Universidade de Aveiro

Ano 2019

Departamento de Biologia

SARA RIBEIRO DA SILVA

Assessment of the toxicity and efficacy of innovative antifouling coatings

Avaliação da toxicidade e eficácia de revestimentos anti-incrustantes

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Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Biologia Marinha, realizada sob a orientação científica do Doutor Roberto Carlos Domingues Martins, Investigador Auxiliar do Departamento de Biologia e Centro de Estudos do Ambiente e do Mar (CESAM) da Universidade de Aveiro e coorientação da Professora Doutora Susana Patrícia Mendes Loureiro, Professora Auxiliar com Agregação do Departamento de Biologia e do Centro de Estudos do Ambiente e do Mar (CESAM) da Universidade de Aveiro.

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agradecimentos

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palavras-chave   Tintas Anti vegetativas; Bio incrustação marinha; Biocidas nano estruturados; Eficácia; Toxicidade

resumo   A bioincrustação marinha em estruturas metálicas marítimas construídas pelo Homem é ainda um problema socioeconómico a nível global. A proteção das estruturas tem sido convencionalmente realizada com recurso a tintas anti vegetativas contendo biocidas orgânicos e/ou metálicos (ex. cobre), cujos efeitos tóxicos em organismos não-alvo têm sido amplamente reportados na literatura. Por forma a contornar este problema ambiental e controlar a libertação dos químicos ao longo do tempo, alguns destes biocidas foram imobilizados/encapsulados em nanomateriais manufaturados. Recentemente foi demonstrado que, quando dispersos em água salgada artificial, os novos nanoaditivos apresentam uma redução apreciável da sua toxicidade e perigosidade para o ecossistema marinho. Desta forma, o presente estudo teve como objetivo avaliar a toxicidade e a eficácia anti vegetativa de 8 tintas modificadas com biocidas livres ou nano estruturados e 1 tinta comercialmente disponível, assim como relacionar estes dados com parâmetros físico-químicos e a libertação de elementos químicos ao longo do tempo. Destas tintas, duas continham biocidas em estado livre (CuPT e DCOIT (nome comercial Sea-Nine), duas com os biocidas na forma "nano" (CuPT@Zn-Al LDH e Sea-Nine@SiNC), três com nano-estruturas "vazias" (Cu-Al LDH, Zn-Al LDH e SiNC), um controlo negativo (sem biocidas) e um controlo positivo (tinta comercial).

resumo (cont.)

A eficácia anti-vegetativa das tintas foi avaliada ao longo de um ano, na marina da Gafanha da Encarnação (Aveiro, Portugal), tendo-se avaliado a evolução das comunidades bacterianas, microfitobentos e de macroorganismos incrustantes. No laboratório, as chapas revestidas foram caraterizadas e, posteriormente, colocadas em aquários para libertar os seus compostos químicos durante 3 meses. Periodicamente, aliquotas de água de cada aquário foram monitorizadas em termos dos efeitos tóxicos em microalgas (inibição de crescimento, *Phaeodactylum tricornutum* e *Tetraselmis chuii*) e crustáceos (letalidade *Artemia salina*), composição elementar (ICP-MS e ICP-OES), quantificação de DCOIT (nas tintas com este composto, via HPLC) e caraterização fisico-química. Globalmente, este estudo mostrou que as tintas com os biocidas em estado livre têm elevada eficácia anti-vegetativa, mas extrema toxicidade. Por sua vez, as tintas anti-vegetativas com nanoaditivos mostraram uma reducão significativa da sua toxicidade e mantiveram (CuPT@Zn-Al LDH) ou até melhoraram (Sea-Nine@SiNC) a eficácia anti-vegetativa contra macrorganismos incrustantes, sobretudo macroalgas, após um ano de imersão em condições reais. Portanto, o encapsulamento/imobilização destes biocidas para aplicação como aditivos anti-vegetativos de tintas parece ser um método bastante promissor em termos ambientais.

abstract

Marine biofouling in man-made metallic immersed structures is still a global socio-economic problem. The protection of structures has been conventionally performed using antifouling paints containing toxic organic and/or metallic biocides (e.g. copper) towards non-target organisms. In order to circumvent this environmental problem and control the chemicals release over time, some of these biocides have been immobilized/encapsulated in engineered nanomaterials. When directly dispersed in artificial saltwater, novel nanoadditives show a noteworthy reduction in their toxicity and hazard to marine ecosystems. Thus, the present study aimed to test the toxicity and antifouling efficacy of 8 modified coatings with free or nanostructured biocides and 1 state-of-the-art coating and to establish a relationship with physicochemical parameters and the release rate of chemicals over time. The tested systems are the following: two coatings containing only free biocides (CuPT and DCOIT (commercially known as Sea-Nine), two with nano-structured biocides (CuPT @ Zn-Al LDH and Sea-Nine @ SiNC), three with unloaded/"empty" nanostructures (Cu-Al LDH, Zn-Al LDH and SiNC), blank reference without biocides and a commercial reference. The anti-fouling efficacy assessment was done at Gafanha da Encarnação marina (Aveiro, Portugal), for a complete year. Bacteria, microphytobenthos and macrofoulers communities were evaluated periodically.

abstract (cont.)

In the laboratory, coated plates were properly characterized in terms of chemical composition and metallographic properties, and then, placed in aquaria and allowed to release their chemical compounds for 3 months. Water samples were taken from each aquarium and monitored for toxic effects on microalgae (growth inhibition, *Phaeodactylum tricornutum* and *Tetraselmis chuii*) and crustaceans (lethality, *Artemia salina*), elemental composition (ICP-MS and ICP-OES), DCOIT quantification (only in paints with this compound via HPLC) and physicochemical characterization. Overall, this study showed that coatings with free biocides showed high antifouling efficacy, but extreme toxicity. The encapsulation of these biocides significantly reduced the coatings toxicity and kept (CuPT @ Zn-Al LDH) or even increased (Sea-Nine @ SiNC) their anti-macrofouling efficacy over an entire year of immersion in the field, being a promising method with environmental benefits.

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1. Introduction

Worldwide marine ecosystems encompass a huge biodiversity, provide several services and goods to humans and, in the last centuries, have been under a growing pressure, being their protection and appropriate management of major importance (Barbier *et. al.*, 2011). Main threats to the marine biodiversity include contamination, recreational activities, resources overexploitation and climate changes, among others, which lead to the habitats perturbation or destruction (Mazaris *et al.*, 2019).

Ecosystems contamination is determined by the presence of some substance(s) that would not occur normally there, or if occurs, is/are detected at concentrations above the natural levels. A place that is contaminated do not experience the negative effects of the detected substance(s), however, a polluted site is characterized by having chemical(s) in levels that may cause harmful effects on the living organisms and ultimately impairing the ecosystems functioning and dynamics.

Basically all pollutants are contaminants, but not all contaminants are pollutants (Chapman, 2007). The release of contaminants into the marine seawater are mainly due to domestic, agriculture and industrial runoff, and to off-shore/coastal activities (e.g. maritime transportation, aquaculture, oil spills, oil and gas exploration) which requires infrastructures free of corrosion and biofouling (Álvarez-Muñoz *et al.*, 2016; Tornero *et. al.*, 2016).

1.1 Biofouling and its impacts

Biofouling corresponds to a natural and progressive accumulation of marine organisms (flora and fauna) in static or mobile submerged surfaces (Avelelas *et. al.*, 2017). Similarly, to other natural ecological successions, biofouling is characterized by different phases (five in this case) in which characteristic organisms colonize the available area, growth and then decline, being then replaced by organisms of the upcoming phase (Figure 1). In the first minutes of the structure's immersion, proteins, polysaccharides, glycoproteins and other organic molecules present in the seawater adsorb to the surface.

Bacteria and, at less extent, some unicellular algae, adhere to the surface after 24 hours. These organisms produce extracellular polymeric substances (i.e. biopolymers such as

oligosaccharides, proteins, glycoproteins and glycolipids; Flemming *et. al.*, 2007). that quickly lead to the formation of a biofilm that affects the porosity, adsorption properties, mechanical stability and other factors of the coatings (Arai *et. al.*, 2009; Almeida *et. al.,* 2007). The biofilm will serve as a conditioning layer and food for the third phase organisms, which includes other microfoulers, like microalgae spores, diatoms or protozoans (Almeida *et. al.*, 2007). The last phase is typically characterized by the macrofoulers, such as barnacles, mussels, macroalgae, tubeworms, bryozoans, hydrozoans, among others (Arai *et. al.*, 2009).
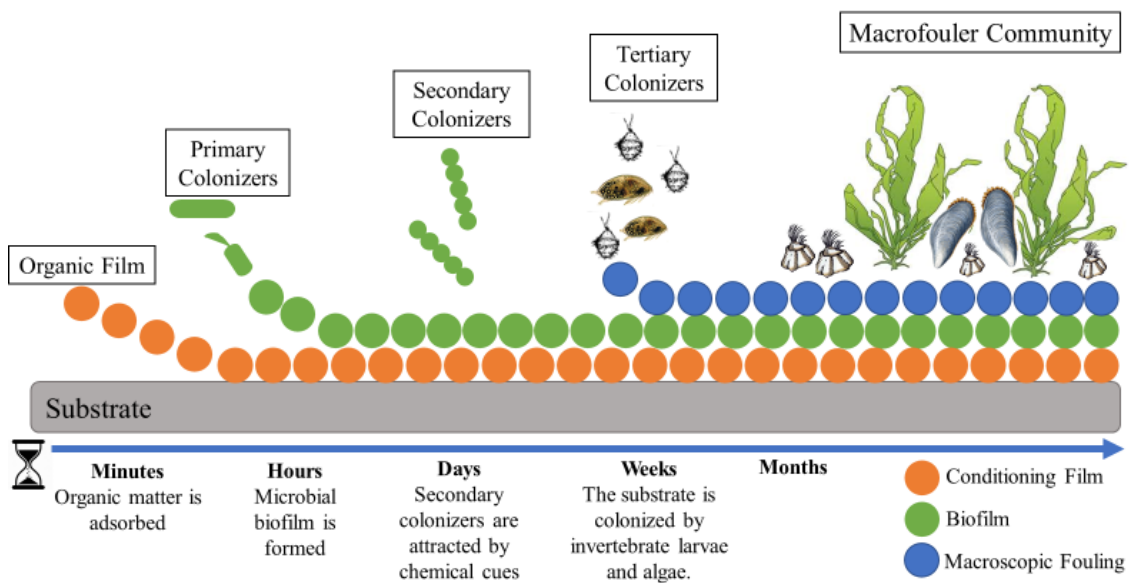
Figure 1: Schematic representation of the biofouling process (adapted from Hellio and Yebra, 2009; Martín-Rodríguez et al., 2015)

In terms of ecological impacts, biofouling associated to the maritime transportation have been responsible by the cross-colonization of species around the world, where exotic fouler species are transported in the ballast waters or hulls surface, competing and, in some cases, leading to the exclusion of native species from the surrounding estuarine/coastal ecosystems to the harbors. This pressure may shift the ecosystems dynamic and stability, which may cause relevant biodiversity and socio-economic losses, particularly when associated to lower production of even exclusion of commercially-valuable species ((Schultz *et. al.*, 2011); Figure 2).

In terms of environmental and economic impacts, biofouling promotes an increase of the surface's roughness and water resistance in mobile structures, and a consequent decrease

of the navigation speed (-15%) and an increase of the fuel consumption (+40%) and greenhouse gases emissions (+90%) (Avelelas *et. al.*, 2017; Yebra *et. al.*, 2004; Figure 2). This process decreases the AF coatings efficacy and also promotes biocorrosion on metallic structures, a phenomena accelerated by metabolites-rich acidic fluids produced and excreted by biofoulers, which may affects the quality and quantity of oil and gas production and the infrastructures stability and, in worst cases, their failure (Li *et. al.*, 2019). It is estimated that annually around 60,000 peoples deaths and a loss around 200€ billion is associated with the toxic emissions from the international maritime navigation, resulting from the effects referred above, as indirect consequences of biofouling (Loto *et. al.*, 2019). Antifouling control and maintenance have also massive costs in the maritime industry worldwide. As an example, it is estimated that around 5-10% of the production costs of global aquaculture are attributed to this problem, roughly US$ 1.5 to 3 thousand million/year (Fitridge *et. al.*, 2012).

Finally, AF protective coatings for maritime metallic infrastructures (e.g. vessels, bridges, aquaculture cages, oil and gas offshore platforms and pipelines) are one of the major sources of chemical contamination of the marine environment. Current AF coatings promote the constant release of chemical components (e.g. antifouling booster biocides) to the surrounding seawater, even in early phases, impacting the ecosystem in all its extension, from the water column to the sediments and their inhabiting organisms (Molnar *et. al.*, 2008; Want *et. al.*, 2018).
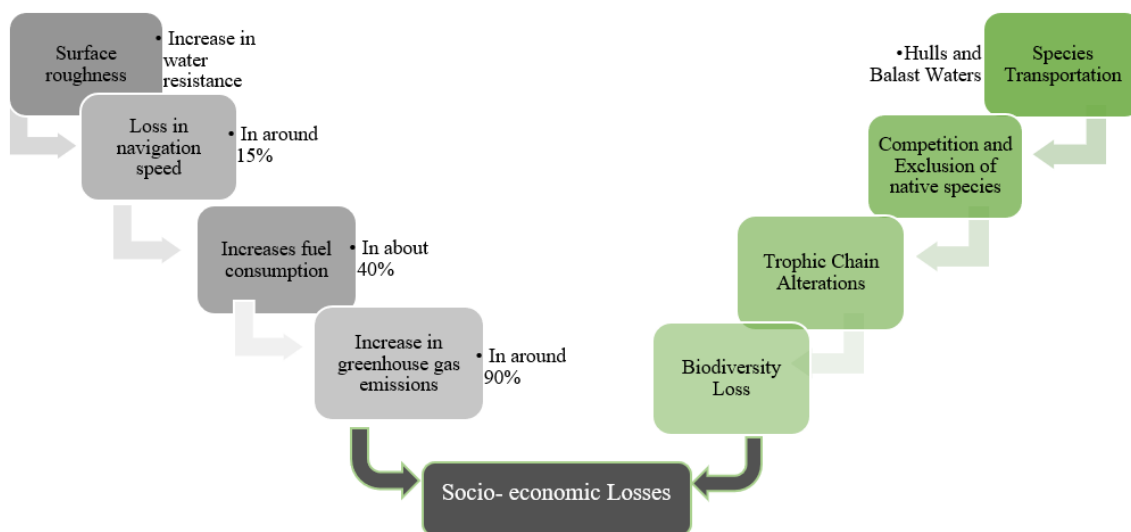
Figure 2: Scheme of fouling impacts on mobile structures and on the biota culminating in socio-economic losses

1.2 Antifouling protection

Human-made maritime infrastructures have been typically protected from biofouling with AF coatings ever since Romans, Greeks, Carthaginians, Phoenicians and later, during the Age of Discovery, Portuguese, Spanish and other Europeans protected vessel's hulls with a wide-range of products, namely, grease, oils, tallow, waxes, resins, oil impregnated with Sulphur or arsenic and, more successfully, copper or lead-sheathing (Nurioglu *et. al.,* 2015) (Figure 3). The technological advances were mostly shaped according to the needs, the type and complexity of the materials used in the structure and know-how of each civilization. Erstwhile, coatings varied between (Arai *et. al.*, 2009; Loto *et. al.*, 2019).

