Ecotoxicology of Antifouling Biocides

With Special Focus on the Novel Antifoulant Medetomidine and Microbial Communities

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ABSTRACT

Marine biofouling, growth on submerged surfaces, is a problem for the commercial shipping industry but also for recreational boat owners. It leads to increased fuel consumption, loss of maneuverability and is a source of invasive species. The common solution to avoid biofouling is to use antifouling paints containing biocides which hinder the fouling organisms from growing on the ship hull. Medetomidine (4-[1-(2,3-dimethylphenyl)ethyl]-1-H-imidazole, also known as Selektope) is used in antifouling paint due to its ability to inhibit settlement of barnacle cyprid larvae. Exposure to medetomidine hinders settlement and metamorphosis to an adult barnacle at 0.2 µg/l (1 nM), a concentration one hundred thousand times lowers than the lethal concentration.

Several studies of possible environmental effects have been performed during the developmental phase of medetomidine as an antifoulant, both on invertebrates and vertebrates. This thesis focuses on the effects on marine microbial communities with studies on short-term toxicity, toxicant-induced succession after intermediate time exposure, long-term microcosm exposure and bioaccumulation. The predicted environmental concentrations (PEC) of medetomidine in different environments have also been established using the MAMPEC model. A worst-case prediction for a Baltic marina generated a water concentration of 0.057 µg/l (0.28 nM). The conclusion for this thesis is that microalgal and bacterial metabolic functions are not affected by medetomidine until very high concentrations (2 mg/l, 10 µM). The same conclusion can be drawn for direct effects on species composition although there is an indication that grazing organisms in the microbial community could be affected, changing their grazing pattern and hence the microalgal species composition. Long-term effects of medetomidine on microbial communities from an antifouling paint were unfortunately surpassed by effects of zinc which was also present in the paint. It can therefore also be concluded that zinc affects both metabolic functions and species composition in microbial communities to a larger extent than does medetomidine.

Keywords: Antifouling biocides, Medetomidine, Microbial communities, Periphyton, Epipsammon, Plankton

POPULÄRVETENSKAPLIG SAMMANFATTNING

Påväxt av havstulpaner, musslor, alger och andra marina organismer på båtskrov har länge varit ett problem för båtägare. Påväxt kan minska manövrerbarheten hos fartyg och fritidsbåtar men framför allt kan den öka bränsleförbrukningen med så mycket som 40 % för ett fartyg.

Den vanligaste metoden för att minimera påväxt är båtbottenfärger innehållande biocider. Biociderna läcker långsamt ut från färgen och hindrar påväxt genom att förgifta de organismer som försöker växa på skrovet. På 70- och 80-talet var den främsta lösningen båtbottenfärger innehållande tenn (tributyltenn, TBT) vilket var effektivt men också mycket skadligt för den marina miljön. Mot slutet av 80-talet hade användningen at TBT förbjudits för fritidsbåtsägare i många i-länder och under 90-talet följde samma förbud för kommersiella fartyg. Sedan 2008 är användning av TBT globalt förbjudet. Den vanligaste biociden i båtbottenfärger idag är kopparoxid. Användning av kopparoxid är dock inte heller helt okontroversiell då koppar lagras i miljön vilket kan leda till framtida miljöproblem.

Den här avhandlingen har ingått i ett större projekt, Marine Paint, där målet var att utveckla en ny effektiv båtbottenfärg med bättre miljöprofil än de färger som finns på marknaden idag. Biociden som Marine Paint fokuserade på, medetomidin, valdes för att den mycket effektivt hindrar påväxt av havstulpaner. Havstulpaner sprids genom larver som söker lämpliga ytor att fästa på. När de hittar en sådan yta limmar de fast sig och utvecklas till vuxna havstulpaner. Medetomidin stör larvens sökande genom att den blir hyperaktiv när den kommer i kontakt med biociden, och simmar iväg istället för att limma fast sig. Denna mekanism skiljer sig markant från övriga biocider i båtbottenfärgen som har en mer generell giftverkan.
Mitt doktorandprojekt har fokuserat på hur medetomidin påverkar alg- och bakteriesamhällen i havet. För att förutspå om och hur marina alger och bakterier skulle påverkas av båtbottenfärger innehållande medetomidin så har jag använt olika långa experiment, från en timma för att simulera akuta effekter till fyra veckor för att simulera en realistisk exponering. Eftersom det inte finns båtbottenfärg med medetomidin på marknaden idag så är det

dock svårt att veta vilka koncentrationer som kan uppstå i miljön och vilka uppmätta effekter som skulle kunna utgöra en miljörisk. De europeiska kemikaliemyndigheterna rekommenderas att miljökoncentrationer beräknas med en matematisk modell som tar hänsyn till biocidens kemiska och fysikaliska egenskaper, i vilken miljö biociden används och hur stor del av båtbottenfärgsmarknaden som biociden har. För medetomidin har det beräknats teoretiska miljökoncentrationer för marinor, hamnar och farleder i Östersjön, EU och OECD länderna. Dessa modeller bygger till stor del på att biociden sprids med tidvatten och strömmar och kan därför underskatta miljökoncentrationen om hänsyn inte tas till lokala förhållanden.

Mina resultat visar att medetomidin inte har någon direkt påverkan på alg och bakteriesamhällen förrän vid väldigt höga koncentrationer. Man kan dock se en liten skillnad i sammansättningen av algsamhällen efter 96 timmars exponering. Detta beror sannolikt på att mycket små ryggradslösa djur som betar av algsamhällena påverkas på samma sätt som havstulpanerna och förändrar sitt beteende. För att får mer information om långtidseffekter av medetomidin så utfördes ett experiment med båtbottenfärg innehållande medetomidin. Tyvärr så visade det sig att den färg som användes hade större effekt på algernas artsammansättning än vad tillsatsen av medetomidin hade.

Effekten av medetomidin har även studerats på flera olika sorters fisk, kräftdjur och blötdjur. Detta har skett både inom Marine Paint projektet men även av företaget I-Tech som arbetar för att få medetomidin godkänt för användning inom EU. De marina arter som är mest känsliga för medetomidin jämförs då mot teoretiska miljökoncentrationer för att avgöra om en säker användning kan ske.

I love deadlines. I like the whooshing sound they make as they fly by.

-Douglas Adams

LIST OF PUBLICATION

This thesis is based on the following papers throughout the thesis referred to by their Roman numerals given below. Paper I is reprinted from Biofouling with permission from Taylor and Francis Ltd. Paper IV is reprinted from Marine Environmental Research with permission from Elsevier Ltd.

I. Ohlauson, C., K. M. Eriksson, and H. Blanck. 2012. Short-term effects of medetomidine on photosynthesis and protein synthesis in periphyton, epipsammon and plankton communities in relation to predicted environmental concentrations. Biofouling 28:491–9

II. Ohlauson, C., and H. Blanck. 2013. A comparison of toxicant-induced succession for five antifouling compounds on marine periphyton in the SWIFT microcosms. Submitted to Biofouling.

III. Ohlauson, C., M. Nydén, M. Hassellöf and H Blanck. 2013. Long-term effects of medetomidine on marine periphyton community structure and functions. Manuscript

IV. Hilvarsson, A., C. Ohlauson, H. Blanck, and A. Granmo. 2009. Bioaccumulation of the new antifoulant medetomidine in marine organisms. Marine environmental research 68:19–24

ABBREVATIONS COMMONLY USED IN THE THESIS

BAF	Bioaccumulation factor
BCDI	Bray-Curtis dissimilarity index
BCF	Bioconcentration factor
BPD	Biocidal product directive
EC50	The concentration causing response a specific effect in 50% of a population of test species
LOEC	Lowest observed effect concentration
NOEC	No observed effect concentration
MAMPEC	Marine antifoulant model to predict environmental concentrations
MDS	Multi dimensional scaling
OECD	Organization for economic co-operation
PEC	Predicted environmental concentration
PNEC	Predicted no effect concentration
SPC	Self-polishing copolymer
TBT	Tributyltin

1 BIOFOULING

Marine biofouling is the undesired attachment and growth of microorganisms, plants and animals on submerged surfaces in the aquatic environment. Immediately after submerging a surface in the marine environment it attracts organic particles, proteins, polysaccharides and glycoproteins, creating an initial organic film that attracts the primary colonizers bacteria and diatoms. Within a week, colonization occurs with spores of macroalgae, protozoa and following them are the larvae of marine invertebrates (Fig. 1) (Yebra et al. 2004). The composition of fouling organisms on a submerged surface varies around the world and is largely influenced by water characteristics such as temperature, salinity and pH which also regulate the amount of fouling generated (Almeida et al. 2007). This diverse fouling community can in some environments increase the hull friction of a ship up to 0.5 % per day affecting both maneuverability of the vessel and the fuel consumption which could be increased with up to 40% during a period of six months (WHOI 1952, Schultz et al. 2011). The increase in fuel consumption can be avoided with antifouling techniques such as antifouling paint (Finnie and Williams 2010). Other problems with biofouling is the transportation of invasive species around the world which harm aquatic ecosystems (Mackie et al. 2004, IMO 2011).

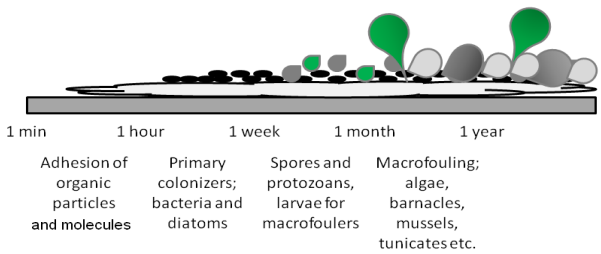


Figure 1. Schematic picture of fouling organism colonization.

2 ANTIFOULING AND THE ENVIRONMENTAL PROBLEM

Antifouling methods can be divided into chemically acting and physically acting. The chemically acting methods reduce fouling through the release of a biocide, which is defined as a substance that is intended to control the effect of harmful organisms with a chemical or biological mode of action (EU 1998). The physically acting methods reduce fouling with physical properties such as hydrophilic or hydrophobic surfaces (Buskens et al. 2012), ultrasound (Guo et al. 2011), oxygen-free layers (Lindgren et al. 2009) etc but this is out of the scope for this thesis.

