A novel bioassay for evaluating the efficacy of biocides to inhibit settling and early establishment of marine biofilms

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Abstract

This paper presents a novel assay that allows a quick and robust assessment of the effects of biocides on the initial settling and establishment of marine photoautotrophic biofilms. The assay integrates the response of the multitude of indigenous fouling organisms, which overcomes a major limitation of existing assays which are largely limited to testing only lab-cultivatable species. The assay was evaluated using eight antifouling biocides, for which full concentration-response curves are presented. The efficacy ranking, based on EC_{98} values from most to least efficacious compound is: copper pyrithione >TPBP >DCOIT >tolylfluanid >zinc pyrithione >medetomidine >copper (Cu^{2+}), while the ecotoxicological ranking (based on E_{C10} values) is irgarol, copper pyrithione >zinc pyrithione >TPBP >tolylfluanid >DCOIT >copper (Cu^{2+}) > medetomidine. The algaecide irgarol did not cause full inhibition. Instead the inhibition leveled out at 95% effect at 30 nmol l^{-1}, a concentration that was clearly lower than for any other of the tested biocides.

Keywords
Photoautotrophic biofilm
Microfouling
Periphyton
Slime
Ecotoxicology
Antifouling biocides

Introduction

Fouling on ships and boats increases the surface roughness of the hull and hence increases its frictional resistance when the vessel moves through water. Such increased resistance leads to higher fuel consumption and associated emissions of exhaust fumes, elevated costs and problems keeping to operational schedules (Almeida et al. 2007). In addition, fouling also facilitates the spreading of invasive species (Piola et al. 2009).

Microbial biofilms ("slime", periphyton) are the first important colonization stage on a clean surface submerged into water, building the basis for later, more mature fouling communities (Almeida et al. 2007). Such biofilms include bacteria, cyanobacteria, microalgae (especially diatoms), fungi and protozoa and are sometimes referred to as "microfouling" (Briand 2009). Already a light slime-layer through which the paint color is still visible causes increases in resistance of around 11%, and a heavy slime-layer can result in up to 20% increase in resistance (Schultz 2007).

The review by Briand (2009) summarizes the scientific literature on the bioassays used for evaluating the efficacy of antifouling biocides over the last 30 years and the authors note a distinct lack of assays that investigate the entire microfouling community. They report further that cultivable microorganisms represent less than 1-5% of the *in situ* biodiversity. Hence, in order to assess antifouling agents under realistic conditions, assays are needed that reflect the high diversity found in microbial fouling communities, which can only be achieved by means of multispecies assays with natural microbial communities. Given the lack of appropriate assessment methodologies, it is not surprising that Molino and Wetherbee (2008) point out that many of today's antifouling coatings fail to inhibit settling and growth of microalgal biofilms or periphyton dominated by diatoms.

Only more realistic assays for evaluating the efficacy of biocides and paints will allow a better understanding and prediction of biocide and paint performance in the real environment, which will aid the environmental risk assessment as well as the development of new biocides and biocidal antifouling paints. In this paper a novel assay using field-collected marine photoautotrophic periphyton as inoculum is described, which integrates effects on settling and the early establishment of biofilms. The assay hence also integrates the response of the multitude of indigenous species that are potential foulers, thereby overcoming the limitations of laboratory and/or assays based on single-species responses.

In short, biofilms were established in the field for approximately three weeks on artificial surfaces (polyethylene terephthalate, PETG), and subsequently harvested. The resulting inoculum was then allowed to colonize pristine PETG-surfaces over 24 hours while exposed to biocides. Exposure is thereafter continued for another 48 hours in order to include effects on the initial biofilm establishment. Chlorophyll *a* content per surface area was used as the endpoint, reflecting total settled biomass, integrating settling and growth.

The assay was evaluated with the following set of biocides: copper (Cu^{2+}), irgarol, DCOIT, copper pyrithione, zinc pyritihone, tolylfluanid, TPBP (borocide), and the novel antifouling biocide medetomidine (Table 1).

Copper is used in coatings as cuprous oxide, copper powder or copper thiocyanate and has been the most commonly used biocide in antifouling paints for a long time (Yebra et al. 2004) and probably still is. The biologically active form of copper in ship paints is its ionic form Cu^{2+} and therefore $CuCl_2$ was used as the test substance. Irgarol is a specifically acting photosystem II inhibiting algaecide and is often used as a booster biocide in combination with copper. DCOIT is a broad spectrum booster biocide that affects all parts of the fouling community including both soft- and hard foulers. The pyrithiones are marketed as broad spectrum antimicrobial biocides and are also used in e.g. plastics, textiles, dry paint and personal care products such as anti-dandruff shampoo (2013). Tolylfluanid is a phenylsulfamide used as a fungicide in agriculture, but has also been applied as an antifouling biocide in paints (Thomas and Brooks 2010). TPBP is an organoborane compound mainly used in Japan (Thomas & Langford 2009) where it has been a common antifouling biocide since 1995 (Mochida et al. 2012).

Medetomidine (4-[1-(2,3-dimethylphenyl)ethyl]-1H-imidazole) is traditionally used within veterinary medicine as a sedative agent (Macdonald et al. 1988). Dahlström and co-workers (2000) discovered that medetomidine inhibits barnacle settling at low concentrations and it is now approved as an antifouling biocide in Japan under the trade name Selektope® (Ohlauson & Blanck 2014).

With the exception of TPBP and medetomidine all the tested biocides are notified under the European Biocidal Product Directive 98/8/EC (European Parliament 1998) as product type 21 (antifouling products).