In the 19th century, copper began to be used in iron-based hulls (instead of wood) however, for a short period of time due to corrosion problems (Arai *et. al.*, 2009; Loto *et. al.,* 2019), being then replaced by mercury in the early 20th century. Despite its good efficacy, availability and price, Hg was also discarded due to its well-known health effects and safety issues (Loto *et. al.*, 2019).

Around 1950s, tributyltin (TBT), an organotin compound, appeared and quickly became very popular on AF coatings. The success of TBT relied on the price and the very high and long-lasting AF efficacy which insured higher half-time of the TBT-based coatings and consecutively savings of time and money (less maintenance and less stops), particularly important for the maritime transportation industry. However, environmental concerns quickly raised when researchers demonstrated its slow degradation, contamination and high persistence on different environmental compartments, high bioaccumulation in organisms from different trophic levels and deleterious effects on non-target organisms even at ng level (e.g. bivalves shell thickening; imposex in gastropods; changes on community composition and structure; (Arai *et. al.*, 2009; Price *et. al.*, 2012); Karlsson *et. al.,* 2010). This compound was banned in vessels with less than 25 m length, by the International Maritime Organization (IMO) during the 80s of the last century, and it was totally banned from use in 2008 (Antizar-Ladislao, 2008; Schultz *et. al.*, 2011).
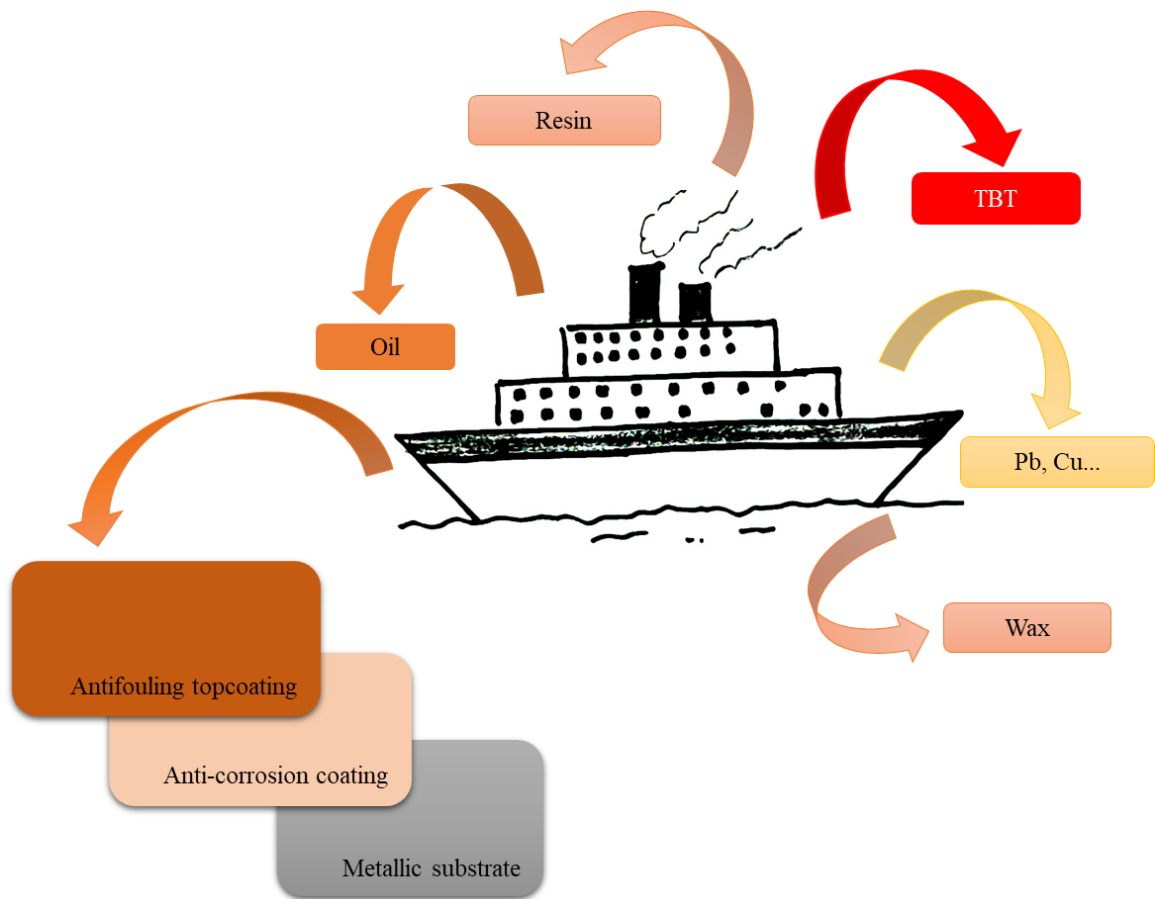
Figure 3: Scheme of antifouling materials/substances used in the past highlighting a simplified painting scheme of metallic surfaces

Currently, maritime metallic infrastructures, namely vessels and bridges, are protected from the aggressive saline conditions through a painting scheme composed by an anti-corrosion coating (primer), a tie-coat and one or more topcoat layers (Figure 3). Topcoats with antifouling characteristics such as tin-free self-polishing antifouling materials, with low surface energy, with silicate, capsaicin or with nano-based properties, and ultra-hydrophobic surfaces have been growing. However, the most common and effective AF topcoats to tackle biofouling are based on coatings containing biocides. AF biocides are chemical substances, naturally-available or synthetically-produced, that control the adhesion and growth of fouler organisms on surfaces of human-made infrastructures (Faÿ *et. al.*, 2010).

Table 1 lists the biocides approved (or under approval) to be used on antifouling coatings in the Europe Union in compliance the Directive 98/8/EC and the Regulation No 528/2012 of the European Parliament. These authorized biocides are supposedly readily degraded, no/low bioavailable and low toxic to non-target organisms (Jacobson *et. al*., 2000), however, concerns regarding the environmental fate and toxicity of some of the so-called booster biocides on non-target marine organisms have been highlighted by several studies (e.g. DCOIT by Figueiredo *et. al.*, (2019); Irgarol by Karlsson *et. al.*, (2010)). Copper oxide is by far the most used biocide in state-of-art biocide-release based AF coatings (Table 1). These coatings correspond to the major anthropogenic source of Cu, other metals and organic compounds in seawater and marine sediments, particularly in harbors and marinas (e.g. Eklund *et. al.*, (2014)), and, therefore, environmental concerns have been raised lately (Srinivasan *et. al.*, 2007).

Table 1: List of antifouling biocides included in the EU category PT21 (27/07/2019)

Substance Name	EC Number	CAS Number	Legal Act	Date of Approval	Expiry Date	Evaluating Competent Authority	Approval Status
4,5-Dicloro-2-octylisothiazol-3(2H)-one (4,5-Dicloro-2-octyl-2H-isothaizol-3-one (DCOIT)	264-843-8	64359-81-5	Regulation (EU) 437/2014	01/01/2016	01/01/2026	NO	Approved
Bis(1-hydroxy-1H-pyridine-2-thionato-O,S)copper (Copper pyrithione)	238-984-0	14915-37-8	Regulation 2015/984	01/10/2016	01/01/2026	SE	Approved
Copper	231-159-6	7440-50-8	Regulation (EU) 2016/1088	01/01/2018	01/01/2026	FR	Approved
Copper thiocyanate	214-183-1	1111-67-7	Regulation (EU) 2016/1090	01/01/2018	01/01/2026	FR	Approved
Dichloro-N-[(dimethylamino)sulphonyl]fluoro-N-(ptolyl)methanesulphenamide (Tolylfluanid)	211-986-9	731-27-1	Regulation (EU) 2015/419	01/07/2016	01/01/2026	FI	Approved
Dicopper oxide	215-270-7	1317-39-1	Regulation (EU) 2016/1089	01/01/2018	01/01/2026	FR	Approved
Medetomidine		86347-14-0	Regulation (EU) 2015/1731	01/01/2016	01/01/2023	GB	Approved

N-(Diclorofluoromethylthio-N',N'-dimethyl-N-Phenylsulfamide (Dichlorofluanid)	214-118-7	1085-98-9	Regulation (EU) 2017/796	01/11/2018	01/01/2026	GB	Approved
N'-tert-butyl-N-cycloropropyl-6-(methylthio)-1,3,5-triazine-2,4-diamine (Cybutryne)	248-872-3	28159-98-0	Regulation /EU) 2016/107			NL	Not Approved
Tyrithione zinc (Zinc pyrithione)	236-671-3	13463-41-7				SE	Under review
Tralopyril		122454-29-9	Regulation (EU) 1091/2014	01/04/2015	01/04/2025	GB	Approved
Zineb	235-180-1	12122-67-7	Regulation (EU) 92/2014	01/01/2016	01/01/2026	IE	Approved

Copper pyrithione (CuPT) is a bactericidal and fungicidal compound with a high efficacy against the biofilm community and mild efficacy against macrofoulers (e.g. (Gutner-hoch *et. al.*, 2018). It is considered neutral and non-persistent in the environment due to its rapid photolysis and its transformation/ degradation into less toxic compounds via interaction with others free metal ions present naturally in seawater (Maraldo *et. al.*, 2004). This biocide is extremely toxic ($EC_{50} < 0.1$ mg/L) towards non-target organisms, such as diatoms (Avelelas *et. al.*, 2017) or early development stages of echinoderms (Gutner-hoch *et. al.*, 2019); it also causes acute effects on corals, crustaceans and fish (Mochida, 2006; Bao *et al.*, 2014; Gutner-hoch *et. al.*, 2019).

Sea-Nine 211™, the trademark of the fungicide DCOIT, is another widely-used booster biocide with high AF efficacy, especially against soft-foulers (Jacobson *et. al.*, 2000). It can be found in Hempel or Sigma coatings (Table 2). In seawater, the half-life of Sea-Nine 211™ is reported to be less than 24 hours (Jacobson *et. al.*, 2000), however a recent study demonstrated that it does not degrade at least for 168 h, in artificial saltwater (Figueiredo *et. al.*, 2019). This biocide is also extremely toxic ($EC_{50} < 0.1$ mg/L) for bacteria, microalgae, diatoms, macroalgae or early development stages of echinoderms (e.g. Fernández-alba *et. al.*, 2002; Devilla *et. al.*, 2005; Bellas, 2006; Ida *et. al.*, 2013; Figueiredo *et. al.*, 2019); it also causes acute effects on rotifers, bivalves, crustaceans and fish at low concentrations (< 1 mg/L) (e.g. (Mochida *et. al.*, 2006; Yamada, 2007; Tsunemasa *et. al.*, 2011; Figueiredo *et. al.*, 2019).

Low degradation and high contamination (at µg/L levels) of European coastal waters and sediments by cybutryne (commercially known as Irgarol 1051) and diuron have been extensively reported (e.g. Readman *et. al.*, 1993; Taylor *et. al.*, 2010)). Both biocides exert acute and/or chronic effects on micro- and macroalgae, several groups of invertebrates and fish at very low doses (e.g. Perina, 2009; Bao *et. al.*, 2011), posing a high environmental risk. As a consequence, cybutryne was already banned from the European market (Table 1) and Diuron was forbidden in some EU countries (Price *et. al.*, 2012).

In order to decrease the toxicity and the early release of booster biocides, researchers from the University of Aveiro and the Portuguese company Smallmatek, proposed their encapsulation or immobilization in engineered nanomaterials[1], such as nanoclays (layered double hydroxides) and silica mesoporous nanocapsules (DCOIT: Maia *et. al.*, 2015; DCOIT and Ag: Figueiredo *et. al.*, 2019; CuPT and ZnPT: Avelelas *et. al.*, 2017). The toxicity of the booster biocides in its free and nano-forms was compared in several marine representative species and, globally, it was shown a toxicity reduction, up to 3 orders of magnitude (Avelelas *et. al*., 2017; Gutner-Hoch *et. al.*, 2018, 2019; Figueiredo *et. al*., 2019). Although the toxicity, fate and behavior of these novel AF additives were already assessed in seawater, few information exist in terms of AF efficacy (Gutner-hoch *et. al.*, 2018 for CuPT and ZnPT forms) and no data is available regarding their field performance in paints (because they were not yet commercially available).

There is in the market multiple references of biocide-release based AF coatings composed by a combination of authorized biocides, usually Cu_2O plus a booster biocide (also called co-biocide), which performance varies between 3 and 7.5 years (Table 2). The coating "Sea Quantum Ultra S" from Jotun (Table 2), a chemically hydrolyzing silyl acrylate AF coating containing 785 g/L of Cu_2O and 66 g/L (3.7% w/w) of CuPT, is widely used in streaming vessels (Takahashi, 2009; Road *et. al.*, 2019) and it was selected as the commercial reference, i.e. a state-of-the-art coating with recognized efficacy, in the context of the present study. Published studies regarding the ecotoxicity of this type of paints are surprisingly scarce, taking into account the high number of commercial biocides-release AF coatings available in the market (Table 2) together with the high

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[1] Nanomaterial definition: "natural, incidental or manufactured material containing particles, in an unbound state or as an aggregate or as an agglomerate and where, for 50 % or more of the particles in the number size distribution, one or more external dimensions is in the size range 1 nm - 100 nm." (EU Recommendation on the definition of a nanomaterial (2011/696/EU)).

toxicity of the booster biocides (as previously highlighted). Karlsson *et. al.,* (2004) showed that a total of five (out of six) tested paints were toxic to macroalgae and/or crustaceans even after two weeks of leakage. Karlsson *et. al.*, (2006) demonstrated that those tested coatings were much more toxic than commercial biocide-free AF paints (polishing paints, Teflon paints, polymer waxes and paints with pepper extract). Later, (Karlsson *et.al.*, 2010) demonstrated that globally ship paints were more toxic than leisure boat paints to bacteria, macroalgae and crustaceans. These authors also showed that one of the tested biocide-free paints was the most toxic from the entire set of tested paints concluding that compounds other than the main active ingredient(s) may be responsible by the observed toxicity (Karlsson *et. al.*, 2010).

