, In press* DOI:http://dx.doi.org/10.1016/j.marpolbul.2013.08.035

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Introduction

Biofouling and antifouling

What is biofouling?

In the marine environment there is a constant on-going competition among sessile organisms for space. A large number of species are dependent on hard surfaces to attach to for their survival. This includes many types of organisms, from unicellular bacteria and microalgae to multicellular organisms such as macroalgae, mussels and barnacles. Available hard surfaces are scarce in the marine environment, in comparison to the number of organisms depending on them and as a consequence all submerged surfaces are targeted by settling organisms in their search for a space to live. When that surface is a man-made structure, e.g. a boat hull, a buoy or an underwater construction, the colonization is most often undesired and referred to as **biofouling**. The colonization of a surface starts as soon as it is submerged with accumulation of organic particles to the surface. This is followed by attachment of microorganisms such as bacteria and microalgae which together with marine fungi and other unicellular organisms form a microbial film, often also referred to as slime or microfouling. This first microbial layer attracts spores from macroalgae and invertebrate larvae who are referred to as macrofoulers (Yebra et al., 2004). The

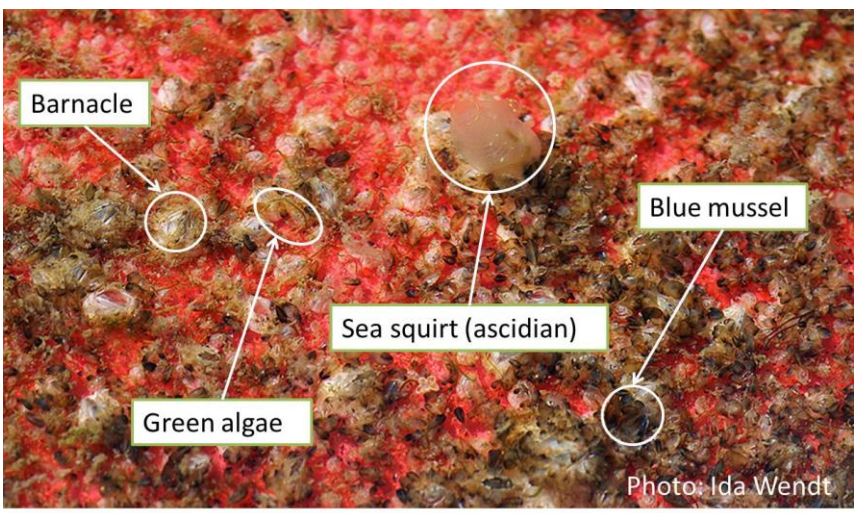


Figure 1. Settling surface with a typical fouling community on the Swedish west coast. The surface is made from PETG and painted with non-biocidal paint. Macrofoulers such as mussels and barnacles are easily visible, while a microscope is needed in order to see microfoulers such as bacteria and diatoms.

composition of the fouling community is not the same in different regions of the world's oceans as it is recruited from the local ecosystems. More than 4000 species has been identified worldwide as biofouling species (Yebra et al., 2004). On the Swedish west coast, the macrofouling community typically includes the barnacle *Amphibalanus improvisus*, the mussel *Mytilus edulis*, macroalgae from the genus *Ulva* and various bryozoan, hydrozoan and ascidian species (Berntsson et al. 2003). This is illustrated in Figure 1 which shows a settling surface that has been submerged in the Gullmar fjord at a depth of 1 meter for a period of little more than two months during summer.

Why is biofouling a problem?

In most human marine enterprises, including transportation, aquaculture and constructions, biofouling is regarded as a nuisance that needs to be eradicated or controlled. For the shipping industry, biofouling brings about high costs as it leads to increased maintenance and dry dock time as well as increased fuel consumption, which in turn cause environmental problems through increased emissions of exhaust fumes. Even a thin slime coverage on a boat hull can have a significant impact on the operational efficiency and the fuel consumption (slime alone reduces the vessel speed with 10-16%) (Schultz, 2007). The annual cost of biofouling for the US Navy has been estimated to 56 million US$, which is mainly due to the increased amount of fuel needed (Schultz et al., 2011). But biofouling is not only an economic problem and a cause of increased emissions, but very much an ecological concern as it is a major vector for the spreading of non-indigenous marine species around the world (Piola et al., 2009). An example is *Amphibalanus improvisus*, the barnacle species most commonly occurring on boat hulls in Swedish waters, which came to northern Europe via fouling on ships from North America (Nellbring, 2005). Non-indigenous species are known to disturb the ecological balances in their new habitats which, if they become invasive, can have severe consequences (Mack et al., 2000). An example of an introduced species that caused considerable damage, both ecological and economical, is the alga *Caulerpa* sp. in the Mediterranean Sea (even though this particular introduction was not mediated through vessel fouling). *Caulerpa* sp. formed large permanent meadows that both caused a reduction in the biodiversity in the areas that where colonised (Francour et al., 2009) and negatively affected the commercial fishing (Klein and Verlaque, 2008). As ecological problems seem to be mostly ignored unless they are coupled to a cost, it has become more and more common to estimate the economic value of ecosystems (Costanza et al., 1997). Consequently, the introduction of harmful non-indigenous species has received a price tag, the United States has for instance estimated the cost of non-indigenous species to 138 billion US$ per year (Dafforn et al., 2011). The growth of living organisms on boat hulls also evokes strong negative feelings among recreational boat owners. Forbidden and highly toxic substances (tributyltin, TBT) have been found in sediment from marinas and in the tissue of snails along the Swedish coast (Magnusson and Samuelsson, 2012,

Nordfeldt, 2007, Magnusson et al., 2008, Magnusson et al., 2009, Cato, 2010, Magnusson et al., 2011) which implies a continuous use of forbidden, but highly efficient, antifouling paints (Cato et al., 2008). It is almost as if the right to have clean hull is valued higher than a healthy marine ecosystem.

What is antifouling?

Antifouling is any method applied to prevent and control biofouling. There are a number of different approaches, the most common being the release of biocides from the painted ship hull (Dafforn et al., 2011). New techniques used or currently under development includes (1) fouling-release coatings for which nanotechnology and/ or polymer science is used to create slippery surfaces with physico-chemical properties that obstruct the attachment of organisms (Callow and Callow, 2011), (2) ultrasound that generates ultrasonic cavitations which prevent settling (Guo et al., 2011) , (3) oxygen-free layers at the hull surface that deter all oxygen demanding life from settling (Lindgren et al., 2009) and (4) electrochemical coatings that regularly changes the pH at the boat surface (Fraunhofer-Gesellschaft, 2012) . This thesis is focused on the efficacy and the ecotoxicity of antifouling biocides.

What is an antifouling biocide?

The word biocide is a composition of the Greek word "bios", meaning "life" and the latin word "cide" that means "to kill". The definition of a biocide used in the most recent update of the European biocide legislation (Regulation (EU) No 528/2012, adopted in spring 2012) is as follows: "any substance or mixture, in the form in which it is supplied to the user, consisting of, containing or generating one or more active substances, with the intention of destroying, deterring, rendering harmless, preventing the action of, or otherwise exerting a controlling effect on, any harmful organism by any means other than mere physical or mechanical action" (European Parliament, 2012). An antifouling biocide is in other words a substance designed to prevent fouling organisms from settling on a surface. This can, but must not necessarily, be made by killing the organism. Antifouling biocides can both be natural occurring metal ions such as copper (Cu^{+}, Cu^{2+}), derivatives from organisms such as capsaicin from Spanish pepper, or synthetic substances. This thesis deals with eight antifouling biocides: copper (Cu^{2+}), tolylfluanid, triphenylborane pyridine (TPBP), 4,5-dichloro-2-*n*-octyl 3(2H)-isothiazolone (DCOIT), irgarol, copper pyrithione, zinc pyrithione and medetomidine.

Efficacy vs. ecotoxicity

The big challenge in the field of antifouling research is not to make an efficient antifouling coating, but to do so without harming the environment. Antifouling paints are designed to release biocides from the paint surface and thereby create a hostile environment for settling organisms at the painted hull. As a consequence, antifouling biocides are also released into the surrounding water and end up in aquatic environments where they can affect non-target organisms (Thomas and Brooks, 2010). Thereby, the biocides from the paint have become an environmental pollutant of ecotoxicological concern. Efficacy and ecotoxicity are two inherent properties of an antifouling biocide; they are two sides of the same coin.

The connection between efficacy and ecotoxicity of antifouling biocides can be illustrated with the history of tri-butyl tin (TBT), one of the most efficacious antifouling biocides ever used. TBT in self-polishing copolymer paints was first applied as an antifouling coating in the 1960's and was soon shown to be extremely efficient at preventing biofouling. In addition to the high efficacy, the coatings were also long-lasting. Its popularity spread worldwide, and TBT was used on the larger part of the world fleet (Yebra et al., 2004). In the mid 1970's, low reproduction and shell malformations among oysters were reported from French oyster producers, and in the early 1980's these effects could be coupled to TBT exposure (Alzieu, 2000). After this, a number of non-target organisms have been declared affected by TBT including phytoplankton, fish and mammals (Fent, 1996). Maybe the most sensational ecotoxicological effect of TBT is the masculinization of female gastropods (imposex) that leads to sterlilty (Gibbs, 2009), which nowadays is the biomarker used for TBT exposure. From the discovery of the severe environmental impact from TBT coatings in the early 1980's, it took almost 30 years before the biocide was globally banned in 2008 (International Maritime Organisation, 2008). One of the reasons behind the severe ecotoxicological consequences of TBT is its persistence in the marine environment, with reported half-life ranging from months to years (Dubey and Roy, 2003), and gastropods exposing imposex are still found in coastal waters despite the international ban (Magnusson and Samuelsson, 2012). The lesson learned from the TBT experience is the importance of evaluating the environmental impact of a biocide prior to its use, and as a consequence we can now see biocidal legislations in most parts of the world (e.g. European Parliament, 1998).

Detailed knowledge of biocidal efficacy and ecotoxicity makes it possible to optimize the use of antifouling biocides

After the ban of TBT, considerable efforts have been put into the research on antifouling strategies. An alternative to antifouling techniques based on physical and mechanical principles is to reduce the amount of antifouling biocides used in the paint to an optimized minimum. The studies in this thesis have been produced within the scope of the research program Marine Paint (2003-2011), which had the overall aim to produce an environmentally optimized paint formulation (Marine Paint, 2012). Part of that work was to design optimized combinations of biocides, optimized in the meaning of being highly effective while at the same time pose the lowest possible environmental risk. The idea is to combine several biocides, the underlying assumption being that a combination of biocides is more efficient than just one or two biocides. As mentioned above, the biofouling community is diverse, both in the number of species present and in the antifouling sensitivity represented by those species. A biocide will not affect all species in the exact same way and at the same concentration. Hence, using only one biocide to prevent settlement and growth of the

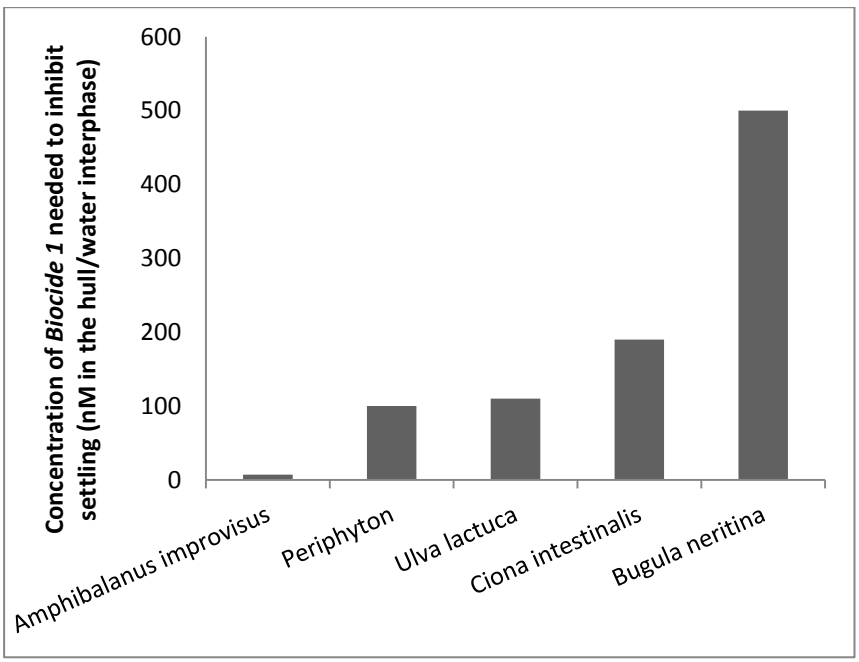


Figure 2. Sensitivity distribution among five fouling species for *Biocide 1*. To inhibit settling of different fouling species, different amounts of a specific biocide are needed. For *Biocide1* the barnacle *Amphibalanus improvisus* is the most sensitive while bryozoan *Bugula neritina* is asssthe least sensitive. To use the amount needed to prevent *Bugula neritina*. from growing on the hull would result in an overdosing for all the other species presented. Different biocides show different sensitivity distributions.

entire fouling community would result in an overdosing for a large part of the community as the concentration needed is driven by the most tolerant species. The sensitivity distribution among five fouling species for *Biocide 1* is illustrated in Figure 2. As can be seen in the figure, the bryozoan *Bugula neritina* is by far the most tolerant species. In order to prevent settlement and growth of all five species, the concentration of *Biocide 1* must therefore be as high as the concentration needed to inhibit *Bugula neritina*, thus overdosing for the other four species. However, the amount needed would decrease significantly if *Biocide 1* were to be combined with a biocide specifically targeting *Bugula neritina*. If a third biocide was added, specifically targeting *Ciona intestinalis*, the amount needed of *Biocide 1* would decrease even further. A combination of biocides can therefore allow for a decrease in concentration of the individual biocide. This is also an advantage in the perspective of tolerance development. As it is more difficult to develop tolerance to a mixture of biocides than to a single biocide, a gradual increase in biocide concentration due to tolerance development can be avoided. This is similar to the principle of multi-target drug treatment used in medicine (Bonhoeffer et al., 1997, Zimmermann et al., 2007, Luni et al., 2010).