Materials and Methods

All studies were conducted during May-September in 2007 to 2010 at the Sven Lovén Center for Marine Sciences, Kristineberg located at the Gullmar fjord (58°15'N, 11°27'E) on the Swedish west coast. All biocides were used in >95% purity and were kindly provided by the respective company (Table 1), except for copper which was purchased as copper(II)chloride from Merck (>98% purity).

Test solutions

The test medium was prepared from filtered (GF/F, Whatman) natural seawater (sampled from 30 metres depth) with the addition of nutrients (0.7 µM PO_4^{2-} and 8 µM NO_3^-), so that the final composition reflects nutrient conditions during a spring bloom (Porsbring et al. 2007).

Dimethyl sulfoxide (DMSO) was used as a co-solvent for irgarol, tolylfluanid, TPBP, copper- and zinc pyrithione and DCOIT at a final concentration of 0.1 vol% (controls as well as biocide treated samples). 0.1% DMSO did not affect settling and growth of the periphyton at the concentration used (Fig. 2, below). Dilution series of the biocides were prepared in DMSO with the concentration intervals adjusted to the steepness of the expected concentration-response curve based on information from previous range-finding experiments. Cu^{2+} and medetomidine were solved in deionized water. All values are reported as nominal concentrations.

Sampling and preparation of test material

The periphyton biofilms were sampled from the bay of Kalvhagefjorden (Lat.N58º14´ Long.11º24´) using artificial substrata made of polyethylene therophtalate (PETG) mounted on polyethylene holders (Blanck and Wängberg 1988). These were exposed in the bay at approximately 1.5 meters depth and allowed to be colonized for 3 to 4 weeks. PETG roughly resembles the surface of a painted boat hull.

The colonized discs were scraped with a razor into filtered (Whatman GF/F) natural seawater (from a depth of 30 metres) and afterwards shaken vigorously in a Pyrex bottle for three minutes to disintegrate the biofilm. The suspension was then sieved through a 100 µm mesh, left standing for 20 minutes and then sieved again to remove those cells that instantly aggregated. The chlorophyll *a* content in this algal suspension was approximately 0.3 µg chlorophyll *a*/ml analysed after extraction in ethanol (Jespersen & Christoffersen 1987) and measured using a 10-AU Turner flouorometer (Turner designs, Sunnyvale California, USA).

Test procedure

Circular 20 mL borosilicate crystallizing dishes (VWR Collection, VWR International, Stockholm, Sweden) were used as incubation vessels. Aliquots of five ml test solution and five ml algal suspension were distributed into each vessel, into which a tailor made rectangular piece of PETG (14*40 mm, 1 mm thick) was inserted as a settling surface. Prior to use the PETG pieces these were gently cleaned in warm water with a mild soap and thereafter rinsed in deionised water over night. Immediately before being inserted into the incubation vessel the PETG pieces were immersed in 70% ethanol for

disinfection and rinsed a final time with deionized water. Each PETG piece had an incision 14 mm from the longer end, which was submerged in the water. The other end was tilted to the edge of the vessel in 45º angle.

Clean PETG pieces were incubated for 24 hours with the biofilm slurry (settling phase), after which the pieces with the newly attached biofilms were transferred to new vessels with fresh test solutions but without biofilm slurry. They were incubated for another 48 hours with a transfer to fresh test solutions after 24hours. The submerged part (1.96 cm^2) of the PETG pieces was sampled after the end of the incubation for further analysis (*chlorophyll a* determination and recording of species composition).

Incubation settings were selected to mimic the average conditions at the Swedish west coast during the early summer season, which is the major period in Scandinavia during which pleasure boats are launched (Berntsson & Jonsson 2003). The incubation vessels were illuminated by fluorescent tubes from above (Osram Lumilux Daylight L 18W/12, Osram, Munich, Germany) to give an average photon flux density of 50 µmol photons m^{-2} s^{-1} at the water surface and with a light-dark regime reflecting the day length during the summer season (16h:8h). The incubation vessels were placed on a shaker with circular movements. Plastic petri-dishes were used as lids to prevent evaporation and avoid contamination. A temperature controlled room was used to achieve a constant temperature of 15°C.

Taxonomic analysis of settled algal species

The species composition of the settled algal community was determined in order to describe the biodiversity of the periphyton communities and to analyse how the test method itself affects species composition and structure. This was done by means of a frequency analysis. In natural habitats the frequency with which a particular species is found typically correlates with its abundance, which was also demonstrated for periphyton communities (Porsbring et al. 2007). Two sets of samples were prepared to compare the communities that established themselves on PETG pieces after three weeks in the environment with the communities that had re-established themselves after the 72 hour test period.

Two samples from each set were preserved in 70% ethanol and stored at 4°C in darkness until species identification. Algae were identified to the species level when possible. Specimens of unknown taxonomical affinity were assigned to groups of higher rank and a numerical archetype that corresponds with a certain set of characters (e.g. shape, size) in our reference files (Dr. Aleksandra Zgrundo, Gdansk University, Poland). In the following, all species, taxonomic groups and algal archetypes will be referred to as taxa.

Taxa were identified and recorded in 50 randomly chosen fields (diameter 0.254 mm) per disc, using differential interference contrast light microscopy at 1000x magnification (Dahl & Blanck 1996). The frequency of each taxon was estimated as the number of fields in which the taxon was observed, thus giving a relative frequency scale for each

taxa from 0 to 50 for each sample. Presented in the following is the sum of duplicate samples (scale 0-100).