Therefore, a remarkable knowledge gap on conventional biocides-release AF paints ecotoxicity and efficacy can be stressed. In order to contribute with new knowledge in this scientific topic, the present thesis will address a holistic assessment of the field AF efficacy, toxicity and release rate of state-of-art and novel nano-based biocides-release AF paints which were formulated for this study.

Table 2: Characteristics of biocides-release based antifouling coatings of the biggest worldwide paint producers (Yebra et. al., 2004; Ameron, 2008; Takahashi, 2009)

Company	Product	Biocide	Performance	Remark
Jotun	Sea Quantum Plus	Cu_2O/ CuPT	5 years	Deep-sea vessel
	Sea Quantum Ultra S	Cu_2O/ CuPT	7.5 years	Steaming vessels
Ameron	ABC-1-2-3 and -4	Ziram/ Cu_2O	3-5 years	
Hempel's MP	Globic 8190081950	Sea-Nine 211™/ Cu_2O	7.5 years	
	Globic NCT	Cu_2O/CuPT	5 years	Deep-sea vessel
International MC	Intersmooth Ecoloflex SPC	ZnPT	5-7.5 years	Deep-sea vessel/ Coastal vessel
	Interswift 655	Cu_2O/ CuPT	3 years	Coastal vessel
Sigma Coatings	Alphagen 10-20-50	Cu_2O/ Sea-Nine 211™	5 years	Deep-sea vessel

1.3   Thesis goals

- Assess the antifouling efficacy of conventional and new modified AF coatings in the field over time;
- Evaluate the toxicity of the coating's leachates on microalgae and crustaceans;
- Characterize the physiochemical properties and chemical composition of the coating's leachates.

1.4 Null Hypotheses

- The efficacy of the coatings is similar to the commercial reference over time;
- The toxicity of the leachates of modified coatings is similar to the blank reference over time;
- The physicochemical composition of the leachates of modified coatings is similar to the blank reference over time.

In order to accomplish these goals and validate these hypotheses, field and laboratorial studies were carried out in plates coated with modified and state-of-the-art coatings. The thesis is organized in three main sections: coatings characterization, coatings' leachates ecotoxicity and chemistry (ecotoxicity and chemistry) and field antifouling efficacy, followed by a holistic data analysis, interpretation and discussion of the collected data.

2. Materials and Methods

2.1 *Tested coatings*

2.1.1 Coating preparation and application

The mild steel plates were prepared and painted on September 2018. A hole was drilled next to each corner to fix the plate and then, edges and surfaces were sanded. Plates were then individually cleaned with HCl and aminas (1:1), for 1 minute, washed with deionized water, degreased using ethanol 96% and dried with compressed air. The primer Safeguard Universal ES from Jotun, which is an epoxy vinyl-base coating, was applied to prevent corrosion, followed by the application of the tie-coat Safeguard Universal from Jotun, in order to promote a good adhesion between the primer and the topcoat. Then, the topcoat was applied above the other layers. A non-commercial top-coat, provided by Jotun, without biocides, was used to prepare a total of eight antifouling systems, by adding free booster biocides (DCOIT as Sea-Nine; Cu pyrithione (CuPT)), nanostructured biocides (Sea-Nine@SiNC; CuPT@Zn-Al LDH) or unloaded nanomaterials (SiNC; Zn-Al LDH; Cu-Al LDH, this one was selected due to the potential antifouling properties associated to the presence of Cu in the structure). Table 3 shows the list of 10 topcoats tested in the framework of this study (8 customized coatings, 1 blank and 1 commercial for benchmarking purposes) and the dry weight percentage of the additive added to the raw coating to prepare the customized formulations.

Table 3: List of additives and percentages used in the modified topcoats

Topcoat tested	Additive	Chemical dry weight %
SiNC	**Engineered nanomaterial**: unloaded mesoporous silica nanocapsules	3
Sea-Nine	**Free booster biocide** 4,5-Dichloro-2-octyl-4-isothiazolin-3-one (DCOIT), commercially known as **Sea-Nine** 211™ (hereinafter abbreviated as Sea-Nine)	10 (3% DCOIT)
Sea-Nine@SiNC	**Nanostructured Sea-Nine**: spherical mesoporous silica nanocapsules loaded with Sea-Nine	10 (3% DCOIT)
Cu-Al LDH	**Engineered nanomaterial**: unloaded copper-aluminum layered double hydroxides	10
Zn-Al LDH	**Engineered nanomaterial**: unloaded zinc-aluminum layered double hydroxides	10
CuPT	**Free booster biocide** copper pyrithione (CuPT)	4
CuPT@Zn-Al LDH	**Nanostructured CuPT**: zinc-aluminum layered double hydroxides loaded with CuPT	4 (as CuPT)
Blank reference	Topcoat not commercially available (without biocides)	0
Commercial reference	Commercial reference Sea Quantum Ultra S (Jotun) containing already two biocides (CuPT and Cu_2O)	No extra additive

2.1.2. Characterization of coated plates

2.1.2.1 Metallography

The characterization of the surface roughness (bottom view) and the coatings thickness (transversal view) of the painted plates was made using a stereo microscope (Nikon SMZ18), which combines macro- and micro-imaging. Roughness was randomly assessed all over the plate at different magnifications (7.5 to 40x). Thickness was monitored in squares of ≈ 2cm x 2 cm of each plate that were previously fix on resin mold. Resin was prepared by mixing 100 g of EpoKwick™ FC Epoxy Resin 20-3453-128 (BUEHLER®) and 25 g of EpoKwick™ FC Epoxy Hardener 20-3453-032 (BUEHLER®). The mold was smeared with a release agent, to facilitate the removal of the dry resin, and a total of roughly 15 mL of the resin mixture was added to the mold which already had the plate. The dried casts were polished using the METASERV 2000 Grinder/Polisher, sandpaper with appropriate granulometry (220, 280, 360, 500 and 600) and MicroPolish™ Alumina 1.0 and 0.3 µm (BUEHLER®) to obtain a smooth surface and a clearer view in the stereomicroscope. Photography and thickness measurement of different sections of all plates were made using the software Nikon SMZ18.

2.1.2.2 Fourier-Transform Infrared Spectroscopy

Fourier-Transform Infrared Spectroscopy (FTIR) analysis consists in the characterization of the chemical properties of the coating via absorption of infrared radiation. This analysis was performed by detaching a small piece of the topcoat with the aid of a scalpel and, whenever possible, individual particles of the topcoat were also analyzed. Spectra were collected in a Perkin Elmer spectrometer Spectrum Two with a UATR TWO unit (Diamond), 64 scans, 4 cm^{-1} resolution, in the region wavelength of 400 ~ 4000 cm^{-1}. The comparation of the plates was made between the Blank reference vs Commercial reference, Cu-Al LDH vs Zn-Al LDH, SiNC vs Sea-Nine@SiNC and CuPT vs CuPT@Zn-Al LDH.

2.2. *Coating leachates characterization and toxicity*

2.2.1. Characterization and behavior of leachates over time

The release of chemicals (biocides, nanostructures or other chemicals present in the formulations) from the tested coatings and the leachates toxicity were assessed during 3 months by placing each plate (area of 216 cm^2; n=1) in a tank containing 16 L of artificial seawater (Ytreberg *et. al.*, 2010), constantly recirculated with a pump Eheim^{TM} Compact On (output of 300 L/h). The experimental design included also an aquarium with only artificial saltwater (no coated plates) to be used as a control of the bio-physico-chemical conditions along the entire period. Aquaria were previously decontaminated (acid wash (HNO$_3$ 5%) and basic wash 5% during 5h each) and washed with distilled water for 24h and then tap water. Artificial saltwater (ASW; salinity 35) was prepared, in the day before, using Tropic Marin® Pro-Reef pharmaceutical grade sea salt (purchased from Tropic Marin®). Every week the following physicochemical parameters were measured: temperature, salinity, pH dissolved oxygen, nitrites and nitrates. From each aquarium, samples of 160 mL of water were taken for chemical analysis (3 x 50 mL for DCOIT and 10 mL for elements), 5 mL for dynamic light scattering measurements, and 24 mL for ecotoxicity tests (pls. see full description in the last section), on time 0, 1, 3, 6, 9 and 12 weeks of immersion. The volume of ASW in the aquaria were adjusted weekly considering the sampling and evaporation.

2.2.1.1. Dynamic Light Scattering

The size (diameter) of the particles/aggregates/agglomerates in suspension over time was assessed by dynamic light scattering (DLS) in order to assess their behavior in a high strength media (ASW). A Zetasizer Nano-ZS (Malvern Instruments, UK) was used for the DLS measurements on sub-samples of 1 mL (in triplicate). Aliquots were sonicated for 10 minutes in an ultrasonic bath (Selecta; 40 kHz), immediately before each reading to avoid the particles settling. This procedure was conducted at CICECO, UA.

2.2.1.2. High Performance Liquid Chromatography

The content of released DCOIT to ASW from aquaria containing the plates coated with Sea-Nine and Sea-Nine@SiNC-based formulations as well as from the aquarium with only ASW was quantified by High Performance Liquid Chromatography (HPLC).

DCOIT was extracted and salts removed following a solid-phase extraction procedure. Cartridges (Finisterre C18 by Teknokroma™ 500 mg/6 mL) were first conditioned with 5 mL of methanol (in duplicate) and then with 5 mL of Milli-Q$^®$ ultra-pure (UP) water (in duplicate). Samples with a total volume of 50 mL were then loaded with a flow of 10 mL/min; then, the falcon was washed with 5 mL of 5% of methanol in UP water and loaded with the same flux the to ensure that all DCOIT was retained in the cartridges. After drying the cartridges, DCOIT was eluted with 5 mL of methanol, with a flow of 1 mL/min, in quadruplicate, and then, cartridges were dried again with nitrogen to force the elution of the analyte. The final volume (20 mL) was corrected with methanol whenever needed. Then, DCOIT concentration was determined in triplicate using a Shimadzu chromatograph, equipped with a SPD-M20A photodiode array UV-VIS detector, and a C18 reversed-phase Teknokroma™ column (TRACER EXCEL 120.5 µm x 25 cm x 0.46 cm) as the stationary phase and acetonitrile/ultra-pure water (90:10, v/v) as the mobile phase (flow rate: 1mL/min; temperature: 35ºC; injection volume: 50 µL). The peak was detected at retention time 6.7 min at 283nm. The calibration curve was performed in every time point using 6 DCOIT standards, dissolved in methanol; the correlation coefficient was higher than 0.999. All samples were filtered using a syringe with 0.22 µm PTFE filter to avoid contamination of the column. The column was washed with deionized water after the injection of each sample. This procedure was conducted at CICECO, UA.

2.2.1.3. Inductively Coupled Plasma Mass Spectrometry

The content of aluminum (Al), iron (Fe), copper (Cu) and zinc (Zn) was monitored by Inductively Coupled Plasma Mass Spectrometry (ICP-MS) in all leachates samples collected over time aiming at understanding if these elements were released from the coatings and there intrinsic constituents or added chemicals. A sample of 10 mL of leachate were collected in triplicate with a syringe filtered with a 0.22 µm cellulose acetate membrane. A total of 40 µL nitric acid (65%) was added to each sample and stored at 4ºC until analysis. Then, samples were diluted with ultra-pure water (1:20) and processed in the LCA, University of Aveiro using a Thermo, model X series.

2.2.1.4. Inductively Coupled Plasma – Optical Emission Spectrometry

Inductively Coupled Plasma – Optical Emission Spectrometry (ICP-OES) technique was also applied on samples from the week 6 to a have an overview of their elemental analysis, namel, alkali metals (lithium (Li), rubidium (Rb), celsium (Cs)), alkaline earth metals (beryllium (Be), strontium (Sr), barium (Ba)), metalloids: boron (B), arsenic (As), antimony (Sb)), nonmetals (phosphorus (P)), transition metals (vanadium (V), chromium (Cr), cobalt (Co), iron (Fe), manganese (Mn) nickel (Ni), copper (Cu), zinc (Zn), cadmium (Cd), post-transition metals (tin (Sn), lead (Pb), aluminum (Al)) and lanthanides (neodymium (Nd), samarium (Sm), europium (Eu), gadolinium (Gd), terbium (Tb), dysprosium (Dy), holmium (Ho), erbium (Er), thulium (Tm), lutetium (Lu), lanthanum (La), cerium (Ce), ytterbium (Yb)). Samples were collected similarly as the ICP-MS. They were also processed in the LCA, University of Aveiro using a Horiba Jobin Yvon, model Activa M. without any dilution.

2.2.2. *Leachates ecotoxicity*

Using three species chosen based on some features such as ecological relevance, laboratorial maintenance, short life cycles and contaminant sensitivity. A target species: *Phaeodactylum tricornutum* (a diatom) and two non- target species *Tetraselmis chuii* (a green microalgae the crustacean *Artemia salina* were performed acute toxicity tests.

The preparation of each pre-culture of the microalgae was performed 4 days before the beginning of each test, using artificial saltwater (ASW) with reverse osmosis water and added artificial salt Tropic Marin® Pro-Reef, to salinity 35, then filtered (0.45 µm) and autoclaved at 120ºC for 20 minutes. The medium to the pre-culture was made by adding 2mL/L of Optimedium to the ASW prepared and 2 mL of each microalgae, in Erlenmeyer's (250 mL) with 150 mL of medium, to enable gas exchange. The microalgae were maintained in laboratory at room temperature (19±1ºC) and photoperiod of 16:8h (light: dark) and agitated daily.

In 24 well plates were added 1 mL of medium (water from the aquarium) and 1 mL of microalgae. Each plate had a negative control (not contaminated ASW; n=8) and two testing coatings (n=8 each), for the microalgae and for the crustacean n=6. On each day (0h, 24h, 48h and 72h) the cell density of the microalgae test was measured via spectrophotometry at 700 nm. To ensure a homogenized measurement, each well was resuspended with the help of a Pasteur pipette immediately before each reading.

2.2.2.1. Crustaceans acute toxicity

The *Artemia salina* dry cysts hydration lasted 30 minutes in 300mL of reverse osmosis water, aeration and lighting. To check the full hydration was collected a sample and under a binocular lens, and then added 700mL of ASW (salinity 35), to the final salinity of 25. The organisms hatched after 16-24 hours. After 24h, the stage 2nd/3rd is archived and those organisms in that stage are used after washed in new ASW. The mortality was observed after 24 and 48h (end of the test) and confirmed under a magnifying glass observation if the immobilization was superior to 10 seconds.