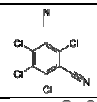
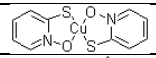
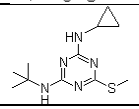
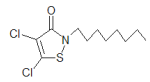
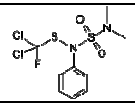
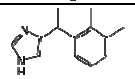
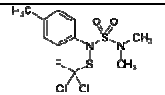
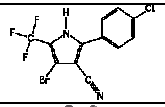
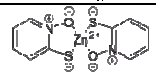
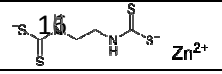
Antifouling methods are not modern inventions only, they have been used since ancient times to protect ship hulls, starting with tar, asphalt and wax derived from nature. Around 700 B.C. it is thought that the use of copper as an antifoulant first started with sheathings on ship hulls, an idea that was in use on and off until the 18th century. In the mid 19th century the first real antifouling paints based on linseed oil, rosin or shellac came in use with copper, arsenic or mercury oxides as biocide. These paints were effective against fouling and were in use until the late 1940s when health and safety concerns about arsenic and mercury were raised which made copper-based paints most popular (Readman 2006, Almeida et al. 2007). In the end of the 1950s a new biocide came in to the antifouling market, tributyltin (TBT), with outstanding antifouling efficiency. The combination of TBT oxide with self-polishing copolymer (SPC) formulations in the 1970s gave an antifouling paint with constant leaching rates, longer service life and resulted in exceptionally smooth ship hulls. By the middle of the 1980s 80% of the commercial fleet were painted with TBT-SPC paint (Abbott et al. 2000). However, the TBT was released into the water and contaminated harbors and coastal areas (Fent 1996). By 1980, negative aspects of TBT usage became noticeable, first in France with shell abnormalities in oysters, and then in England with development of male genitalia in female gastropods (imposex) causing reproductive failure (Abbott et al. 2000). France was also the first country to enforce limitations on TBT usage, followed by England and most industrialized countries in the 1980s (Champ 2000). In 1999 the International Maritime Organization (IMO) adopted a convention prohibiting all application of TBT-containing antifoulants on ships by 1st of January 2003

and total prohibition by 1st of January 2008. The convention would however not come into force immediately. It required that 25 states, representing 25% of the worlds merchant shipping tonnage, consented (Champ 2003). This was achieved on the 17th of September in 2008 when the convention thus came into force (IMO 2008).

Following the TBT ban, self-polishing copolymer paints (SPC) and controlled depletion paints are still the most used technologies to protect ship hulls from fouling (Almeida et al. 2007). The SPC are based on an acrylic polymer matrix with pendant groups, usually copper or zinc but also organosilicones in the form of silyl (Finnie and Williams 2010). The pendant groups and additional co-biocides in the paint are released through hydrolysis or ion exchange which is followed by erosion of the paint layer. Controlled-depletion paints (ablative/erodible paints) are in general based on a water-soluble binder combined with metallic pigments and polymers to control erosion of the paint. The biocides are released at a constant rate together with the soluble binder and can therefore be better controlled than in self-polishing paints. One drawback is however that the controlled depletion paints need a higher biocide concentration to maintain efficacy (Almeida et al. 2007).

Copper oxide is the main antifouling biocide used today (Thomas and Brooks 2010). Questions have however been raised about the environmental consequences of Cu ions since high levels in the environment have been reported for areas with large boating activity (Singhasemanon et al. 2009). A ban or phase out have been discussed in two states in USA (Carson et al. 2009, Prichard 2010, *Senate Bill 5436: Prohibiting copper in antifouling paints used on recreational water vessels* 2011). Before the TBT ban, copper and TBT was used in combination with high efficacy. To fill the gap after TBT, several new co- biocides were developed (Hellio 2010). The most common co-biocides in use today are chlorothalonile, copper pyrithione, dichlofluanide, tralopyril, cybutryne, tolylfluanide, DCOIT, zinc pyrithione and zineb. Please refer to Table 1 for the chemical structure, name and synonyms, CAS-number and mode of action for the co-biocides.

Table 1. Name, synonyms, CAS-number, chemical structure and mode of action for antifouling co-biocides

chlorothalonile	2,4,5,6-tetrachloro-isophthalonitrile CAS: 1897-45-6		Inhibits thiol-containing enzymes, causing depletion of glutathione reserves leading to oxidative stress. Disturbs ATP production (Cima et al. 2008).
copper pyrithione (Copper Omadine®)	copper 2-pyridinethiol-1-oxide CAS: 154592-20-8		Disrupts proton gradients over cell membranes (Al-Adham et al. 1998).
cybutryne (Irgarol 1051)	2-metylthio-4-tertbutylamino-6-cyclopropylamina-s-triazine CAS:28159-98-0		Photosystem II inhibitor, blocking of electron transport in the D1 protein (Hall et al. 1999).
DCOIT (Sea-nine 211™)	4,5-dichloro-2-octyl-3(2H)isothiazolone CAS: 64359-81-5		Disruption of metabolic pathways by inhibition of dehydrogenase enzymes, consumption of glutathione reserves, inhibiting respiration and ATP synthesis (Williams 2007).
dichlofluanide (Preventol A4-S™)	N,N-dimethyl-N'-phenylsulfamide CAS: 1085-98-9		Inhibits thiol-containing enzymes by forming disulfide bridges. Inhibits mitochondrial Ca^{2+} accumulation (Hertel et al. 1981).
medetomidine (Selektope™)	4-[1-(2,3-dimethyl phenyl)ethyl]-1-H-imidazole CAS:86347-14-0		Stimulation of the octopamine receptor in invertebrates causing hyperactivity (Lind et al. 2010) α_2-adrenoreceptor agonist in vertebrates (Scheinin et al. 1989).
tolylfluanide (Preventol A5-S™)	N-dichlorofluoromethyl thio-N',N'-dimethyl-N-p-tolylsulfamide CAS: 731-27-1		See dichlofluanide.
tralopyril (Econea™)	4-bromo-2-(4-chlorophenyl)-5-(trifluoromethyl)-1H-pyrrole-3-carbonitrile CAS: 122454-29-9		Thought to uncouple oxidative phosphorylation in mitochondria, resulting in disruption of ATP production. Based on information regarding closely related biocide chlorfenapyr (Rand 2004).
zinc pyrithione (Zinc Omadine™)	zinc 2-pyridinethiol -1-oxide CAS: 13463-41-7		See copper pyrithione.
zineb	zinc ethane-1,2-diylbis(dithiocarbamate) CAS: 12122-67-7		Disrupts aminoacids preventing protein and enzyme production. Multisite inhibitors (Isaac 1999).

Antifouling paints containing biocides have caused unwanted environmental consequences both in the past and in the present (Strand et al. 2003, Garaventa et al. 2006, Neira et al. 2011, Biggs and D'Anna 2012). Substances in use today may still pose a risk to marine life and their use have been questioned (Konstantinou and Albanis 2004). Cybutryne (Irgarol 1051) has been shown to induce tolerances in microalgal communities at locations exposed to the substance (Blanck et al. 2009, Eriksson et al. 2009), to severely affect periphyton, plankton and zooplankton communities in large scale mesocosms (Mohr et al. 2008) and to bioaccumulate in algae (Dyer et al. 2006). Copper oxide concentrations exceed water quality standards in several areas (Singhasemanon et al. 2009) and have led to reductions in the number of boats allowed in marinas (Biggs and D'Anna 2012). Elevated copper concentrations in sediment have been shown to reduce biodiversity and biomass in macrobenthic communities (Neira et al. 2011), and cause olfactory dysfunction in fish (Dew et al. 2012).

Several countries and regions that regulate the use of antifouling biocides are now re-evaluating regulatory approvals. In EU, a re-registration of all substances that are to remain on the market is required. In New Zealand, recommendations to phase-out several biocides were issued following a revised risk assessment which left copper, copper pyrithione, dichlofluanide, tolylfluanide, zinc pyrithione and zineb on the market (Forlong 2013).

3 ANTIFOULING IN THE REGULATORY WORLD

Antifouling biocides and the products in which they are used are regulated by legislations in many parts of the world. In Europe the use is controlled by the Directive 98/8/EC concerning the placing of Biocidal products on the market (EU 1998), usually called BPD. An EU directive has to be implemented in each member state legal system but leaves some room for interpretation to the member state. However, in September 2013 the BPD is replaced by a regulation: "Regulation (EU) No 528/2012 concerning the making available on the market and use of biocidal products" (EU 2012), which immediately comes into force in the member states and leaves no room for national interpretations. Before the BPD only a handful of European countries regulated the use of antifouling products so the

implementation of the BPD in all member states changed the market drastically. An antifouling biocide on the market in 2002 with a producer that submitted a notification to undergo BPD registration and submit a registration dossier by March 2006 was included in the review program and allowed to remain on the market. If not, the biocide was phased out by September 2006 at the latest. In the end, 10 biocides were included in the review program for antifouling biocides (tolylfluanide, dichlofluanide, copper thiocyanate, dicopper oxide, copper, zineb, zinc pyrithione, copper pyrithione, cybutryne and DCOIT) (EU 2003). To this date, no antifouling biocides in the review program have been approved or banned for usage. All new biocides have to go through the same system, but are not allowed to be put on the market until their dossier passes the evaluation. Therefore, no new antifouling biocides have reached the EU market since 2002. The required core data can be divided in eight sections with main focus on physical/chemical properties, analytical methods, efficacy, mammalian toxicology and ecotoxicology. The core data is used to generate risk assessments for human health and the environment together with exposure and emission predictions.

The environmental risk assessment procedure for antifouling biocides in EU is described in two documents; Technical guidance document of risk assessment (ECB 2003) and Emission scenario document for antifouling products in the OECD countries (Van der Aa and Van der Plassche 2004a). Basically the risk assessment is a comparison of an effect threshold concentration for the most sensitive endpoint in the ecotoxicological data set, key study, with the predicted environmental concentrations (PEC). The PEC for an antifouling substance depends on market share, the leaching rate from the ship hull, degradation rate, partitioning between environmental compartments and the hydrographical properties of the environment for which the PEC is calculated. The Marine Antifouling Model to Predict Environmental Concentrations, MAMPEC, was developed in 1999 specifically for PECs of antifouling biocides (Van Hattum et al. 1999, van Hattum et al. 2002). The predicted no effect concentration (PNEC) is derived from the key study EC_{50} or no observed effect concentration (NOEC) which is divided with an assessment factor based on how many studies that are available. The PEC/PNEC ratio must be below 1 for an EU approval.

USA (United States Environmental Protection Agency, US EPA), Australia (Australian Pesticides and Veterinary Medicines Authority, APVMA) and New Zeeland (New Zeeland Environmental Protection Authority, NZ EPA) have similar requirements as EU for antifouling biocides and products. However, the data requirements and environmental risk assessment procedures differs slightly. For example the US EPA does base the risk quotients (RQ) on estimated environmental concentration and toxicity values for fish, invertebrates, plants and algae, instead of assessment factors. The RQ is compared to levels of concern which address the risk for acute or chronic effects on non-target species. If the level of concern is exceeded additional regulatory actions or risk mitigation actions are triggered (EPA 2013).