Chlorophyll a as measure of growth
In order to estimate algal and cyanobacterial biomass, chlorophyll *a* was extracted from each colonized PETG piece using ethanol (96%) (Jespersen & Christoffersen 1987) for 20-24 hours at room temperature and analysed using a10-AU Fluorometer (Turner designs, Sunnyvale California, USA).Visual inspection of the discs using an epifluorescence microscope after the chlorophyll *a* extraction showed that there were only a very few cells that still contained any visible fluorescent pigment.

Data treatment
The relative inhibition of settlement and growth was calculated in relation to the arithmetic mean of the DMSO-containing controls (n=10). Concentration-response functions where determined by applying "best-fit" approach (Scholze et al. 2001) were ten different regression models were applied to each pooled data set and based on statistical criteria the regression model that described the observed data best was selected. The reason for using this method was to describe the full concentration-response curve from low to high effect and the "best-fit" improved the accuracy of the fit. From the selected regression curves, EC_{10}s, EC_{50}s and EC_{98}s were derived and the corresponding 95% confidence intervals were estimated using the standard Wald-based approach implemented in SAS (proc nlin, SAS vers. 9.1. Calgary, US).
The final concentration-response models selected were:

Generalized Logit 2: $E(conc) = 1 - \frac{1}{(1 + \exp(\theta_1 + \theta_2 \times \log_{10}(conc))^{\theta_3}}$

Morgan-Mercier Flodin (MMF): $E(conc) = 1 - \frac{1}{1 - (\theta_1 \times conc)^{\theta_2}}$

with $\theta_1, \theta_2, \theta_3$ being the model parameters, *E(c)* the estimated effect in a range of 0 to 1 caused by a given concentration *conc*.

Results and Discussion

Even a single settled diatom cell of any species can potentially quickly colonize and cover large areas (Molino et al. 2009). For a paint to be ineffective in preventing microfouling it is hence sufficient that a single taxon that is present in the environment in which a boat is used, is tolerant to the applied antifouling biocide. It is hence insufficient to restrict testing to selected single species.

We therefore developed a rapid assay with a high test capacity that allows to quantify the efficacy of antifouling biocides to inhibit settlement and growth of natural marine biofilms. Periphyton communities sampled from the environment were used to prepare an inoculum that was allowed to re-settle on a pristine surface and then establish a new biofilm under controlled exposure. The assay hence integrates the response over three days for all the different taxa present in natural periphyton communities. The presented approach using a multispecies assay increases the chance that the most tolerant taxa are

present and hence the recorded efficacy is a better estimate of the actual field situation and the true efficacy of a biocide incorporated in a painted boat hull.

In temperate waters such as the Swedish west-coast where the present studies were performed, it should be possible to perform the assay during the whole year. However, colonisation and growth in the field are substantially lower during the winter season with lower light intensities and lower temperatures and hence a longer colonisation phase in the field would be needed, in order to accumulate a sufficient amount of biomass for the preparation of the inoculum.

Settlement as well as initial biofilm establishment and growth is included in the assay as those processes are critically important for the overall biofilm development and hence for the microfouling of submerged surfaces (Briand 2009). Therefore, after the initial settling over the first 24 hours the PETG pieces were transferred to new biocide solutions without inoculum where the assemblages were allowed to develop and grow for another 48 hours. Choosing a 72 hours incubation period means that one experiment (including preparations, implementation, and endpoint analysis) fits into a standard working week which has practical advantages.

Noteworthy, periphyton communities are considered non-target entities, providing valuable ecosystem services such as primary production and nutrient cycling when the biofilm is not living attached to man-made surfaces such as boats and ship hulls. Hence the new novel assay was also used for estimating low-effect concentrations such as the EC_{10} as additional information for a PNEC determination. In the following, we will discuss the assay as such, and the efficacy and ecotoxicity of the biocides to periphyton communities will be presented. Full concentration response curves are presented for eight biocides: copper (Cu^{2+}), irgarol, DCOIT, copper pyrithione, zinc pyritihone, tolylfluanid, TPBP (borocide) and medetomidine.

Biodiversity and structure of the biofilms after re-settling

PETG surfaces were used as a settling surface for the biofilms, for sampling the biofilms in the field and during the assay itself. The structure of assemblages developed on the PETG surfaces after re-settling (Fig. 1) shows the typical biodiversity structure of a algal biofilm community, similar to natural assemblages and periphyton established on glass surfaces used in previous studies (Porsbring et al. 2007). The biofilms comprise around five abundant and around fifty rarely occurring taxa.

To detach cells from environmental samples and prepare inoculum for the assay a “scrape, shake and sieve” method was used. The main disadvantage of scraping the surface with sharp tools to remove the biofilm is related to presence of mechanically damaged cells and underrepresentation of some taxa (Patil & Anil 2005). We hence examined the efficiency of our method for detaching cells from the surface under a microscope. As expected, specimens tightly attached to the surface such as diatoms from

the *Cocconeis* genus (Molino & Wetherbee 2008) were difficult to scrape off and get into the suspension and some cells remained on the surface.

The frequency analysis of algae in natural communities revealed a replacement of several dominant taxa after their re-establishment on the PETG surfaces (Fig. 1). The overall taxa composition of the re-established experimental communities had fewer dominant and rare taxa than the field samples. For example, the opportunistic diatom *Cylindrotheca closterium* and a pennate diatom (labelled "no 236") was clearly favoured by the test conditions, their frequency of occurrence increased from about 10 in the environmental samples to about 75 in the re-established communities. However, the attached diatom *Cocconeis* sp. was present also in the re-settled communities, although at lower abundances, which is expected for such immature biofilms. The number of taxa present under laboratory conditions was approximately the same as the number of taxa found in environmental samples, although analyses based on duplicate samples makes the results indicative only. Only one of the taxa frequent in the environmental samples was not recorded in the re-settled communities (labelled "unidentified algae 210").