2.2.2.2. Microalgae growth inhibition effects

The tests were performed by following the guideline ISO 10253.2016. The growth inhibition of each species was calculated at 72 hours was, following the equation:

$$\% \text{ Ir} = \frac{(\mu c - \mu T)}{\mu c} x100$$

% Ir: percent inhibition in average specific growth rate;

- *μC:* mean value for average specific growth rate (μ) in the control group;
- *μT:* average specific growth rate for the treatment replicate):

2.2.2.3 Statistical analysis

Data normality and homoscedasticity were tested with the Shapiro-Wilks tests. If these assumptions were verified, a one-way analysis of variance (one-way ANOVA) test was performed using the software *SigmaPlot v.12.5* to evaluate significant differences among treatments and both commercial reference and the coating without biocides ($p<0.05$). Additionally, leachates toxicity was also compared between coatings modified with biocides in its free and nano-forms (Sea-Nine vs Sea-Nine@SiNC; CuPT vs CuPT@Zn-Al LDH; CuPT vs Cu-Al LDH and CuPT@Zn-Al LDH vs Cu-Al LDH). In the presence of differences, a Tukey test followed the ANOVA. If data failed the normality and/or the homoscedastic assumptions data was transformed (i.e., square, Ln, Log_{10}, reciprocal, exponential and square root). If these transformations did not comply with the aim for normality and homoscedasticity, a one-way ANOVA on Ranks was run, followed by a Dunn's test.

2.3. *Field efficacy testing*

2.3.1 Experimental set-up

The antifouling efficacy of tested paints was evaluated by placing an experimental set up in the marina of Gafanha da Encarnação (40.628320, -8.735606) (Figure 4), on October 2018, containing the 9 coatings (1 reference without AF biocides, 1 state-of-the-art coating and 7 test AF formulations with different additives; pls. cf. Table 3), in triplicate, in a total of 27 panels. The place was chosen based on parameters, such as water renovation (close to the "mouth" of the marina and the entrance of the Ria de Aveiro lagoon), solar orientation (south), water depth, constant salinity, in fully agreement with the guideline ISO 15181-1:2007. The depth below the surface of the water was variable according to the tides, without records of emersion periods. The set-up was not protected with cages to mimic predation as in real conditions. The marina is a private area with controlled access ensuring the security of tested system over the entire immersion time.
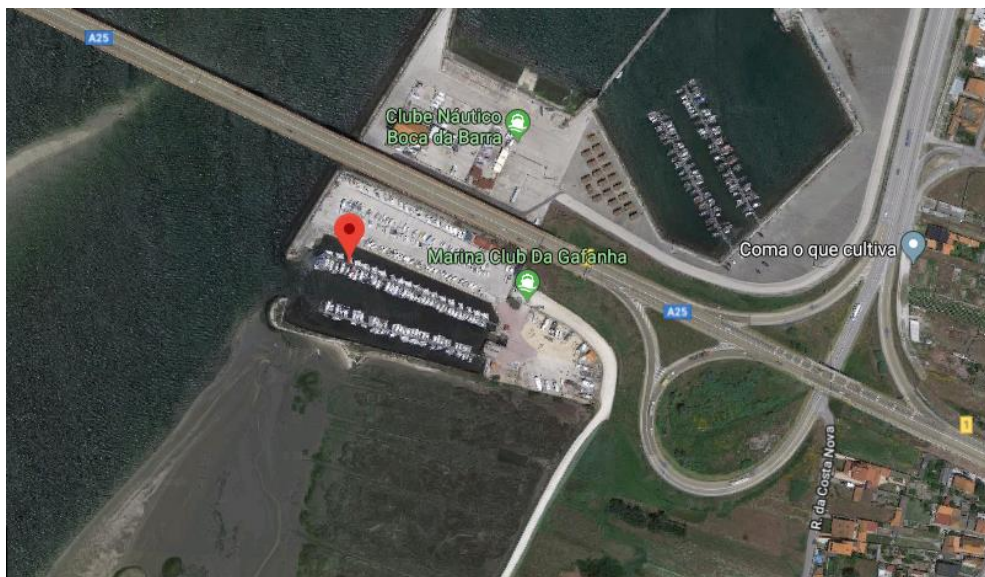
Figure 4: Localization of the field experimental setup, marina of Gafanha da Encarnação, Aveiro, Portugal

Figure 5: A: Immersion of the system containing 7 out of 9 coatings (Commercial reference, CuPT, CuPT@Zn-Al LDH, Sea-Nine, Sea-Nine@SiNC, SiNC and Cu-Al LDH); B: Immersed Larger system; C: Dry smaller system (with the coatings Zn-Al LDH and Blank reference; D: Immersed smaller system

The 12 x18 cm plates were tied in a plastic net with 21 cm between each coating and 1 cm between each replicate (n=3), positioned vertically and perpendicularly to the bottom (Figure 5). Unlike other similar studies (Cassé *et. al.*, 2006), the experimental set-up was permanently immersed following the abovementioned ISO guideline, and suspended at approximately 25 cm above the sediment. The largest system, containing the Commercial reference, CuPT, CuPT@Zn-Al LDH, Sea-Nine, Sea-Nine@SiNC, SiNC and Cu-Al LDH coated plates, was fixed between two PVC pipes, pendant in hooks to the platform with the objective of allowing the plates to go up and down consonant the amplitude of the tides. The smaller system, containing the unloaded Zn-Al LDH (a low-toxic nanomaterial without biocides; ex. Avelelas *et. al.,* 2017) and Blank reference coated plates, was immersed five meters forward of the other, in the same conditions of the other (one-meter depth, southern-dominant solar exposure). A separation of the coatings in two different systems was chosen to avoid the effects of the biocides leaching from the coatings without any biocides. Each column (with three similar plates) is composed by the same coating, to avoid cross contamination of the leachates.

2.3.2 Sampling

Biological sampling included microbiological and microphytobenthos (diatoms). Microfouling was sampled periodically, according to the expected settlement of foulers: first (11/10/2018), third (25/10/2018), sixth (15/11/2018), ninth (06/12/2018) and twelfth (27/12/2019) week of immersion. Then, plates were photographed once a month to evaluate the presence of fouling, particularly macrofouling.

In each period, a sample of 3 x 3 cm ($9cm^2$) was taken for bacteria (1, 3 and 6 weeks of immersion) and microphytobenthos (1, 3, 6, 9 and 12 weeks of immersion), collected by gentle scraping without damaging the paint surface. The sampling area was randomly using the software Research Randomizer. Every sampling was made in a different area each week, as the numbers never repeated itself in the same plate, for the same biological endpoint (Figure 6).
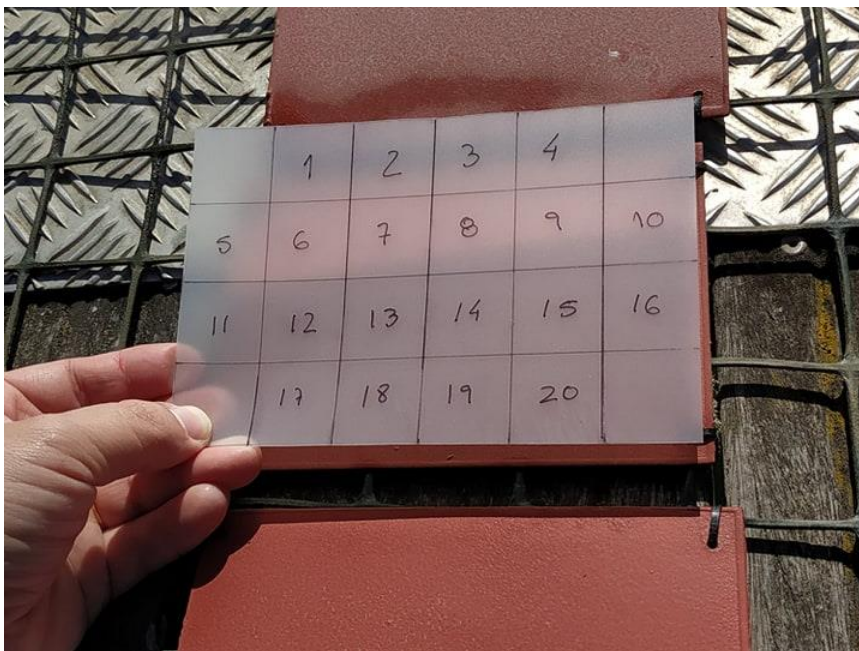
Figure 6: Auxiliary sheet to locate randomized areas for collecting both bacteria and microphytobenthos samples.

The sampling area was previously watered with 1 mL of 0.22 µm filtered natural seawater collected in the marine for both bacteria and diatoms. Water was filtered in order to remove particles and microorganisms present in water. In the case of the samples for microphytobenthos, the sampling area was gently scraped with the tip of a Pasteur pipette

(without damaging the coating); then, the sample was collected with the pipette to a microtube and the final volume adjusted to 1.5 mL. Bacteria were collected by swabbing the surface with a sterilized swab, which was then cut to a length of 2.5 cm and placed on a sterilized 2 mL microtube. The material was carefully sterilized among samples to avoid cross-contamination. Samples were preserved in liquid nitrogen and then stored in a -80ºC ultra-freezer until the DNA extraction.

Relevant seawater physico-chemical parameters were measured weekly. To measure the salinity was used a refractometer (V^2 refractometer). The dissolved oxygen and temperature were obtained by an oximeter (WTW Oxi 330 Oxygen meter with CellOx 325 sensor). The pH was measured using a Hanna HALO pH probe (with Bluetooth® Smart technology-Hanna Instruments).

2.3.3 Characterization of the microbiological communities

In order to characterize the bacterial communities in the first six weeks of immersion, sample pools (n=1 per coating system) were submitted to a step of DNA extraction and a Polymerase Chain Reaction (PCR) followed by a Denaturing Gradient Gel-Electrophoresis (DGGE).

The total genomic DNA extraction and respective PCR were performed during November 2018, following the protocol provided by PureLink™ Microbiome DNA Purification Kit Used Guide (Rectal or Environmental Swab Samples). The extracted DNA was kept in the refrigerator at 4 ºC. Before each use, they were slowly thawed with ice to avoid thermal shock and DNA damage. The PCR, a critical step to confirm the correct amplification of the samples and markers, was accomplished using a C1000Touch™ Thermal Cycler. The PCR was performed using a reaction mixture (25 µL) containing 12.50 µL NZYTaq 2 x Green Master Mix (2.5 mM $MgCl_2$; 200 µM dNTPs; 0.2 U µL^{-1} DNA polymerase, NZYtech, Portugal), 10.00 µL of ultrapure water and primers, namely 0.75 µL of 799F_GC (forward primer) and 0.75µL of / 1115R (reverse primer) (cf. Table 4). The program for PCR reaction included the following heating steps: 95ºC for 7 min, 35 cycles of 95ºC for 30 (denaturing), 53ºC for 1 min (annealing), 72ºC for 1 min (extension), and 72ºC for 30 min. The PCR products were then charged in 1.5% agarose gel to perform the electrophoresis, a technique which creates many copies of a particular DNA region *in vitro* by amplifying the 16S rDNA region (Muyzer, De Wall and

Uitterlinden, 1993). The DGGE followed the protocol of Bio-Rad DCODE gel electrophoresis gel system. This was performed on a D-Code Universal Mutation Detection System (Bio-Rad) with in 1xTAE buffer (Sigma-Aldrich, Germany) at 60ºC, 90V, for 1 h. The DNA molecular weight marker *Methylobacterium sp*. N355 (GeneRuler™ DNA ladder Mix – Thermo Fisher Scientific, USA) was used as a reference standard. In each gel, a combination of sample pools was made, as follows: week 1 vs. week 3, week 1 vs. week 6 and week 3 vs. week 6 (note: samples can be compared within each gel, however gels should not be compared due to methodological constraints). In the end of this step, the gel was stained in a solution of 0.5 µg mL^{-1} ethidium bromide (Sigma,USA) for 20 min., and then washed in distilled water. An UV light with the imaging Molecular Imager® Gel Doc™ XR+ System (Bio-Rad, USA) was used to capture the gel images.

Table 4: Primers tested for 16S rRNA gene amplification (Chelius and Triplett, 2001)

Forward primer name	Reverse primer name	Forward primer sequence (5'-3')	Reverse primer sequence (5'-3')	Annealing T(ºC)
799F_GC	1115R	AACMGGATTA GATACCCKG	AGGGTTGCGCTCG TTG	53

For DGGE analysis, banding patterns similarity was analyzed with the Bionumerics Software (Applied Maths, Belgium). A classification analysis (i.e. dendrogram) was run using the Pearson coefficient based on the densiometric curve values. Bacterial diversity was estimated by the richness index (S), Shannon index of diversity (H') (Shannon and Weaver, 1949) and Pielou's evenness index (J), calculated using PRIMER v6 software (Primer-E Ltd, Plymouth, UK).

2.3.4 Characterization of the microphytobenthos communities

Fluorescence measuring techniques are often used to analyze the levels of chlorophyll *a* present in photosynthetic organisms, and also to assess the microalgal biomass, via pulse amplitude modulated (PAM) fluorometry (Herlory, Richard and Blanchard, 2007). Thus, in the present study, microphytobenthos communities of the biofouling early stages were characterized in terms of biomass, photosynthetic yield and abundance. Samples collected in the field were rapidly analyzed in the laboratory in terms of fluorescence. Then, samples were fixed in lugol (1%). Fluorescence measurements were performed in a Multiple Excitation Wavelength Chlorophyll Fluorescence Analyzer MULTI-COLOR-PAM, which is a very sensitive instrument to photosynthesis parameters, in suspended solutions of algae, cyanobacteria and other photosynthetic forms. Records had a dark and light phase, which included a modulated measuring light (ML) at wavelength of 440 nm and actinic light (AL) white color (the most adequate for this kind of samples). To assess these parameters was used the software PamWin-3 (Walz, Germany). It measures in the darkness: the minimum fluorescence (F_0), as proxy for biomass values, maximum fluorescence (F_m), variable fluorescence (Fv), the maximum light utilization efficiency of photosystem II (PS II) (Fv/Fm) $\left(\frac{Fv}{Fm} = \frac{Fm-0}{Fm} \right)$ and Y(II), the effective photochemical quantum yield of PS II (Taylor *et al.*, 2005). The F_0, Fm, and Y(II) were measured to obtain the yield and biomass of the samples.

The diatoms abundance was also determined by direct counting using the Neubauer chamber, in triplicate.

2.3.5 Characterization of the macrofoulers communities

In the field, photo sampling was performed, first by following the sampling times of mentioned above and then began to take place monthly up until one year of immersion. The software ImageJ was used to estimate the area covered by each major fouling components: biofilm, macroalgae and macroinvertebrates over time.

2.4. *Multivariate data analysis*

Multivariate analysis was used to discriminate groups of coatings in terms of efficacy and toxicity over the first 3 months of immersion.

