A novel rapid assay for evaluating the efficacy of biocides to inhibit the development of marine photoautotrophic biofilms

Abstract

In this paper a novel settlement and growth assay is presented that uses field-collected marine photoautotrophic biofilms as inoculum. The multitude of indigenous species that are the potential foulers are therefore included in the assay, which overcomes the limitation of testing only those species that can be cultivated in the laboratory. The assay was evaluated using eight antifouling biocides. The methodological considerations are discussed thoroughly and full concentration response curves are presented for all tested biocides. 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% already at 30 nmol l^{-1}, at a concentration that is clearly lower than for any other of the tested biocides.

Keywords
Photoautotrophic biofilm
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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. Increased resistance leads to increased fuel consumption and associated emissions of exhaust fumes, increasing costs and problems keeping to operational schedules (Almeida et al. 2007). In addition, fouling also aids in the spreading of invasive species (Piola et al. 2009).

The magnitude of the increase in frictional drag depends on the fouling type and coverage, estimations indicate that heavy calcareous fouling could result in up to 80% increase in resistance (Schultz 2007). However already a light slime-layer through which the paint colour is still visible could cause increases in resistance by around 11%, and a heavy slime-layer where the paint colour is difficult or impossible to determine can result in up to 20% increase in resistance (Schultz 2007). Microbial biofilms ("slime") are the first important colonization stage on a clean surface in water that builds up the fouling community (Almeida et al. 2007). Such biofilms include bacteria, cyanobacteria, microalgae (especially diatoms), fungi and protozoa and are sometimes referred to as "microfouling" (Briand 2009).

The review by Briand (2009) summarizes the laboratory bioassays reported in the scientific literature over the last 30 years and noted a lack of assays investigating the entire microfouling community and although the number of settling assays and publications in this research area have increased dramatically there is still a lack of more realistic settling assays for microfoulers. Briand (2009) further reports that cultivable microorganisms represent only <1-5% of the *in situ* diversity found in the natural environment. To reflect the high diversity found in microbial fouling communities multispecies assays are needed to eg estimate the efficacy and ecotoxicity of natural and man-made compounds that are evaluated as potential antifouling agents.

Molino and Wetherbee (2008) point out that many antifouling coatings today fail to inhibit settling and growth of microalgal biofilms or periphyton dominated by diatoms. A more realistic assay for evaluating the efficacy of biocides and paints will allow a better understanding and prediction of biocide and paint performance under realistic conditions, which will aid the development of new biocides and paints. In this paper a novel settlement and growth assay that uses field-collected marine photoautotrophic periphyton as inoculum is described. Hence the multitude of indigenous species that are the potential foulers are included in the assay, which overcomes the limitation of testing only those species that can be cultivated in the laboratory. The assay were 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 part of copper in coatings is its ionic form Cu^{2+} and therefore $CuCl_2$ was used as the test substance. Irgarol is a specifically acting 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 eg 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 also 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®.

With the exception of TPBP 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 at Sven Lovén Center for Marine Sciences, Kristineberg (58°15'N, 11°27'E) by the Gullmar fjord on the Swedish west coast during May-September in 2007, 2008, and 2010. All biocides were in qualities >95% and kindly provided by the companies (Table 1) except for copper were copper(II)chloride (Merck) were used. Note that the companies had no influence on the actual work or on the interpretation of the results.

Test solutions

The test medium was made from filtered (GF/F, Whatman) natural deep-seawater (from a depth of 30 metres) with the addition of nutrients to avoid nutrient limitations. The final concentrations (0.7 µM PO_4^{2-} and 8 µM NO_3^-) reflect nutrient conditions during a spring bloom (Porsbring et al. 2007).

Co-solvents were used to ease the distribution of the biocides; dimethyl sulfoxide (DMSO) for irgarol, tolylfluanid, TPBP, copper- and zinc pyrithione and DCOIT and deionised water for Cu^{2+} and medetomidine. Yet, in all treatments (controls and treatments) DMSO were present at a concentration of 100 ul/l (0.1%). DMSO has previously been shown to have a low toxicity to microalgae (Okumura et al. 2001) which were confirmed as DMSO did not affect settling and growth of the periphyton at the concentration used (data not shown). 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.

No chemical analysis of biocides in the test solutions was made and hence nominal concentrations are reported.

Sampling and preparation of test material

Periphyton (the biofilms) were sampled from the bay of Kalvhagefjorden (Lat.N58º14´ Long.11º24´) using artificial substrata made of polyetylene therophtalate, glycol-modified (PETG) mounted on polyethylene holders(Blanck and Wängberg 1988) at approximately 1.5 meters depth (Blanck & Wängberg 1988). PETG roughly resembles the surface of many self-polishing release systems and is easy to handle and make into many replicate sampling surfaces (75*14 mm) with high precision.