Similar biodiversity changes have been described previously even for mechanically undisturbed intact biofilms, when they were grown under controlled laboratory conditions (Mitbavkar & Anil 2008;Porsbring et al. 2007). The reasons for such alterations might be changes in grazing pressure, slightly elevated nutrient levels, light, temperature, water movements and the fact that no new taxa is introduced during the incubation phase in the lab.

Inoculum, variability and reproducibility

The cell density in the inoculum varied somewhat as the growth on the surfaces deployed at sea was not constant over time. As the cell densities (biomass) might affect the bioavailability of the tested biocides, attention was therefore given to prepare inocula with similar biomasses for the experiments (0.3 µg chl *a* /ml), which was sufficient to record robust chlorophyll *a* data after the 72 hour incubation. The final biomasses in the controls after 72 hours were 0.7 µg chlorophyll *a*/plate in average. The absolute minimum was 0.3 µg chlorophyll *a*/plate, absolute maximum was 1.2 µg chlorophyll *a*/plate, with a mean coefficient of variance (CV) of 11%, as shown for all the 30 controls presented in Figure 2. This is comparable to what is usually observed in the 96 hours multispecies SWIFT assay that analyses the toxicant-induced succession in pre-settled biofilms (Porsbring et al. 2007). The CV of the settling assay is slightly lower than the CV of 30% that was observed previously in short-term periphyton tests that recorded the inhibition of photosynthesis (Arrhenius et al. 2004). In order to account for the variability in the assay, all results are expressed as relative growth or biomass compared to the controls.

Examples of the quality of the effect data recorded are shown in Figures 2 and 3 where the efficacy of copper pyrithione and irgarol to inhibit periphyton settlement and growth is presented. Note that different symbols represent data from different experiments and hence different natural communities that were used as inocula. Still there is a clear overlapping of concentration response curves, allowing pooling the data for the

concentration-response analysis. This high reproducibility makes the assay suitable to comparatively assess the efficacies of antifouling compounds to inhibit marine periphyton communities.

Chlorophyll a as growth indicator

The total biomass is of primary interest for estimating antifouling efficacy, not the individual taxon making up the biofilm. Extracted chlorophyll *a* was hence used as the endpoint of choice, as this closely reflects the total biomass and it also integrates settling and growth. The use of a Turner fluorometer makes the analysis quick and easily accessible and even small amounts of settled biomass (0.002 µg clorophyll *a* cm^{-2}) could be detected. However, other methods to analyze chlorophyll *a* on a surface could also be applied, such as e.g.PAM- fluorometry (Eggert et al. 2006), although this requires more sophisticated instruments and handling. Analysis of the whole pigment composition using HPLC (Porsbring et al. 2007) is more time-consuming and requires larger amounts of samples. Note that chlorophyll *a* only reflects the algal and cyanobacterial part of the micro fouling community and it should be pointed out that other endpoints could be applied to cover other organismal groups in the microfouling community, e.g. fluorescent dyes such as DAPI could be used for bacterial counts (Leroy et al. 2008).

Biocide efficacy and ecotoxicity

All tested biocides did inhibit settlement and growth of periphyton communities although at different concentrations as expected from their intended use and primary target organisms (Table 1). High-effect concentrations are of primary interest when evaluating efficacy i.e. the lowest concentration that inhibits fouling completely. As the calculated concentration-response curve is asymptotic, we chose the EC_{98} values as the effect level that indicates efficacy. For the ecotoxicity evaluation the situation is the opposite and relevant effect levels are EC_{10} values as statistically more robust surrogates for the commonly applied NOEC values. Additionally, the commonly used EC_{50} values are reported. An overview of all concentration-response curves is given in Figure 3, EC_{10}, EC_{50} and EC_{98} and their 95% confidence intervals are given in Table 2, together with the concentration-response models and their corresponding parameter estimates.

The efficacy ranking based on EC_{98} values from the most to the least efficacious compound is as follows: copper pyrithione > TPBP > DCOIT > tolylfluanid > zinc pyrithione > medetomidine > copper (Cu^{2+}). The algaecide irgarol did not cause a full inhibition of settlement and growth but the inhibition leveled out at 95% at an approximate concentration of 30 nmol l^{-1}, and did not increase further. The ecotoxicity ranking at the 10% and 50% effect level was identical with the follow order from most to least toxic biocide; irgarol, copper pyrithione > zinc pyrithione > TPBP > tolylfluanid > DCOIT > copper (Cu^{2+})> medetomidine.

Copper pyrithione exerts toxicity by disrupting the cell membrane (Dinning et al. 1998) and/or through apoptosis as a consequence of increased intracellular metal ion concentrations (Mann & Fraker 2005). Although copper pyrithione was the most efficient

biocide (EC_{98} = 50 nmol l^{-1}), irgarol was the biocide with the highest ecotoxicity with an EC_{10} of 0.07 nmol l^{-1}, compared to 1.9 nmol l^{-1} for copper pyrithione. This is due to the different shapes of the concentration-response curves. For a biocide that generates a steep curve, there will be a short concentration span between a low-effect concentration (e.g. EC_{10}) and a high-effect concentration (e.g. EC_{98}), as was seen for copper pyrithione. A biocide that has a flat concentration-response curve will on the other hand generate effect concentrations that are farther apart, which can be seen for irgarol. As a consequence, the toxicity ranking differs between the different effect levels.