The efficacy (coverage of biofilm, macroalgae and macrofoulers; yield, biomass and abundance of microphytobenthos) and toxicity (diatom and microalgae growth inhibition; crustacean acute toxicity) data matrix per coating in each sampling period was normalized transformed and the Euclidean distance matrix calculated between variables. The dissimilarity matrix was analyzed using ordination analysis, with principal coordinates (PCO). Most correlated physicochemical variables (Spearman rho>0.7) were superimposed as blue vectors. This statistical analysis was performed using the software PRIMER & PERMANOVA v.6.

3. Results

3.1.2.1 Metallography

The images obtained via a stereo microscope (Nikon SMZ18) of the surface of each painted plate are represented with letters in Figure 7. The images revealed that the roughness of the surface was higher in the coatings containing nanomaterials (C, D, E, H and I). Coatings with free booster biocides have a smooth surface, as well as the controls (Commercial and Blank reference).

A: Commercial reference	*B: Blank reference*	*C: Cu-Al LDH*
D: Zn-Al LDH	*E: CuPT@Zn-Al LDH*	*F: CuPT*
G: Sea-Nine	*H: SiNC*	*I: Sea-Nine@SiNC*
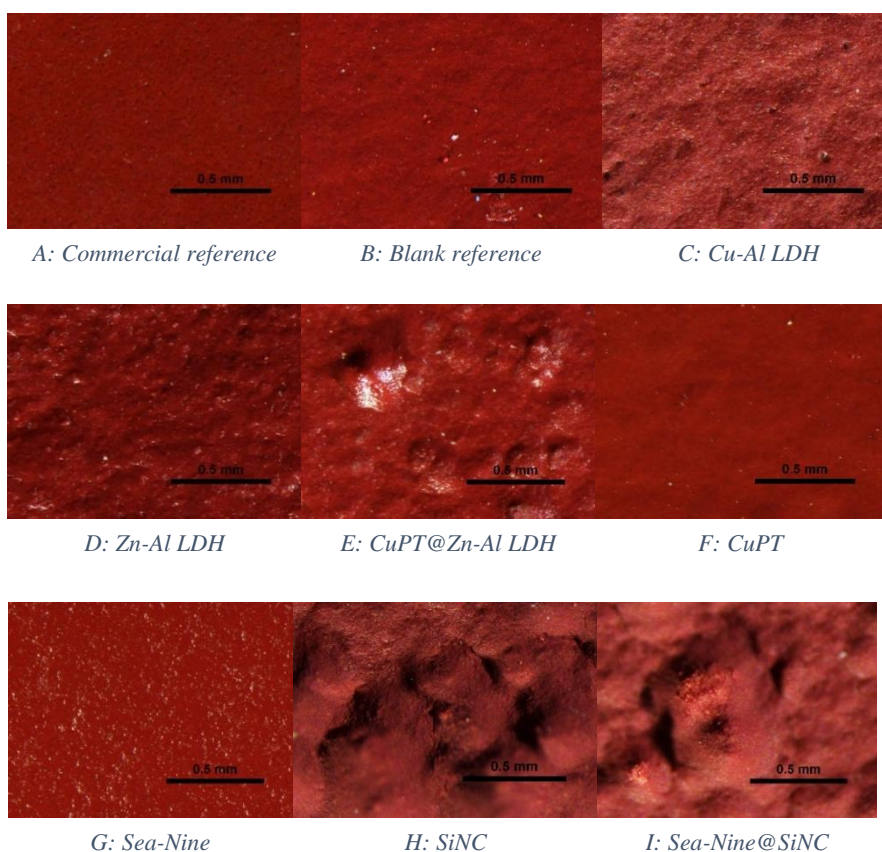
Figure 7: Top view of the coatings surface using a stereo microscope

The images obtained via a stereo microscope (Nikon SMZ18) of the cross section of each painted plate are represented with letters in Figure 8. In the images is possible to distinguish clearly the plate (black), the primer (beige) and the topcoat (red). The images revealed that the thickness varies between the different coatings.

The Blank reference (B), Zn-Al LDH (D) and CuPT (F) demonstrate a clear and homogeneous separation between the primer and the top-coat. The CuPT@Zn-Al LDH (E) seems to reveal absence of the primer layer and the topcoat which is not clearly seen in CuPT@Zn-Al LDH coating (E).

Coatings with silica nanocapsules (H and I) were not homogenously attached to the primer, possibly due to the presence of air in the moment of application or big aggregates of additives. However, this was not a relevant factor for the efficacy of the coating.

A: Commercial reference	*B: Blank reference*	*C: Cu-Al LDH*
D: Zn-Al LDH	*E: CuPT@Zn-Al LDH*	*F: CuPT*
G: Sea-Nine	*H: SiNC*	*I: Sea-Nine@SiNC*
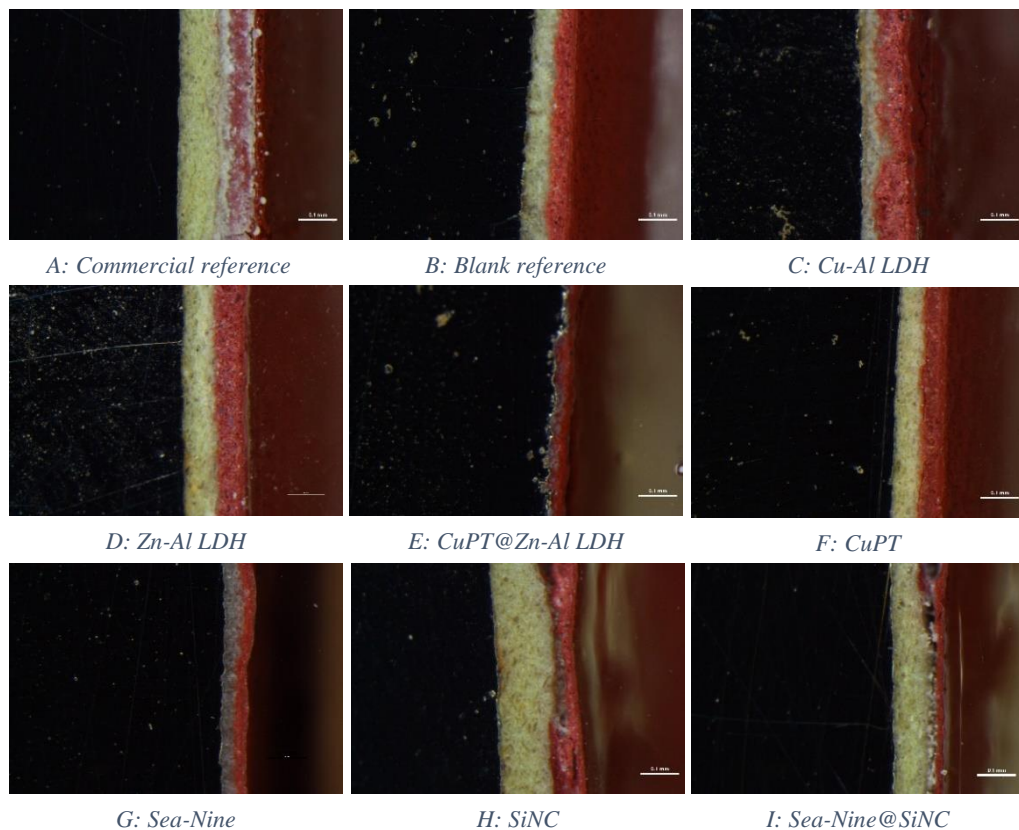
Figure 8: Images of the cross section of each plate coated using stereo microscope (Nikon SMZ18) (magnification 13.5nm)

3.1.2.2 FTIR

a) Comparison between the Blank reference (no biocides) and the Commercial reference (Sea Quantum Ultra S)

Broadness in the region between 700-400 cm^{-1} is indicative of the presence of inorganic fillers, namely metallic oxides (Figure 9A). Also, a sharp peak around 878 cm^{-1} reveals the presence of other filler, inorganic carbonate, in the blank reference spectrum. The presence of organic aliphatic components of solvents and binders is demonstrated by C–H asymmetric and symmetric stretching (2860-2950 cm^{-1}) and bending vibrations (1350-1480 cm^{-1}) (Figure 9A). Peaks around 1730 and 1155 cm^{-1} may refer to C=O and C-O stretching vibrations of ester groups and broad peaks at 3300 cm^{-1} are characteristic of O–H group vibrations (Figure 9A). The spectrum of the commercial reference is weaker in terms of peak intensity, biocides are not traceable, and, as expected, no main chemical differences are noticeable (Figure 9A) since the blank reference is the raw formulation (without active ingredients) that Jotun uses to produce the Sea Quantum Ultra S (commercial reference).

b) Comparison between the coating with Cu-Al LDH-nitrate and Zn-Al LDH-nitrate

Differences between spectra of the topcoat with copper- or zinc-based LDH are not perceptible (Figure 9B). The presence of LDH material is not traceable when compared to the blank reference (spectra not shown; cf. Figure 9A).

c) Comparison between the coating containing SiNC and the biocide (Sea-Nine) encapsulated in nanocapsules mesoporous of silica (Sea-Nine@SiNC)

Figure 9C shows the spectra of the coating containing empty silica nanocapsules and the respective powder of this nanomaterial, while Figure 9D shows the FTIR spectra of the encapsulated form of the biocide Sea-Nine, as well as the powder form of the free biocide respectively. Figure 9E shows the spectra of the SiNC topcoat, the loaded form of the silica nanocapsules (Sea-Nine@SiNC) and the blank reference. The presence of SiO$_2$ capsules is confirmed by the characteristic Si–O stretching and rocking bands, located at 1066 and 444 cm^{-1}, respectively, in the SiNC_particle and Sea-Nine@SiNC spectrum.

On the other hand, the presence of Sea-Nine in the coating is not confirmed although some peaks are coincident.

d) Comparison of coatings containing the biocide CuPT and the biocide (CuPT) immobilized in LDH (CuPT@Zn-Al LDH)

Figure 9F represents the spectra of the blank reference, as well as the free biocide CuPT topcoat and the biocide immobilized (CuPT@Zn-Al LDH). The presence of copper pyrithione biocide was detected in both coatings containing free and intercalated CuPT (in Zn-Al LDH). This could be confirmed by the presence of aromatic C-H stretching around 3020-3098 cm^{-1} and C=C stretching at 1544 cm^{-1}, and C-N stretching at 1195 cm^{-1} from the pyridine backbone.
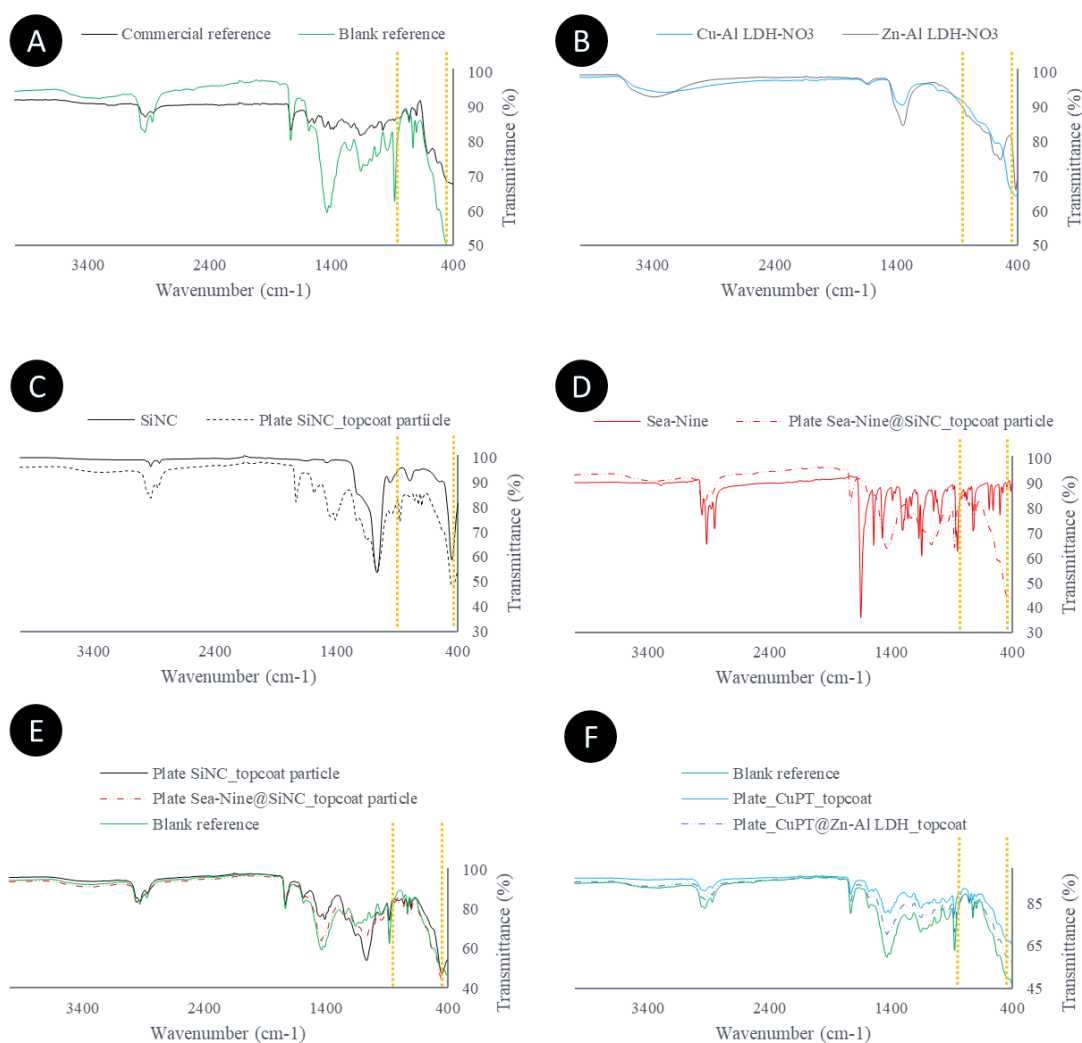
Figure 9: FTIR representation of the coatings topcoats and particles. A: FTIR spectra of Commercial reference and Blank reference; B: FTIR spectra of coating containing Cu-Al LDH-nitrate and Zn-Al LDH-nitrate; C: FTIR spectra of SiNC powder and SiNC topcoat; D: FTIR spectra of Sea-Nine powder and Sea-Nine@SiNC topcoat; E: FTIR spectra of SiNC top-coat, Sea-Nine@SiNC top-coat and Blank reference; F: FTIR spectra of CuPT topcoat and CuPT@Zn-Al LDH topcoat with Blank reference as reference.