The colonised discs were scraped with a razor into filtered (Whatman GF/F) natural deep-seawater (from a depth of 30 metres) and holders and discs were shaken rigorously in a Pyrex

bottle for three minutes to detach cells from the periphyton. The solution was 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 were 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 dish (WVR Collection, VWR International, Stockholm, Sweden) were used as incubation vessels. Aliquots of five ml of test solution and five ml of algal suspension were distributed in the vessels. In each vessel a piece of PETG were inserted to serve as settling surface. Specially designed rectangular discs of PETG were prepared with an incision 14 mm from one end. The end with the incision was placed in the water and the other end tilted to edge of the vessel (angle 45º). After incubation the settled part (1.96 cm^2) could easily be removed for further analysis of growth and species composition. In addition this aided the daily change of test solutions as these discs easily could be transferred to new vessels with fresh solutions. Before use the discs were gently cleaned in warm water with a mild soap and thereafter rinsed in deionised water over night. Before being inserted into the incubation vessel the discs were immersed in 70% ethanol for disinfection and rinsed with deionized water and placed on Kleenex paper tissues.

Incubation conditions were selected to mimic the average situation at the Swedish west coast during the summer season which is the major settling season in Scandinavian waters. The vessels were illuminated from above by fluorescent tubes (Osram Lumilux Daylight L 18W/12, Osram, München, Germany) to give an average photon flux density of about 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 (16:8). 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.

Settling was allowed for 24 hours after which the discs were transferred to new vessels with fresh test solutions but without biota. The growth continued for another 48 hours with daily transfer to new vessels with fresh test solutions.

Frequency analysis of different taxonomic groups of algae

To describe the periphyton communities and how the test method itself affects species composition and structure, a frequency analysis was made. In natural habitats frequency typically correlates with abundance and the same was showed for periphyton communities studied previously. Two sets of samples were prepared to compare the communities colonised for three weeks in the environment and the communities that had re-established during the 72 hours test period. Two samples from each set were preserved in 70% ethanol and stored at 4°C in darkness until species identification. Different algal taxonomic groups were identified to the species level when possible. Specimens of unknown taxonomical affinity were assigned to groups of higher rank and given numbers which corresponded with a certain set of characters (eg, shape, size, habit of growth) in our reference files (Dr. Aleksandra Zgrundo, Gdansk University, Poland). In the following, all species and taxonomic groups will be referred to as taxa only. 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 where the taxon was observed, thus giving a relative scale of frequency of taxa from 0 to 50 for each analysed sample. Presented in the paper is the sum of duplicates (scale 0-100).

Chlorophyll a as growth indicator

To estimate algal and cyanobacterial biomass, chlorophyll *a* was extracted from the individual colonised discs 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 few cells that still contained any visible fluorescent pigment.

Data treatment

The relative inhibition of settling and growth were 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" improves 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

A rapid assay with high test capacity to estimate the efficacy of antifouling biocides to inhibit settlement and growth of marine biofilms has been developed. In the assay, multispecies periphyton communities sampled from the environment were used to prepare an inoculum that was allowed to re-settle on new surfaces under controlled exposure. The assay hence integrates the response over three days for all the different taxa present in natural periphyton communities. This is a clear advantage of such a multi-species system in comparison to studies with individual species, since even a single settled diatom cell of any species can potentially quickly colonise and cover large areas (Molino et al. 2009). Thus it is enough that only one taxon is tolerant to the used biocide, for a paint to be ineffective in preventing microfouling. The present approach using a multispecies assay increases the chance that the most tolerant taxa are present and hence the estimated efficacy is closer to the actual field situation and the true efficacy on a painted boat hull.

Noteworthy, when not living attached to man-made surfaces such as boat and ship hulls the periphyton communities are to be considered as non-target species. Hence from this novel assay for estimating efficacy in addition also information about the hazard concentrations (eg EC_{10} or EC_{50}) of the same compound are available. The method as such will be discussed 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.

Settling surfaces

For this settlement and growth assay PETG surfaces were used for sampling biofilms in the field as many self-polishing release systems are acrylate-based and PETG roughly resembles a painted surface. Direct comparisons of periphyton assemblages from different sites, seasons or treatments on the species level is problematic as their species composition changes depending on season, biofilm age etc. However, the structure of assemblages developed on the PETG surfaces (Fig. 1) shows patterns that are typical of natural assemblages and periphyton established on the glass surfaces that were used in previous studies, ie the biofilms comprise a few abundant species and many rare ones (Porsbring et al. 2007).

Detachment method and taxa composition

To detach cells from environmental samples and prepare inoculum for the assay a "scrape shake and sieve" method was developed. The main disadvantage of scraping the surface with sharp tools to remove the biofilm is related to presence of mechanically damaged cells or underrepresentation of some taxa (Patil & Anil 2005). However, in order to achieve a rapid test this method was chosen, together with rigorous shaking. Initially, the efficiency of our method for detaching cells from the surface was examined using 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. The frequency analysis of algae in natural communities revealed a replacement of 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 rarer taxa than the field samples. For example, the frequency of *Cocconeis* sp. decreased, while the opportunistic diatom *Cylindrotheca closterium* and a pennate diatom (labelled "no 236") were clearly favoured by the test conditions as their frequency increased from about 10 in the environmental samples to about 75 in the re-established communities.