An efficient biocide mixture can either be attained by testing all possible biocide combinations and concentrations *in vitro* (which from practical perspectives is not feasible), or by using mixture toxicity predictions performed *in silico* (i.e. using computer). However, for the later alternative a sound understanding of the sensitivity patterns in the fouling community is required, or put the other way around; a detailed understanding of biocide efficacy. If the concentrations needed to prevent settling of the fouling species are known, mixtures with a high efficiency towards the fouling community can be predicted mathematically. For evaluation of the environmental impact of such a mixture, knowledge of the ecotoxicity of the biocides to non-target marine organisms is also needed. Both types of information can be extracted from a concentration-response curve, which will be further discussed below.

Target and non-target organisms

Just as efficacy and ecotoxicity are two inherent properties of an antifouling biocide, marine species can be both target and non-target organism depending on where they live. A macroalga growing on a boat hull is a target organism that needs to be removed, while the same species growing on the bottom underneath the boat is a non-target organism, worthy of protection. Organisms that are not found on the hull, e.g. planktonic- and sediment burrowing species, are regarded as more exclusively non-target organisms (Figure 3).

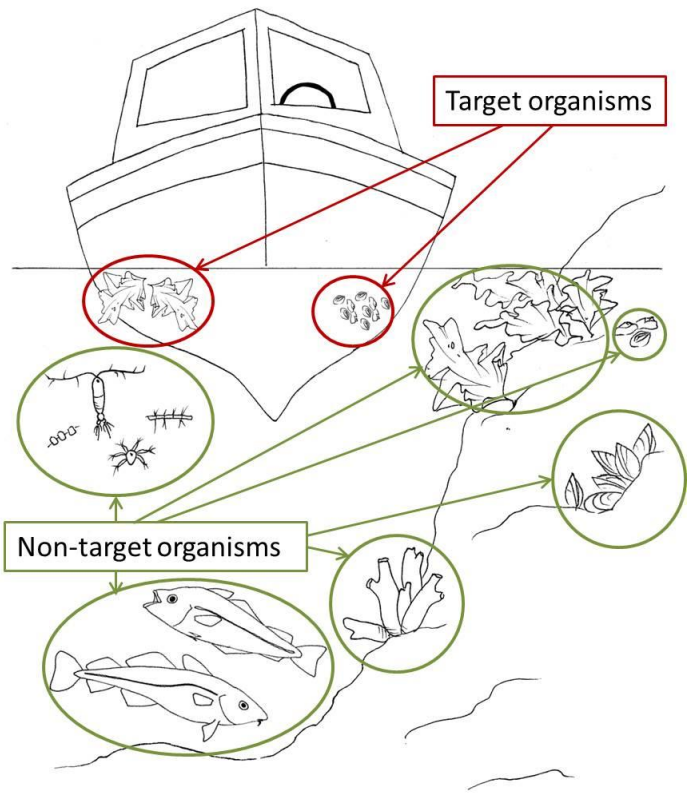


Figure 3. Target and non-target organisms. Sea lettuce and barnacles can be both target and non-target organism depending on where they grow. Illustration: Ida Wendt

The informative concentration-response curve

There is a lot of information that can be extracted from a concentration-response curve, providing it has a high enough resolution in the entire span from 0 to 100% effect (Figure 4). The upper part of the curve provides information on the biocidal efficacy, i.e. what concentration that is needed to entirely prevent activity or settling of a certain fouling species. The lower part of the curve provides information about

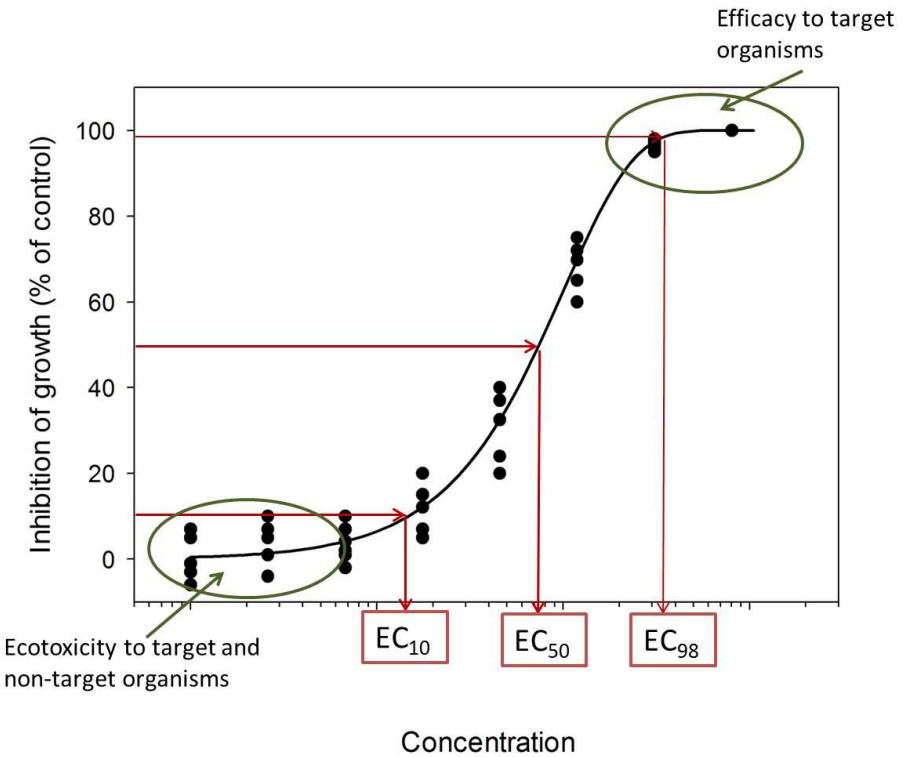


Figure 4. The concentration-response curve provides information on both efficacy, and ecotoxicity. Effect concentrations for different effect levels can be derived from the concentration-response curve. In the figure inhibition of growth is given as an example, but any suitable endpoint can be used, such as e.g. mortality.

the ecotoxicological properties of the biocides. In European biocidal legislation, the ecotoxicity of a biocide is specified as its acute effects on fish, invertebrate and algae combined with its environmental fate (European Parliament, 1998). However, what is meant by ecotoxicity in the present text is a number of biocidal properties, such as the "safe" concentration of the biocide, the concentration at which the biocide starts to have an effect, the concentration range that provokes lower effects, etc. When the mathematical function of the curve is defined, different effect levels can be calculated,

such as the EC50 which corresponds to the concentration at which 50 % of the tested population, or the activity, is affected.

A major part of the work summarized in this thesis has been carried out to describe the concentration-response relationship between antifouling biocides and organisms both considered as target and non-target organisms (further information is given under *Methodological considerations*). Thus, the work contributes to the understanding of the efficacy of the antifouling biocides (the information given in the upper part of the concentration-response curves) as well as the sensitivity of the marine community (information given in the lower part of the concentration-response curve).

Biology of organisms studied

The studied organisms were selected either because of their relevance as target organisms, i.e. they are found growing on boat hulls, or as non-target organisms subjected to a substantial risk of being exposed to antifouling biocides in their natural habitat.

Sea lettuce, *Ulva lactuca* (Linnaeus)

The marine alga *Ulva lactuca*, known as sea lettuce, is commonly found in the littoral zone around the world (e.g. Messyasz and Rybak, 2009, Hofmann et al., 2010, Teichberg et al., 2010, Wang et al., 2010). The thallus of *Ulva lactuca* is only two cells thick, but can grow up to a meter or more in length in exceptional cases. It attaches to the substrata by extensions of the cells at its base (Raven et al., 1999). *Ulva lactuca* is isomorphic, which means that it has an alternation of generations with diploid sporophytes and haploid gametophytes, but morphologically the two generations are very similar (Figure 5). *Ulva lactuca* reproduces through the two types of motile, reproductive bodies; the quadriflagellated zoospores produced by the

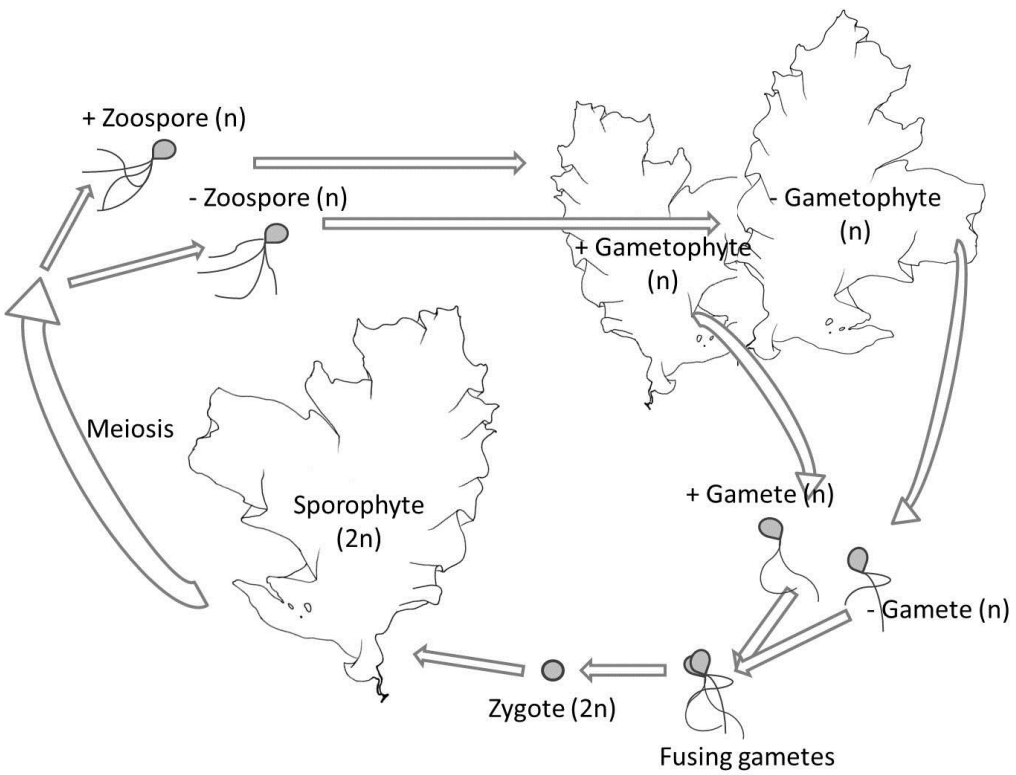


Figure 5. *Ulva lactuca* life cycle, modified from: Raven et al. (1999).

sporophyte, and the biflagellated gametes produced by the gametophyte. Zoospores tend to settle in groups, i.e. they have a gregarious settlement (Callow et al., 1997) and they display a negative phototaxis. The gametes are on the other hand positively phototactic but display negative phototaxis after fusion and zygote formation (Callow and Callow, 2000). A number of cues has been found to affect the settlement of zoospores and includes chemical cues such as the presence of saturated fatty acids, biological cues in the form of microbial biofilms and physio-chemical cues such as surface free energy (Callow and Callow, 2000). The reproduction in *Ulva lactuca* is not restricted to any special areas of the thallus, but all vegetative cells can transform to form sporangium (in the sporophyte) or gametangium (in the gametophyte) in which the zoospores or gametes are formed (Niesenbaum, 1988). Each vegetative cell can give rise to many spores, and this gives *Ulva lactuca* an enormous capability to spread and colonize new surfaces, which is probably one of the reasons behind its wide distribution. The high reproductive output is also the furthermost reason why species from the *Ulva* genus are so successful as fouling species, they are the most commonly occurring macroalgae on boat hulls (Callow and Callow, 2000, Mineur et al., 2008). Due to the high spore production, the easy handling and high relevance in fouling, species from the genus *Ulva* are frequently used within antifouling research (e.g. Briand, 2009, Gudipati et al., 2005, Callow et al., 2002). Why *Ulva lactuca* was the species of choice in the presented studies instead of its filiform relatives, e.g. *Ulva intestinalis*, was its ability to survive and thrive in culture conditions.