3.2. Coating leachates

3.2.1. Characterization and behavior of leachates over time

3.2.1.1 DLS

Table 5 resumes the average diameter of the suspended particles in each aquaria considering all sampling times. Figure 10 represents the average diameter of the particles in suspension over time in the different aquaria. In some samples, large aggregates/agglomerates (sub-micron sized) were detected in the leachates over time, indifferently of the main additive of the tested coatings. The sample control (ASW) does not have any plate, only artificial salt water.

Table 5: Resume of samples size (average of the sampling times)

Sample	Size (average) (nm)
Control (ASW)	539.6
Blank reference	478.1
Commercial reference	294.8
Cu-Al LDH	408.1
CuPT	545.9
CuPT@Zn-Al LDH	362.5
Zn-Al LDH	598.0
Sea-Nine	452.4
Sea-Nine@SiNC	617.2
SiNC	505.1
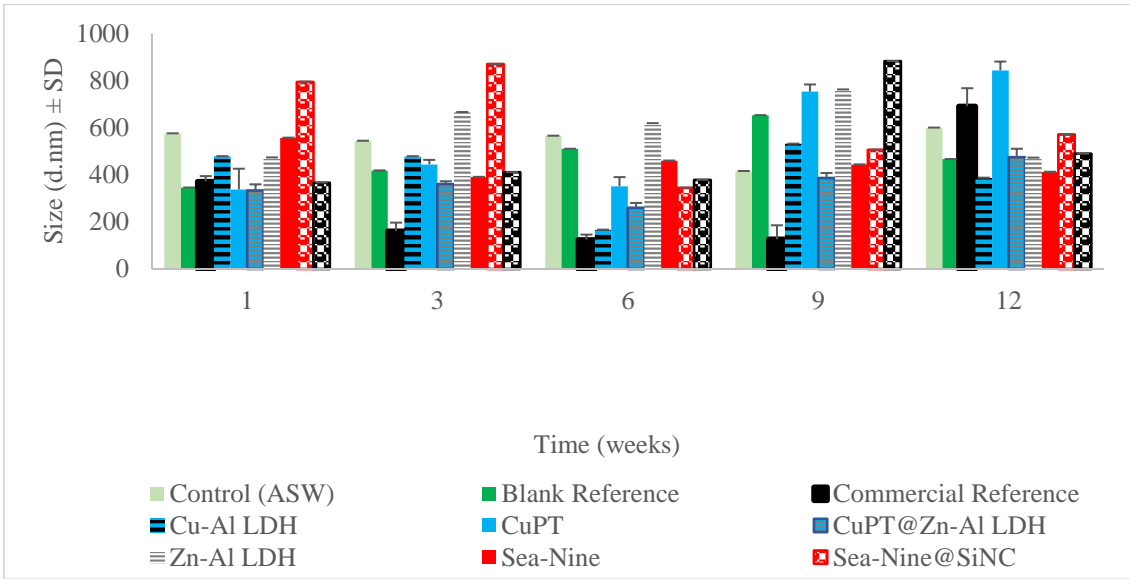
Figure 10: Representation of leachates size (average) of each testing coating over time

3.2.1.2 HPLC

Table 6 shows the concentrations of Sea-Nine obtained by HPLC, in leachates of the coatings containing Sea-Nine (both free and encapsulated forms), as well in the aquarium with only artificial saltwater). The presence of Sea-Nine in the leachates was confirmed at 6.7 minutes of retention time. As expected, Sea-Nine was not detected in the control samples over time. The coating with Sea-Nine released the biocide over time, which is not detected in the leachates of the coating with encapsulated Sea-Nine.

Table 6: Concentration of Sea-Nine (µg/L) in artificial seawater and leachates of Sea-Nine@SiNC and Sea-Nine coatings between 0 and 12 weeks of immersion, detected by HPLC (n=3). "N/d" – Not detected.

Sampling time (weeks after immersion)	Artificial seawater	Leachates coating	
		Sea-Nine@SiNC	Sea-Nine
0	N/d	N/d	N/d
1	N/d	N/d	0.12±0.10
3	N/d	N/d	0.17±0.01
6	N/d	N/d	0.36±0.02
9	N/d	N/d	0.15±0.00
12	N/d	N/d	0.29±0.01

3.2.1.3 ICP-MS

The ICP-MS analysis revealed that the concentration of aluminum was below the detection limit of the equipment (except in leachates of Sea-Nine@SiNC on time 9 (22 ± 1 µg/L) (Figure 11). Figures 12 to 14 shows the results of Cu, Fe and Zn in leachates of all coatings over 12 weeks of immersion. Levels of Cu are very high in leachates of the Commercial reference (> 1 mg/L). Cu levels tend to increase over time in most cases, particularly in the CuPT, CuPT@Zn-Al LDH and Cu-Al LDH coatings (Figure 12). Basal levels of Fe are quite constant until 6 weeks of immersion, however some leachates tend to be richer on Fe after 9 weeks of immersion (Figure 13). Globally, levels of Zn tend to increase over time in all leachates, in particular, the case of the leachates of the coating containing CuPT immobilized in Zn-Al LDH (Figure 14).
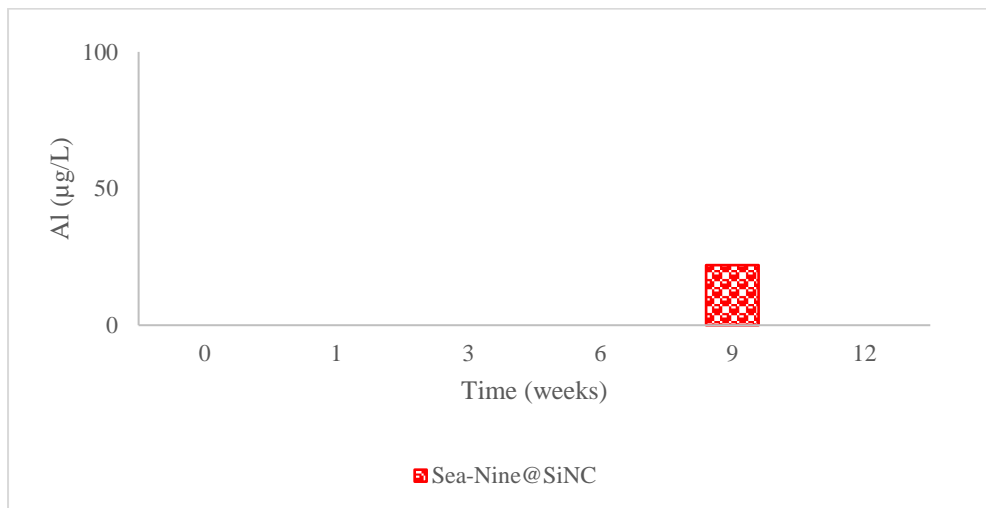
Figure 11: Aluminum concentration over time in coating's leachates for 12 weeks of immersion in laboratorial conditions (no bar: samples below the detection limit of the equipment).
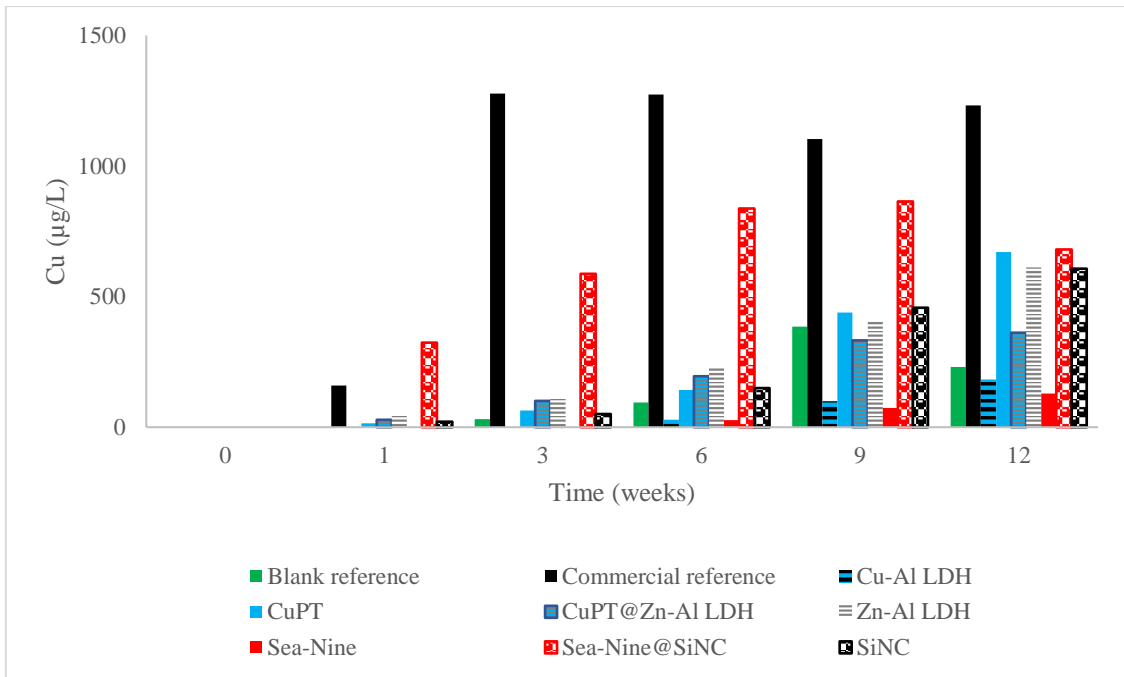
Figure 12: Copper concentration over time in coating's leachates for 12 weeks of immersion in laboratorial conditions (no bar: samples below the detection limit of the equipment).
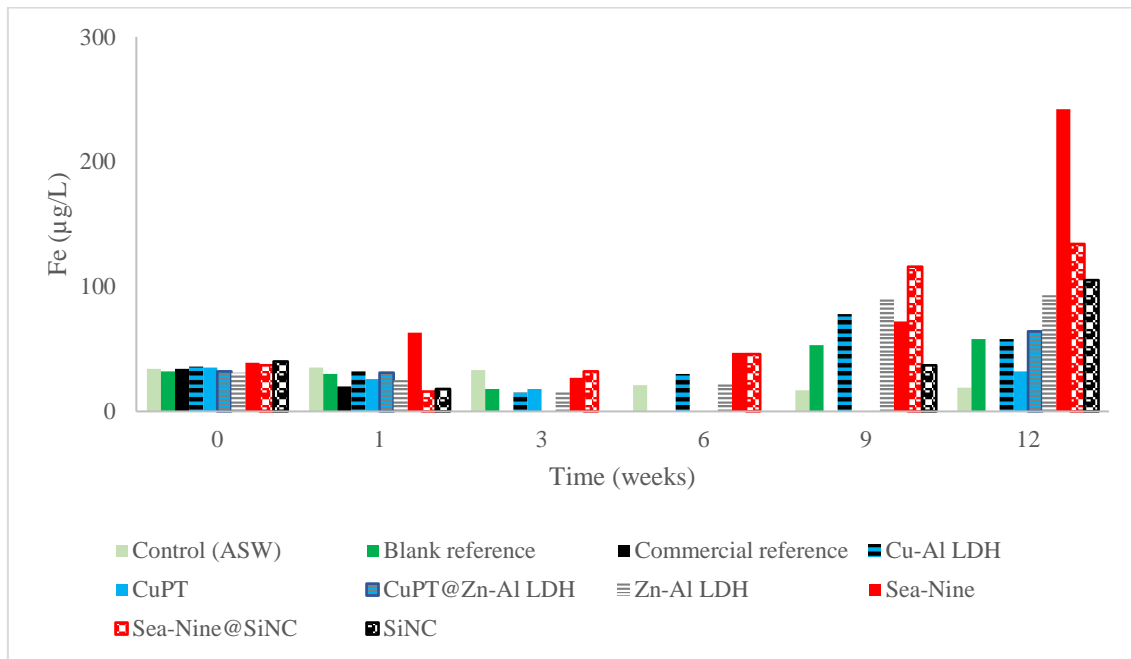
Figure 13: Iron concentration over time in coating's leachates for 12 weeks of immersion in laboratorial conditions (no bar: samples below the detection limit of the equipment).
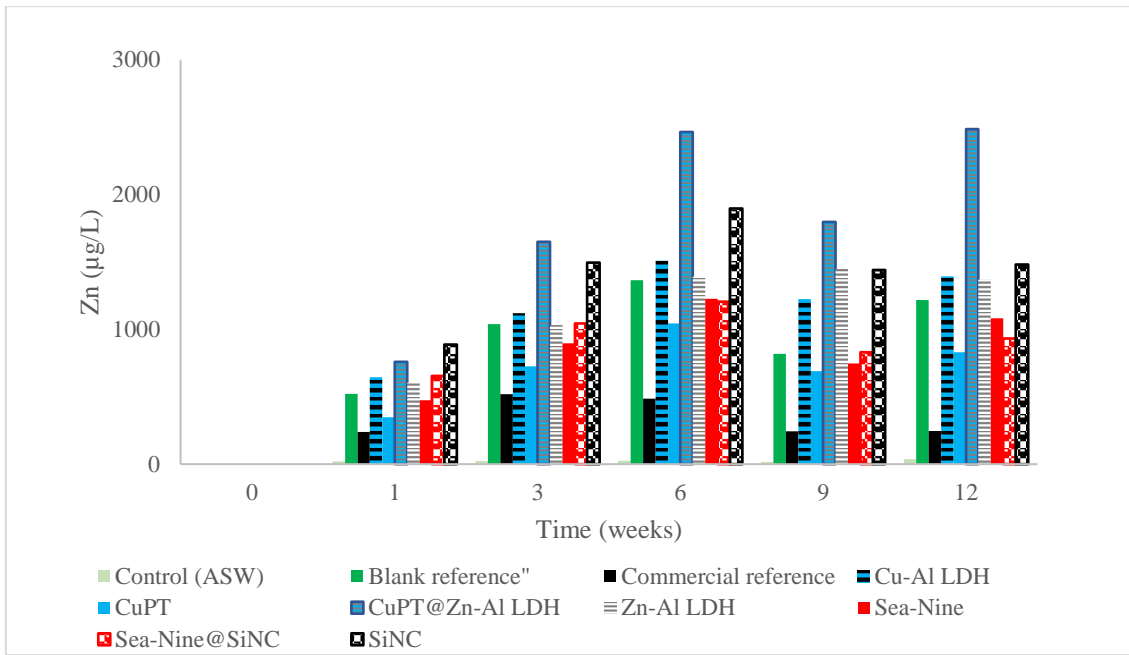
*Figure 14: Zinc concentration over time in coating's leachates for 12 weeks of immersion in laboratorial conditions
(no bar: samples below the detection limit of the equipment).*