Similar changes in dominant taxa have been described previously when mechanically undisturbed intact biofilms were grown under controlled laboratory conditions (Mitbavkar & Anil 2008;Porsbring et al. 2007). The reasons for these alterations might be changes in grazing pressure, nutrient levels, light, temperature, water movements and the fact that no new taxa is introduced during the incubation phase in the lab. However, it should be emphasized that 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"). Even the prostrate tightly attached taxon *Cocconeis* sp. was present in the re-settled communities, although at lower abundances which is expected for such immature biofilms.

However, it should be emphasized that 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 abundant in the environmental samples was missing from the re-settled communities (labelled "unidentified algae 210"). Even the tightly attached taxon *Cocconeis sp* was present in the re-settled communities, although at lower abundances which is expected for such immature biofilms.

Inoculum cell density and assay duration

A cell density in the inoculum that resulted in sufficiently high biomasses to record robust chlorophyll *a* data after a short period of time was used. Such high cell densities (biomass) might affect the bioavailability of the tested biocides. Particular attention was therefor given to prepare inocula with similar biomasses between replicates and repeated experiments.

Settlement as well as growth are included in the assay as both processes are important from a fouling perspective (Briand 2009). Therefore PETG surfaces were allowed to be colonised for the first 24 hours with the inoculum present, and then transferred to new biocide solutions without inoculum where the assemblages were allowed to develop and grow for another 48 hours. This timing has some important practical advantages: the biomasses after 72 hours are high enough to record chlorophyll *a* data with high precision, even small amounts of settled biomass (0.002 µg clorophyll *a* cm^{-2}) could be determined with high precision. Additionally, using a 72 hours incubation period means that one experiment (including preparations, implementation, and endpoint analysis) fits into a standard working week.

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 the endpoint of choice, as this closely reflects the total biomass and integrates settling and growth. The use of a Turner fluorometer makes the analysis quick and easily assessable. However, other methods to analyze chlorophyll *a* on a surface could also be applied, such as eg 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, ie fluorescent dyes such as DAPI could be used for bacterial counts (Leroy et al. 2008).

Variance and reproducibility

The mean coefficient of variance (CV) of the untreated controls is 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 (Porsbring et al. 2007) and in settling assays with *Ulva lactuca* (Wendt et al. 2013b). Furthermore the variance is also lower than the CV of 30% observed previously in tests on short-term inhibition of photosynthesis in marine periphyton (Arrhenius et al. 2004).

The quality of the effect data recorded with the assay is also shown in Figure 2 and 3 where the efficacy of copper pyrithione and irgarol to inhibit periphyton settlement and growth are 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 perfectly suitable to comparatively assess the efficacies of antifouling compounds to inhibit marine periphyton communities.

Ecotoxicity and efficacy

All tested biocides did inhibit settlement and growth of periphyton communities although at different concentrations as could be expected from their intended use (Table 1). 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 approximately concentration of 30 nmol l^{-1}, and did not increase further.

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 (eg EC_{10}) and a high-effect concentration (eg 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 ecotoxicity ranking at the 10% and 50% effect level were identical from most to least toxic; irgarol, copper pyrithione > zinc pyrithione > TPBP > tolylfluanid > DCOIT > copper (Cu^{2+})> medetomidine.

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 antifouling 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 a tolerable exposure might allow them to settle. 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 have exerted a selection pressure in the marine environment. This, of course, has strong implications for the efficacy of irgarol in 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 test. 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.

Concluding remarks

The described assay uses field sampled periphyton communities that are tested in natural water for estimating 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, there is no immediate need for extensive cultivation efforts, but instead the communities can be sampled directly from the field. 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 duration is only 72 hours, and light and temperature is 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}, ie at concentrations that are clearly lower than for any other of the tested biocides.

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References

Arch Chemicals (2013). Omadine^{tm}.
http://www.archchemicals.com/Fed/BIO/Products/Brand/Omadine.htm . 8-7-2013.

Almeida,E., Diamantino,T.C. & de Sousa,O. (2007) Marine paints: The particular case of antifouling paints. Prog Org Coat. 59, 2-20.

Araujo,C.V.M., Diz,F.R., Lubian,L.M., Blasco,J. & Moreno-Garrido,I. (2010) Sensitivity of Cylindrotheca closterium to copper: Influence of three test endpoints and two test methods. Sci Tot Environ. 408:3696-3703.

Arning,J., Matzke,M., Stolte,S., Nehen,F., Bottin-Weber,U., Boschen,A., Abdulkarim,S., Jastorff,B. & Ranke,J. (2009) Analyzing Cytotoxic Effects of Selected Isothiazol-3-one Biocides Using the Toxic Ratio Concept and Structure-Activity Relationship Considerations. Chem Res Toxicol. 22:1954-1961.