In Asia, a very important geographical area for the shipping industry, most countries do not regulate the use of antifouling products and do not require registrations for the biocide that will be used. There might be chemical notification systems similar to the European chemical notification system, REACH, if the biocide is imported to the country for further use. Japan and China are two exceptions where the notification systems require both human and environmental risk assessments for antifouling biocides.

Generally, most other geographical regions do not have any legislation for antifouling biocides and products, or do not regard antifouling biocides as biocides at all. A standard for risk assessment of antifouling biocides (ISO/PRF 13073-2) is however under development by the International Organization for Standardization (ISO) and could be used as a minimal requirement globally.

4 NEXT GENERATION'S ANTIFOULING BIOCIDE

The search for new antifouling biocide(s) has been ongoing for several years both in the academic and the industrial community. A number of substances with antifouling properties have been discovered but to be a solution to the fouling problem, several criteria have to be fulfilled such as compatibility with an antifouling paint, economically feasible and a good environmental profile. One initiative to develop a new antifouling biocide was the MISTRA

funded Marine Paint program which focused on the substance medetomidine. The program will be presented briefly below and the outcome will be addressed further in the discussion of this thesis.

4.1 MEDETOMIDINE

Medetomidine, (4-[1-(2,3-dimethylphenyl)ethyl]-1-H-imidazole), is an α_2-adrenoreceptor agonist that is used as a sedative and analgesic in human and veterinary medicine (Macdonald et al. 1988, Virtanen et al. 1988). Medetomidine also reduces barnacle settling, which was discovered by a group of Swedish researchers in 1998 when settling of the cyprid larvae and metamorphosis into adult barnacles was inhibited at 0.2 µg/L (1 nM), a concentration 100 000 times below the lethal concentration for barnacle cyprid larvae (Dahlström et al. 2000). The mode of action was first hypothesized to be connected to regulation of the cement gland in the barnacle cyprid larvae (Dahlström et al. 2005), but it was later discovered that medetomidine evoked hyperactivity in the cyprid larvae (Lind et al. 2010). The hyperactivity disturbs normal settling behavior, including the thorough exploration of the surfaces available for settling. When subjected to medetomidine the cyprid larvae cannot conclude the exploration due to hyperactivity and swims away. The mode of action has now been demonstrated to be through the invertebrate-specific octopamine receptor (Lind et al. 2010).

4.2 MARINE PAINT

The discovery of medetomidine´s antifouling properties eventually lead to the initiation of the research program Marine Paint in 2003. Marine Paint was a collaboration between research groups at the University of Gothenburg and Chalmers University of Technology. The program was financed by the Swedish Foundation for Strategic Environmental Research (MISTRA) during nine years. The aim of the program was to develop new and effective antifouling paints that were more environmentally friendly than those in use at present. Marine Paint 1 encompassed three research areas:

- Fundamental Research on the interactions between the barnacles and medetomidine to provide essential knowledge of mechanisms and mode of action.
- Paint Formulation on the behavior of medetomidine in antifouling paint, the amount needed to generate efficacy and how release from the paint could be controlled.
- Ecotoxicology of medetomidine on possible environmental risks with medetomidine.

During Marine Paint 2, the main focus was to find efficient and environmentally sustainable biocide combinations for all types of fouling organisms. The Marine Paint research program was summarized at the end of Marine Paint 2 in a final report (Backhaus and Arrhenius 2012).

4.3 MARINE PAINT ECOTOXICOLOGY

The ecotoxicological strategy in Marine Paint was to generate a thorough environmental risk assessment of medetomidine for invertebrates, fish (Hilvarsson 2007, Lennquist 2010) and microbial communities. Invertebrates (mollusks and crustaceans) and fish were chosen based on previous knowledge of medetomidine's ecotoxicological effects while algae and bacteria were included to cover important trophic levels with no known sensitivity to medetomidine.

5 AIM AND APPROACH

Marine microbial communities are involved in two crucial ecological processes in the marine food web, degradation of organic matter to inorganic material and primary production of organic material. These are functions that if altered would affect the whole ecosystem. The aim with this thesis was therefore to investigate the effects of medetomidine on microbial communities in the marine environment at environmentally realistic concentrations.

The main focus areas were:

- Short-term effect (hours) on photosynthesis and bacterial production in plankton, epipsammon and periphyton communities.
- Intermediate effects (days) on periphyton community structure.
- Comparison of medetomidine effects with other antifouling biocides.
- Long-term effects (weeks) on photosynthesis, bacterial production and structure in periphyton communities.
- Bioaccumulation in periphyton communities.
- Development of predicted environmental concentrations (PECs).

A community approach was chosen since they provide the opportunity to expose several species with different sensitivity in their natural context, thus increasing the ecological realism. The communities used; plankton, periphyton and epipsammon, differ in characteristics and species composition. Plankton communities are composed of free-floating organisms while epipsammon and periphyton communities are associated with substrata where they are attached by extracellular polysaccharides (EPS) to form a biofilm matrix. Epipsammon are microbial communities of microalgae, bacteria, fungi and grazers that are found on sand grains in wave-exposed environments (Dahl and Blanck 1996). Periphyton are microbial communities with the same type of constituents but attached to submerged surfaces in the aquatic environment (Sladeckova 1962), in our studies sampled on artificial substrata. The differences between the communities used influence the ecological functions represented but also the bioavailability of toxicants and therefore the sensitivity to external

factors such as biocides. Previous studies have shown that plankton, epipsammon and periphyton communities differ in their sensitivity to herbicides (Bonilla et al. 1998).

The initial studies on microalgal communities and medetomidine in **Paper I** focused on short-term effects (hours) on photosynthesis and bacterial protein synthesis in plankton, epipsammon and periphyton communities. Even though algae and bacteria are thought to lack the adrenergic receptors that medetomidine influence, effects may occur through reactions with related receptors in the G-protein coupled receptor family or with other unknown targets. The hypothesis for these studies was that medetomidine would not affect the functional endpoints investigated.

Following the short-term studies of functional endpoints a semi-static test system, SWIFT, was used to investigate structural effects on the community and toxicant-induced succession (TIS) (Porsbring et al. 2007). Exposure to chemicals can with time cause TIS where sensitive organism or species are eliminated while more tolerant species are favored. This affects the structure and possibly functions of the whole community. Periphyton communities were exposed to medetomidine or one of four other antifouling biocides (chlorothalonile dichlofluanide, tolylfluanide and zinc pyrithione) during 96 hours (**Paper II**). The other substances were chosen as comparisons to medetomidine based on their usage and the lack of microbial community response information. The hypothesis for this study was that medetomidine would not affect the community structure directly but that there was a potential for indirect effects on grazing organisms (meiofauna).

With a combination of functional and structural endpoints a long-term microcosm study over four weeks (Blanck and Wängberg 1988) was also performed to evaluate the effect on a colonizing periphyton community, when medetomidine being delivered continuously through a model paint system using ZnO nanoparticles to control the release (**Paper III**). The hypothesis behind this study was that release of medetomidine from the paint system would be controlled by zinc nanoparticles and that indirect effect of medetomidine on periphyton communities would occur.

To address the fate of medetomidine in marine organisms a bioconcentration/bioaccumulation study with periphyton communities and invertebrates was performed (**Paper IV**). Information regarding medetomidine behavior and if was absorbed or adsorbed in periphyton communities was regarded as important to further understand transport in the marine food web.

6 EXPERIMENTAL PROCEDURES

6.1 FIELD SAMPLING

Three types of algal and bacterial communities; plankton, epipsammon and periphyton were used for ecotoxicological testing. The communities were all sampled from the coastal ecosystem near Sven Lovén Center for Marine Sciences Kristineberg at the Gullmar fjord on the west coast of Sweden (Fig. 2).

Plankton samples were taken at two, four and six m depth with a Rüttner sampler and pooled to attain a representative sample of the plankton community (Bonilla et al. 1998). Epipsammon communities were sampled at a wave-exposed sub-tidal sandy shore at 0.5 m water depth where the upper centimeters of sand were collected. Several samples were pooled, sieved through a 500 µm mesh net and mixed to achieve a representative selection of the epipsammon community (Dahl and Blanck 1996). Periphyton community samples were collected by letting a periphyton film form on submerged glass discs (1.5 cm^2) mounted on polyethylene holders (Blanck and Wängberg 1988) at 1.5 meters depth. Depending on what test system the periphyton would be used for, the age of the biofilm differed from one week (SWIFT) to approximately three weeks (short-term tests), slightly modified by weather and growth conditions. The age of the periphyton film and the colonization and growth conditions influence the thickness of the film. For SWIFT a very thin film is used since it will develop further during the test, while a more mature and thicker film is suitable for the short-term testing. The glass discs were always gently cleaned on all sides except the colonized one, and sorted to achieve a homogenous set of glass discs for further testing.

Figure 2. Location of the Lovén Center Kristineberg.

6.2 SHORT-TERM TEST SYSTEM (hours)

In algal and bacterial communities, photosynthesis and protein synthesis are examples of essential metabolic processes. Effects on such functions indicate that further structural and functional changes might occur in the community after extended exposure to the toxicant (Clements 2000).

6.2.1 Photosynthetic activity

In the sampled microalgal communities the rate of photosynthesis was measured with radio labeled bicarbonate ($H^{14}CO_3$) (Blanck and Wängberg 1988). The algal communities were incubated at ambient temperature with a photon flux density of 100 µmol photons $m^{-2}s^{-1}$. The total volume of the test solutions including toxicant was five ml for the phytoplankton and two ml for the epipsammon and periphyton communities. After one h of pre-incubation with medetomidine (**Paper I**), $H^{14}CO_3$ were added and the radioactivity was adjusted to allow sufficient labeling of the communities in spite of any difference in biomass and activity. The $H^{14}CO_3$ was allowed to incorporate (two h in **Paper I**, 15 min in **Paper III**) after which the reaction was terminated with formaldehyde (at a final concentration 2% v/v). Unincorporated bicarbonate was driven off by acidification and air bubbling for plankton and epipsammon while the periphyton were acidified and dried. Periphyton cells were lysed with DMSO to facilitate release of radio labeled carbon and the amount of incorporated carbon was measured by liquid scintillation counting. The amount of incorporated ^{14}C was compared to controls that were not exposed to medetomidine, and therefore regarded as having 100% photosynthetic activity. For more details of the method see **Paper I** and **III**.