Copepod, *Acartia tonsa* (Dana)

Copepods (Figure 6) are the most abundant multicellular organism on earth (Humes, 1994) and among these *Acartia tonsa* is a commonly occurring pelagic species. It lives in coastal waters worldwide (Cervetto et al., 1995) where it in periods can dominate the copepod community completely (Heinle, 1966). *Acartia tonsa* has its origin in the American Pacific Ocean and was brought to European waters in early 1900, most probably via vessel ballast water (Selander, 2005). *Acartia tonsa* is a nocturnal vertical migrator and more abundant in the surface waters during night, when the feeding activity also is at its peak (Cervetto et al., 1995). *Acartia tonsa* can shift between suspension feeding (used for small, non-moving preys such as algae) and raptorial feeding (used for capture of large, moving preys such as ciliates), which can

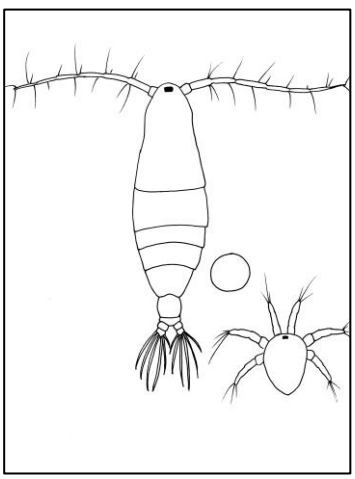


Figure 6. Copepod female, egg and nauplius. Illustrtion: Ida Wendt

be a competitive advantage as it thus can utilize many different food sources (Jonsson and Tiselius, 1990). The species has also displayed cannibalism when starved (Gaudy, 1974). Mating occurs through attachment of a spermatophore to the female genital segment after which the female remains fertile for more than 10 days (Parrish and Wilson, 1978). Females of *Acartia tonsa* have a high and continuous egg production and are free spawners, i.e. the eggs are released into the water instead of held in egg pouches, which facilitates measurements of egg production and hatching success. The number of eggs produced per female is strongly coupled to the nutritional value of the food, and the algal genus *Rhodomonas* is a good diet from a nutritional perspective (Støttrup and Jensen, 1990). The eggs hatch into nauplius larvae which then progress through six naupliar stages and five copepodite stages before they become sexually mature adults. The reproduction cycle from egg to adult takes about three weeks. *Acartia tonsa* is easily held in culture which allows for year-round experimental activity and the species is recommended by ISO for marine ecotoxicological assessments (International Organization for Standardization, 1999). The copepods used in the work in **paper III** are originally from cultures from the Danish Institute for Fisheries Research, Charlottenlund, Denmark (Støttrup et al., 1986).

Periphyton

There are many definitions of periphyton in the literature, but one that more or less summarize them all is the following definition: "*algae, bacteria, other associated microorganisms, and non-living organic matter attached to any submerged surface.*" (Biology Online, 2013). When growing on boat hulls periphyton is also often referred to as *biofilms*, *slime* or *microfouling*. The periphyton community is dominated by unicellular organisms, primarily heterotrophic bacteria, cyanobacteria, diatoms (Figure 7) and unicellular grazers in the form of ciliates and flagellates, but multicellular organisms such as nematodes, small crustaceans and invertebrate larvae are also found within the periphyton (Railkin, 2004). Together they form a community on a micro-scale with interactions both between trophic levels, e.g. grazing and predation, and within tropic levels in the form of competition. Since these important ecological interactions all are represented in small and easily manageable unity, periphyton communities have been used within ecotoxicological testing as a more ecological relevant alternative to single-species testing (Arrhenius et al., 2006, Eriksson et al., 2009, e.g. Blanck et al., 1988).

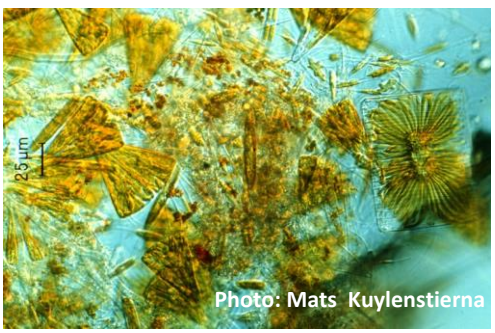


Figure 7. Periphyton community from the Gullmar fjord on the Swedish west coast, what can be seen is mainly diatoms.

Antifouling biocides studied

In this thesis, eight antifouling biocides have been studied: DCOIT, medetomidine, TPBP, tolylfluanid, copper, irgarol, zinc pyrithione and copper pyrithione. Except for TPBP, they all are evaluated under the European Biocidal Product Directive 98/8/EC (BPD) (European Parliament, 1998) for use in EU. The biocides were selected either because they are already in use as antifouling biocides, or they were considered likely to pass the evaluation under the BPD. The evaluation process is up to the present still not completed. Please refer to Table 1 for additional information about the biocides. In the paragraphs below, the applications, mode of action and environmental characteristics of the studied biocides are described.

DCOIT

DCOIT (4,5-dichloro-2-octyl-1,2-thiazol-3(2H)-one) is an isothiazolinone that is used as a broad spectrum booster biocide in antifouling paints where it affects both soft- and hard fouling species (Jacobson and Willingham, 2000). The molecule diffuses easily through cell membranes and cell walls (Morley et al., 2007) and cause oxidative stress in the cell followed by necrosis (Figure 8) (Arning et al., 2008). The toxic mechanism is suggested to be both through the formation of free radicals (Chapman and Diehl, 1995) and by blocking the oxidative defence system. DCOIT inhibits glutathione reductase by irreversible binding to the enzyme active centre, and thereby decreases the amount of cellular glutathione (Arning et al., 2008, Arning et al., 2009, Morley et al., 1998,

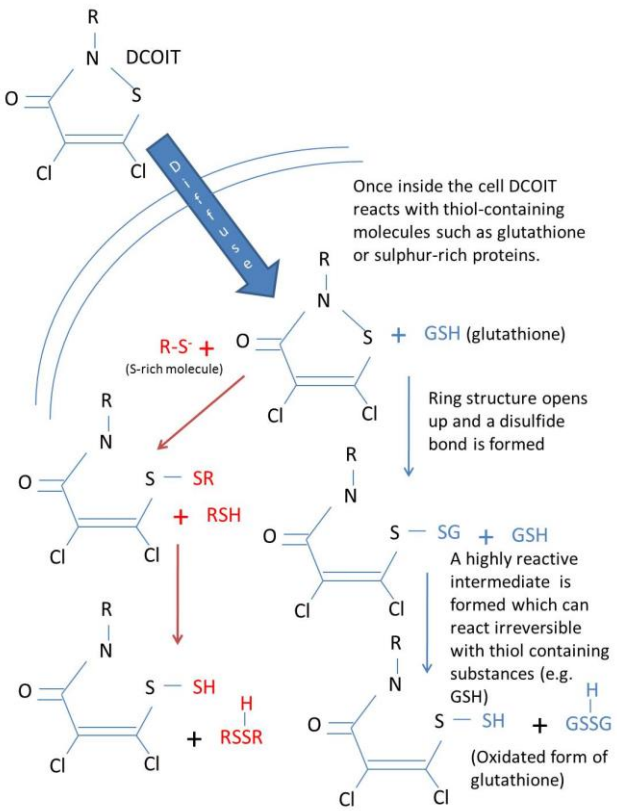


Figure 8. Mode of action of DCOIT. DCOIT enters the cell where it reacts with thiol-containg molecules. Summarized from: Morley et al. (2007), Morley et al. (1998), Arning et al. (2008)

Morley et al., 2007). DCOIT is easily biodegraded with reported half-life in natural seawater between less than 24 hours (Jacobson and Willingham, 2000, Thomas et al., 2003) and 3 days (Callow and Willingham, 1996). Abiotic degradation of DCOIT through hydrolysis and photolysis is considerably slower, and the main route of dissipation in the environment is therefore biological (Norwegian Climate and Pollution Agency, 2010).

Medetomidine

Medetomidine (4-[1-(2,3-dimethylphenyl)ethyl]-1H-imidazole) is traditionally used within veterinary medicine as a sedative agent. Dahlström et al. (2000) discovered that medetomidine inhibits barnacle settling at low concentrations, and consequently attracted the attention to its use in antifouling applications. The biocide is now approved for use as an antifouling biocide in Japan and Korea under the trade name Selektope®, and it is undergoing evaluation in EU (I-Tech 2013). Medetomidine belongs to the chemical group imidazoles, and functions as an α_2-adrenoceptor agonist. In crustaceans, medetomidine binds to octopamine receptors, which is the invertebrate analogue to the adrenoceptor. In barnacles medetomidine inhibits the settling process. By activating the octopamine receptor, medetomidine causes enhanced kicking activity in the swimming legs of the cyprid larvae, thus preventing the larvae from staying at the settling surface (Lind et al. 2010). Whether the octopamine receptor is present in copepods is not known, neither is there any known target receptor for medetomidine in algae.

TPBP

TPBP (triphenylborane pyridine, also known as Borocide®) is an organoborane compound and it is used as an antifouling biocide mainly in Japan (Thomas and Langford, 2009), where it has been the predominant antifouling biocide since 1995 (Mochida et al., 2012). The mechanism behind its toxicity is unknown and the lack of studies is probably due to its limited distribution on the antifouling market. Abiotic degradation through both hydrolysis and photolysis has been shown to occur in natural seawater (Zhou et al., 2007). The highest reported environmental concentration is 21 pg l^{-1} (0. 065 pM) from a fishing port in Japan (Mochida et al., 2012).

Tolylfluanid

Tolylfluanid (dichloro-n-[(dimetylamino)sulfonyl]fluoro-n-(p-tolyl)sulfonamide) is a halogenated sulfonamide derivate that is used as fungicide in agriculture (FAO, 2003), but lately also as an antifouling biocide in paints (Thomas and Brooks, 2010). The mode of action is poorly understood but the toxicity of tolylfluanid is possibly driven by the sulfonamide functional group ($–S(=O)_2-N(CH_3)_2$). Sulfonamides inhibit the synthesis of folate (vitamin B9) since they are structurally analogues of p-aminobenzoic acid (pABA) and therefore inhibit the enzyme dihydropteroate synthase (DHPS) which is part of the folate synthesis pathway (Brain et al., 2008). Folate is an essential co-factor during DNA-synthesis and repair. In plants, folate also plays a role

in the biosynthesis of chlorophyll and in the photorespiration pathways (Hanson and Roje, 2001). Due to the structural similarities, it is also possible that the mode of action of tolylfluanid is similar to that of dichlofluanid, which is through inhibition of thiol-containing enzymes by forming disulfide bridges (Johansson et al., 2012).

Irgarol

Irgarol 1051 (N-cyclopropyl-N'-(2-methyl-2-propanyl)-6-(methylsulfanyl)-1,3,5-triazine-2,4-diamine), also known under the trade name Cybutryn, is a *s*-triazine herbicide widely used in antifouling paints, although European countries such as Sweden, Denmark and UK have restricted its use (Thomas et al., 2002). The mode of action of irgarol is inhibition of the photosynthesis (Hall Jr et al., 1999) which is attained through binding to the plastoquinone (Q_B) binding-niche at the D1 protein in photosystem II (PSII) (Tietjen et al., 1991). This leads to blocking of the electron transfer and inhibition of the D1 protein turnover (Jansen et al., 1993). It is also known that PSII inhibiting herbicides causes oxidative stress through production of reactive oxygen species. This production of radicals is assumed to be the cause of cell death in exposed plants rather than starvation following the inhibition of electron transfer (Rutherford and Krieger-Liszkay, 2001, Fufezan et al., 2002). Photosynthetic organisms exposed to triazine herbicides are also known to increase their content of chlorophyll and accessory photosynthetic pigments. This effect is known as the greening effect and is assumed to be a compensation mechanism for the loss of photosynthetic efficiency (Hatfield et al., 1989, Koenig, 1990, Boura-Halfon et al., 1997).

