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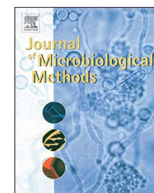

A multi-step approach for testing non-toxic amphiphilic antifouling coatings against marine microfouling at different levels of biological complexity

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ABSTRACT

Marine biofouling on artificial surfaces such as ship hulls or fish farming nets causes enormous economic damage. The time for the developmental process of antifouling coatings can be shortened by reliable laboratory assays. For designing such test systems, it is important that toxic effects can be excluded, that multiple parameters can be addressed simultaneously and that mechanistic aspects can be included. In this study, a multi-step approach for testing antifouling coatings was established employing photoautotrophic biofilm formation of marine microorganisms in micro- and mesoscoms. Degree and pattern of biofilm formation was determined by quantification of chlorophyll fluorescence. For the microcosms, co-cultures of diatoms and a heterotrophic bacterium were exposed to fouling-release coatings. For the mesocosms, a novel device was developed that permits parallel quantification of a multitude of coatings under defined conditions with varying degrees of shear stress. Additionally, the antifouling coatings were tested for leaching of potential compounds and finally tested in sea trials. This multistep-approach revealed that the individual steps led to consistent results regarding antifouling activity of the coatings. Furthermore, the novel mesocosm system can be employed for advanced antifouling analysis including metagenomic approaches for determination of microbial diversity attaching to different coatings under changing shear forces.

1. Introduction

Biofouling is defined as the unwanted colonization of surfaces with temporary or permanent adhesion of micro- and macroorganisms. In the marine environment after formation of a conditioning layer, this process starts with microfouling by diatoms and bacteria, which form biofilms on the surfaces. The process continues with the settlement of larvae and spores of invertebrates (e.g. molluscs) and macroalgae, respectively. These organisms develop thick layers of biomass upon their metamorphosis and cause macrofouling, although marine biofouling does not strictly follow this described succession. On ship hulls biofouling causes an increase in hydrodynamic drag, which leads to increased fuel consumption causing immense economic problems. Furthermore, the regular cleaning of ship hulls creates additional costs and downtimes (Railkin, 2003). Similar economic damage applies for the biofouling on cages of offshore fish farms (Fitridge et al., 2012). For this reason, there is a clear economic pressure for the use of antifouling coatings. For avoiding the adverse effects of previous antifouling coatings on the environment, such as endocrine effects of tributyltin (Champ, 2001) and for preventing accumulation of toxic amounts of copper (Dafforn et al., 2011), coatings of the future should be completely non-toxic. For this, advanced coating technology is required, which should take a deep understanding of the biological processes into account.

The initial biofilm formation by photoautotrophic diatoms and heterotrophic bacteria involves mutual metabolic interactions. While the diatoms produce dissolved and particulate organic carbon for the bacterial metabolism, those can also deliver metabolites that are essential for certain diatoms (Amin et al., 2012). After initial adhesion and possible rearrangement of the diatom cells on the surface to favorable areas, long-term attachment is accomplished through further excretion of extracellular polymeric substances (Higgins et al., 2003; Hudon and Legendre, 1987; Mitbavkar and Anil, 2007; Underwood and Paterson, 2003). The extracellular polymeric substances excreted by diatoms can mediate the attachment to both hydrophilic and hydrophobic surfaces (Klein et al., 2014; Kopanska et al., 2014). Marine heterotrophic bacteria can also permanently attach by excretion of extracellular polymeric substances (Anderson, 1995; Harder et al.,

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2002; van Loosdrecht et al., 1990; Wieczorek et al., 1995). Co-occurrence of bacteria and diatoms can lead to a synergistical increase in extracellular polymeric substance production (Agogué et al., 2014). Bacterial biofilms can stimulate attachment of diatoms, spores of macroalgae and larvae of fouling invertebrates leading to specific compositions of the organismic communities (Joint et al., 2007; Lau et al., 2005; Mieszkin et al., 2013; Shikuma et al., 2016).

An important approach for the development of non-toxic antifouling strategies are self-polishing fouling-release coatings with specific surface properties. Coatings can have homogenously hydrophilic or hydrophobic surfaces (Cho et al., 2011). Hydrophobic or super-hydrophobic coatings were discussed to be effective since attachment of hydrophilic polymers may be hindered (Jung and Bhushan, 2009; Neinhuis and Barthlott, 1997). Hydrophobic poly(dimethylsiloxane) (PDMS)-based coatings showed excellent fouling release properties because of their low surface energy with low modulus and elasticity (Beigbeder et al., 2008; Brady and Singer, 2000; Wynne et al., 2000). Nevertheless, many hydrophobic coatings can be colonized by diatoms forming biofilms with bacteria and other microalgae, which can stimulate macrofouling (Cassè and Swain, 2006; Holland et al., 2004; Marszalek et al., 1979; Sweat et al., 2017). As an alternative, amphiphilic coatings containing both hydrophilic and hydrophobic groups have been shown to reduce biofilm formation and improve fouling release of a wide range of micro- and macroorganisms (Eduok et al., 2017; Gudipati et al., 2005; Lejars et al., 2012; Pieper et al., 2007; Weinman et al., 2009).