6.2.2 Bacterial protein synthesis

The rate of bacterial protein synthesis was measured as incorporation of ^{3}H-L-leucine using the centrifugation method by Smith and Azam (1992), slightly modified with regard to leucine concentrations and experimental containers. Aliquots of the sampled bacterioplankton community were used directly in the protein-synthesis assay. For the epipsammon and periphyton communities, bacteria had to be extracted from their substrata (sand or glass discs) by mild sonication in filter-sterilized seawater. A subsample of 10 g sand from the pooled and sieved epipsammon communities or 20

representative glass discs with periphyton communities were sonicated. The samples were then centrifuged and the supernatant was used for the assay. With the addition of medetomidine solution or filter-sterilized seawater a final sample volume of 1.7 ml was obtained for all communities. The bacterial communities were incubated at ambient temperature in scintillation vials in **Paper I** and in micro-centrifuge tubes in **Paper III**. After one h of pre-incubation with medetomidine (Paper I), ^{3}H-L-leucine was added and the communities were allowed to incorporate leucine for two h in **Paper I** and one h in **Paper III**. The incorporation was terminated with trichloro-acetic acid (TCA), centrifuged and the supernatant discarded to remove unincorporated ^{3}H-L-leucin. TCA was then added to clean the pellet, the samples were centrifuged again, and the supernatant discarded to leave only incorporated ^{3}H-L-leucine. The amount of incorporated ^{3}H-L-leucine was measured by liquid scintillation counting. The amount of incorporated ^{3}H-L-leucine was compared to controls that were not exposed to medetomidine and regarded as having 100% bacterial protein synthesis. See **Paper I** and **III** for more details of the method.

6.3 THE SWIFT TEST FOR TOXICANT-INDUCED SUCCESSION

Exposure to contaminants over a certain time can lead to toxicant-induced succession in a community (Blanck 2002). This was investigated by the SWIFT periphyton test, a semi-static system where immature periphyton communities are exposed to a toxicant during 96 hours. Changes in community structure were characterized by pigment profiles derived from HPLC (High Performance Liquid Chromatography) measurements of pigment extracts (Porsbring et al. 2007). Pigment profiles are surrogate data for community structure, reflecting aspects of biomass, physiological status and species composition. Fifteen periphyton discs per treatment were distributed in glass containers (10 x 15 x 5 cm) filled with 300 ml of test medium. The test medium consisted of filtered seawater (GF/F, Whatman) with elevated concentrations of phosphate and nitrate to 0.7 µM and 8 µM respectively (Wängberg and Blanck 1990). The containers were incubated on a shaking table in a climate chamber with continuous light and a temperature corresponding to the ambient water temperature at the sampling site. The test media were exchanged every 24 h. The test was terminated after 96 h, and seven discs were taken for pooled pigment

analysis (Porsbring et al. 2007). Structural differences between communities were quantified with the Bray-Curtis Dissimilarity Index (BCDI) (Bray and Curtis 1957). The BCDI is scaled between 0 and 1 where 0 represents maximal similarity and 1 maximal dissimilarity. Concentration-effect curves are generated through comparison of all treatments to an average control community based on the averages of all individual pigment values in the control samples (n = 6-8). Non-metric Multi Dimensional Scaling (MDS) (Clarke 1993) was used to plot the differences in pigment composition between the substances tested. The square-root transformed pigment data were normalised to the total pigment content of the sample and then pair-wise compared using the Bray-Curtis dissimilarity index. The resulting BCDI distances between all treatments were used for the MDS (Porsbring et al. 2007). A stress value show how well the BCDI have been preserved in the MDS, a value below 0.1 indicates a good ordination. For more detailed description of the test system see **Paper II**.

6.4 LONG-TERM FLOW-THROUGH PERIPHYTON MICROCOSM

Long-term toxicity of medetomidine to periphyton was investigated in an indoor flow-through aquaria system similar to the one described by Blanck and Wängberg (1988). Algae and bacteria from natural seawater were allowed to colonize the surfaces in the aquaria during four weeks under the selection pressure of medetomidine. Natural seawater was continuously pumped from three meters depth with an air-driven Teflon-membrane pump, through a nylon net (1 mm mesh) and into 19 aquaria (22 liters each). The water was distributed by a flow distributor modified for radial symmetry, which kept a continuous water flow through each aquarium (Molander et al. 1992). Mean water residence time in the aquaria was approximately 90 min.

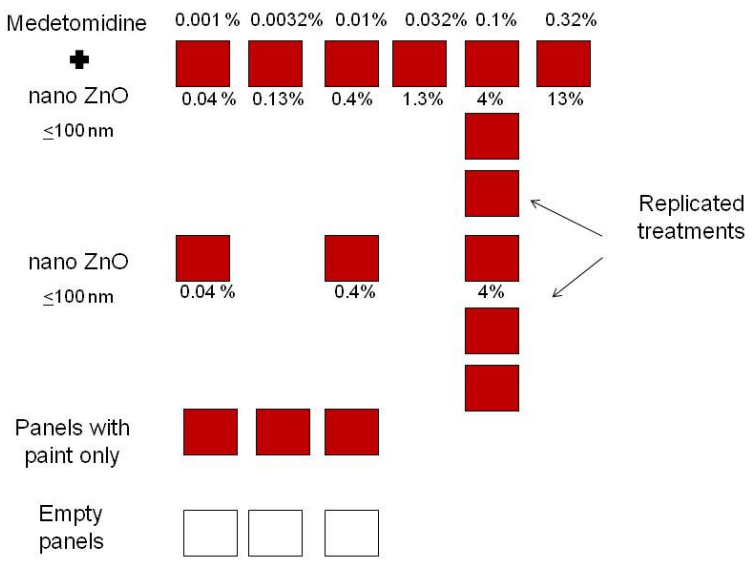


Figure 3. Treatment design for the long-term microcosm experiment with medetomidine and ZnO nanoparticles released from painted panels.

Medetomidine was delivered from a prototype ablative antifouling paint containing medetomidine and ZnO nanoparticles in different combinations to generate 11 treatments (Fig 3). Medetomidine is coordinated by metal ions (Fant et al. 2006) and adsorbs strongly to metal particles, especially CuO and ZnO nanoparticles (Shtykova et al. 2009, Trojer Andersson 2012). This property is beneficial for paint formulation and was used to control the release of medetomidine. The paints were applied to PETG (polyethylene terephthalate) panels and placed along the sides of the aquaria (Fig 4).

Each aquarium also contained glass discs (100 discs à 1.5 cm^2) arranged vertically along the sides of the aquaria, for organisms from the incoming water to settle on. After four weeks of colonization the periphyton glass discs were sampled and used for further analysis of photosynthetic activity (see 5.2.1), bacterial protein synthesis (see 5.2.2) and pigment composition (see 5.3). For further information see **Paper III**.

6.5 BIOACCUMULATION

Bioaccumulation of medetomidine was studied in a semi-static test system similar to the SWIFT test system. 15 glass discs with mature periphyton communities were incubated in a glass container (10 x 15 x 15 cm) together with 300 ml of test solution containing ^{14}C-labelled medetomidine to a concentration of 20 µg/l (100 nM). Test solutions were exchanged daily. To avoid extensive growth of the periphyton community during the test period light intensity was kept to 25 µmol m^{-2} s^{-1}. The light:dark regime and temperature was adjusted to ambient conditions. Uptake and elimination of medetomidine were investigated during 96 h. Analyses of accumulated medetomidine were performed using liquid scintillation counting. For a more detailed description please refer to **Paper IV**.

6.6 ENVIRONMENTAL EXPOSURE MODELING

The environmental exposure was modeled using the Marine Antifouling Model to Predict Environmental Concentrations, MAMPEC (Van Hattum et al. 1999, van Hattum et al. 2002). The model can provide predictions of environmental concentrations of antifouling biocides in five generalized marine environments; commercial harbor, estuarine harbor, marina, open sea and shipping lanes. The MAMPEC model version 2.5 was used to calculate medetomidine PECs for harbor, shipping lane and marina environments with three model scenarios; MAMPEC default, OECD (used for EU BPD) (Van der Aa and Van der Plassche 2004a) and Baltic (Koivisto 2003) adjusted for Baltic Sea specific properties. The input parameters used for the medetomidine compound settings are presented in detail in Table 2 and Table 3.

Leaching rate for medetomidine was calculated using the mass balance method developed by the European Paint Industry (CEPE) “Paints and varnishes – Modeling of biocide release rate from antifouling paints by mass-balance calculation” (Van der Aa and Van der Plassche 2004b). Input parameters used for the leaching rate calculations are presented in table 3. A hypothetical antifouling paint formulation containing 0.1% medetomidine, a realistic concentration in a final antifouling paint, was used. The leaching rate for a self polishing paint with a lifetime of 12 months was calculated to

61 ng cm^{-2} day^{-1}. The application factor, i.e. the percentage of ship hulls painted with a medetomidine-containing paint, was set to 20 percent.

Table 2. Medetomidine parameters used for the PEC calculations in the MAM-PEC model.

Parameter (unit)	
Molecular mass (g/Mol)	200.28
Vapor pressure (Pa)	$1.86x10^{-4}$
Solubility (g/m^3)	200
Octanol/Water coefficient (-log Kow)	2.9
Koc (-log Koc)	$3.5x10^{-1}$
Henry's coefficient (Pa x m^3/mol)	$1x10^{-5}$
Melting temperature (C°)	116.6
pKa	7.1
Biological degradation water (1/d)	$6.3x10^{-3}$
Biological degradation sediment	0
Hydrolytical degradation water	0
Hydrolytical degradation sediment	0
Photolytical degradation water	0
Photolytical degradation sediment	0

Table 3. Input values used for calculations of medetomidine leaching rate.

Parameter	**Value**
Paint type	Self-polishing
Density (kg/dm^3)	1.52
Volume solid content (%)	47
Biocide content of the active ingredient (mass fraction)	0.995
Biocide content of the wet paint (mass percent)	0.1
Nominal lifetime of the paint (months)	12
Specific dry film thickness (µm)	80
Fraction of the active ingredient in the dry film released during lifetime	0.9

7 SIGNIFICANT FINDINGS

7.1 SHORT-TERM DIFFERENCES IN SENSITIVITY

The acute effect of medetomidine on basic physiological functions such as photosynthesis and protein synthesis was studied in epipsammon, periphyton and phytoplankton communities (**Paper I**). As described previously, the mode of action of medetomidine is not known in algae and bacteria but nonetheless effects on physiology could occur through unidentified targets. To safeguard against unwanted effects it is therefore necessary, also to study non-target organisms with unknown sensitivity. The study showed that short-term exposure, three hours, caused minor effects on photosynthetic activity and bacterial protein synthesis (Fig. 4 and 5). Photosynthesis in the periphyton community seems to be stimulated at the lower concentrations tested (0.02-0.63 mg/l, 0.1-3 µM) and inhibited only at the highest test concentration 2.0 mg/l (10 µM), although none of these effects were statistically significant with the test method used. In phytoplankton, the photosynthetic activity significantly decreased to 86% of the control community at the highest test concentration 2.0 mg/l (10 µM). No significant effect on photosynthesis was observed in the epipsammon community.