Copper

The use of copper based antifouling paints has increased significantly after the ban of TBT. In 2004 copper was the most commonly used antifouling biocide, which still is the situation (Yebra et al., 2004, KEMI, 2011). Copper is an essential metal for many organisms. In plants, copper is associated with the enzyme plastocyanin which is involved in the photosynthetic electron transfer (Taiz and Zeiger, 2010) and in crustaceans it is part of the oxygen binding blood protein haemocyanin (Hebel et al., 1997). However, at elevated concentrations copper becomes toxic. The main toxicity mechanism is assumed to be oxidative stress which is caused both through the formation of reactive oxygen species and through a reduction of the antioxidant capacity in the cell (Wu et al., 2009, Knauert and Knauer, 2008), but copper also binds to the sulfhydryl groups on proteins and thereby disrupt the protein structure and inhibit their function (Letelier et al., 2005). Copper is also known to inhibit algal photosynthesis and growth (Reed and Moffat, 1983, Bond et al., 1999, Lewis et al., 2001, Gatidou and Thomaidis, 2007, Han et al., 2008) and to disrupt cell membranes (Webster and Gadd, 1996).

Zinc pyrithione and copper pyrithione

Zinc- and copper pyrithione (2(1H)-pyridinethione, 1-hydroxy-, zinc/copper(2+) salt) are metal chelates of two pyrithione rings (C_5H_5NOS) bonded to a central metal ion via zinc/copper-oxygen bridges (Dinning et al., 1998a). The central metal ion can be exchanged with other cations such as Na^+, Fe^{2+} and Mn^{2+}, all with different complex strength. The order of complex strength is believed to be as follows: Na<Fe<Mn<Zn<Cu. The speciation of pyrithione in natural seawater is therefore dependent on both the concentration of pyrithione, the concentration of metals and the presence of other organic- and inorganic ligands (Dahllöf et al., 2005). Zinc pyrithione will therefore to a large extent be transchelated into copper pyrithione in contact with water containing copper. Moreover, if zinc pyrithione is present in an antifouling paint together with copper, e.g. Cu_2O, all pyrithione leakage will be in the form of copper pyrithione (Grunnet and Dahllöf, 2005). Both pyrithiones are photodegradable with half-lives less than one hour in full sunlight (Thomas and Brooks, 2010). However, in seawater the wavelengths reported to be most active in photodegradation of zinc- and copper pyrithiones (320-355 nm) are extinct within the first two meters of depth and therefore the pyrithiones are potentially accumulated in sediments (Maraldo and Dahllöf, 2004a). Due to technical difficulties, only few attempts have been made to measure environmental concentrations of zinc- and copper pyrithione, but zinc pyrithione has been found in UK waters at a concentration of 105 nM, and copper pyrithione has been detected in harbour sediments in Japan (Thomas and Brooks, 2010). The pyrithiones are marketed as broad spectrum antimicrobial biocides. Except for use in antifouling paints, the pyrithiones are also used in e.g. plastics, textiles, dry paint and personal care products such as anti-dandruff shampoo (Arch Chemicals, 2013). There are two possible ways through which pyrithiones can cause toxicity in a cell: (1) membrane disruption through complex binding between the metal ion and the phosphate head group of the membrane lipids (Figure 9) (Dinning et al., 1998a) (2) apoptosis as a consequence of increased zinc/copper ion concentration within the cell (Mann and Fraker, 2005).

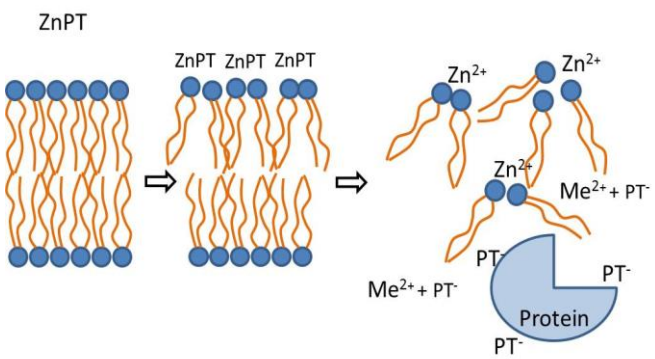
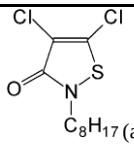
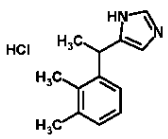
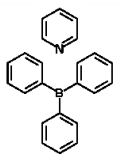
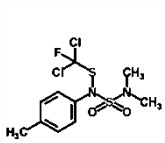
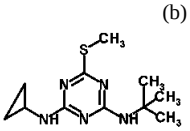
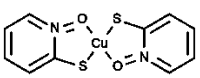
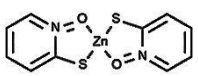


Figure 9. Suggested mode of action for zinc pyrithione at the bacterial outer cell membrane. Modified from: Dinning et al. (1998a).

Table 1. Substance identity and key physical-chemical characteristics of biocides investigated.

Substance name	Structure	Systematic name	Synonymes	CAS no.	Molecular weight (g mol^{-1})	Purity	Log K_{OW}
DCOIT	 (a)	4,5-dichloro-2-octyl-1,2-thiazol-3(2H)-one	Sea-Nine 211 Kathon	64359-81-5	282.2	>95%	2.8
Medetomidine	 (b)	4-[1-(2,3-dimethylphenyl)ethyl]-1H-imidazole hydrochloride	Selektope® Catemine	86347-15-1	236.7	>99%	3.13
TPBP	 (b)	Triphenylborane pyridine	Borocide® Pyridine-triphenylborane	971-66-4	321.2	>99%	5.52*
Tolylfluanid	 (b)	Dichloro-n-[(dimetylamino) sulfonyl]fluoro-n-(p-tolyl) sulfonamide	Euparen Preventol	731-27-1	347.3	>99%	3.93
Irgarol	 (b)	N-cyclopropyl-N'-(2-methyl-2-propanyl)-6-(methylsulfanyl)-1,3,5-triazine-2,4-diamine	Cybutryne	28159-98-0	253.4	>97%	2.8
Copper	$CuCl_2 \cdot 2H_2O$ (b)	Copper(II) chloride dehydrate		7447-39-4	134.5	>99%	
Copper pyrithione	 (c)	2(1H)-pyridinethione, 1-hydroxy-, copper(2+) salt	Copper OMADINE™	14915-37-8	315.9	>95%	0.97
Zinc pyrithione	 (c)	2(1H)-pyridinethione, 1-hydroxy-, zinc(2+) salt	Zinc OMADINE™	13463-41-7	317.7	>95%	0.97

[a] Thomas et al. 2003
[b] chemspider.com
[c] chemistry.about.com
* log K_{OW} for triphenylborane (TPB)

Aim and approach

The overall aim of this thesis was both to evaluate the efficacy of antifouling biocides and to describe the ecotoxicity of the same biocides to marine organisms. A detailed understanding of the biocidal efficacy enables an optimized choice of biocides for paint designs, and knowledge of the ecotoxicity is crucial in order to evaluate the environmental impact of the biocides as well as the final paint product. More specifically, the focus within this thesis is to evaluate the efficacy and the ecotoxicity of the eight antifouling biocides DCOIT, tolylfluanid, TPBP, copper, irgarol, copper pyrithione, zinc pyrithione and medetomidine to marine macroalgae (*Ulva lactuca*) and microalgae communities (periphyton). To extend the ecotoxicological evaluation beyond target species, the ecotoxicity of DCOIT, TPBP and medetomidine to copepods (*Acartia tonsa*) was also determined.

For evaluation of efficacy, a settling and growth approach has been chosen. The sole purpose of an antifouling biocide is to prevent settlement and growth of fouling organisms, therefore the choice of endpoint for efficacy evaluation is rather straight forward, i.e. inhibition of settling and growth. For evaluation of ecotoxicity the choice of approach is a bit less obvious. Within ecotoxicology, the ideal endpoint is the most sensitive biological process or system that affects the long-term survival of the species within their ecological niches. That is to say how the population can tackle the prevailing abiotic and biotic conditions and survive in its habitat. Abiotic conditions are e.g. light, temperature, salinity, nutrients and oxygen saturation while the biotic factors consist of e.g. predation pressure, interspecies competition and food availability. Endpoints that affect the ability of the long-term survival are also referred to as sub-lethal endpoints and may include essentially any biological process, although photosynthesis, respiration, reproduction, growth and behaviour are among the more common. For attached species, the ability to settle on a surface and go through metamorphosis from a pelagic form to a sessile form is crucial for survival. Consequently, inhibition of settling is highly relevant for ecotoxicological as well as efficacy evaluations. For the fouling organisms studied, i.e. *Ulva lactuca* and periphyton communities, the settling and growth approach is suitable. However, the copepod *Acartia tonsa* is a pelagic animal and effects on reproduction were therefore chosen as sub-lethal endpoint for the measurement of ecotoxicological effects on this group.

The specific aims for the individual studies included in this thesis were:

Paper I
The aim of this study was to evaluate the efficacy and ecotoxicity of the five antifouling biocides DCOIT, tolylfluanid, TPBP, copper and medetomidine to the marine macroalgae *Ulva lactuca*. Settlement and growth was chosen as the most appropriate endpoint, and part of the aim was therefore also to develop a zoospore settlement assay, modified from prevailing methods and optimized in the aspects of feasibility, swiftness, and accuracy.

Paper II
The aim of this study was twofold: both to develop a settling assay using natural microfouling communities (periphyton) and to investigate the effect of antifouling biocides on periphyton using the new method. The eight biocides studied were: DCOIT, tolylfluanid, copper pyrithione, zinc pyrithione, TPBP, copper, medetomidine and irgarol.

Paper III
The aim of this study was to evaluate the ecotoxicological effects of antifouling biocides on planktonic species. *Acartia tonsa* was chosen as a representative of the pelagic copepod community. The ecotoxicity of DCOIT, TPBP and medetomidine was quantified both as mortality and as EC_X of egg production, thereby enable comparison of endpoint sensitivity.

Paper IV
The aim of this study was to elucidate weather a population of the marine macro algae *Ulva lactuca* had developed a tolerance towards the algaecide irgarol, and if so, to quantify that tolerance. The population were from a site in the Gullmar fjord where tolerance development has been observed in periphyton communities.

Methodological considerations

The methods used in the thesis will be discussed on a general level to give an insight to the reasoning behind the choice of methods. For more detailed methodological descriptions, please refer to the corresponding paper.

Cultured vs. field-sampled test organisms

Cultured organisms and organisms sampled directly from the mouth of the Gullmar fjord have been used for the experiments. Both types of approaches have their merits. Field-sampled organisms can be argued to have a higher ecological relevance in that they represent what is actually occurring in the marine ecosystem, but they also

Figure 10. Cultures of *Rhodomonas salina* in plastic bags with vigorous air bubbling to avoid sedimentation of the algal cells (a), cultivation of *Acartia tonsa* in open 100 L plastic barrels with air supply through gentle bubbling (b), incubation of adult *Acartia tonsa* in cell culture flasks during the mortality assay (c).

present a higher variance. Periphyton communities sampled from the field will all differ slightly in their tolerance since no communities will have exactly the same species composition. However, the variation in sensitivity to biocides among periphyton communities from different sites and years has been found to be no larger than a factor of 2-3 (Arrhenius et al., 2004). It might be more difficult to establish the cause of a certain biological response in a more complex system such as periphyton communities than in a less complex system such as one-species testing. Nevertheless, periphyton communities indisputably represent the pool of species from which a microfouling community is recruited. By using settling surfaces in the field made from the plastic polyetylene therophtalate, glycol-modified (PETG), there is a good probability of sampling the composition of species actually occurring on boat hulls as PETG roughly resembles the surface of self-polishing paints. In contrast, the copepods investigated (*Acartia tonsa*) was reared in culture instead of collected from the field (Figure 10a-b). Cultured organisms ensure for a steady provision of experimental organisms and present a lower variance (lower genetic variability) since they most often are either clones (e.g. algae), or inbreeded (e.g. *Acartia tonsa*). Even though a low variance within the cultured population facilitates comparisons between experiments, it also results in a lower ecological relevance. As the individuals from a cultured population have not been subjected to the same natural selection as their natural counterparts, they may have a different functional and/or morphological diversity compared to populations from the field. However, the sensitivity to heavy metals have been shown not to differ between cultured and natural populations of *Acartia tonsa* (Sosnowski and Gentile, 1978), suggesting that cultured animals can be used for adequately estimating the sensitivity in natural populations. *Ulva lactuca* represent something between the two previous presented test systems. Since the thalli were sampled from the field, the *Ulva lactuca* used in the experiments represents genotypes truly existing in the ecosystems. Additionally, by keeping the collected thalli in culture after sampling (Figure 11a) the experimental activities were prolonged beyond the field season. It was also because of its ability to survive and thrive under cultural conditions that *Ulva lactuca* was chosen as test species instead of its filiform relatives, such as *Ulva intestinalis*.