The biocides zinc pyrithione, TPBP, tolylfluanid and DCOIT, have an intermediate efficacy as well as ecotoxicity, and their concentration-response curves all fall within roughly one order of magnitude (Fig. 3). The large disparity of the efficacy of copper pyrithione and zinc pyrithione is somewhat unexpected. As zinc pyrithione transchelates into copper pyrithione when copper is present (Dahllöf et al. 2005) the two pyrithiones were expected to have roughly similar EC_{98} values, since copper is naturally occurring in seawater (Hirose 2006). This was however not the case and zinc pyrithione was shown to be considerably less efficient than copper pyrithione with EC_{98}s of 50 and 29,400 nmol l^{-1} respectively. This is caused by the substantially flatter concentration-response curve of zinc pyrithione in the upper effect range. However also when comparing EC_{10}s and EC_{50}s copper pyrithione was clearly more ecotoxic.

DCOIT and tolylfluanid exert their effects via cytotoxic modes of action that potentially can affect many different organisms. DCOIT causes oxidative stress once it has diffused through the cell membrane (Arning et al. 2009) and tolylfluanid disrupts folate synthesis (Brain et al. 2008) and inhibits thiol-containing enzymes by forming disulfide bridges (Johansson et al. 2012). The mode of action of TPBP is unknown, but the biocide is known to affect microalgae growth at concentrations slightly lower than recorded in the present study with periphyton communities (see Table 3 for details).

The two biocides least efficient at preventing settlement and growth of periphyton communities were medetomidine and copper. Medetomidine binds to adrenergic receptors (Savola et al. 1986) and the current data hence confirm the absence of a specific mode of action of medetomidine (Wendt et al. 2013b; Ohlauson et al. 2012). Copper is known as an inefficient biocide for prevention of algal growth, which is why it is most often applied together with so called “booster biocides” in antifouling paints (Yebra et al. 2004). Several fouling algal species are known to be copper tolerant (Reed & Moffat 1983;Voulvoulis et al. 1999;Wendt et al. 2013b) and the high EC_{98} value (42 µmol l^{-1}) reported in the present study also indicates the presence of highly copper tolerant taxa in the periphyton communities investigated.

Five percent of the exposed algae managed to settle and grow even at irgarol concentrations as high as 1000 nmol l^{-1} (Fig. 3 and 4). Irgarol inhibits photosynthesis by blocking the electron transport in PSII (Hall et al. 1999). However, exposure to irgarol does not necessarily kill the algae, and they hence might still settle under irgarol exposure. Hence, the 5% of the control chlorophyll *a* level that is observed after 72 hours exposure to irgarol might stem from the algae that settled during the first day of

incubation. Additionally it is known that PSII inhibiting herbicides can induce the so-called greening effect, as a compensation mechanism in photosynthetic organisms exposed to sub-lethal concentrations of PSII inhibiting herbicides (Hatfield et al. 1989;Koenig 1990;Boura-Halfon et al. 1997).

Furthermore, irgarol is known to have caused pollution-induced community tolerance (PICT) after years of exposure to irgarol (Blanck et al. 2009). This was observed in the most irgarol-contaminated sites in the Gullmar fjord area. It is therefore possible that irgarol-tolerant species or genotypes, as shown by Eriksson and co-workers (2009) might be present also in the communities sampled for this study, and a fraction of the taxa might be tolerant enough to survive also at high irgarol exposures. This is further supported by settling studies performed in later years (2010) resulting in higher irgarol tolerance in the tested periphyton communities (Fig. 4). These findings of increased tolerance in the microbial communities coincide with an irgarol tolerance found in a population of the macroalga *Ulva lactuca* from the same geographical area (Wendt et al. 2013a). These findings, together with previous findings of irgarol tolerance (Blanck et al. 2009) are clear indicators that irgarol exerts or has exerted a selection pressure in the marine environment. This might limit the use of irgarol for preventing microfouling.

Comparisons with other studies that report effect data for biocides are mostly limited to a comparison of EC_{50}-values, as other effect levels are usually not reported. In Table 3, the available data from assays with benthic diatoms are summarized. For some biocides the single species assays are actually more sensitive (copper, DCOIT, and TPBP) and for others the presented periphyton assay is clearly more sensitive (pyrithiones and irgarol). However by using a broad range of algal taxa sampled from the field the multitude of indigenous species that are the actual foulers is included in the presented settling assay. Hence the data generated are more realistic, with a broad range of sensitivities/tolerances, and are therefore better suited for describing the efficacy of a certain biocide for inhibiting microfouling in the marine environment.

Conclusion and summary

The described assay uses field sampled periphyton communities that are tested in natural water for quantifying the efficacy and ecotoxicity of antifouling biocides. It is fairly simple to perform, relatively rapid, has a high test capacity and produces robust data. The high biodiversity that is characteristic for natural microbial biofilms is maintained in the assay. Compared to a standardized assay, it does not rely on extensive cultivation efforts, but instead the communities can be sampled directly from the field after three to four weeks of colonization. By using PETG as artificial substrata for colonisation, most relevant fouling species will be included. However, compared to the situation in the field, the assay relies on higher cell densities and nutrient levels, the exposure time is only 72 hours, and light and temperature are controlled.

All eight antifouling compounds inhibited the settlement and growth of slime communities. Their efficacies (EC_{98}s) vary as expected from their modes of actions, in the order copper pyrithione > TPBP > DCOIT > tolylfluanid > zinc pyrithione >

medetomidine > copper Cu^{2+}. Irgarol did not fully inhibit the settlement and growth but inhibited the biofilm development to 95% already at 30 nmol l^{-1}, i.e. at concentrations that are clearly lower than for any other of the tested biocides.