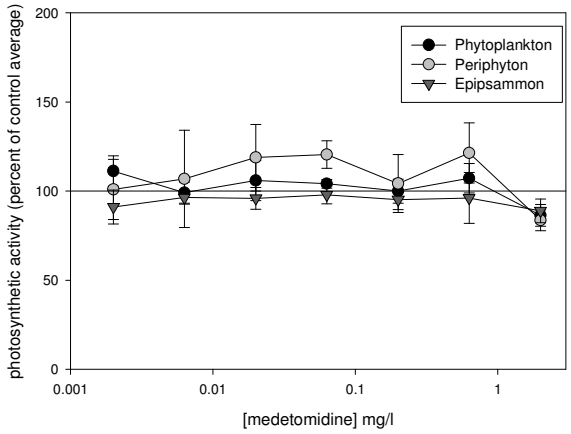


Figure 4. Photosynthetic activity of phytoplankton, epipsammon and periphyton communities during medetomidine exposure, expressed as a percentage of the control average. Error bars = SD, statistical significance (p 0.05).

Bacterial protein synthesis was slightly but not significantly inhibited for all communities at 2.0 mg/l (10 µM) medetomidine. Despite the lack of significant effects, the periphyton community seems to be most sensitive for this endpoint with a decreasing trend starting at 0.2 mg/l (1 µM) and ending at 83% protein synthesis activity compared to the control community at 2 mg/l (10 µM) (Fig. 5).

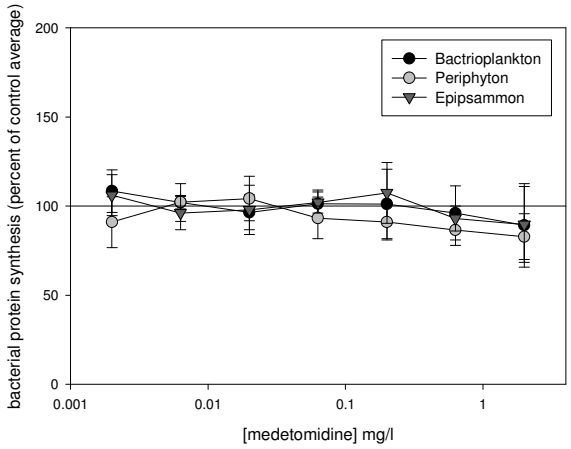


Figure 5. Bacterial protein synthesis of phytoplankton, epipsammon and periphyton communities during medetomidine exposure, expressed as a percentage of the control average. Error bars = SD, statistical significance (p 0.05).

Two crucial physiological functions, photosynthesis and bacterial protein synthesis, were mostly unaffected in the microbial communities following short-term exposure to medetomidine. However, some small differences in response between the communities were indicated. An increase in photosynthetic activity compared to the control community was seen for periphyton but not the other communities. As described above the increase was not statistically significant, but in spite of this it might be a sign of a response specific for the periphyton community. However, the response is not of any larger ecotoxicological interest since the concentration where it might occur is very high. The most medetomidine-sensitive organisms of relevance for periphyton algae and bacteria are the crustaceans with reported effects on behavior at 0.2-10 µg/l (1-50 nM) (Dahlström et al. 2000, Krång and Dahlström 2006). Since the increased algal activity was observed at 20 µg/l (0.1 µM) it would be possible that meiofauna (40 µm-1 mm

invertebrates) were affected. Meoifauna graze on bacteria, diatoms and protozoa (Rzeznik-Orignac and Fichet 2012) and a decreased grazing pressure could be the reason behind the measured increase in photosynthetic activity. For unknown reasons, any such grazing-mediated effect seems stronger in periphyton than in the epipsammon and not at all evident for bacteria. The variation in the response could be influenced by differences in medetomidine bioavailability or variations in the species present.

It can be concluded that the medetomidine concentration required to affect photosynthesis and bacterial protein synthesis is at least 10,000-fold higher than what is needed at the ship hull to prevent barnacle larvae from settling, 0.2 µg/l (1 nM) (Dahlström et al. 2000).

7.2 ANTIFOULANT-INDUCED SUCCESSION

In **Paper II** the succession of a periphyton community during exposure to five antifouling biocides was studied with changes in community composition estimated as changes in pigment profiles. The biocides used were chlorothalonile, dichlofluanide, medetomidine, tolylfluanide and zinc pyrithione. Chemical name, structure and mode of action are given in Table 1. Chlorothalonile, dichlofluanide, tolylfluanide and zinc pyrithione are all fungicides used as antifouling biocides, mainly against microfouling. They are often used in combination with biocides like copper oxide acting on hard fouling.

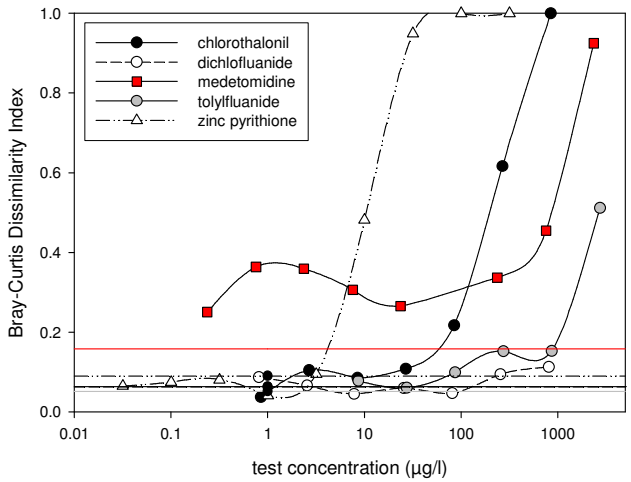


Figure 6. Effects on periphyton pigment profiles from exposure to five different antifouling biocides. The BCDI values represent dissimilarity to an average control community represented by the horizontal lines; black for chlorothalonile and dichlofluanide, red for medetomidine, grey for tolylfluanide and dash-dotted for zinc pyrithione.

The 96 hour SWIFT microcosm generated large variations in the periphyton community structure depending on which biocide and the concentrations the community was exposure to (Fig. 6). Medetomidine has no known mode of action in algae or bacteria. Despite this, differences from the control community were noted between 0.8 and 7.6 µg/l (4-40 nM) and then again above 240 µg/l (1.2 µM). The first response (0.8-7.6 µg/l, 4-40 nM) is thought to be due to grazers since that corresponds to the range where certain crustaceans and their behavior is affected (Dahlström et al. 2000, Krång and Dahlström 2006). The mechanism behind the change in pigment profiles above 240 µg/l (1.2 µM) is more difficult to explain. However, the effect range matches the concentration range where small effects on photosynthesis and bacterial protein synthesis could be discerned although not statistically significant (Fig 4 and 5).

Chlorothalonile affected the pigment composition at concentrations exceeding 85 µg/l (320 nM) which was surprising since previous studies on algae have reported effects in the 500 µg/l (1.9 µM) range (Cox 1997). The effects could however be secondary responses caused by toxicity to grazing

organisms as hypothesized in the previous section. Mollusks show a much higher sensitivity (EC_{50} ~ 8 µg/l, 30 nM) (Konstantinou and Albanis 2004, Bellas 2006) than algae, and it is possible that other invertebrates are more sensitive as well. Dichlofluanide on the other hand showed no effect up to the highest concentration used, 800 µg/l (3.2 µM), which could be expected based on previous studies of algal physiology (Johansson et al. 2012), while tolylfluanide started to affect community composition at 270 µg/l (0.8 µM) which is lower than previously described for *Saccharina latissima* (Johansson et al. 2012). Zinc pyrithione had the steepest dose-response curve with no effect on community composition at 3.2 µg/l (10 nM) and maximum dissimilarity possible at 32 µg/l (0.1 µM) and above. Since zinc pyrithione affect photosynthesis already at 0.6 µg/l (2 nM) (Maraldo and Dahllof 2004) this was not an unexpected response.

Non-metric multidimensional scaling (MDS) (Clarke 1993), was used to depict differences and changes of the periphyton pigment profiles (Fig. 7) and thus estimate physiological changes and the succession caused by antifoulants in the periphyton communities. MDS can be described as a map over how distant or dissimilar objects are from each other. Hence the outcome is dependent on all communities included in the ordination. As can be seen in Figure 5 the communities exposed to chlorothalonile, dichlofluanide, medetomidine and tolylfluanide respectively, were quite similar, while the periphyton community developed in the opposite direction during zinc pyrithione exposure. Substances with different modes of actions might generate different responses within an organism group or community. However, since the mode of action only describes what happens within the organism, the effect on community composition could also be the similar if the same sensitive species are eliminated by compounds with different modes of action. Such an effect on the community level could be described as ecological mode of action (Arrhenius 2005).

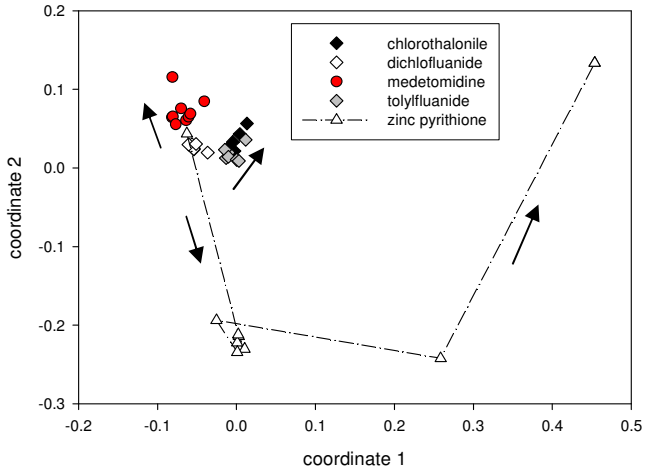


Figure 7. MDS ordination of all periphyton pigment. The distance matrix was compiled using Bray-Curtis dissimilarity index. Stress: 0.08. Arrows indicate development direction with increasing test substance concentration.

The pigment profiles used in **Paper II** are an indirect measurement of changes in community composition which affects at what resolution a response can be seen. Since the specific pigments are redundant in many algal species differences can at most be described to class or phylum level. A more detailed picture of the community response could have been achieved with taxonomical data. Taxonomical analyses cannot always describe the community composition to species level either but it would have been a significant complement.

