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Effect of biocidal coatings on microfouling: *In vitro* and *in situ* results

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ABSTRACT

Coatings containing booster biocide (diuron and tolylfluanid) and copper thiocyanate were exposed to marine bacteria and diatoms. The effect of biocides included in coatings on cells adhesion and biofilm formation was studied in different conditions of immersion. *In vitro*, two marine bacterial strains (*Pseudoalteromonas* sp. and *Bacillus* sp.) and two diatom strains (*Cylindrotheca closterium* and *Amphora* sp.) were used in mono-species culture. *In situ* (French Atlantic Ocean, Lorient Harbour), the colonization process of natural microfouling onto coatings were evaluated. Confocal laser scanning microscopy was used to observe and quantify cells adhesion on the coatings.

The results demonstrated that biocidal coatings are more active against diatom attachment (inhibition) than against bacterial adhesion (no effect). Microalgae were more sensitive to coating. The results between *in vitro* and *in situ* assays showed a difference in behaviour according to the mode of presentation of biocide (biocidal solution or coating) or the environment (mono-species culture or marine consortium).

1. Introduction

Biofouling is a multi-step process, which includes adsorption of dissolved organic molecules, colonization by prokaryotes, eukaryotes and recruitment of invertebrate larvae and algal spores [1]. The substratum properties and the presence of biofilms (bacteria and microalgae principally) are among the most important factors determining the attachment of biofouling species (algae, barnacles, mollusks…) [2]. Prevention of fouling on immersed structures implies persistent maintenance involving intensive hard work and use of antifouling (AF) strategies. Most of them concern the use of biocide-containing antifouling paints [3,4]. These have traditionally linked to the leaching of booster biocides and copper to inhibit the growth of biofouling.

The evaluation of antifouling efficiency of coatings requires prolonged immersion times (several months to years) and the results are seasonal [5]. Consequently, laboratory-based assays have been developed [6–10] and a large panel of test organisms are used in antifouling bioassays. Ideally, AF compounds are tested against organisms embroiled in each step of colonization: bacteria, fungi, microalgae, macroalgae and invertebrates (cypris and adult barnacle) [7,11–13]. Nevertheless, assays with macro-organisms are regarded as the most representative of antifouling activity. For example, the barnacle, *Balanus amphitrite*, and the macroalga *Ulva sp*. are two main models organisms used for adhesion bioassays [14–19]. However, some papers show the interest to study bacteria or diatoms to assess the coatings performances [5,20,21].

The development of bioassays based on bacteria and diatoms represents an interest for various purposes. Marine biofilms are the first step of colonization process. It directly interacts with macro-fouling expansion [22]. Moreover, biofilms can decrease the efficiency of antifouling paints by perturbing direct interactions between the macrofouling and the coatings surface [23]. So, the quantitative and qualitative description of biofilms (ie number of species present, their thickness and biomass) on the antifouling coatings could be precious indications to microbiologists and AF coating manufacturers [6,24,25]. Another reason involves the rapidity of their answer (from a few hours to days). Generally, biocides are evaluated in solution against a mono-species culture (use of multiwall plates or discs diffusion). The growth of organisms is estimated and parameters as Effective Concentrations (EC), Lethal Doses (LD) or Inhibitory Concentration (IC) affecting 50% of the organism tested are quantified. Results enable to confront the relative efficiency of biocides under the same conditions [6].

In vitro bioassays concerning the cells adhesion and biofilms formation are realized under static conditions or flow chambers system. This approach consists in incorporating tested molecules in culture media and in observing the adhesion on a glass slide (Fig. 1A). Contrariwise, the present study proposes a different method (Fig. 1B). The studied biocides are included in a polymeric matrix called varnishes, and the bacterial and diatoms adhesion on biocidal varnishes is evaluated. The investigations are achieved by laboratory assays (*in vitro*) and field immersion trials (*in situ*).

For this, three biocides were chosen: diuron, tolylfluanid and copper

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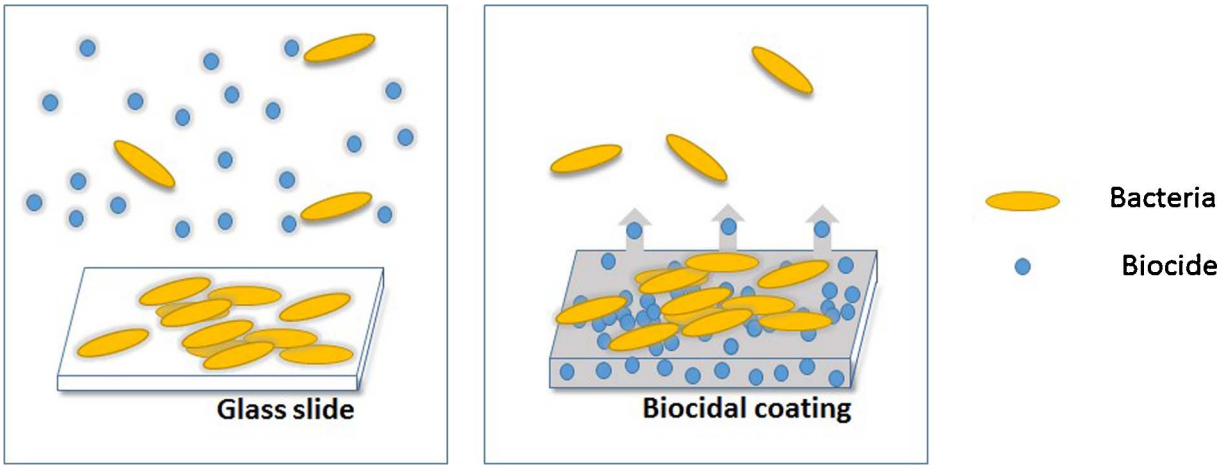

Fig. 1. Evaluation of biocides on the formation of bacterial biofilms. A) Biocides are introduced in the growth medium. B) Biocides are included in a coating.