The endpoint used in the present study, relative chlorophyll *a* of the samples in relation to the untreated controls, reflects effects on the algal and cyanobacterial part of the microfouling community only. Other endpoints could be applied to also include other organismal groups in the microfouling community, e.g. fluorescent dyes such as DAPI could be used for bacterial counts (Leroy et al. 2008).

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Table and figure captions

Table 1. Selected antifouling biocides included in the study. For copper, the most bioavailable form is proposed to be its divalent ionic form Cu^{2+}. The commonly used copper compounds in antifouling paints are mentioned in the table as these are the compound actually put into the paint.

Table 2. Effect concentrations for inhibition of settlement and growth of marine periphyton communities exposed to antifouling biocides during 72 hours. Additionally concentration-response models and parameter estimates θ_1, θ_2, *and* θ_3 (see Material and Methods for equations). CI – approximate 95% confidence interval.

Table 3. Efficacy data for the tested biocides evaluated using single species assays with fouling diatoms *Cylindrotheca closterium, Amphora coffeaeformis* and *Skeletonema costatum*. No data for medetomidine were found.

Figure 1. Relative abundances of taxa in environmental samples colonized for three weeks and in experimental communities after three days (72 hours). Taxa are sorted according to decreasing abundances in the experimental communities. The sums of abundances in duplicate samples are given.

Figure 2. Efficacy of copper pyrithione to inhibit settlement and growth of periphyton communities. The different symbols indicate three independent experiments (circles from 17/07/07, triangles from 18/09/07, and squares from 30/08/10). Open symbols represent controls (n=10 per experiment, black are the true controls with DMSO added (0.1%) and grey are the filtered seawater controls (FSW)) and filled symbols represent treatments with copper pyrithione (n=3).

Figure 3. Concentration–response curves for eight antifouling biocides. Substances are labeled in order of increasing EC_{50} values. The horizontal lines indicate 10, 50, and 95% inhibition respectively.

Figure 4. Efficacy of irgarol to inhibit settlement and growth of periphyton communities. The black symbols indicate three independent experiments (circles - 27/08/07, triangles - 04/09/07, and squares - 09/06/08). The grey diamonds are from 30/08/10; note these data are not included in the fitted curve. The shift of the curve to the right is indicative of increased tolerance. Open symbols represent controls (n=10) and filled symbols represent treatments (n=3).

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Table 1. Selected antifouling biocides included in the study. For copper, the most bioavailable form is proposed to be its divalent ionic form Cu^{2+}. The commonly used copper compounds in antifouling paints are mentioned in the table as these are the compound actually put into the paint.

Antifouling compound	IUPAC name*	Cas number	Molecular weight (g/mol)	Main target(s) according to manufacturer	Manufacturer (Europe)
Cu^{2+} Cu_2O, CuSCN, Cu-powder	copper(2+)	7440-50-8	170.5	Soft and hard foulers	Merck (among others)
Copper pyrithione, Copper OMADINE™, CuPT	2(1H)-Pyridinethione, 1-hydroxy-, copper(2+) salt	14915-37-8	315.9	Broad spectrum antimicrobial	Arch Chemicals
Zinc pyrithione, Zinc OMADINE™, ZnPT	2(1H)-Pyridinethione, 1-hydroxy-, zinc(2+) salt	13463-41-7	317.7	Broad spectrum antimicrobial	Arch Chemicals
DCOIT, Kathon™ 287T N Biocide, Sea-Nine 211 N	4,5-dichloro-2-octyl-1,2-thiazol-3-one	64359-81-5	282.2	Broad spectrum (bacterial slime, algae, barnacles, seaweed, and other marine organisms)	Dow Chemicals - Rohm&Haas
Irgarol 1051, Cybutryne	2-N-tert-butyl-4-N-cyclopropyl-6-methylsulfanyl-1,3,5-triazine-2,4-diamine	28159-98-0	253.4	Algae	Ciba Specialty Chemicals GmbH
Medetomidine, Catemine, Selektope	5-[1-(2,3-dimethylphenyl)ethyl]-1H-imidazole hydrochloride	86347-15-1	236.7	Hard foulers	I-Tech AB
Tolylfluanid, Preventol A5S, Euparen	4,5-dichloro-2-octyl-1,2-thiazol-3-one	731-27-1	347.2	A broad range of fouling organisms	Lanxess
TPBP (triphenylboron-pyridine), Borocide®P, KH 101	pyridine; triphenylborane	971-66-4	321.2	Soft and hard foulers, Broad-spectrum antimicrobial	Arch Chemicals