3.2.1.4 ICP-OES

A full elemental analysis was carried in leachate samples after 6 weeks of immersion, via ICP-OES. Several elements were found to be below the detection limit, namely, Be, Al, V, Cr, Co, Ni, As, Cd, Sn, Sb, Cs, La, Ce, Pr, Nd, Sm, Eu, Gd, Tb, Dy, Ho, Er, Tm, Yb, Lu and Pb. Figure 15 shows the concentration of the remain elements. Zn, P, B and Sr were found in mg/L levels; no apparent enrichment was detected between samples apart in some case for Cu and Zn (as previously detected in the ICP-MS analysis).
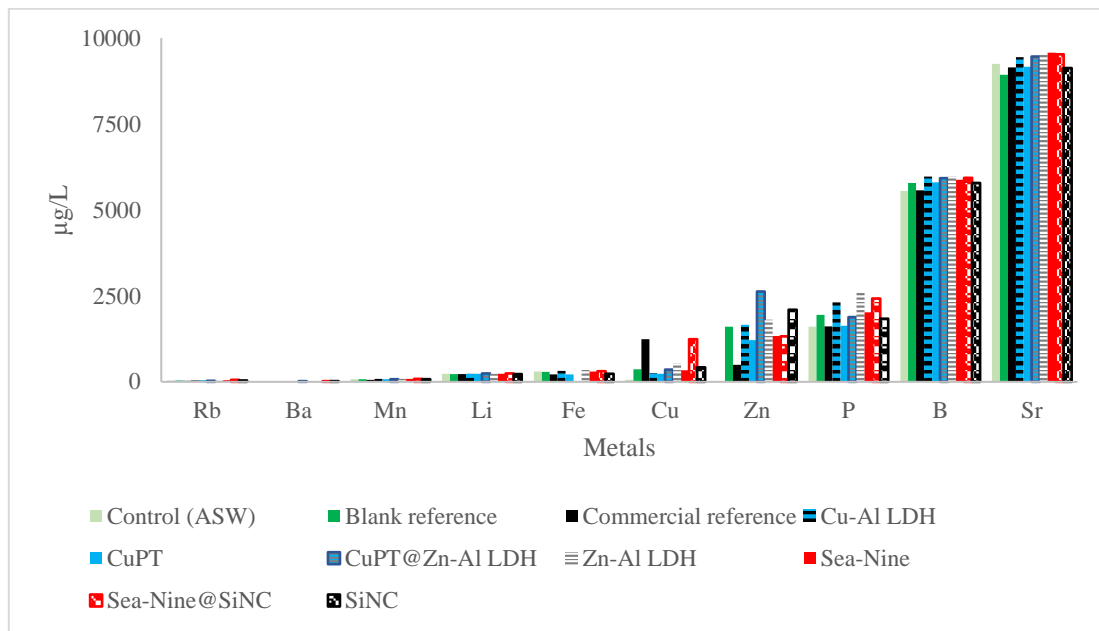
Figure 15: Concentration of the elements present on each coating sample from time 6, obtained via ICP-OES (no bar: samples below the detection limit of the equipment).

3.2.2 Ecotoxicity of leachates

3.2.2.1 Crustacean acute toxicity

Figure 16 shows the results of the coating leachates acute toxicity towards the microcrustacean *Artemia salina*. From the testes coatings, including the control with only ASW, those that did not induced any toxicity at all times sampled, were removed from the caption. Leachates from the coating containing the free biocide Sea-Nine revealed acute toxicity on these crustaceans (100% mortality) as early as in the first week of immersion and afterwards. Leachates from remaining coatings caused no significant acute effects on *A. salina* (maximum of 10% in the case of the commercial reference on time 1).
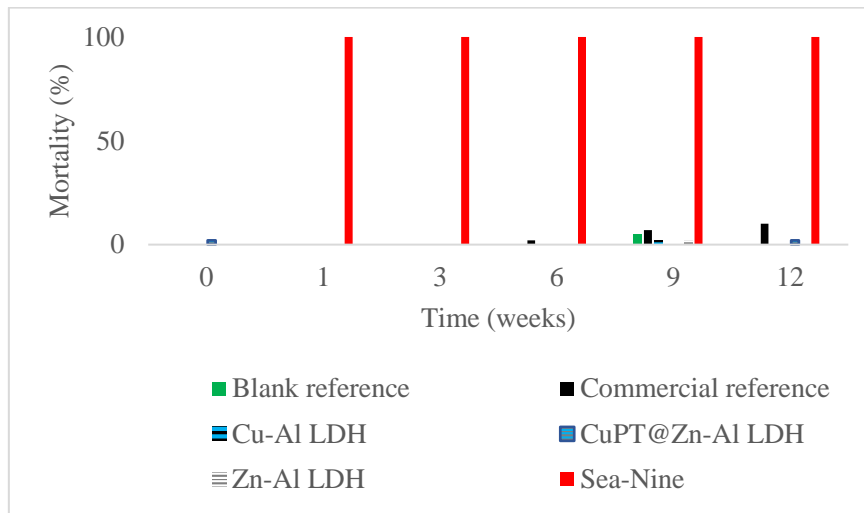
Figure 16: Acute toxicity of the tested coatings leachates (up to 12 weeks of immersion) in Artemia salina.

3.2.2.2. Microalgae growth inhibition effects

Figure 17 shows the *Tetraselmis chuii* growth inhibition results after 72 h exposure to the coatings leachates generated during 12 weeks of plates immersion in ASW. Leachates from the coating containing free Sea-Nine showed toxic effects since time 0; additionally, the growth inhibition effects were significantly different when compared with the equivalent nanoform (Sea-Nine@SiNC). The most toxic leachates, over time, were those released from the commercial reference, CuPT and Sea-Nine, significantly different from the blank reference. Coatings containing copper revealed significant differences from the blank reference since week 3, and between them since week 6. Leachates from coatings having nanostructured biocides exhibited much lower toxicity than the free booster biocides and also the blank reference, for this non-target species.
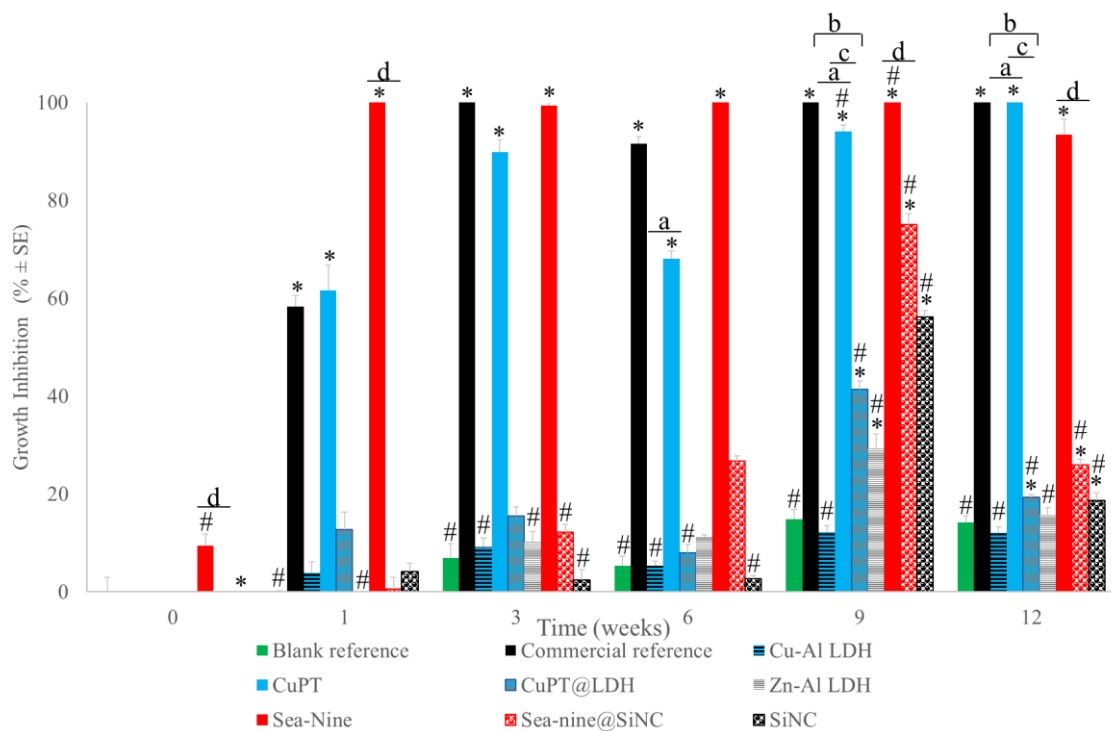
*Figure 17: Tetraselmis chuii growth inhibition caused by tested coatings leachates (up to 12 weeks of immersion). *: Blank reference vs treatments; #: Commercial reference vs treatments; a: Cu-Al LDH vs CuPT; b: Cu-Al LDH vs CuPT@Zn-Al LDH; c: CuPT vs CuPT@Zn-Al LDH*

Figure 18 shows the results of the 72 h exposure of *Phaeodactylum tricornutum* to the leachates over time. On time 0, none of the leachates coatings caused growth inhibition. The most toxic leachates, over time, were the commercial reference and both biocides in free state (CuPT and Sea-Nine), right after 1 week of immersion. It is noted that the encapsulation of the biocides reduces their toxicity as expected, reaching maximum a 50% growth inhibition. The statistical analysis confirmed the differences between the three most toxic coating leachates and the remaining tested paints.
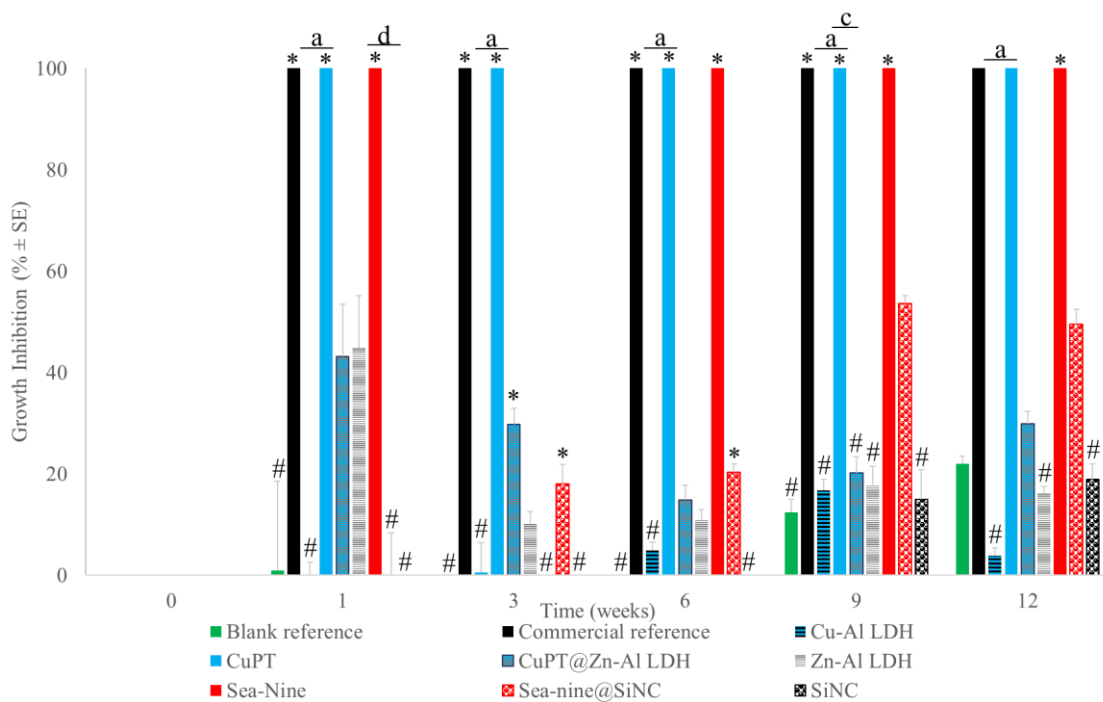
Figure 18: Phaeodactylum tricornutum growth inhibition caused by tested coatings leachates (up to 12 weeks of immersion). *: Blank reference vs treatments; #: Commercial reference vs treatments; a: Cu-Al LDH vs CuPT; c: CuPT vs CuPT@Zn-Al LDH; d: Sea-Nine vs Sea-Nine@SiNC.

3.3 Field efficacy testing

Table 7 shows the physicochemical parameters in the field during the microfouling sampling period (first 3 months).

Table 7: Physicochemical parameters measured in the marina's seawater during the first three months of immersion.

Sampling Time (week)	Day (dd/mm/yy) + Time	Dissolved Oxygen (%)	pH	Salinity	Temperature (ºC)
1	11/10/2018 (15:40h)	85.7%	7.98	34	19.1
3	25/10/2018 (15:34)	76.0%	7.91	32	17.8
6	15/11/2018 (12:05h)	68.0%	7.98	36	15.4
9	06/12/2018 (13:04h)	75.6%	7.84	33	14.3
12	27/12/2018 (12:32h)	76.0%	7.87	35	13.8

The formation of a transparent biofilm was confirmed by the microbiological samples, since the first week of immersion, but not visually. The appearance of macrofouler species was verified being progressive and variable according to the tested coating. Macroalgae appeared in some plates after 9 weeks of immersion, as in the Blank reference, Cu-Al LDH Sea-Nine@SiNC and SiNC, and progressively increased over time. In its turn, marine invertebrates, such as ascidians and bryozoans (Figure 19), started to appear after 28 weeks of immersion, in the blank reference coating (without biocides).

Figure 19: Photographic record of biofouling in blank reference plates showing the presence of ascidians and bryozoans (whitish organisms) and macroalgae (green or brown).

The blank reference and the coating with Zn-Al LDH showed the lowest AF efficacy and started failing from week 9 (blank reference) and 12 (Zn-Al LDH) proven trough the weeks for macroalgae appearance and the last percentage coverage registration. All remaining coatings had a macrofauna coverage of 0% after a half year of immersion and less than 5% after a year of immersion (Figure 20 and 21).

After a year of immersion, the commercial reference had low macroalgae coverage (~6%). The modified coating with the most similar behavior were Cu-Al LDH, CuPT and Sea-Nine-SiNC, which had a maximum macroalgae percentage coverage of ~34%.

Coatings with free biocides had no records of macrofauna attached to the surface of the plates over the entire period, however plates were covered with macroalgae up to ~25% in the case of CuPT and ~52% for Sea-Nine, after a year of immersion. The AF efficacy of the coatings with novel nanoadditives increased in the case of Sea-Nine@SiNC (macroalgae coverage decreased to ~34%; residual presence of invertebrates ~0.3%), or it was similar in the case of CuPT@Zn-Al LDH coating (~27% of macroalgae percentage coverage). The coating with Cu-Al LDH had a performance similar to Zn-Al LDH, with a total of macroalgae coverage of ~56% and ~53% respectively, and SiNC reached a ~89% after a year of immersion.