For testing the efficiency of novel coatings, these have to be exposed to fouling organisms under marine conditions. However, before such sea trials are performed, it is advisable to test coatings under laboratory conditions to reduce costs and to avoid the release of potentially toxic compounds. First steps in testing antifouling coatings are usually performed with quantifying biofilms formed by diatom or bacterial cultures, e.g. by staining nucleic acids with fluorescent dyes (Vesterlund et al., 2005) or by spectrophotometric or fluorimetric quantification of chlorophyll (Galhenage et al., 2017; Sommer et al., 2010; Zhou et al., 2014). Although bacteria-diatom co-cultures under photoautotrophic conditions would be more realistic than single mono-cultures, there are only a few test systems using such co-cultures (Briand, 2009; Buhmann et al., 2012). In general, studies with defined organisms in mono- or co-culture give first indications about antifouling effects and may allow mechanistic insight, but are environmentally not so relevant because the natural communities are much more complex. In turn, sea trials are environmentally relevant, but mechanistic studies are difficult to perform. While both test systems are important, they leave a gap. Thus, the goal of this study was to establish a multi-step approach that addresses different levels of biological complexity. The focus was on the development of a mesocosm step addressing microfouling.

For the microfouling test system with defined cultures, the photoautotrophic diatoms *Amphora coffeaeformis* and *Phaeodactylum tricornutum* and the heterotrophic bacterium *Alteromonas macleodii* were chosen. *A. coffeaeformis* was chosen as it is well known to attach to fouling-release coatings (Cassè and Swain, 2006; Zargiel and Swain, 2014). Attachment of *A. coffeaeformis* is more pronounced on hydrophobic surfaces than on hydrophilic surfaces (Finlay et al., 2002; Hodson et al., 2012; Hunsucker and Swain, 2016). In contrast, *P. tricornutum* is not a regularly reported fouling species but its adhesive structures and its morphological adaptability may enable attachment to particular surface structures (Buhmann et al., 2016; Dugdale et al., 2006; Tesson et al., 2009). *A. macleodii* was chosen because *Alteromonodaceae* are reported to belong to the first bacteria settling on newly immersed surfaces (Dang et al., 2008; Salta et al., 2013). Further, *Alteromonodaceae* together with representatives from the *Rhodobacteraceae* and *Flavobacteraceae* are described to frequently co-occur and can be able to interact with diatoms mutualistically (Amin et al., 2012; Buchan et al., 2014; Grossart et al., 2005; Seymour et al., 2017).

For the mesocosms, a novel device was constructed that could fill some of the gaps between microcosm and sea trial. The mesocosm should contain an algal-bloom enrichment culture based on a natural organismic community. Additionally, it should allow the application of shear forces. Finally, it should allow parallel testing of at least three replicates of different coatings. For the sea trials, a marine site with cold water was chosen, at which the potential coatings could be exposed to a representative environment under static conditions. The exposures took place in spring and summer time, when the growth pressure in cold sea water is the highest. Prior to the antifouling assays, toxicity assays have to be performed because the antifouling effect should not be based on inhibiting compounds (e.g. unbound monomers) that leach from the coatings. Therefore, it is important to perform leaching assays before performing the antifouling assays with attached biomass. In our study, the ISO-certified assay with bioluminescent bacteria (DIN EN ISO11348-2-L52) was adapted for *A. macleodii*. This assay is based on the fact that energy-demanding bioluminescence is an indicator of an active physiological state, which is very sensitive to toxic chemicals. The establishment of the three-step approach for antifouling assays on different levels of biological complexity (I. microfouling, II. mesocosm, III. sea trials) was performed with newly designed amphiphilic coatings and with commercial non-toxic antifouling coatings.

2. Material and methods

2.1. Coating preparation and analysis

2.1.1. Preparation of coated substrates

All formulations, amphiphilic silicone binders (provided by Evonik Resource Efficiency GmbH) and commercial paints, were applied on microscope glass slides (fully frosted Microscopic slides, Fischer Scientific, U.S.A.) using a film applicator (Simex GmbH, Germany). The solvent was allowed to evaporate slowly at room temperature (RT) for 24 h. Wet film thickness was ~300 µm. Application on PVC sheets was done, using an air sprayer Jet 3000 RP (1.4 RP, SATA, Germany) with 2 bar air pressure. Wet film thickness was ~300 µm. The solvent was allowed to evaporate slowly at room temperature for 24 h.

2.1.2. Polishing analysis

The polishing rate was determined by measuring the reduction in film thickness of a coating film over time. Glass slides were coated by applying the coating composition as a radial stripe on the glass slide using a film applicator. The thickness of the dry film was calculated from the weight loss. The glass slides were mounted in a glass capsule. Artificial sea water was applied at a constant flow rate adjusted to 8 knots and a water temperature of 23 °C ± 2 using a Julabo water circulator (Julabo, Germany). Glass slides were taken out at a regular interval for measuring the film thickness as weight loss after drying them overnight at 60 °C.

2.2. Media and growth conditions for diatoms and bacteria

Diatom cultures of *P. tricornutum* strain UTEX 646 were kindly provided by Peter Kroth (Konstanz, Germany). The common fouling diatom *A. coffeaeformis* strain CCAP1001/1 was obtained from SAMS Research Service Ltd. (Scotland). Both strains were verified as axenic and cultivated in artificial sea-water salts (SW) containing f/2-medium as described previously (Zecher et al., 2015). SW-f/2 medium, pre- and main-cultures of diatoms and bacterial strains were prepared as described previously (Zecher et al., 2015). For growth of *Alteromonas macleodii* strain DSM6062 and its bioluminescent derivative with pBBR1MCS-5::*luxCDABE* (this work), 0.2% (w/v) tryptone and 0.1% (w/v) yeast extract was added. For *A. macleodii* [pBBR1MCS-5::*luxCDABE*], 20 µg ml^{-1} of gentamycin was added during incubations for plasmid stability.

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