The use of cultured populations is often argued to facilitate the experimental procedure since it ensures a continuous provision of experimental organisms. From an overall perspective this is true, but not without exceptions. Biological organisms are not machines, and their responses to their environment can never truly be predicted. Cultures crash for no obvious reason, and the health status of the organisms as well as the reproductive output varies on grounds that may seem incomprehensible. From personal experience, it has also been noted that the production of spores of *Ulva lactuca* follows the lunar cycle. This is a behaviour described from the field (Sawada, 1974), and apparently the response to the monthly phases remains in culture even though the tidal influences are removed. Although the phenomenon is highly fascinating, it is also highly inconvenient if the purpose of the cultivation is to achieve

a high and continuous spore production. In spite of the fact that cultivation of organisms is coupled to a high workload, it is most often a more time efficient alternative than sampling specific species from the field. Field sampling is limited by the weather conditions and restricted to the (at times very short) time windows in which the species and/or specific life stage is accessible.

Figure 11. Cultivation of *Ulva lactuca* in flow-through aquaria (a), spore-release from *Ulva lactuca* thallus (b).

The settlement assays

For organisms living attached to a surface, settlement and growth are key processes. They are also highly relevant for antifouling efficacy, and were chosen as endpoints in our assays. To quantify the settlement and growth, the amount of chlorophyll *a* was chosen as a measurement of the attached biomass. By using chlorophyll *a*, the least sensitive photosynthetic species/individual is taken into account since an absence of chlorophyll *a* in the samples indicates that all algae and cyanobacteria are dead (the last chlorophyll *a* represents the most tolerant photosynthetic species/individual).This makes chlorophyll *a* measurements very suitable for efficacy evaluation. However, by using chlorophyll *a* we only measure the effects on photosynthetical organisms, and for the periphyton communities this implies that any effects on the non-photosynthetic part of the community are neglected. In addition, for ecotoxicological evaluations in periphyton communities, the total amount of chlorophyll *a* is a rather crude endpoint since it does not provide any information of changes in the community structure. A more sensitive endpoint would be to measure number of species and abundance as that captures early effects on changes in the community. Pigment composition has previously been used as an efficient and easily used tool for surveying the relative

species abundance (Porsbring et al., 2007). A change in the pigment composition indicates elimination of species or increase/decrease in species abundance, i.e. early changes that influence the survival of a species in its ecological niche.

The total incubation time in the settling assays was set to 72 hours. The algae were allowed to settle during the first 24 hours after which the experimental solutions were changed and thereby all non-attached organisms were removed. The following 48 hours of incubation allowed for effects on growth to be captured in addition to effects on the settling capability. For antifouling efficacy, inhibition of growth is equally important as inhibition of settling since it does not take more than a few individuals out of many thousands that manage to settle and grow to create a biofouling problem. This is especially of concern for macrofoulers, e.g. individuals of *Ulva lactuca* can grow as large as one meter or more in extreme cases (Raven et al., 1999). On the other hand, hundreds of individuals that manage to settle but not grow are not as problematic.

Periphyton

For testing settlement and growth of natural communities of microfoulers, i.e. periphyton communities, the Scrape, Shake and Sieve (SSS) -method was developed (Figure 12). Historically, antifouling assays using microorganisms have mainly been single-species tests (Briand, 2009) and there has been a need for more realistic bioassays for microfoulers using multispecies rather than single species. In addition, assays involving diatoms have been deemed especially important in the antifouling research as many antifouling coatings fail against microalgae (Briand, 2009). By using communities established in the field as start material, the SSS-assay includes a high biodiversity and species with differential sensitivity to toxicants. The experimental approach resembles the reattachment of cells on new substrata after sloughing of a periphyton community due to e.g. nutrient depletion. This can be compared to settlement of pelagic life stage of a sessile macrofouler (such as *Ulva lactuca*) which undergoes metamorphosis and thereafter becomes permanently attached. A detailed description of the periphyton settling assay can be found in **Paper II**.

Ulva lactuca

Zoospores were chosen for evaluation of settlement and growth in *Ulva lactuca* (Figure 11b). In contrast to gametes, zoospores do not need to fuse prior to settling which facilitates the experiments considerably. However, one could argue that asexual reproductive bodies represent a smaller sensitivity range than sexual reproductive bodies because of the higher genetic variability that comes with sexual reproduction. Variability in sensitivity is important to take into consideration since a coating-design based on the settling of a sensitive reproductive body is not guaranteed to prevent settling of a less sensitive reproductive body. No studies have been published that

Figure 12. Demonstration of the SSS-method: sampling on polyetylene therophtalate (PETG) surfaces in the field (a), scraping the settled microfouling community (periphyton) from the PETG surface (b), collection of the scraped-off organisms (c), shaking a bottle containing PETG surfaces to resuspend attached cells (d), sieving to remove quickly aggregated cells (e), re-settling of microorganisms onto new PETG-surfaces while exposed to biocides in the lab (f).

compares the sensitivity of zoospores and gametes to biocides. Hence, it is unclear which of the two reproductive types that is the best for antifouling testing. Therefore practical reasons, such as quick settling, seems to be the underlying factor for choosing zoospores in most studies, as well as in this thesis.

It was deemed as important that the entire settled biomass was analysed in order to ensure that no attached spore was neglected. Therefore, the prevailing method for settlement estimations where parts of the total settled area are selected and analysed for attached spores and germlings using a microscope (Briand, 2009), was left out as an alternative. In addition, zoospores of *Ulva* tend to settle in clusters (Callow et al., 1997, Callow and Callow, 2000), which makes it difficult to select representative areas for enumeration. Hence, analysis of the entire settled surface area and the whole biomass of settled spores and germlings improve the accuracy of the settlement

estimates why chlorophyll *a* measurements were chosen. To avoid loss of settled biomass, the chlorophyll *a* extractions were made directly in the incubation vials. This approach has previously been applied in studies of fouling release surfaces (e.g. Sommer et al., 2010, Chaudhury et al., 2005), but not for biocidal testing. A detailed description of the *Ulva lactuca* settling assay can be found in **Paper I**.

The *Acartia tonsa* ecotoxicological assays

Mortality

The experimental procedures for mortality testing in *Acartia tonsa* followed the procedures stated in the international standard for determination of acute lethal toxicity to marine copepods (International Organization for Standardization, 1999). Males and females were exposed to the biocides in a static exposure design without food (Figure 10c). The gender distribution was not standardized and consequently the numbers of males and females differed between samples. It has been shown that females are slightly more tolerant than males to biocide exposure (Medina, 2002). This is believed to be due to the slightly larger body size in females and the egg production through which females can eliminate some of the toxicants. However, this difference in sensitivity is only significant during the first 24 hours of exposure, and no difference is detectable after 48 hours (Medina, 2002). In the study presented in **Paper III**, the incubation time was set to 48 hours.

Egg production

Biocidal effects on the egg production were investigated in a semi-static test using only females. In *Acartia tonsa*, the process from ingested food to produced egg takes approximately 24 hours. The number of eggs produced by a female is therefore a product of her nutritional status from the day before, i.e. the food availability and the quality of the ingested food (Støttrup and Jensen, 1990). Females were therefore incubated together with the microalga *Rhodomonas salina* at a concentration previously established to induce maximum egg production in female *Acartia tonsa* (data not published). To prevent algal sedimentation on the bottom of the incubation bottles that would render the food inaccessible, the bottles were incubated attached to a slowly rotating plankton wheel. *Rhodomonas* sp. is known to be of a high food quality to copepods (Støttrup and Jensen, 1990), and is also recommended by the ISO standard (International Organization for Standardization, 1999). Even though it is not strictly necessary for the egg production, the females were incubated with algae also during the second day of incubation. This was done for two reasons; to maintain the same type of exposure scenario as during the first day, and to minimize egg cannibalism as copepods are known to eat their eggs when starved (Gaudy, 1974). A

detailed description of the methods used for copepod mortality and egg production studies can be found in **Paper III**.

The biocidal exposure routes differed between the two copepod experiments in that mortality was measured in the absence of food, while egg production was measured when food was present. In nature, copepods experience periods of full food saturation (during phytoplankton blooms) as well as periods of starvation (between blooms). An ecologically realistic exposure scenario for pelagic copepods should consequently include both food situations, like in **Paper III**. Since the bioaccumulation of a hydrophobic biocide depends on whether the organism is exposed via water and/or food (Magnusson and Tiselius, 2010), bioaccumulation rates will differ between different experimental setups. However, it is completely meaningless to measure effects on egg production in a system without food since the accumulation of carbon is a requirement for egg production. As the process of food accumulation and egg production are so tightly linked with one another, it is not possible to distinguish whether effects are caused by a higher physiological vulnerability or by a higher bioaccumulation of the biocide.

Test conditions

Natural seawater from the Gullmar fjord (marine tapwater from 30 m depth) was used in all experiments to achieve as natural test environments as possible for the organisms. Using water from below the pycnocline entails a rather stable water with a more or less constant salinity of 32 and smaller fluctuations in water chemistry compared to surface waters. The water chemistry affects the toxicity and bioavailability of the biocides, and therefore variability in water chemistry would result in variability in biocidal toxicity. To overcome variability between experiments and allow for inter-experimental comparisons, results were always related to controls from the same experiment. The water was prefiltered with GF/F to remove particles and then filter sterilized using a 0.2 µm membrane filter (Pall Corporation, VWR international, Stockholm, Sweden). For those assays where addition of unwanted particles could not be controlled, i.e. all assays were algal cells were added (the periphyton assay and the egg production assay), additional filter sterilization of the water was regarded unnecessary. All tests were made in 15°C and with a day-night regime of 16 hours light and eight hours darkness, which were set to mimic typical conditions during the boating season in Swedish waters. However, the light intensity differed between experiments and had a higher photon flux density in the algal tests to make sure the algae had sufficient light intensity (40-60 µmol photons $m^{-2}s^{-1}$, PAR) to sustain photosynthesis and growth.

Dimethyl sulfoxide, DMSO, was used as co-solvent in all experiments since it was the only co-solvent investigated that could dissolve copper pyrithione and zinc pyrithione. For copper and medetomidine however, DMSO reacts with the biocides and forms complexes, why instead deionized water was used as solvent for those two biocides. To enable future mixture predictions as well as comparisons of efficacy and toxicity of different biocides, DMSO was added to the experimental water (the final water solution used in the experiments) of copper and medetomidine in order to achieve identical DMSO consecration in all samples. No differences in toxicity were detected to periphyton communities exposed to copper with DMSO and copper without DMSO. It was therefore concluded that the amount of DMSO added to the experimental water (100µl l^{-1}) was not enough to affect the bioavailability and toxicity of the copper ions. Biocidal stock solutions in DMSO were prepared before experimental use and stored in -18°C for maximum 4 weeks.

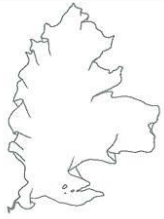
Main findings and discussion

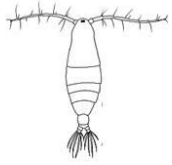
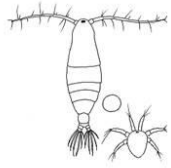
The antifouling biocides investigated within this thesis (DCOIT, medetomidine TPBP, copper, tolylfluanid, irgarol, copper pyrithione and zinc pyrithione) all caused different effect levels on the studied organisms, which is to be expected due to the different modes of action of the biocides and the differences in biology of the organisms. A summary of the effects of the eight antifouling biocides is provided in Table 2. In the following section the main findings will be presented and discussed, please refer to corresponding paper for a more detailed presentation of the results.