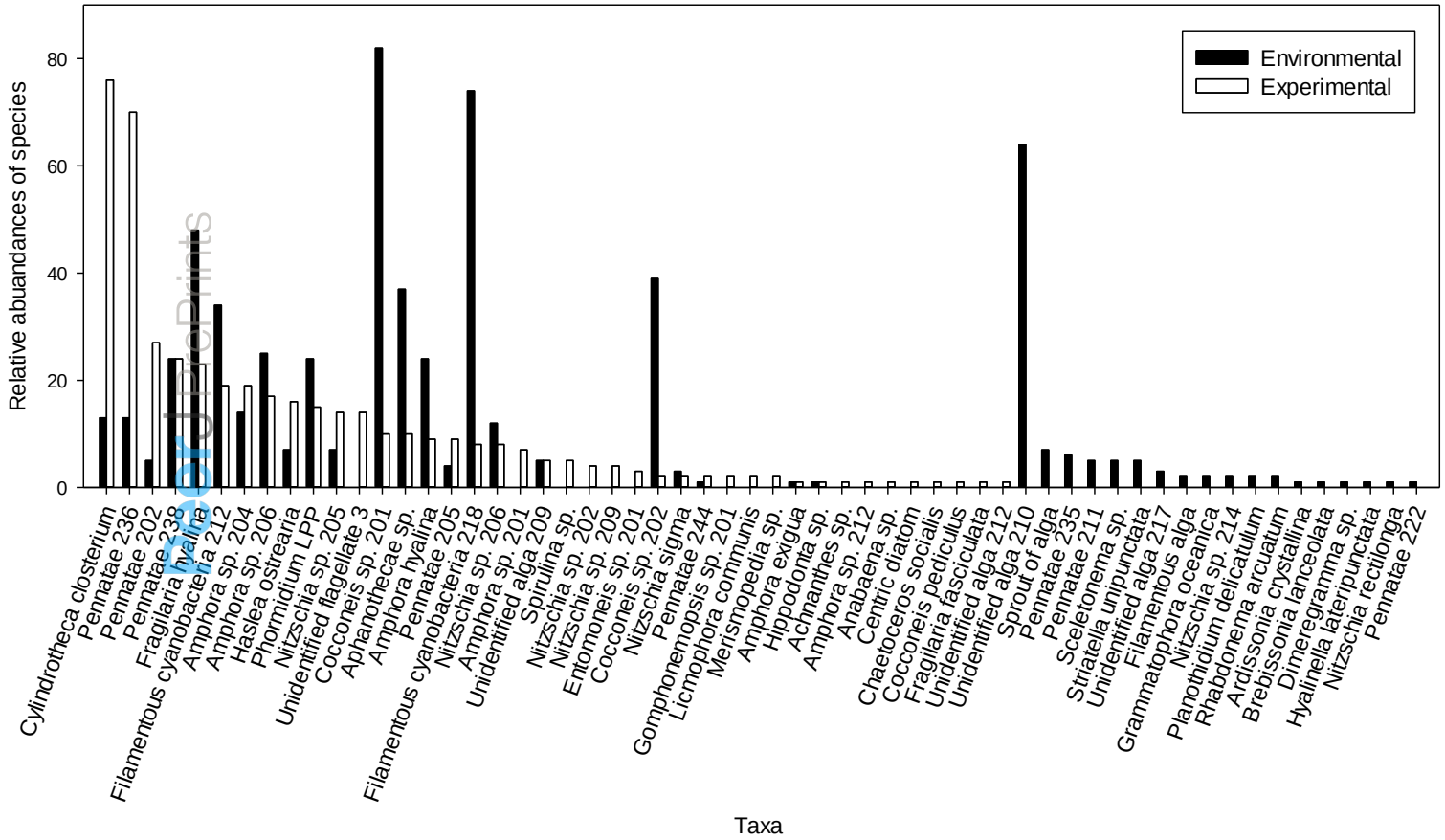
Table 2. Effect concentrations for inhibition of settlement and growth of marine periphyton communities exposed to antifouling biocides during 72 hours. Additionally concentration-response models and parameter estimates θ_1, θ_2, *and* θ_3 (see Material and Methods for equations). CI – approximate 95% confidence interval.

Substance	EC_{10} [95% CI] (nmol l^{-1})	EC_{50} [95% CI] (nmol l^{-1})	EC_{98} [95% CI] (nmol l-1)	Model	θ_1	θ_2	θ_3	θ_{max}	θ_{min}	No. of pooled datasets
Cu	550 [480-630]	1,100 [920-1,300]	41,500 [13,800-93,300]	Generalized Logit II	-537.369	199.912	0.010	1	0	2
Copper pyrithione	1.9 [1.3-2.7]	6.4 [5.3-7.5]	50 [20-130]	Generalized Logit II	-3.469	4.205	1.062	1	0	3
Zinc pyrithione	3.8 [2.0-6.2]	28 [19-40]	29,400 [2,300-8,700]	Generalized Logit II	-2.899	3.434	0.314	1	0	2
DCOIT	13 [5.9-30]	93 [62-140]	3,000 [630-8,300]	Morgan-Mercier Flodin	5.067	2.573		1	0	2
Irgarol	0.07 [0.03-0.1]	0.96 [0.7-1.4]	Not reached	Generalized Logit II	-0.056	1.922	1.137	0.957	0	3
Medetomidine	1,900 [1,000-2,900]	4,400 [3,400-5,700]	35,000 [11,000-98,000]	Generalized Logit II	-23.605	6.716	0.564	1	0	2
Tolylfluanid	5.4 [2.7-11]	65 [44-100]	5,200 [930-15,000]	Morgan-Mercier Flodin	3.694	2.041		1	0	3
TPBP	4.4 [2.2-8.0]	35 [27-44]	440 [130-860]	Generalized Logit II	-4.591	2.219	2.567	1	0	2

Table 3. Efficacy data for the tested biocides evaluated using single species assays with fouling diatoms *Cylindrotheca closterium*, *Amphora coffeaeformis* and *Skeletonema costatum*. No data for medetomidine were found.