The succession patterns caused by chlorothalonile, dichlofluanide and tolylfluanide that appeared similar in Figure 7, can be distinguished more from each other, by leaving out zinc pyrithione data from the MDS analysis (Fig. 8). Figure 8 show that the chlorothalonile and tolylfluanide exposed communities actually developed in different directions. This difference can be explained further by the pigment profiles of the communities. The tolylfluanide exposed community had higher ration of the light-harvesting pigment diadinoxanthin (DD) to the total amount of xanthophyll measured (diadinoxanthin and diatoxanthin (DT) calculated as DD/(DD+DT). Diadinoxanthin is transformed to diatoxanthin as a photoprotective

response (Van Leeuwe et al. 2008) in the xanthophyll cycle. The change in pigment ratio could be caused by disturbances to the xanthophyll cycle by changing light conditions (Van Leeuwe et al. 2008) or by photosystem II inhibitors (Porsbring et al. 2007). The photosystem II inhibition explanation does however seem less plausible since chlorophyll α decrease with increased exposure.

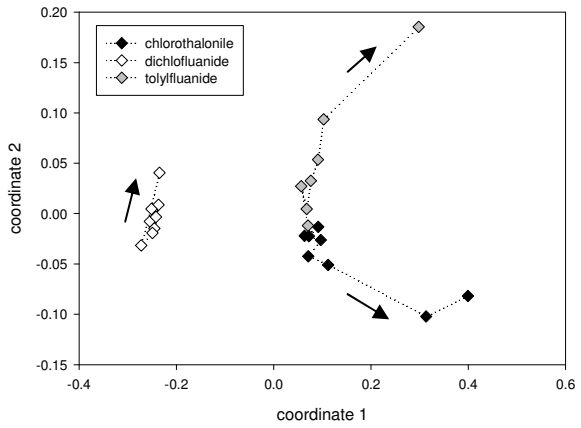


Figure 8. MDS ordination of chlorothalonile, dichlofluanide and tolylfluanide periphyton pigment profiles. The distance matrix was compiled using Bray-Curtis dissimilarity index. Stress: 0.09. Arrows indicate development direction with increasing test substance concentration.

7.3 LONG-TERM EFFECTS ON PERIPHYTON COMMUNITIES

The final study of periphyton communities and medetomidine with longer test duration and possibly increased sensitivity was a four-week flow-through microcosm study presented in **Paper III**. The aim here was to see how a realistic exposure of medetomidine leaching from a paint matrix would influence functional and structural parameters in a periphyton community colonizing surfaces under the influence of leachates from the painted surfaces (Fig. 9). The paint base used was a prototype non-biocidal ablative paint.

Medetomidine has been shown to coordinate with metal ions (Fant et al. 2006) and adsorbs strongly to metal particles, especially CuO and ZnO nanoparticles (Shtykova et al. 2009). These properties are beneficial for paint formulation and can be used to control the release of medetomidine

which is why zinc oxide nanoparticles were added to the prototype paint. However, when the zinc levels in the microcosm system were analysed to follow the release of zinc ions from the nanoparticles in the experimental paint, it was evident that the original paint base used was not zinc free. Due to different views of zinc´s role, the term "biocide-free" can be misinterpreted. In regulatory terminology and paint formulation, zinc is considered a substance of concern and a pigment with specific roles in controlling paint polishing rates - but not a biocide. This view is under discussion both in science and regulation with environmental and ecotoxicological effects under scrutiny (Karlsson and Eklund 2004, Ytreberg et al. 2010, KemI 2012).

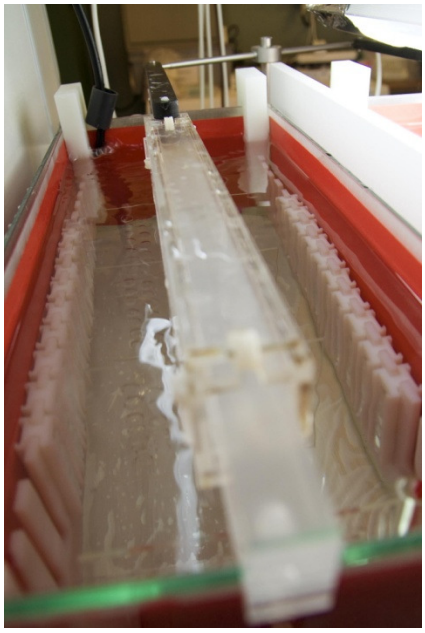


Figure 9. Aquarium with painted panels, holders for glass discs and stirrer. Photo Per Johansson.

The periphyton communities in the microcosms with painted panels thus had established under a selection pressure not only of medetomidine and nano-ZnO, but also at high "background" concentrations of zinc from the paint itself. The chemically verified medetomidine concentrations in the aquaria was 0.4 ng/l (0.002 nM) for the 0.1% medetomidine treatments and could have been 1.4 ng/l (0.007 nM) in the treatment with 0.38% medetomidine, if we assume a linear increase with increasing medetomidine concentration in the paint. The concentration of zinc was measured to 61-140 µg/l (0.9-2 µM) depending on the paint treatment (background in incoming seawater 3.7 µg/l (56 nM)).

In general, all treatments with painted panels had significantly lower photosynthetic activity but higher rates of bacterial protein synthesis. The structure of the communities, measured as pigment profiles, in the same treatments were also affected in comparison to the control communities

not exposed to the painted panels. A relationship to the measured labile zinc concentration can be observed for all endpoints (Fig. 10 and 11).

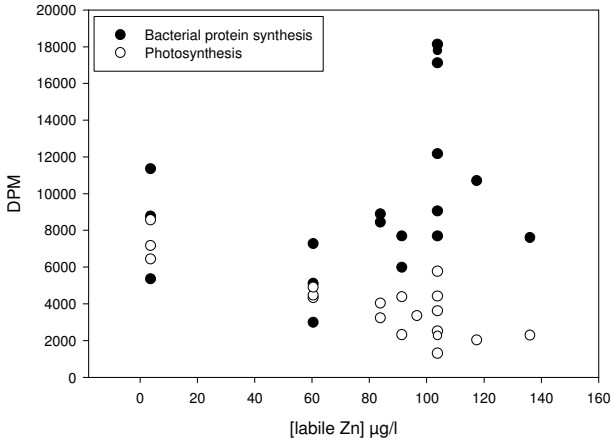


Figure 10. Bacterial protein synthesis and photosynthesis measurements correlated to labile zinc concentrations.

The unintended elevated zinc concentrations in the microcosm treatments might have caused zinc effects that overshadow any long-term effects of medetomidine. It may also interfere with the speciation and thus the availability of free medetomidine. A slight trend towards a change in community composition (pigment profiles) might be visible in Figure 11 for the treatments with ZnO nanoparticles and medetomidine. However since the measured zinc concentration also increase with the increasing medetomidine concentration it is impossible to clarify whether this is caused by medetomidine or zinc.

Similar microcosm studies with zinc on river periphyton communities have decreased algal and bacterial biomass at 8-30 µg/l (0.1-0.5 µM) (Paulsson et al. 2000, 2002) in river water with phosphorus-limited primary production. The mechanism behind these biomass effects is thought to be lowered phosphorus availability due to zinc-phosphorous interactions. At concentrations above 650 µg/l (9.7 µM) zinc disrupts photosynthesis more directly (Paulsson et al. 2000). A direct comparison of the test systems is difficult to make since the environments studied differ in nutrient limitations. However, both of these effects, reduced biomass and photosynthesis, are consistent with the reduced photosynthetic activity

seen in **Paper III**. The bacterial protein synthesis results do not correspond to the reported effect on river bacteria measured as inhibition of bacterial DNA synthesis in the Paulsson et al study (2000). The mechanism behind the increased bacterial protein synthesis seen in some treatments is difficult to clarify. Although, it has been reported that zinc concentrations around 30 µg/l (0.5 µM) can increase bacterial protein production (Hassen et al. 1998). Another possibility is that the periphyton community was affected at colonization and that present algae were more sensitive to zinc than the bacteria which would result in a periphyton community more dominated by bacteria. This is supported by the observation that the periphyton community in the paint treatments were atypical in appearance upon inspection, with little pigmentation and large amounts of extracellular polysaccharides. The community composition (pigment profiles) in the medetomidine microcosm study show that all paint containing treatments differed from the control communities (Fig. 11)

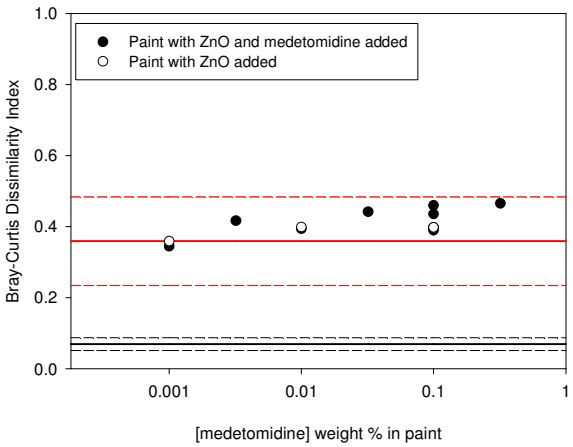


Figure 11. Effects of medetomidine antifouling paint on the pigment composition of periphyton communities. Results of pigment analyses presented as BCDI compared to unpainted control panels (black solid line, black dashed lines represent 95% confidence limit). Painted panels without nano-ZnO and medetomidine represented by red solid line, red dashed lines represent 95% confidence limit.

This long-term study intended to determine possible effects of medetomidine on colonizing periphyton communities resulted in a study on the effects of a zinc containing antifouling paint. Without any additions to the paint, the zinc concentrations in the water was elevated 16 times, which

resulted in decreased photosynthetic activity, increased bacterial protein synthesis and changed the community composition to a large extent.

7.4 PREDICTIONS OF ENVIRONMENTAL CONCENTRATIONS

PEC for medetomidine in water and sediment have been determined both for scientific and regulatory purposes using a variety of models and scenarios. In **Paper I** PECs are calculated for a marina, a harbor and a shipping lane environment using the MAMPEC model and three types of scenarios (MAMPEC default, OECD (BPD) and Baltic) (Van Hattum et al. 2002, Koivisto 2003, van der Aa and van der Plassche 2004b). The PEC values used are average water concentrations and average sediment concentrations after ten years based on input values presented in **Paper I**. What input data and which PEC values that should be used for risk assessment in a regulatory setting have been under discussion for years and are still under debate. The results presented in table 4 are based on medetomidine emissions from ship and boat hulls in-service with a realistic marker share for a new antifouling biocide.

Table 4. Results of the PEC calculations for the total medetomidine concentration in water (average concentration) and in sediment (average concentration after ten years).