Efficacy of antifouling biocides

In the design of novel antifouling paints it is desirable to minimize the amount of biocides used, both from an environmental perspective and to minimize costs. A detailed understanding of the efficacy of different biocides on fouling organisms provides information on the minimum concentration needed of each biocide at the hull to prevent settling. This understanding of efficacy is also essential for an optimized choice and combination of biocides for paint design purposes. As discussed in the introduction, a mixture of biocides allows for a lower concentration of individual biocides without affecting the overall efficacy. For efficacy evaluation, it is primarily the highest effect concentrations that are of interest, i.e. the concentrations that prevents fouling entirely. Those concentrations are found in the upper part of the concentration-response curve (Figure 4). The mathematically calculated concentration-response curves are asymptotic, and consequently the concentration corresponding to 100% effect would be indefinitely high. Therefore, the EC_{98}-values have been chosen as the concentration at which the request of total inhibition was considered achieved. It is important to keep in mind that the biocide with the highest efficacy not necessarily needs to be the biocide with the highest ecotoxicity, provided that ecotoxicity is defined as effects at low concentrations. This is due to the different shapes of the curves. A steep curve will result in an EC_{10} value more close to the EC_{98} value than a shallow curve. This can be illustrated in the case with biocidal efficacy and ecotoxicity in periphyton (Figure 14) were copper pyrithione was found to be the most efficacious biocide at preventing settlement and growth (i.e. the lowest EC98 value, 50 nM), but irgarol had effects on periphyton at lower concentrations (i.e. a lower EC_{10} value, 0.1 nM), this will be discussed further below.

Table 2. Summary of the effects of the eight antifouling biocides DCOIT, medetomidine, TPBP, tolylfluanid, copper, irgarol, copper pyrithione and zinc pyrithione on the target organisms *Ulva lactuca* and periphyton communities, and the non-target organism *Acartia tonsa*. The efficacy/ecotoxicity quotient (EC_{98}/EC_{10}) is given and indicates slope steepness. All concentrations are given in nM, n.d. = not determined.



	***Ulva lactuca* L.** Inhibition of settling and growth (72h)				**Periphyton** Inhibition of settling and growth (72h)				***Acartia tonsa* D.** Mortality (48h)			***Acartia tonsa* D.** Egg production (48h)		
	EC_{10} (nM)	EC_{50} (nM)	EC_{98} (nM)	EC_{98}/EC_{10}	EC_{10} (nM)	EC_{50} (nM)	EC_{98} (nM)	EC_{98}/EC_{10}	LC_{10} (nM)	LC_{50} (nM)	EC_{50}/EC_{10}	EC_{10} (nM)	EC_{50} (nM)	EC_{50}/EC_{10}
DCOIT	28	83	230	8.2	13	93	3000	230	27	62	2.3	27	74	2.7
Medetomidine	Inhibited only at the concentraion of 100000				1900	4400	35000	18	0.3	540	1800	1.2	720	600
TPBP	300	400	1000	3.3	4.4	35	440	100	14	67	4.8	0.3	3.3	2.5
Tolylfluanid	32	80	180	5.6	5.4	65	5200	960	Not tested			Not tested		
Copper	880	2000	n.d.	n.d.	550	1100	42000	76	Not tested			Not tested		
Irgarol	Increased chlorophyll a content				0.1	1.0	n.d.	n.d.	Not tested			Not tested		
Copper pyrithione	22	38	110	5	1.9	6.4	50	26	Not tested			Not tested		
Zinc pyrithione	n.d.	47	680	n.d.	3.8	28	29000	76400	Not tested			Not tested		

Biocidal efficacy towards Ulva lactuca

Seven out of the eight biocides had an inhibiting effect on settlement and growth of the macroalga *Ulva lactuca*, namely copper, tolylfluanid, DCOIT, TPBP, copper pyrithione, zinc pyrithione and medetomidine. Surprisingly, the only algaecide tested (irgarol) did not inhibit settlement nor growth, but instead caused an increase in chlorophyll *a* levels in the exposed samples, but this will be further discussed below (see *Irgarol tolerant Ulva lactuca*). The remaining seven biocides that inhibited settlement and growth of *Ulva lactuca* zoospores did so with different efficacies (Table 2, Figure 13). In **Paper I** the effect of all biocides except the two pyrithiones are described in detail.

Copper pyrithione was the biocide that most efficiently inhibited settlement and growth i.e. had the lowest EC_{98} value (110 nM). The mode of toxicity of the pyrithiones is primarily disruption of membrane integrity, which in itself causes disruptions of important electrochemical gradients over the membranes, e.g. the pH-gradient essential for ATP formation (Dinning et al., 1998a, Dinning et al., 1998b). It was hypothesized before the onset of the experiments that no larger differences would be found in efficacy between the two pyrithiones as zinc pyrithione is known to transchelate into copper pyrithione as soon as copper ions are present in the medium (Dahllöf et al., 2005), and copper is naturally occurring in seawater (Hirose, 2006). This was however not the case. Zinc pyrithione was less efficient in preventing settlement and growth for both *Ulva lactuca* (Figure 13) and periphyton communities (Figure 14). At the same time zinc pyrithione caused higher inhibition at low concentrations than copper pyrithione for *Ulva lactuca*, and from that perspective can be said to have a higher ecotoxicity. However, as can be seen in Figure 13, the inhibition does not go below 45%, not even at concentrations as low as 1.6 nM. The effects of zinc pyrithione were tested in two separate experiments which both showed the same pattern with a high inhibition already at low concentrations. That the concentration-response curve levels out at 45% inhibition do not make sense from an ecotoxicological perspective, but as the high inhibition at low concentrations were confirmed in two experiments, the results cannot be ignored. At present, I regret being unable to provide a plausible explanation to the observed pattern.

DCOIT and tolylfluanid affected zoospore settlement and growth at almost the same concentration, i.e. the concentration-response curves of the two biocides overlapped (Figure 13), but tolylfluanid was found to have a lower EC_{98} (i.e. a somewhat steeper curve), and tolylfluanid was therefore concluded to be the second most efficacious biocide. The efficacy of DCOIT is probably due to its ability to easily diffuse through cell membranes (Morley et al. 2007) and well within the cell cause cell death (Arning et al., 2008). This is a rather general mechanism of cytotoxicity that can affect many different organisms. The mode of action of tolylfluanid is somewhat more specific than that of DCOIT as tolylfluanid is likely to disrupt the folate (vitamin B9) synthesis

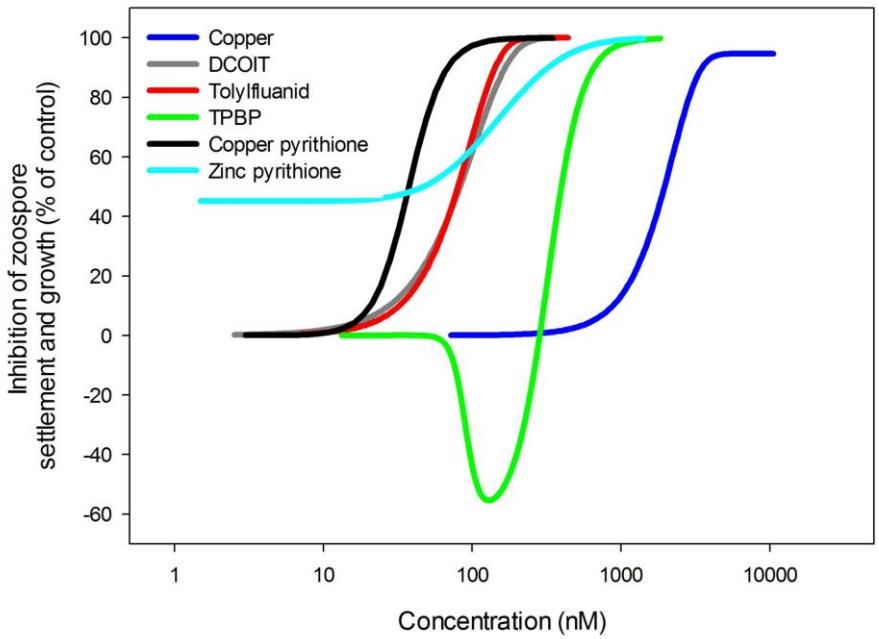


Figure 13. Effects of the six antifouling biocides copper, DCOIT, tolylfluanid, TPBT, copper pyrithione and zinc pyrithione on settling and growth of *Ulva lactuca* zoospores.

(Brain et al., 2008). Folates are however biomolecules that are essential in many different biological processes (Hanson and Roje, 2001), and so, a disrupt folate synthesis results in severe cellular dysfunction. A possible inhibition of thiol-contaning enzymes, as Johansson et al. (2012) suggested as a probable mode of action of tolylfluanid, is also a general mechanism of toxicity with a potential to affect many different organisms.

The least efficacious out of the tested biocides to prevent settlement and growth of *Ulva lactuca* was medetomidine, of which extreme concentrations (100,000 nM) were needed to achieve inhibition (Table 2). Medetomidine binds specifically to adrenergic receptors (Savola et al., 1986). Even though it is known that catecholamines, e.g. adrenaline and dopamine, are physiological active compounds in higher plants (Kulma and Szopa, 2007), and dopamine has been found to function as a grazing deterrent in algae (Van Alstyne et al., 2006), no algal analogous to the adrenergic receptor has been described. There is always a possibility that medetomidine can affect other receptors similar to the adrenergic receptors, e.g. other G-protein coupled receptors. However, from the results presented in **Paper I** and **Paper II** together with previous findings where medetomidine had low or no effects in algal systems (Ohlauson et al.,

2012), it can be concluded that medetomidine does not have a specific target-receptor in algae.

TPBP shows intermediate efficacy compared to the efficacious copper pyrithione and the non-efficacious medetomidine, and causes hormesis around 200 nM. Hormesis is a well-known toxicological effect where stimulations occurre at low levels of the toxicant. The mode of action of TPBP is unknown and it is therefore difficult to have any speculation of what can be the underlying cause of the stimuli. Copper did not fully inhibit settlement and growth of *Ulva lactuca* zoospores. Even at the excessively high concentration of 10,000 nM, 5% of the exposed spores managed to settle and grow, which indicates that part of the population is highly copper tolerant. High copper tolerance has previously been reported in other *Ulva* species (Reed and Moffat, 1983, Andrade et al., 2004). To sum up, the high efficacy of copper pyrithione, and partly also of tolylfluanid and DCOIT, is probably due to their high, general cytotoxicity while the low efficacy found for medetomidine can be coupled to its highly specific mode of action and the lack of algal target receptors for medetomidine.

Biocidal efficacy towards periphyton

Although not all eight biocides are intended to control microfoulers according to the manufactures, all biocides (DCOIT, medetomidine, TPBP, tolylfluanid, copper, irgarol, zinc- and copper pyrithione) inhibited settlement and growth of periphyton communities (Figure 14, **Paper II**). Copper pyrithione was the most efficacious biocide to inhibit settlement and growth of periphyton with an EC_{98} of 50 nM (Figure 14). It is a bit surprising that irgarol, which is a specific algaecide that inhibits photosynthesis, did not show the highest efficacy. Additionally, irgarol did not fully inhibited settlement and growth of the periphyton communities but the inhibition levelled out at 95% at an approximately concentration of 30 nmol l-1, and hereafter it did not increase with increasing concentrations. The mode of action of copper pyrithione is through disruption of the cell membranes and disruption of electrochemical gradients (Dinning et al., 1998a, Dinning et al., 1998b). This is a rather general mode of action and copper pyrithione exercise toxicity towards many different species ranging from bacteria to fish (Kobayashi and Okamura, 2002, Mochida et al., 2006, Dahllöf et al., 2005, Maraldo and Dahllöf, 2004b). Apparently, affecting cellular processes that all organisms are dependent on is a more efficient toxicity mechanism towards the algae than a specific inhibition of photosynthesis. However, the algaecide irgarol presents a higher ecotoxicity than copper pyrithione towards periphyton as it provokes higher biological effects at lower concentrations, i.e. irgarol had the lowest EC_{10} value (Figure 14, Table 2). This is an example of a case where the slope of the curve is crucial, and illustrates the importance of full definition of the whole concentration-response curve. If the efficacy/toxicity evaluation had been tackled from a NOEC/LOEC perspective, information on the variation in toxicity ranking at different effect levels would have been lost. NOEC (no