Arrhenius,Å., Grönvall,F., Scholze,M., Backhaus,T. & Blanck,H. (2004) Predictability of the mixture toxicity of 12 similarly acting congeneric inhibitors of photosystem II in marine periphyton and epipsammon communities. Aquat Toxicol. 68:351-367.

491 Blanck,H., Eriksson,K.M., Gronvall,F., Dahl,B., Guijarro,K.M., Birgersson,G. & Kylin,H. (2009) A
492 retrospective analysis of contamination and periphyton PICT patterns for the antifoulant irgarol 1051,
493 around a small marina on the Swedish west coast. Mar Pollut Bull. 58:230-237.

494 Blanck,H. & Wängberg,S.Å. (1988) Validity of an ecotoxicological test system: short-term and long-
495 term effects of arsenate on marine periphyton communities in laboratory systems. Can J Fish Aquat
496 Sci. 10:1807-1815.

497 Boura-Halfon,S., Rise,M., Arad,S. & Sivan,A. (1997) Characterization of mutants of the red microalga
498 *Porphyridium aerugineum* (Rhodophyceae) resistant to DCMU and atrazine. Phycologia. 36:479-487.

499 Brain,R.A., Ramirez,A.J., Fulton,B.A., Chambliss,C.K. & Brooks,B.W. (2008) Herbicidal Effects of
500 Sulfamethoxazole in *Lemna gibba*: Using p-Aminobenzoic Acid As a Biomarker of Effect. Environ
501 Sci Technol. 42:8965-8970.

502 Briand,J.F. (2009) Marine antifouling laboratory bioassays: an overview of their diversity. Biofouling.
503 25:297-311.
504
505 Dahl,B. & Blanck,H. (1996) Pollution-induced community tolerance (PICT) in periphyton
506 communities established under tri-n-butyltin (TBT) stress in marine microcosms. Aquat Toxicol.
507 34:305-325.

508 Dahllöf, I., Grunnet, K., Haller, R., Hjorth, M, Maraldo, K., and Petersen, D. G. (2005) Analysis, fate
509 and toxicity of zinc- and copper pyrithione in the marine environment. 550. Copenhagen, Denmark.
510 TemaNord.
511
512 Dahlstrom,M., Martensson,L.G.E., Jonsson,P.R., Arnebrant,T. & Elwing,H. (2000) Surface active
513 adrenoceptor compounds prevent the settlement of cyprid larvae of *Balanus improvisus*. Biofouling.
514 16:191-203.
515
516 Dinning,A.J., Al-Adham,I.S.I., Eastwood,I.M., Austin,P. & Collier,P.J. (1998) Pyrithione biocides as
517 inhibitors of bacterial ATP synthesis. J Appl Microbiol. 85:141-146.

518 Eggert,A., Haubner,N., Klausch,S., Karsten,U. & Schumann,R. (2006) Quantification of algal biofilms
519 colonising building materials: chlorophyll a measured by PAM-fluorometry as a biomass parameter.
520 Biofouling. 22:79-90.

521 Eriksson,K.M., Clarke,A.K., Franzen,L.G., Kuylenstierna,M., Martinez,K. & Blanck,H. (2009)
522 Community-Level Analysis of psbA Gene Sequences and Irgarol Tolerance in Marine Periphyton.
523 Appl Environ Microbiol. 75:897-906.

524 European Parliament (1998) Directive 98/8/EC of the european parliament and of the council of 16
525 February 1998 concerning the placing of biocidal products on the market. In Council EPa (Ed.),
526 98/8/EC. Official Journal of the European Communities. 24.4.98.

527 Fay,F., Linossier,I., Carteau,D., Dheilly,A., Silkina,A. & Vallee-Rehel,K. (2010) Booster biocides and
528 microfouling. Biofouling. 26:787-798.

529 Hall,J., Giddings,J.M., Solomon,K.R. & Balcomb,R.R. (1999) An Ecological Risk Assessment for the
530 Use of Irgarol 1051 as an Algaecide for Antifoulant Paints. Crit Rev Toxicol. 29:367-437.

531 Hall,L.W., Killen,W.D., Anderson,R.D., Balcomb,R. & Gardinali,P. (2009) Ecological risk of Irgarol
532 1051 and its major metabolite in coastal California marinas and reference arease. MarPollut Bull.
533 58:702-710.

534 Hatfield,P.M., Guikema,J.A., Stjohn,J.B. & Gendel,S.M. (1989) Characterization of the Adaptation
535 Response of *Anacystis nidulans* to Growth in the Presence of Sublethal Doses of Herbicide. Curr.
536 Microbiol. 18:369-374.

537 Hirose,K. (2006) Chemical speciation of trace metals in seawater: a review. Analyt Sci. 22:1055-1063.

538 Jespersen,A.M. & Christoffersen,K. (1987) Measurements of Chlorophyll-A from Phytoplankton
539 Using Ethanol As Extraction Solvent. Archiv fur Hydrobiologie. 109:445-454.