Scenario and Environment	PEC Water (ng/L)	PEC Sediment ng/g dw
Baltic		
Harbor	2.3	3.1×10^{-5}
Shipping lane	4.2×10^{-4}	2.7×10^{-9}
Marina	57	7.8×10^{-4}
Default		
Harbor	0.44	3.4×10^{-5}
Shipping lane	3.6×10^{-4}	2.7×10^{-8}
Marina	2.4	4.6×10^{-5}
OECD (BPD)		
Harbor	1.1	1.3×10^{-5}
Shipping lane	3.4×10^{-4}	4.4×10^{-10}
Marina	1.5	7.3×10^{-5}

PECs are highly influenced by the scenario used for calculations, and on the environment per se since the contamination pressure is very different e.g. in a shipping lane where ship just pass by and in a harbor where they are moored in shallow waters. Water exchange rate is the one parameter in the

scenario which controls the outcome, even in the OECD (BPD) and Baltic scenarios where the contribution of tidal water exchange has been decreased (**Paper I**). Even when the half-life of a substance is altered substantially the resulting PEC remains more or less the same. This is problematic since persistence of the active ingredient has a central role in chemical risk assessment.

Other input values that have a large effect on the PEC are leaching rate of the biocide and market share. The leaching rate model developed by the European council of the paint, printing ink and artist's colors (CEPE) and presented in chapter **6.6** is regarded to generally overestimate the leaching rate since it is assumed that 90% of the biocides is released during the lifetime of the paint (IMO 2009). Based on measured leaching rates for several substances a correction factor of 2.9 is recommended to generate more realistic leaching rates (IMO 2009). The paint used in **Paper I**, was a yacht paint with 12 months service-life, hence 90% of the medetomidine would be released during 12 months. Since little was known about the leaching rate of medetomidine no correction factor was applied. For comparative reasons a market share of 90% is recommended for MAMPEC determinations under BPD. However, since medetomidine is not on the European market a 20% market share was considered more realistic for a new antifouling biocide and therefore used in **Paper I**.

To put the results from **Paper I-III** in an ecological context, the effect concentrations can be compared to the predicted environmental concentrations (Table 5). The worst-case PEC, 0.057 µg/l (0.28 nM) is at least a factor ten lower than the first effect concentration observed, a change in community composition from the SWIFT test system. The short-term endpoints, photosynthesis and bacterial production, were much less sensitive, and to have an acute effect on periphyton metabolism would require a concentration 35 000 × the PEC-value. The intermediate length endpoint (days) showed some indirect effects of medetomidine exposure at 15 × PEC while the long-term exposure only displayed zinc effects.

Table 5. Comparison of effect concentrations and worst-case PEC_{water}.

Endpoint	Effect concentration µg/l	PEC_{water} µg/l
acute photosynthesis	2000	0.057
acute protein synthesis	2000	(Baltic
community composition(96h)	0.8	marina)
chronic photosynthesis	n.d	
chronic protein synthesis	n.d	
chronic community comp.	n.d	

7.5 MODE OF ACTION OF MEDETOMIDINE IN ALGAE AND BACTERIA

Based on previous knowledge about medetomidine's mode of action, there is no known receptor or mechanism of action in microbial communities. Still, medetomidine effects are observed at high concentrations.

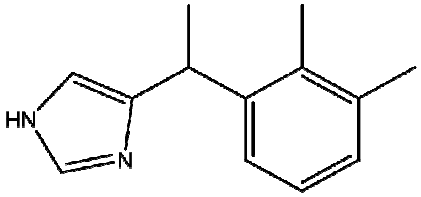


Figure 12: Structure of medetomidine.

At present the effects of medetomidine can be explained by baseline toxicity which is common for all organic molecules. Baseline toxicity is basically decreased biological activity caused by disturbance of cell membranes reactions by foreign organic molecules (Rand 1995). The building blocks of medetomidine might give some insight since imidazole rings are components in many natural substances such as histamine, purine and nucleic acids but they are also commonly used in pharmacological substances both for their biological activity and to improve solubility and pharmacokinetic properties (Shalini et al. 2010). Imidazoles also have strong tendency to bind transition metals (Trojer et al. 2013) and may therefore interfere with the transport, bioavailability and biochemistry of essential metals. Since the effects observed in microalgae and bacteria (**Paper I-III**) occurred at much higher concentrations than reported for organism groups with octopamine or adrenergic receptors the direct membrane or metal interaction might be involved in the mode of action in algae and bacteria.

7.6 FATE OF MEDETOMIDINE IN PERIPHYTON AND INVERTEBRATES

Bioaccumulation of medetomidine was studied in **Paper IV**. Focus was on the uptake and elimination of radiolabelled medetomidine in periphyton communities, blue mussels (*Mytilus edulis*), *Abra nitida* (a sediment-burrowing mollusk) and brown shrimp (*Crangon crangon*). Bioconcentration or bioaccumulation studies can be used as a decision basis for labeling a substance as bioaccumulative or not. In the EU a substance with a bioconcentration factor (BCF) or bioaccumulation factor (BAF) at 2000 l/kg or above is used for this decision (ECHA 2012). This applies for studies performed according to specific approved guidelines, for example OECD or OPPTS (Office of Prevention, Pesticides and Toxic Substances), and in a laboratory certified according to good laboratory practice (GLP). However, guideline studies like these are usually performed on fish and seldom published. Since medetomidine already have a known half-life in fish (5.5 h) (Horsberg et al. 1999) a study on other marine species was thought to be of higher interest. In **Paper IV** the bioconcentration factor (BCF) was determined for periphyton, *Crangon crangon* and *Mytilus edulis* while the bioaccumulation factor (BAF) was determined for *Abra nitida.* BCF is defined as the ratio of the contaminant concentration in the tissue of an organism to the concentration in the water, and BAF as the ratio of the contaminant in the tissue (C_B) to the concentrations in any compartments (such as water, sediment, food) relevant for the main uptake routes from the environment (Arnot and Gobas 2006). The BCF and BAF values are presented in table 6.

Table 6: BCF, BAF and half-life for the organisms exposed to medetomidine.

Parameter	**Species/Community**			
	Periphyton	*Mytilus edulis*	*Abra nitida*	*Crangon crangon*
Bioconcentration factor	1195	134	2.6[a]	2.8
Half-life $t_{½}$ (h)	<1	<6	96-120	6-24

[a]bioaccumulation factor

Due to the significantly higher BCF value for periphyton in combination with an elimination time of less than an hour further investigations regarding the behavior of medetomidine in the periphyton community were made.

Whether medetomidine is rapidly mobile in and out of the organisms within the community or strongly adsorbed cannot be made certain based on the study performed. The behavior seen is most likely due to a combination of large surface area and high lipid content (Wang et al. 1999) in the periphyton community. The uptake fits a two-compartment bioconcentration model (Newman 1995) with a rapid initial uptake during the first 30 minutes to fou hours followed by a secondary slower uptake during the following 8 to 48 hours. This is an indication that surface adsorption takes place (Fig. 13). The rapid elimination of medetomidine with a $t_{1/2}$ of less than one hour strengthens the view that most of the medetomidine was adsorbed on the surface, and that only a small fraction was taken up by the organisms since the concentration decreased immediately when exposure ended (Fig. 14). 10 % of medetomidine does however remain in the periphyton after 48 hours in clean water. This could be caused by a new steady-state with re-uptake of the medetomidine released to the water phase (average water concentration 1.7 µg/l, 8.5 nM). It could also be caused by interactions with something in the biofilm, for example transitional metals which would cause a strong bond (Trojer et al. 2013).

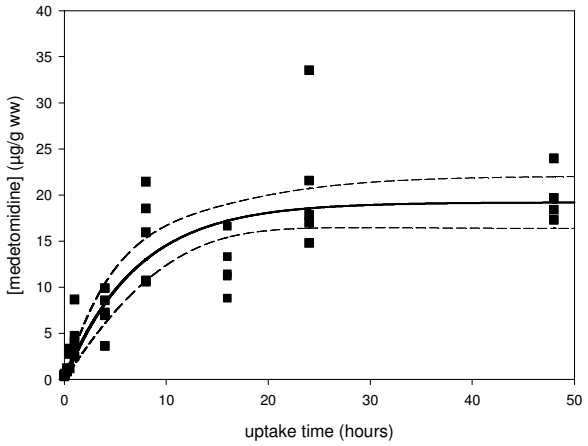


Figure 13: Uptake of medetomidine in periphyton communities exposed to a nominal concentration of 20 µg/l (0.1 µM) of medetomidine.

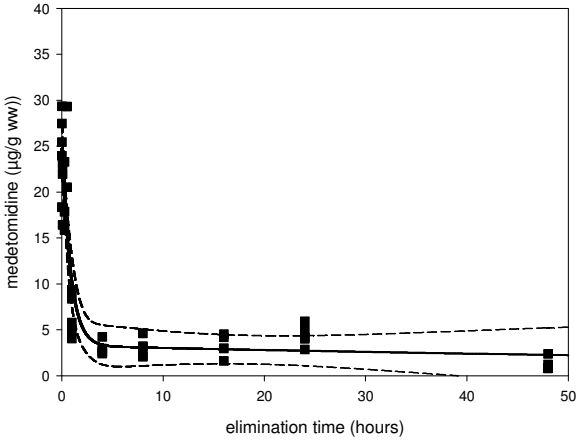


Figure 14: Elimination of medetomidine from periphyton communities pre-exposed to a nominal concentration of 20 µg/l (0.1 µM) of medetomidine for 48 h.

7.7 METHODOLOGICAL CONSIDERATIONS

The methods used in the **Paper I-IV** are all previously published and most of them are established. All methods do have their strengths and weaknesses; the short-term endpoints used might be more suitable for substances with a mode of action specific for those endpoints while the SWIFT and long-term systems would have been strengthened with taxonomical analyses since pigment profiles are less specific than taxonomical data. A strength with the approach for this thesis work is that a wide range of endpoints and a progression of exposure have been used; from adhesion and short-term metabolism to toxicant-induced succession and finally a (semi-)long-term and more realistic exposure. The approach can of course not fully predict possible future effect on microbial communities but it does cover many sensitive functions.

One limitation that is evident in **Paper I** and **II** is the lack of analytical data for the chemicals used. Even though several attempts were made to analyze medetomidine from these test systems methodological problems made the results inadequate. The bioaccumulation study (**Paper IV**) was performed with radiolabelled medetomidine and chemical analysis was therefore not required since liquid scintillation counting could be used to confirm test concentrations. The method used to prepare test solutions with medetomidine was however the same in **Paper I, II** and **IV**. Since the analyzed test concentration in **Paper IV** did not differ more than 25% from the nominal concentration the same should also be true for the medetomidine concentrations in **Paper I** and **Paper II**.

8 DISCUSSION

8.1 FROM ACUTE TO CHRONIC EXPOSURE

Based on previously known effects of medetomidine; pigmentation effects in fish (Karlsson et al. 1989) and the high sensitivity of barnacle larvae (Dahlström et al. 2000), studies on microbial communities could at first seem slightly redundant. However, the periphyton community is viewed as an early warning system for a number of disturbances of both natural and anthropogenic origin and is the first contact point with pollutants in many environments (Sabater et al. 2007). It is also obvious from the history of environmental problems that it is chemicals with unexpected or unknown mechanisms that are detected as pollution problems in the environment after long time. It is therefore essential to focus also on the unexpected, and do it conclusively.