Antifouling Biocide	**Test species**	**Endpoint**	**Tox value (nmol l^{-1})**	**Present study 72h-EC_{50} (nmol l^{-1})**
Cu	*C. closterium*	Population growth, 72h-EC_{50}	157 [1]	1070
Copper pyrithione	*A. coffeaeformis*	Population growth, 96h-LC_{50}	158 [2]	6.4
Zinc pyrithione	*A. coffeaeformis*	Population growth, 96h-LC_{50}	94 [2]	28
DCOIT	*A. coffeaeformis*	Viability, 96h-LC_{50}	12 [3]	93
Irgarol	*S. costatum*	Population growth, 120h-EC_{50}	1.8 [4]	1.0
Tolylfluanid	*C. closterium*	Population growth, 72h-EC_{50}	2016 [5]	65
TPBP	*S. costatum*	Population growth, 72h-LC_{50}	6.9 [6]	35

Refs; [1](Araujo et al. 2010), [2](Turley et al. 2005), [3](Willingham & Jacobson 1993), [4](Hall et al. 2009), [5](Fay et al. 2010), [6](Okamura et al. 2009).



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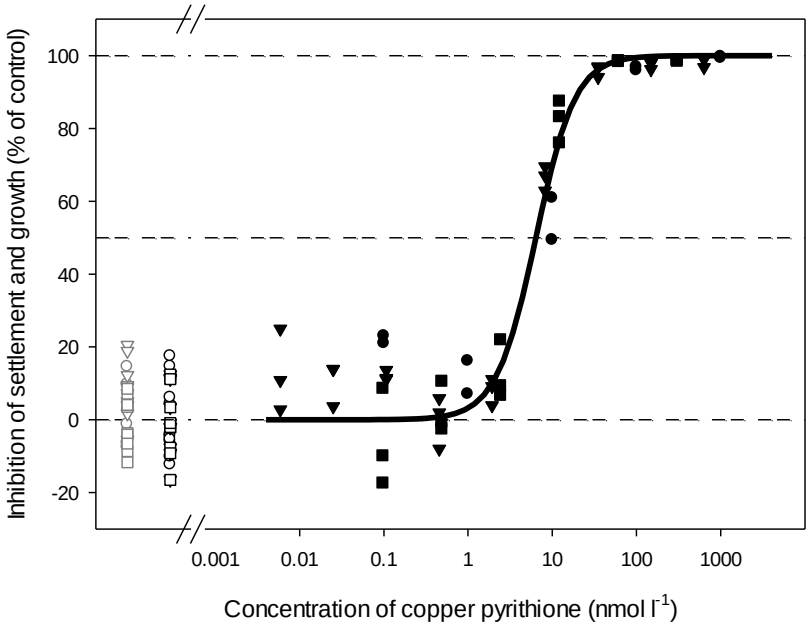

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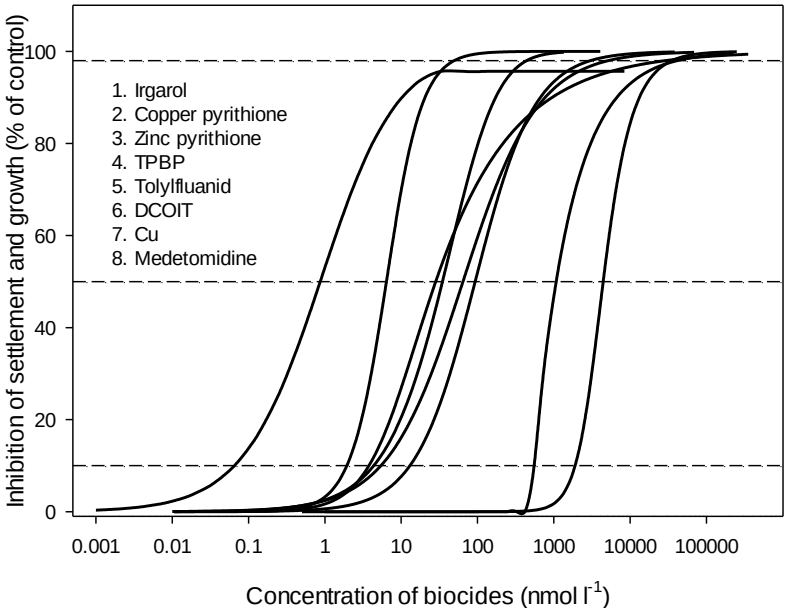

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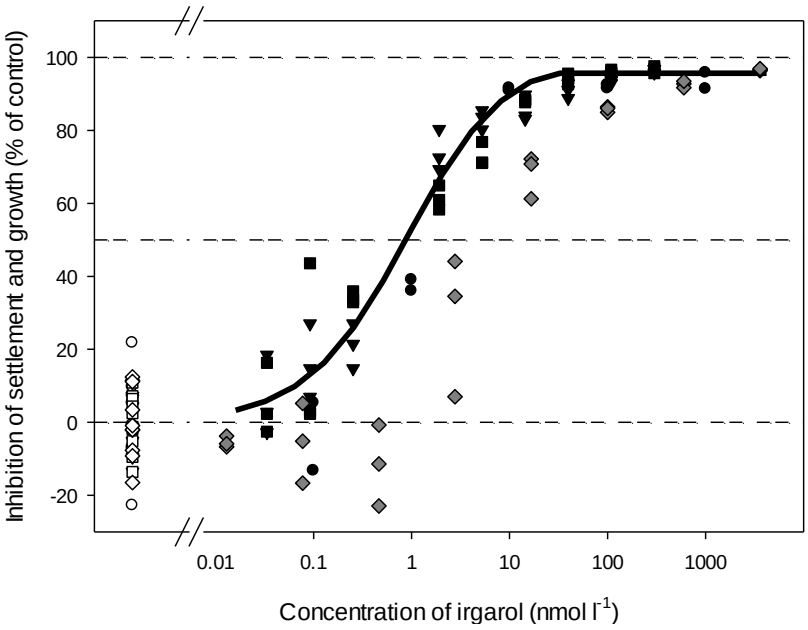

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