540 Johansson,P., Eriksson,K.M., Axelsson,L. & Blanck,H. (2012) Effects of Seven Antifouling
541 Compounds on Photosynthesis and Inorganic Carbon Use in Sugar Kelp *Saccharina latissima*
542 (Linnaeus). Arch Environ Contam Toxicol. 63:365-377.

543 Koenig,F. (1990) Shade Adaptation in Cyanobacteria - Further Characterization of *Anacystis* Shade
544 Phenotype As Induced by Sublethal Concentrations of Dcmu-Type Inhibitors in Strong Light.
545 Photosynth Res. 26:29-37.

546 Leroy,C., barre-Ladrat,C., Ghillebaert,F., Compere,C. & Combes,D. (2008) Effects of commercial
547 enzymes on the adhesion of a marine biofilm-forming bacterium. Biofouling. 24:11-22.

548 Macdonald,E., Scheinin,H. & Scheinin,M. (1988) Behavioral and Neurochemical Effects of
549 Medetomidine, A Novel Veterinary Sedative. European J Pharmacol. 158:119-127.

550 Mann,J.J. & Fraker,P.J. (2005) Zinc pyrithione induces apoptosis and increases expression of Bim.
551 Apoptosis. 10:369-379.

552 Mitbavkar,S. & Anil,A.C. (2008) Seasonal variations in the fouling diatom community structure from
553 a monsoon influenced tropical estuary. Biofouling. 24:415-426.

554 Mochida,K., Onduka,T., Amano,H., Ito,M., Ito,K., Tanaka,H. & Fujii,K. (2012) Use of species
555 sensitivity distributions to predict no-effect concentrations of an antifouling biocide, pyridine
556 triphenylborane, for marine organisms. Mar Pollut Bull. 64:2807-2814.

557 Molino,P.J., Campbell,E. & Wetherbee,R. (2009) Development of the initial diatom microfouling
558 layer on antifouling and fouling-release surfaces in temperate and tropical Australia. Biofouling.
559 25:685-694.

560 Molino,P.J. & Wetherbee,R. (2008) The biology of biofouling diatoms and their role in the
561 development of microbial slimes. Biofouling. 24:365-379.
562
563 Ohlauson,C., Eriksson,K.M. & Blanck,H. (2012) Short-term effects of medetomidine on
564 photosynthesis and protein synthesis in periphyton, epipsammon and plankton communities in relation
565 to predicted environmental concentrations. Biofouling. 28:491-499.

566 Okamura,H., Kitano,S., Toyota,S., Harino,H. & Thomas,K.V. (2009) Ecotoxicity of the degradation
567 products of triphenylborane pyridine (TPBP) antifouling agent. Chemosphere. 74:1275-1278.

568 Okumura,Y., Koyama,J., Takaku,H. & Satoh,H. (2001) Influence of organic solvents on the growth of
569 marine microalgae. Arch Environ Contam Toxicol. 41:123-128.

570 Patil,J.S. & Anil,A.C. (2005) Quantification of diatoms in biofilms: Standardisation of methods.
571 Biofouling. 21:181-188.

572 Piola,R.F., Dafforn,K.A. & Johnston,E.L. (2009) The influence of antifouling practices on marine
573 invasions. Biofouling. 25:633-644.

Porsbring,T., Arrhenius,A., Backhaus,T., Kuylenstierna,M., Scholze,M. & Blanck,H. (2007) The SWIFT periphyton test for high-capacity assessments of toxicant effects on microalgal community development. J Exp Mar Biol Ecol. 349:299-312.

Reed,R.H. & Moffat,L. (1983) Copper Toxicity and Copper Tolerance in Enteromorpha-Compressa (L) Grev. J Exp Mar Biol Ecol. 69:85-103.

Savola,J.M., Ruskoaho,H., Puurunen,J., Salonen,J.S. & Karki,N.T. (1986) Evidence for Medetomidine As A Selective and Potent Agonist at Alpha-2-Adrenoceptors. J Auton Pharmacol., 6:275-284.

Scholze,M., Boedeker,W., Faust,M., Backhaus,T., Altenburger,R. & Grimme,L.H. (2001) A general best-fit method for concentration-response curves and the estimation of low-effect concentrations. Environ. Toxicol. Chem. 20:448-457.

Schultz,M.P. (2007) Effects of coating roughness and biofouling on ship resistance and powering. Biofouling. 23:331-341
.
Thomas,KV. & Langford,K. (2009) The analysis of antifouling paint biocides in water, sediment and biota. Ecotoxicology of antifouling biocides (eds Arai, Harino, M. Ohji & W. Langston), Springer, Tokyo.

Turley,P.A., Fenn,R.J., Ritter,J.C. & Callow,M.E. (2005) Pyrithiones as antifoulants: Environmental fate and loss of toxicity. Biofouling. 21:31-40.

Voulvoulis,N., Scrimshaw,M.D. & Lester,J.N. (1999) Alternative antifouling biocides Appl Organometal Chem. 13:135-143.