The approach in this thesis, with acute to chronic testing of microbial communities despite the initial results indicating little risk, was used to evaluate if an important part of the marine ecosystem would be affected by medetomidine. Since microalgae and bacteria perform important ecosystem functions, a severe change in the microbial community might affect the whole marine food web if the functionality of the community is lost. To investigate the actual risk for microbial communities if medetomidine would be used as an antifouling biocide, acute exposure only was not considered enough to confirm whether or not medetomidine may pose a risk to the microbial community. The generation time for organisms in the microbial communities are in general relatively short, ranging from hours to days. The short-term exposure therefore covers a substantial part of the life-cycle. Furthermore, both a 96-hour and a four week microcosm study were included to verify the risk with intermediate and more or less chronic exposure. Neither of these studies showed any adverse effects from medetomidine exposure at predicted environmentally relevant concentrations. The most sensitive effect observed, a change in pigment profile at fourteen times the worst-case PEC, was most likely a secondary effect caused by an impact on algal-grazing unicellular protozoans or multicellular invertebrates.

8.2 FATE AND BEHAVIOR OF MEDETOMIDINE

Bioaccumulation studies with medetomidine indicate that the substance quickly binds to the periphyton community surface area during exposure and 90% is rinsed off when exposure ends. This adhesion is probably due to either ion interactions with carboxyl-groups in the biofilm (Dahlström et al. 2004) or the affinity of the medetomidine imidazole group to transition metals and their nanoparticles (Shtykova et al. 2009, Trojer et al. 2013). However, little effects of medetomidine have been seen on the periphyton community so the adhesion does not seem to influence the community under acute or chronic exposure. The increased concentration of medetomidine in the periphyton could of course affect the grazing micro- or meoifauna which could be reflected as medetomidine-mediated changes in species composition of the microalgae. The detected changes, measured as changed pigment profiles, are first observed at the concentration range (0.8 µg/l, 4 nM) which is known to affect larval behavior of barnacles. While this concentration might occur on the ship hull it is not in the range of the predicted environmental concentrations (0.057 µg/l, 0.28 nM) which makes this concern less plausible for the overall marine environment. There is no other indication that medetomidine bioaccumulates in the organism groups tested; crustaceans, mollusks (**Paper IV**) and fish (unpublished information, table 7).

8.3 PREDICTED ENVIRONMENTAL CONCENTRATIONS

One of the difficulties with testing chemical substances on organisms with unknown sensitivity is that you can never really verify a low risk since there might always be a more sensitive organism or endpoint somewhere.

A way to address this uncertainty in environmental risks assessment is to use assessment factors. The basic principle is the more data available for a substance, the lower additional factor is used to calculate the predicted no effect concentration (PNEC). If only the minimal amount of required data (acute data for three taxonomical groups) is available for a substance that will be used in the marine environment, a factor of 10 000 is divided with the available effect data (EC_{50} or NOEC) to generate the PNEC. If additional laboratory data with longer exposure times and more organism groups is available the assessment factor can be lowered to a minimum of 10. The

reasoning behind this system is that with more information available on more species, the uncertainty of the assessment is reduced.

One crucial component in the environmental risk assessment is the predicted environmental concentration (PEC). As previously described the PEC depends on a number of factors; environmental, chemical and emission calculations (leaching rate, market share etc). The PEC generated is therefore highly dependent on the input values used. The environmental parameters used for the scenarios are generally an average of a range of harbors and marinas and might therefore not be representative at all for certain areas. The Baltic scenario, for example was developed with the special conditions of the Baltic Sea in mind, with little tidal influence since most of the water exchange in the default and OECD scenarios are tide driven. With this change in environmental parameters the Baltic scenario usually generates the highest PECs.

On the other hand, some of the chemical parameters used in the MAMPEC model were shown to have a surprisingly small impact on the PEC (**Paper I)**. Since the degradation rate used in **Paper I** was based on the amount of substance lost during aerobic and anaerobic transformation in an aquatic sediment system some predictions were made to see how much the degradation rate influenced the PEC. Based on the importance put on degradability in environmental risk assessment it was startling that half-life's from 0.1 to 100 000 days only increased the PEC with 11% at most. This approach is severely underestimating the potential long-term impact of slowly degrading biocides.

8.4 ECOTOXICOLOGICAL EFFECTS OF MEDETOMIDINE

At least 22 ecotoxicological endpoints from medetomidine exposure have been generated in a variety of organisms and presented in scientific publications as part of the Marine Paint project. Due to the high sensitivity in barnacles one focal point was effects on other marine invertebrates with reproduction, behavior and bioaccumulation as endpoints (Bellas 2006, Bellas et al. 2006, Krång and Dahlström 2006, Hilvarsson et al. 2009). Since medetomidine was known to affect fish pigmentation (Karlsson et al. 1989) another focal point was acute and chronic effects in various fish species

(Bellas et al. 2005, Lennquist and Forlin 2006, Hilvarsson et al. 2007, Lennquist et al. 2008, 2010, 2011). The third focal point was the work on microbial communities presented in this thesis.

In addition to the Marine Paint data, a regulatory dossier containing about the same amount of ecotoxicological data has been compiled by I-Tech AB with the purpose of registering medetomidine as an antifouling biocide within the EU. The Marine Paint generated data and some comparative endpoints provided by I-Tech are presented in table 7. A comparison show that the most sensitive endpoints are in the same range; periphyton pigment profile, digging activity in sediment-living mollusks, respiration/pigmentation in some fish species with effect concentration at 0.5-1 µg/l (2.5-5 nM) and early-life stage effects in fish and survival/reproduction in a crustacean with effect concentration at 3.2-10 µg/l (16-50 nM). There is a large discrepancy between the effect concentration for the 72-hour diatom study (*Skeletonema costatum*) and the 96-hour periphyton community microcosm which is explained by the endpoints: growth inhibition, which is a population response, versus a more sensitive change in pigment profile. Then again, if the diatom study would be used for risk assessment an assessment factor would be applied which would lower it with at least a factor 10, depending on the amount of data. The PNEC would then be 50 µg/l (0.3 µM) which is close to the range where the non-secondary effects of medetomidine were seen in the SWIFT study.

The fish endpoints on the other hand have more similar effect concentrations. Generally the most sensitive endpoints have been detected for the early-life stages (larvae and juvenile fish) even though the sensitivity varies between species. For some endpoints, such as pigmentation, variation within the same species is also evident.

Table 7: Ecotoxicological endpoints of medetomidine.

Test organism	Endpoint	Effect conc(µg/l)	Origin
Microbial communities	acute photosynthesis	2000	Paper I (Ohlauson et al. 2012)
	acute protein synthesis	2000	Paper I (Ohlauson et al. 2012)
	pigment profiles. (96h)	0.8	Paper II (Ohlauson and Blanck 2013)
	chronic photosynthesis	n.d	Paper III (Ohlauson and Blanck, submitted)
	chronic protein synthesis	n.d	Paper III (Ohlauson and Blanck, submitted)
	chronic pigment profiles	n.d	Paper III (Ohlauson and Blanck, submitted)
Algae			
Skeletonema costatum	growth inhibition	500	I-Tech AB (unpublished)
Mollusks			
Abra nitida	burrowing activity	86	Bellas et al. 2006
	reworking activity	0.9	Bellas et al. 2006
Blue mussel	scope for growth	n.d	Hilvarsson 2007
(*Mytilus edulis*)	embryonic development	n.d	Hilvarsson 2007
Pacific Oyster (*Crassostrea gigas*)	embryonic development	2500	I-Tech AB (unpublished)
Crustaceans			
Corophium volutator	mate search behavior	10	Krång and Dahlström 2006
	survival and reproduction	32 µg/kg	I-Tech AB (unpublished)
Americamysis bahia	survival and reproduction	10	I-Tech AB (unpublished)
Daphnia magna	immobilization	4500	I-Tech AB (unpublished)
Fish			
Turbot	respiration	0.42	Hilvarsson 2007
(*Psetta maxima*)	pigmentation	0.42	Lennquist et al. 2008
	EROD	100 µg/kg	Lennquist et al. 2008
Lumpfish	respiration	1	Bellas et al. 2005
(*Cyclopterus lumpus*)	pigmentation	0.8	Bellas et al. 2005
Atlantic cod	respiration	>240	Bellas et al. 2005
(*Gadus morhua*)	EROD	>12	Lennquist et al. 2008
Three-spined stickleback	swimming activity	10	Hilvarsson 2007
(*Gasterosteus aculeatus*)	feeding behavior	20	Hilvarsson 2007
Sheephead minnow	early-life stage devel.	3.2	I-Tech AB (unpublished)
(*Cyprinodon variegates)*	bioaccumulation	BCF 1	I-Tech AB (unpublished)
Rainbow trout	pigmentation	1	Lennquist et al. 2010
(Oncorhynchus mykiss)	pigmentation	25	I-Tech AB (unpublished)
	EROD	1	Lennquist et al. 2008
	growth hormones	>1.2	Lennquist et al. 2011

9 CONCLUDING REMARKS AND OUTLOOK

The studies performed on microbial communities for this thesis show that algal and bacterial metabolic functions are not affected by medetomidine except at very high concentrations (2 mg/l, 10 µM). The same conclusion can be drawn for direct effects on species composition. There is however indications that organisms grazing on the community are affected, which might cause a secondary food-web effect on algal species composition. This possible change in species composition occurs at fourteen times the predicted environmental concentration for a Baltic marina, which should be considered a worst-case prediction. Further insight on long-term exposure effects of medetomidine on community composition from realistic antifouling paint concentrations were unfortunately overshadowed by effects of zinc which was also present in the antifouling paint. It turns out that the antifouling paint without any added medetomidine affected the species composition to the degree that no medetomidine effects were detectable. One conclusion that can be drawn from this is that with regard to medetomidine and antifouling paints other substances might be of more concern when it comes to microbial community effects.

To conclude on possible effects of medetomidine in marine environments all other information should of course also be taken into account. The Marine Paint data identify some sensitive endpoints in sediment dwelling mollusks, sediment living crustaceans and fish respiration and pigmentation. These endpoints have been taken into account also for the regulatory evaluation of medetomidine and similar studies have been used to describe predicted no effect concentrations for the marine environment.

With regards to all the information on medetomidine generated from Marine Paint and the I-Tech information presented here it seems that the most sensitive endpoints in the marine environment have been identified to facilitate an environmental risk assessment of medetomidine as an antifouling biocide.

10 ACKNOWLEDGEMENTS

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