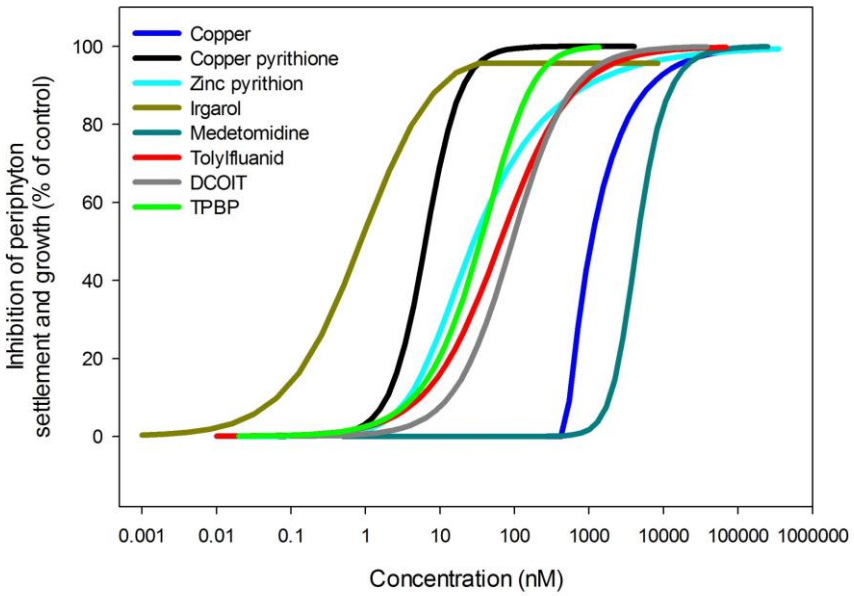


Figure 14. Effects of the eight antifouling biocides copper, copper- and zinc pyrithione, irgarol, medetomidine, tolylfluanid, DCOIT and TPBP on settling and growth of periphyton communities.

observed effect concentration) is the highest concentration at which no statistical significant effect is observed compared to controls, and LOEC (lowest observed effect concentration) is the lowest concentration that causes a statistical significant biological response compared to controls. Both values are strongly dependent on the experimental design and vary with the number of concentration tested and how widely spread these concentrations are as well as with number of replicates and controls. Using this approach, irgarol would be ranked as the biocide with the highest toxicity, and the fact that copper pyrithione pose a higher toxicity (efficacy) at higher effect levels would not be revealed.

The steepness of the slope of the concentration-response curves can also be used as a tool for a quick assessment of the efficacy/ecotoxicity quotient for a biocide. The steeper the curve, the shorter is the concentration interval between high and low toxicity (efficacy/ecotoxicity). The "perfect" antifouling biocide has a high efficacy at the hull-water interface, but quickly degrades into non-toxic metabolites as soon as it leaves the surface. However, a biocide for which the biological effect quickly diminishes with decreased concentration is not far from that ideal. Of course, that is under the assumption that the biocide undergoes degradation in the marine environment. As seen in Table 2, the efficacy/ecotoxicity quotient, defined as the

EC_{98}/EC_{10} quotient, differs between *Ulva lactuca* and periphyton for the different biocides, and the lowest quotient is not linked to the biocide with the highest efficacy. For the periphyton community for example, medetomidine has the lowest efficacy/ecotoxicity quotient, but it is the least efficacious biocide and large amounts would be needed to prevent settlement and growth of the microbial community. Nevertheless, how quickly the toxicity of a biocide declines with decreased concentration is of importance from an environmental point of view.

The efficacy of the investigated biocides differed between the macroalga and the periphyton community, as can be seen in Table 2 and in Figure 13 and 14. The largest difference in biocidal efficacy can be seen for zinc pyrithione and tolylfluanid where the EC_{98} values are much higher for the periphyton communities than for *Ulva lactuca*. Taking tolylfluanid as an example; while it was the second most efficacious biocide at preventing settlement and growth of *Ulva lactuca* with an EC_{98} value of 180 nM, it inhibited settlement and growth of periphyton communities first at a concentration almost 30 times higher at an EC_{98} of 5200 nM. This highlights the problem in using a single biocide for preventing a large number of fouling organisms (see also Figure 2). If we were to use tolylfluanid by itself in an antifouling paint, we would need a concentration of at least 5200 nM at the surface-water interface in order to prevent microfoulers from growing, thereby overdosing by an order of 30 for *Ulva lactuca*.

Ecotoxicity of antifouling biocides

In ecotoxicological evaluations and comparisons, it is the lower part of the concentration-response curve that is of interest. Relevant effect levels are EC_{10} or lower, rather than EC_{98} since a concentration where the entire population is affected is of no interest from an environmental protection point of view. The number of species that is of interest for ecotoxicological evaluation of antifouling biocides also multiplies in comparison to number of species that are of interest for efficacy evaluations. For efficacy evaluation of an antifouling biocide, it is only the species that occur on the boat hull that are of interest. For ecotoxicological evaluations however, all species in the entire marine ecosystem that potentially can be affected are of concern. In this thesis, key groups of marine primary producers and marine primary consumers are represented.

The ecotoxicity of antifouling biocides to Acartia tonsa

Living in coastal areas in the upper part of the water column, the copepod *Acartia tonsa* is subjected to a substantial risk of being exposed to antifouling biocides from boat hulls. The antifouling biocides DCOIT, TPBP and medetomidine all causes mortality and inhibits egg production in *Acartia tonsa* (**Paper III**), although to different extent (Table 2). Before the onset of the experimental work, the working

hypothesis was that egg production, being a sub-lethal endpoint, would be more sensitive than mortality, which often is the case (e.g. Lotufo, 1997, Hook and Fisher, 2001, Sunda et al., 1987). However, in **Paper III**, this was shown to be the case for only one out of the three tested biocides (Table 2, Figure 15). Both DCOIT and medetomidine inhibit egg production at roughly the same concentration as where mortality occurs (Figure 15), which indicates that the egg production is not specifically affected. The decrease in number of produced eggs is rather a consequence of an increased mortality among the females. However, at high concentrations of medetomidine mortality and egg production seems to be decoupled. At 100,000 nM, the egg production is completely inhibited even though complete mortality does not occur. Medetomidine binds to the octopamine-receptor in crustaceans (Lind et al., 2010), and increased levels of the neurotransmitter octopamine is known to inhibit egg production in the prawn *Macrobranchium rosenbergii* (Tinikul et al., 2009). It seems as if medetomidine does have a specific effect on egg production in *Acartia tonsa*, but only at extreme concentrations.

In contrast, TPBP consistently inhibited egg production at lower concentrations than lethal concentrations. Hence, the inhibition of egg production in females exposed to TPBP is not a consequence of an increased mortality, but TPBP affects the egg production specifically. TPBP was also the most toxic biocide to *Acartia tonsa* of the three biocides tested (Figure 15). The mechanisms behind the toxicity of TPBP is poorly understood, but part of the higher toxicity can probably be coupled to the high log K_{OW} value for TPBP (5.5, Table 1) which suggests that TPBP is more readily bioaccumulated than DCOIT and medetomidine and hence result in higher internal concentrations. None of the biocides affected the egg hatching success, i.e. the eggs produced also hatched to the same extent in all treatments. Hence, these biocides do not affect the egg viability in *Acartia tonsa*.

From the results presented in **paper III**, it can be concluded that egg production is not a sensitive endpoint in *Acartia tonsa* from a general point of view, even though it differs between different biocides. Within ecotoxicology, and especially for environmental hazard assessments, it is desirable to choose endpoints that are sensitive and affect the long-term survival of a population (Breitholtz et al., 2006). The number of endpoints that can be used is huge, and it can be chosen from all levels of biological organization, which makes the selection of endpoint challenging (Dahl et al., 2006). Endpoints, apart from reproduction, that have been found to be more sensitive than acute toxicity in copepods are e.g. grazing (Hjorth et al., 2006), developmental time, growth rate and RNA content (Dahl et al., 2006). Hence, for an ecotoxicological evaluation in *Acartia tonsa*, egg production might not be the most suitable endpoint if it is measurements of the most sensitive parameter that is desired.

Ecotoxicological comparison of the three antifouling biocides DCOIT, TPBP and medetomidine

An ecotoxicological comparison of the three antifouling biocides DCOIT, TPBP and medetomidine for the three test-systems *Ulva lactuca*, *Acartia tonsa* and periphyton is shown in Figure 15. DCOIT exert approximately the same toxicity to all tested organisms and all tested endpoints (seen in the conformity of the concentration-response curves), while both TPBP and medetomidine are more toxic to *Acartia tonsa* than to the studied algae (Table 2). Medetomidine, as earlier mentioned, binds to the octopamine-receptor in crustaceans (Lind et al., 2010) and since no analogue receptor is known in algae, it is not surprising that medetomidine affects *Acartia tonsa* at lower concentrations than the algae. Before the onset of the experiments, it was hypothesized that medetomidine would affect egg production in *Acartia tonsa* at concentrations far lower than lethal concentrations. Previously, medetomidine has been shown to cause hyperactivity at concentrations 100,000 times lower than lethal concentrations (Lind et al., 2010, Dahlström et al., 2000). However, egg production was not more sensitive than mortality, except at the extreme concentration of 100,000 nM.

Vaal et al. (1997) argued that species sensitivity varies less between species, and especially closely related species, than the toxicity of differently acting toxicants to the same species. Thus, the biological effect of one specific toxicant would be more accurately extrapolated between species than the biological effect of one toxicant to another toxicant within the same species. That the biological effect of one toxicant cannot be extrapolated to another toxicant is supported by the results presented in Figure 15, where the three different biocides clearly induce different responses within the same species. However, the argument of intra-species extrapolation can only be applied for DCOIT, where we do not see any large difference in toxicity between the studied organisms. This is probably due to the general mode of action of DCOIT (inducing cell death through oxidative stress (Arning et al., 2008)). Both medetomidine and TPBP differ in their toxicity to the investigated organisms with *Acartia tonsa* being the most sensitive organism to both biocides (Table 2, Figure 15). The highly specific mode of action of medetomidine (binding to adrenergic receptors (Savola et al., 1986)) is probably the explanation to the different responses in the different organisms. For TPBP, the mode of action is not known, but following the line of argument, it is probably a more specific mode of action than a general toxicity since it gives rise to different responses in the different organisms. Nevertheless, it should be emphasized that only three organisms have been investigated within the scope of this thesis. From the open literature a much larger distribution of sensitivities among marine species can be found for these three biocides (Table 3). This advocates for some pre-caution when extrapolating sensitivities between species. It should also be stressed that the algae investigated within this thesis are all fouling species, and therefore are highly relevant when it comes to efficacy testing. However, from a risk point of view, they are but a few of many marine algae that are part of the marine

ecosystem, and as can be seen from the comparisons made in Table 3, not the most sensitive species.

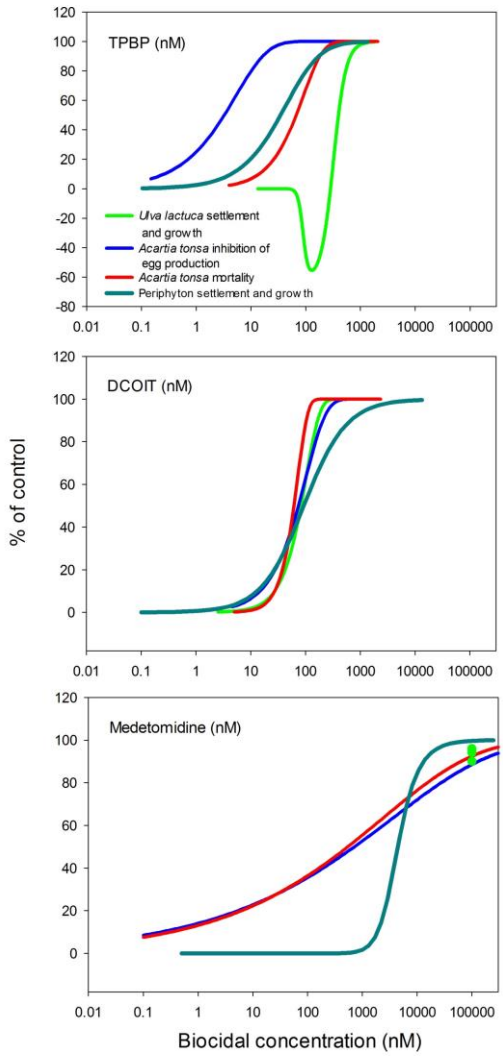


Figure 15. Ecotoxicity of the three antifouling biocides TPBP, DCOIT, and medetomidine for the organisms *Ulva lactuca, Acartia tonsa* and periphyton communities. The different curves represents the different test-systems exposed to each biocide. Since medetomidine did not cause any inhibition of *Ulva lactuca* until the extreme concentration of 100,000nM, no curve-fit could be produced.

Table 3A. Effects of the antifouling biocide DCOIT to aquatic plants and crustaceans reported in the open literature.