Wendt,I., Arrhenius,Å., Backhaus,T., Hilvarsson,A., Holm,K., Langford,K., Tunovic,T. & Blanck,H. (2013a) Extreme irgarol tolerance in an *Ulva lactuca* L. population on the Swedish west coast. Mar Pollut Bull. 76(1-2):360-364.

Wendt, I., Arrhenius, Å., Backhaus, T., Hilvarsson, A., Holm, K, Langford, KH, Tunovic, T, and Blanck, H. (2013b) Effects of Five Antifouling Biocides on Settlement and Growth of Zoospores from the Marine Macroalga *Ulva lactuca* L. Bull Environ Contam Toxicol. 91(4):426-432.

Willingham, G. L. and Jacobson, A. H. Efficacy and environmental fate of a new isothizolone antifoulant. p. 14.1-14.13. 1993. Paint Research Association International Centre for Coatings Technology. the third Asia-Pacific conference of the paint research association. International Centre for Coatings Technology.

Yebra,D.M., Kiil,S. & Dam-Johansen,K. (2004) Antifouling technology - past, present and future steps towards efficient and environmentally friendly antifouling coatings. Prog Org Coat. 50, 75-104.

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]	3000 [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**	**Toxicity 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).

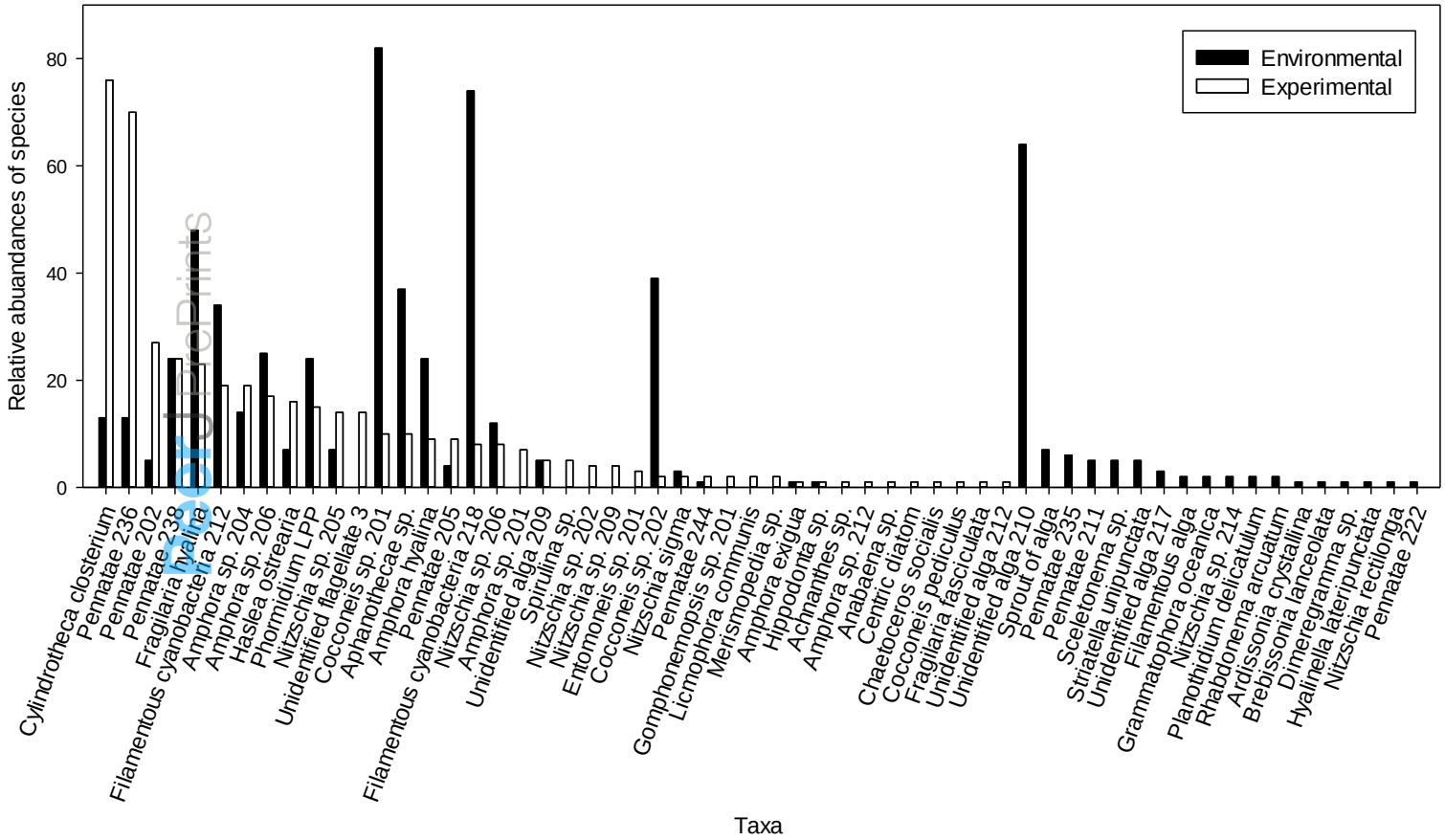

Figure 1. Frequency of taxa in environmental samples colonised for three weeks and in experimental communities after three days (72 hours). Taxa are sorted according to decreasing frequency in the experimental communities. The sums of frequency in duplicate samples are given.