Table 1
Effect of Diuron on microbenthic community from rivers and lakes.

	Effects	Reference
River	• Reduction of bacterial viability and abundance.	[36]
	• Reduction of biovolume of diatoms, chlorophyll and photosynthetic efficiency.	
	• Affect the algal community composition.	[37]
	• No impact on bacterial density.	
	• Impact on the heterotrophic part of the biofilm.	[38]
	• No effect on the autotrophic community.	
	• Increase in biofilm biomass and change in community structure	[39]
	• Effects on phototrophic biofilms	[40]
	• Increase in diatom mortality.	[41]
	• Inhibition of photosynthetic efficiency and capacity.	
	• No effect on bacterial viability.	
Lake	• Negative impacts on bacterial viability and abundance.	[42]
	• Growth Inhibition.	[43]

thiocyanate. The first two are booster biocides. Diuron has been regulated due to it toxicity against the no-target organisms and it ability to accumulate in aquatic environment. Tolylfluanid has been approved by the European Union [26]. Both biocides were selected for their known antifouling efficiency [27]. Herbicides can adversely affect photosynthesis in microalgae at relatively low levels via blocking of the electron transport in the photosystem II [27,28]. Their impact can increase under complex mixture conditions [29–31]. Copper thiocyanate was selected as authorized biocide because of it acute toxicity against macrofoulers principally. It has been approved [32]. However, it's toxicity against other marine organisms have been demonstrated [33–35]. The published data dealt with principally on the effects of these three biocides on river and lake communities, particularly for diuron (Table 1). To a lesser extent, their toxicity has been studied against marine bacteria and diatoms (Tables 2 and 3). However, studies focused principally on the effect of biocidal solutions on bacterial viability and abundance, the reduction of biomass and growth inhibition. No data are published on the efficiency of these biocides included in varnishes on marine environment.

The capacity to form a biofilm on biocidal varnish was therefore investigated by Confocal Laser Scanning Microscopy (CLSM). Two marine bacteria isolated from a natural biofilm (*Pseudoalteromonas* sp. and *Paracoccus* sp.) and two diatoms (*Cylindrotheca closterium* and *Amphora* sp.) were selected as the test organisms for *in vitro* assays, whereas a natural community was presented for *in situ* immersion. The aims of this study were: i) to quantify the biofilm (bacteria and/or microalgae) developed on biocidal coatings, ii) to assess the sensibility of bacteria and diatoms exposed to coatings, iii) to compare *in vitro* and *in situ* experiments, iv) to value the antifouling activity of biocides included in coating.

2. Material and methods

2.1. Materials

Diuron (N-[3,4-dichlorophenyl]-N,N-dimethylurea), tolylfluanid (N-([Dichlorofluoromethyl]Thio)-N´, N´- Dimethyl-N-P-Tolylsulfamide) N-[dichloro(fluoro)methyl]sulfanyl-N-(dimethylsulfamoyl)-4-methylaniline, copper thiocyanate and all formulation ingredients were supplied by Nautix Compagny.

Table 2
Effect of biocides on marine bacteria.

Biocide	Effect	Species	Reference
Diuron	Growth inhibition	*Vibrio* sp. (EC_{50} > 300 µg/mL) *Pseudovibrio denitricans* (EC_{50} > 300 µg/mL) *Rhodobacteracea bacterium* (EC_{50} = 107.1 µg/mL)	[44]
		Navicula forcipata (EC_{50} = 0.027 µg/mL)	[30]
	Bioluminescence inhibition	*Vibrio Fischeri* (EC_{50} = 12.74 µg/mL)	[34]
	Cell attachment and biofilm inhibition	*Pseudoalteromonas* sp. (10 µg/mL) *Vibrio Vulnificus* (10 µg/mL)	[45]
Tolylfluanid	Growth inhibition	*Vibrio* sp. (EC_{50} > 300 µg/mL) *Pseudovibrio denitricans* (EC_{50} > 300 µg/mL) *Rhodobacteracea bacterium* (EC_{50} > 300 µg/mL)	[44]
	Cell attachment and biofilm inhibition	*Pseudoalteromonas* sp. (10 µg/mL) *Vibrio Vulnificus* (10 µg/mL)	[45]
Copper	Adhesion inhibition ($CuSO_4$)	*Flavobacteriaceae* (EC_{50} > 500 µM) *Polaribacter* sp. (EC_{50} = 300 µM) *Pseudoalteromonas* sp. (EC_{50} > 500 µM) *Paracoccus* sp. (EC_{50} = 15 µM) *Pseudoalteromonas* sp. (EC_{50} > 500 µM)	[8]
	Bioluminescence inhibition ($CuSO_4$)	*Vibrio Fischeri* (12.74 µg/mL)	[34]