Type of organism	Species	Endpoint	Effect level	Conc. (nM)	Reference
Microalga	*Emiliania huxleyi*	Growth	EC_{50} (72h)	1	Devilla et al. 2005
Macroalga	*Ulva intestinalis*	Spore settling	EC_{50}(120h)	7	Willingham and Jacobson 1993
Microalga	*Selenastrum capricornutum*	Growth	EC_{50} (72h)	11	Fernandez-Alba et al. 2002
Mysid shrimp	*Americamysis bahia*	Mortality	LC_{50} (96h)	17	Shade et al. 1993
Shrimp	*Farfantepenaeus aztecus*	Mortality	LC_{50} (96h)	44	Shade et al. 1993
Prawn	*Penaeus japonicas*	Mortality	LC_{50} (96h)	45	Yamada 2007
Copepod	*Acartia tonsa*	Mortality	LC_{50} (48h)	62	Paper III
Copepod	*Acartia tonsa*	Egg production	EC_{50} (120h)	66	Hjorth et al. 2006
Macroalga	*Fucus serratus*	Germination and growth	EC_{50} (72h)	69	Braithwaite and Fletcher 2005
Copepod	*Acartia tonsa*	Egg production	EC_{50} (48h)	74	Paper III
Macroalga	*Ulva lactuca*	Settling and growth	EC_{50} (72 h)	83	Paper I
Microalga	*Skeletonema costatum*	Growth	EC_{50} (96h)	92	KemI 1992
Microbial community	Periphyton community	Settling and growth	EC_{50} (72h)	93	Paper II
Zooplankton	Zooplankton community	Grazing	EC_{50} (1h)	118	Hjorth et al. 2006
Copepod	*Acartia tonsa*	Egg production	EC_{50} (72h)	136	Hjorth et al. 2006
Copepod	*Tigriopus japonicus*	Mortality	LC_{50} (24h)	270	Yamada 2007
Microalga	*Scenedesmus vacuoatus*	Growth	EC_{50} (24h)	310	Arrhenuis et al. 2006
Barnacle	*Balanus amphitrite*	Settling inhibition	EC_{50} (24h)	780	Willemsen et al. 1998
Barnacle	*Balanus amphitrite*	Mortality (nauplii)	LC_{50} (24h)	1200	Willingham and Jacobson 1993
Macroalga	*Hormosira banksii*	Germination	EC_{50} (72h)	1500	Myers et al. 2006

Table 3B. Effects of the antifouling biocide TPBP to aquatic plants and crustaceans reported in the open literature.

Type of organism	Species	Endpoint	Effect level	Conc. (nM)	Reference
Copepod	*Acartia tonsa*	Egg production	EC_{50} (48h)	3.3	Paper III
Microalga	*Skeletonema costatum*	Growth	EC_{50} (72h)	7	Okamura et al. 2009
Microalga	*Selenastrum capricornutum*	Growth	EC_{50} (72h)	24	Okamura et al. 2003
Microbial community	Periphyton community	Settling and growth	EC_{50} (72h)	35	Paper II
Copepod	*Acartia tonsa*	Mortality	LC_{50} (48h)	67	Paper III
Copepod	*Tigriopus japonicus*	Mortality	LC_{50} (24h)	340	Yamada 2007
Macroalga	*Ulva lactuca*	Settling and growth	EC_{50} (72 h)	400	Paper I
Brine shrimp	*Artemia salina*	Mortality	LC_{50} (48h)	400	Okamura et al. 2009
Prawn	*Penaeus japonicas*	Mortality	LC_{50} (96h)	460	Yamada 2007
Duckweed	*Lemna gibba*	Growth	EC_{50} (168h)	3400	Okamura et al. 2003

Table 3C. Effects of the antifouling biocide medetomidine to aquatic plants and crustaceans reported in the open literature.

Type of organism	Species	Endpoint	Effect level	Conc. (nM)	Reference
Barnacle	*Balanus improvisus*	Settling inhibition	~ 100% affected (6-8d)	1	Dahlström et al. 2000
Amphipod	*Corophium volutator*	Search behaviour	LOEC (24h)	42	Krång and Dahlström 2006
Barnacle	*Balanus improvisus*	Hyperactivity	Not reported	100	Lind et al. 2010
Copepod	*Acartia tonsa*	Mortality	LC_{50} (48h)	540	Paper III
Copepod	*Acartia tonsa*	Egg production	EC_{50} (48h)	720	Paper III
Microalgae	Periphyton community	Settlement and growth	EC_{50} (72h)	4400	Paper II
Microalgae	Phytoplankton community	Photosynthesis	16% reduction	8400	Ohlauson et al. 2012
Barnacle	*Balanus improvisus*	Mortality	~ 100% affected (6-8d)	100000	Dahlström et al. 2000
Macroalga	*Ulva lactuca*	Settling and growth	Inhibition of settling	100000	Paper I
Microbial community	Periphyton community	Photosynthesis	no effect	42000	Ohlauson et al. 2012
Microalgae	Epipsammon community	Photosynthesis	no effect	42000	Ohlauson et al. 2012

Irgarol tolerant *Ulva lactuca* and periphyton

As an herbicide commonly used in antifouling paints, irgarol has been found in coastal waters around the world (Konstantinou and Albanis, 2004, Hall Jr et al., 1999). On the Swedish west coast, irgarol was found to induce community tolerance in periphyton communities over a 10 year period from 1994 to 2004 (Blanck et al., 2009). In **Paper IV** the effects of irgarol were investigated on settlement and growth of the marine macroalga *Ulva lactuca* from the mouth of the Gullmar fjord in Sweden, the same geographical area where tolerance development was observed in periphyton communities by Blanck et al., (2009). The tested *Ulva lactuca* population managed to both settle and grow in irgarol concentrations as high as 2000 nM, a clear indication of a strong tolerance in the tested population. What is more, five percent of the exposed algae in the periphyton communities investigated in **Paper II** managed to

settle and grow at irgarol concentrations of 1000 nM. This indicates that irgarol tolerant species or genotypes, as shown by Eriksson et al. (2009), were present in the communities, even though they originated from sites previously regarded as unpolluted. Furthermore, the tolerance in the periphyton communities increased over time and was stronger during the period when tolerance was detected in the *Ulva lactuca* population than during previous years.

Compared to other marine macroalgal species, the tolerance observed in the *Ulva lactuca* population is an extreme tolerance. E.g. zoospores from *Ulva intestinalis* could not survive a concentration of 200 nM (Scarlett et al., 1997), a concentration that is an order of magnitude lower than the highest concentration at which zoospores from *Ulva lactuca* still managed to settle and grow. In addition, the chlorophyll *a* content in the exposed samples was higher than in the controls. The observed increase in chlorophyll *a* was most likely the result of an increased settlement of zoospores in combination with an increase in chlorophyll *a* concentration per germling (the so-called greening effect). Together with the extreme tolerance this resulted in chlorophyll *a* concentrations almost as high as 400% of the control values in some of the irgarol treated samples.

There is no tolerance mechanism towards PSII inhibitors described in macroalgae, but possible mechanisms described in other photosynthetic organisms are increased GSH conjugation (Anderson and Gronwald, 1991, Gray et al., 1996), point mutation in the D1 protein where serine264 is substituted with glycine (e.g. Bettini et al., 1987, Oettmeier, 1999, Devine and Shukla, 2000, Kumata et al., 2001). An increased D1 protein turnover has been proposed as tolerance mechanism in periphyton communities (Eriksson et al., 2009). The observed tolerance in *Ulva lactuca* indicates that either the investigated thalli, or ancestors to the investigated thalli, have been exposed to irgarol in their natural environment. It can tentatively be concluded that irgarol exerts selection pressure on seaweeds in the marine environment, as previously demonstrated for periphyton microalgae (Blanck et al., 2009). This is supported by the findings of irgarol tolerant periphyton presented in **Paper II**.

That both periphyton and zoospores can survive and settle in irgarol concentrations as high as 2000 nM (zoospores) is not only an indication of the presence of irgarol as a marine pollutant, but it also raises doubts about the efficacy of irgarol as an algaecide. This also highlights the problematic situation caused by tolerant species for antifouling coating design, since it does not take more than one tolerant genotype to establish and cover a hull. If one tolerant zoospore manage to settle and grow into a full thalli it will both induce a drag by itself, but maybe more important, it will be the source of new tolerant spores. As discussed previously, tolerant species force an overdosing for all other, less tolerant species and thereby an unnecessary high use of biocides.

Conclusions

The overall aim of this thesis was to describe the efficacy as well as the ecotoxicity of the eight antifouling biocides DCOIT, medetomidine, TPBP, tolylfluanid, copper, irgarol, zinc pyrithione and copper pyrithione.

The efficacy and ecotoxicity of the investigated antifouling biocides varies depending on organism. However, for both the periphyton communities and *Ulva lactuca* copper pyrithione was the most efficacious biocide to prevent settlement and growth. The efficacy ranking and the ecotoxicity ranking are not always interconnected, which is due to differences in the slope of the concentration-response curves. For *Ulva lactuca*, copper pyrithione was consistently the most toxic biocide at all effect levels and had the lowest EC_{98} as well as EC_{10} value. But for the periphyton community there was a non-consistency between the different effect levels; copper pyrithione showed the highest efficacy, i.e. it was the most toxic at high effect levels (highest EC_{98} value), while irgarol was the biocide with the highest ecotoxicity, i.e. had the highest toxicity at low effect levels (highest EC_{10} value). The least efficacious antifouling biocide for both test systems was medetomidine, it was also the biocide with the lowest ecotoxicity, both for the algal species and for the copepod *Acartia tonsa*. The most toxic biocide for *Acartia tonsa* was TPBP which in addition was the only biocide of the ones tested that specifically affected the egg production.

The algaecide irgarol had unexpected effects on settlement and growth of *Ulva lactuca* zoospores. While irgarol inhibited settlement and growth of the periphyton communities, zoospores from *Ulva lactuca* displayed higher chlorophyll *a* levels in irgarol exposed samples than in controls. The extreme tolerance towards irgarol in the *Ulva lactuca* population supports previous studies that the biocide constitutes a selection pressure in the marine environment, and that its use in antifouling paints has made its imprint on the marine ecosystem.

The results from this thesis, in the form of a detailed understanding of the biological effects of antifouling biocides, facilitate a clever choice of biocides in the design of antifouling paints. Hopefully, the results will thereby also contribute to a decrease in the total use of antifouling biocides. The well-defined concentration-response curves can also be used in a number of other applications where either biocidal efficacy or ecotoxicity is of interest. Such applications are e.g. prediction of mixture toxicity, both within efficacy evaluations (paint formulations) and ecotoxicological hazard assessments where the infamous “cocktail effect” is an upcoming concern. A well-defined concentration-response curve allows for more correct ecotoxicological comparisons than single effect concentrations can provide (e.g. NOEC, LOEC, ECx) since the concentration-response curve permits for comparisons between any of effect levels and thereby also visualizes any differences in toxicity ranking between effect levels.

Future Perspectives

An optimal design of an antifouling paint, and thereby also a minimal use of antifouling biocides, requires (among other things) a detailed knowledge of the biological response of the entire fouling community. Even though the number of species that are of interest is limited to those growing on boat hulls, it is still a very large number of species. The work within this thesis contributes with knowledge on fouling microalgae and macroalgae. Effects on other fouling species, such as barnacles, ascidians and bryozoans have been covered within the scope of the Marine Paint program (Marine Paint, 2012), but other needs to be included. For Swedish waters, evaluation of efficacy of antifouling biocides on the common blue mussel (*Mytilus edulis*) would be a logical next step as it is one of the most commonly occurring species in fouling communities on the Swedish west coast.

Ecotoxicological evaluation of marine pollutants, in which antifouling biocides are included, is a never ending project. The lists of effects on the marine life grows steadily with the number of studies performed and as new compounds are taken into use the number of potential pollutants increases. Among the biocides investigated in this study, the knowledge gaps of the ecotoxicological effects of the antifouling biocides medetomidine and TPBP are larger than for the other, more studied biocides. For medetomidine, since it is known to target the octopamine receptor, continued studies on non-target crustaceans would be interesting and could contribute to a better understanding of its potential environmental effects. Endpoints that would be of special interest are those coupled to the role of octopamine, such as locomotion and behaviour. For TPBP, in addition to add to the knowledge of its ecotoxicological effects, it would be interesting to elucidate the underlying mechanism of toxicity. It would also be interesting to follow up on the effects of zinc pyrithione on settlement and growth of *Ulva lactuca*, and investigate whether the observed pattern of high inhibition at low concentrations holds, and if so also elucidate the underlying mechanism.

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