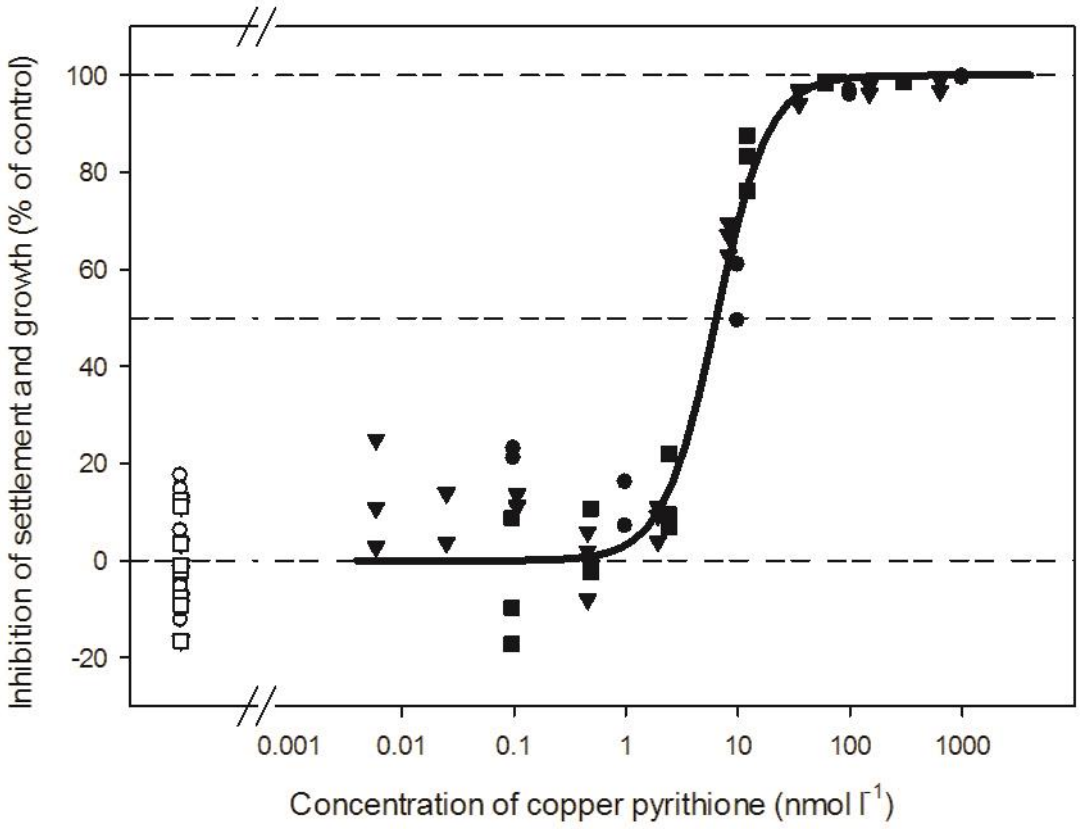

Figure 2. Efficacy of copper pyrithione to inhibit settlement and growth of periphyton communities. The different symbols indicate three independent experiments (circles from 17/07/07, triangles from 18/09/07, and squares from 30/08/10). Open symbols represent controls (n=10 per experiment) and filled symbols represent treatments with copper pyrithione (n=3).

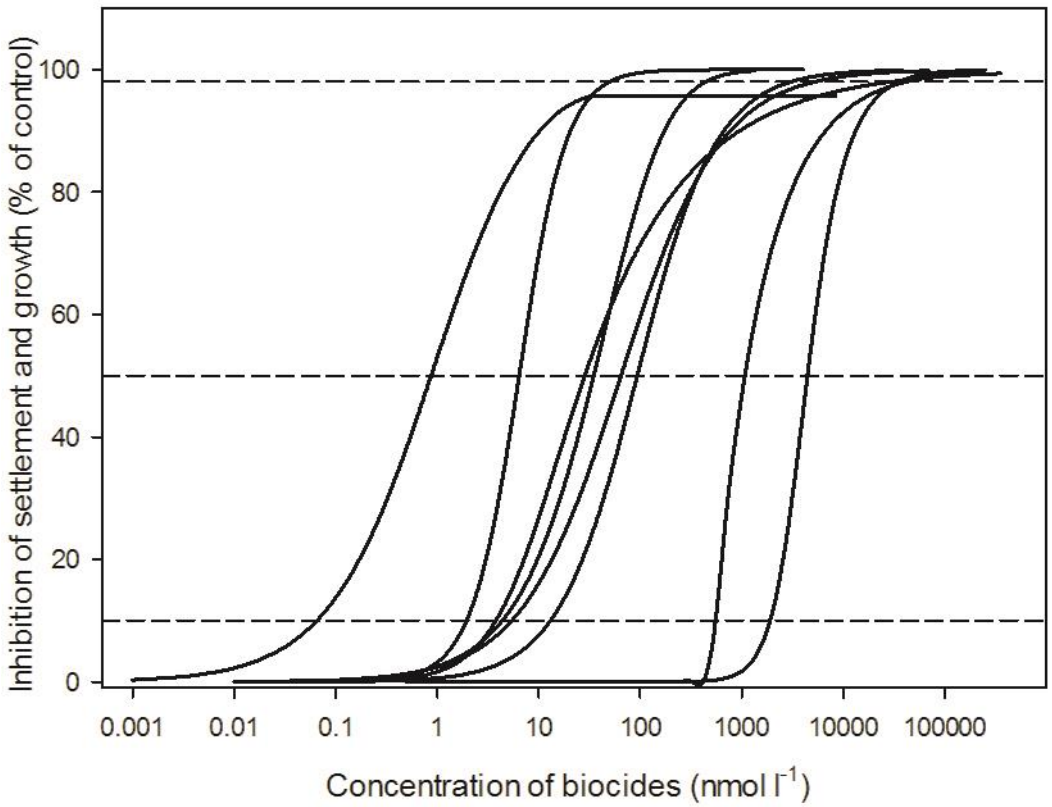

Figure 3. Concentration–response curves for eight antifouling biocides. Substances are labeled in order of increasing EC_{50} values. The horizontal lines indicate 10, 50, and 95% inhibition respectively.

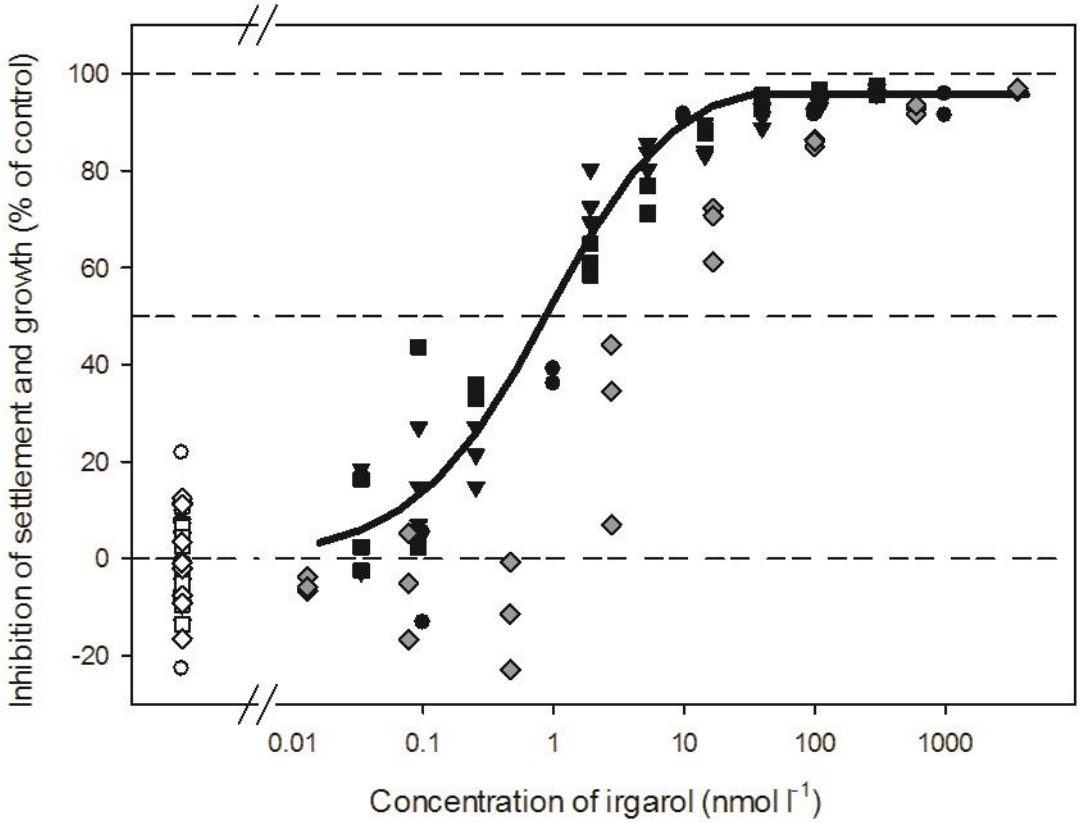

Figure 4. Efficacy of irgarol to inhibit settlement and growth of periphyton communities. The black symbols indicate three independent experiments (circles - 27/08/07, triangles - 04/09/07, and squares - 09/06/08). The grey diamonds are from 30/08/10; note these data are not included in the fitted curve. 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).