Figure 20: Compilation of the percentage of the field parameters (Biofilm, Macroalgae and Invertebrates) over time. The Biofilm is represented by 0% transparency, macroalgae: 50% transparency and Invertebrates: 75% transparency. First 20 weeks of immersion.
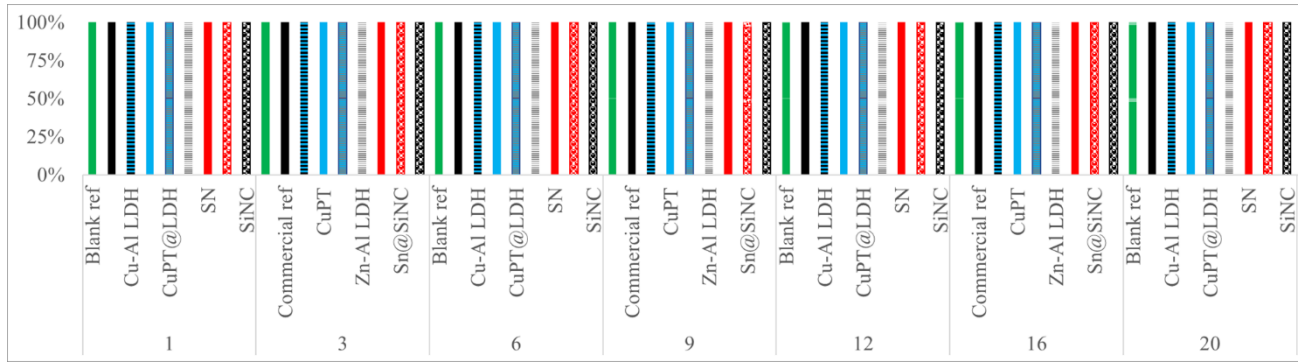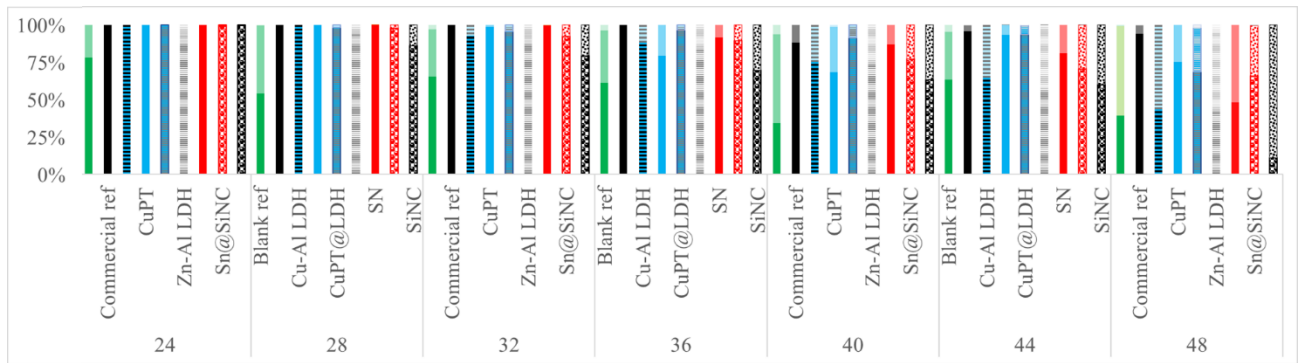

Figure 21: Compilation of the percentage of the field parameters (Biofilm, Macroalgae and Invertebrates) over time. The Biofilm is represented by 0% transparency, macroalgae: 50% transparency and Invertebrates: 75% transparency. Continuation from Figure 25 until a year of immersion.

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3.3.1 Characterization of the microbiological communities

The following dendrograms were obtained through the PCR-DGGE technique, which combine the three sampling times: week 1 vs week 3, week 3 vs week 6 and week 1 vs week 6.

Figure 22 represents the comparison of the microbiological samples of week 1 and week 3. The dendrogram splits most microbiological samples in two major affinity groups: samples from week 1 (which included also the sample of the commercial reference from the third week) vs week 3, with a similarity intragroup of 75% or higher. Richness, and diversity in all plates were low in the first and third weeks (Table 8), ranging from 4 (blank reference, Zn-Al LDH and Sea-Nine – week 1) and 15 for Cu-Al LDH (week1). The Pielou index (J) demonstrated a tendency to high evenness in all coatings in both sampling periods.

Figure 23 represents the comparison of microbiological samples of week 3 and week 6. A biological affinity group with more than 85% of similarity can be depicted grouping most samples from week 3 (apart from the bacteria collected in surface of the coating with empty SiNC) and two samples from week 6, the commercial reference and CuPT. According to the biotic indices (Table 9), these samples share a low species richness and diversity, probably denoting a high AF efficacy against the bacterial film. Bacterial communities from sixth week are more heterogenous, being characterized by higher species richness, diversity and equitability comparing with the third week, apart from the commercial reference and Sea-Nine samples. The species richness between these two weeks revealed more discrepant values, with a minimum value of 4 (CuPT – week 3) and a maximum of 37 (SiNC – week 6), with this sample also being the most diverse (3.49).

Figure 24 represents the comparison between the microbiological samples from week 1 and week 6. In this dendrogram it is possible to realize that is formed a group with the most similar samples, composed by the samples with lower bacterial richness and diversity. Represented by the samples: CuPT (W6), CuPT@Zn-Al LDH (W6). Sea-Nine@SiNC (W6), commercial reference (W1), CuPT (W1), Sea-Nine (W6), blank reference (W1), Zn-Al LDH (W1), commercial reference (W6) and Sea-Nine (W1), CuPT@Zn-Al LDH (W1) and Sea-Nine@SiNC (W1). The remaining samples have lower similarity between them but have higher bacterial diversity and richness in the two weeks compared. The obtained indexes revealed that between the first and sixth week of

immersion the species richness of some coatings increased in most of the samples and in other decreased (Table 10). It is noted that for the blank reference and Zn-Al LDH the species richness indicates a higher increase than in the commercial reference, CuPT and Sea-Nine samples (generally the better in terms of AF efficacy) between the first and sixth week. Microbiological richness decreased in Cu-Al LDH, CuPT@Zn-Al LDH and SiNC coatings.

After six weeks of immersion, it is possible to note an effect of the coatings in the microbiological communities in the samples results. The samples with biocides show lower bacterial richness and diversity comparing with the ones without biocides.
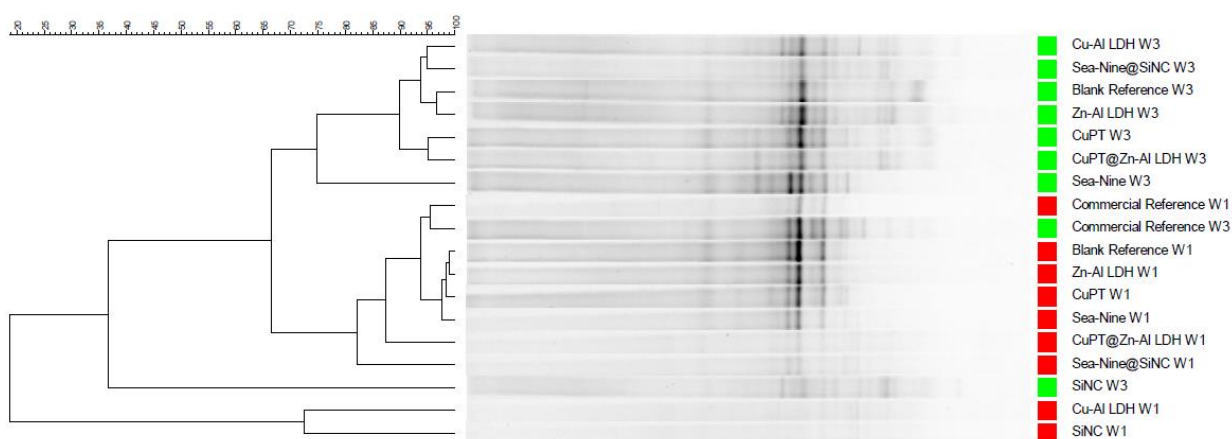
Figure 22: Dendrogram of PCR-DGGE comparing the samples from week 1 and week 3. Week 1 represented by the color red and week 3 represented by the color green.

Table 8: Biotic indices based on the microbiological communities settled in the tested coatings after 1 and 3 weeks of immersion: S(richness); J(evenness) and H'(diversity)

Samples (week 1)	S	J	H'	Samples (week 3)	S	J	H'
Blank reference	4	0.80	1.10	Blank reference	8	0.73	1.52
Commercial reference	7	0.71	1.39	Commercial reference	9	0.80	1.75
Cu-Al LDH	15	0.88	2.39	Cu-Al LDH	12	0.82	2.03
CuPT	7	0.74	1.44	CuPT	9	0.74	1.63
CuPT@Zn-Al LDH	13	0.86	2.20	CuPT@Zn-Al LDH	8	0.83	1.72
Zn-Al LDH	4	0.76	1.05	Zn-Al LDH	10	0.75	1.73
Sea-Nine	4	0.78	1.08	Sea-Nine	9	0.82	1.80
Sea-Nine@SiNC	12	0.84	2.09	Sea-Nine@SiNC	10	0.83	1.90
SiNC	10	0.95	2.18	SiNC	12	0.90	2.24
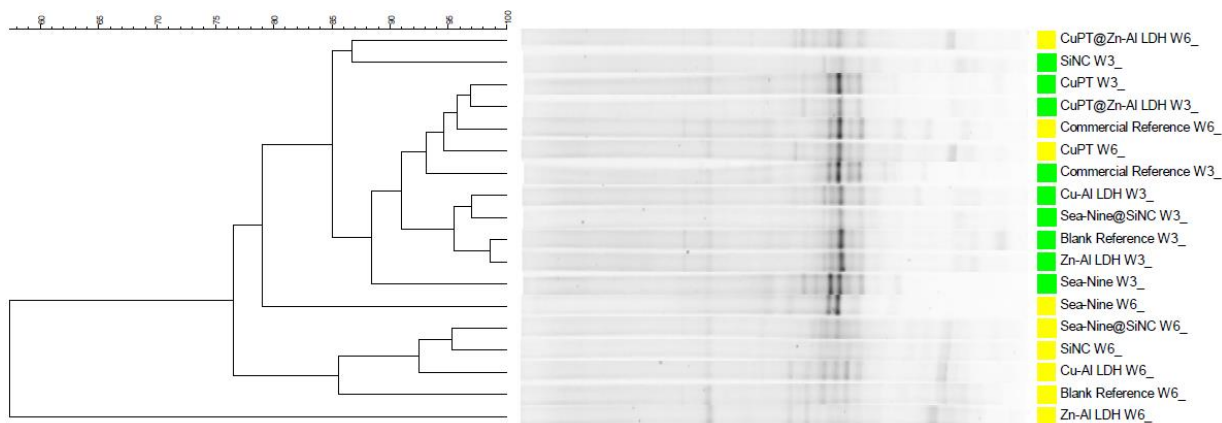
Figure 23: Dendrogram of PCR-DGGE comparing the samples from week 3 and week 6. Week 3 represented by the color green and week 6 represented by the color yellow.

Table 9: Biotic indices based on the microbiological communities settled in the tested coatings after 3 and 6 weeks of immersion: S(richness); J(evenness) and H'(diversity)

| Sample (week 3)	S	J	H'	Sample (week 6)	S	J	H'
Blank reference	9	0.89	1.95	Blank reference	21	0.97	2.94
Commercial reference	9	0.89	1.95	Commercial reference	8	0.88	1.82
Cu-Al LDH	9	0.89	1.95	Cu-Al LDH	15	0.94	2.54
CuPT	4	0.90	1.25	CuPT	10	0.93	2.14
CuPT@Zn-Al LDH	6	0.91	1.63	CuPT@Zn-Al LDH	19	0.97	2.85
Zn-Al LDH	9	0.89	1.97	Zn-Al LDH	27	0.96	3.17
Sea-Nine	9	0.91	2.01	Sea-Nine	6	0.77	1.38
Sea-Nine@SiNC	11	0.95	2.27	Sea-Nine@SiNC	21	0.95	2.89
SiNC	19	0.96	2.82	SiNC	37	0.97	3.49
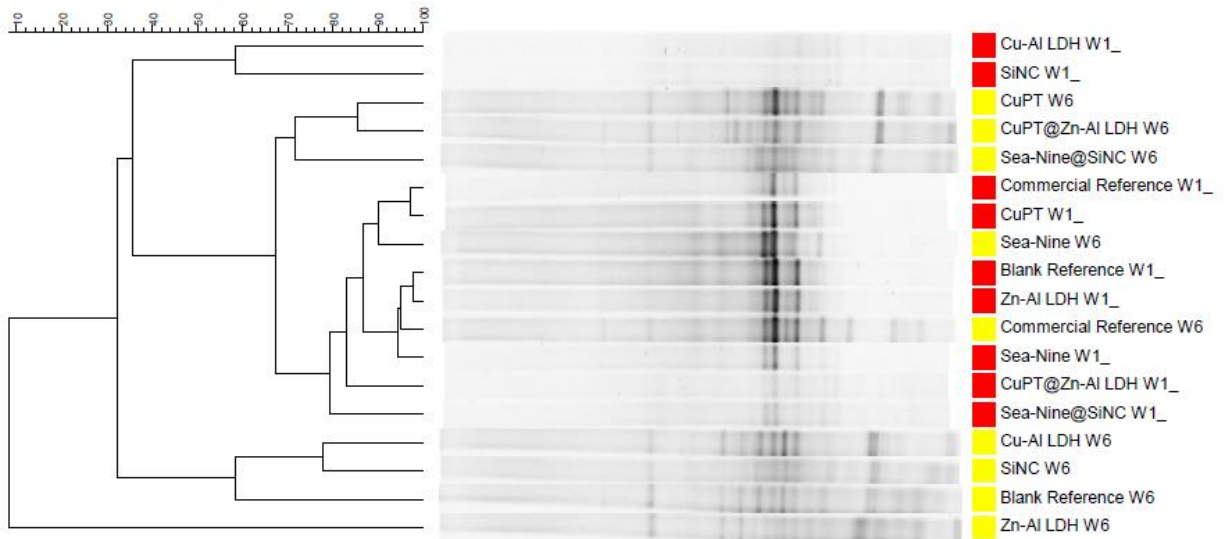
Figure 24: Dendrogram of PCR-DGGE comparing the samples from week 1 and week 6. Week 1 represented by the color red and week 6 represented by the color yellow.

Table 10: Biotic indices based on the microbiological communities settled in the tested coatings after 1 and 6 weeks of immersion: S(richness); J(evenness) and H'(diversity)

Samples (week 1)	S	J	H'	Samples (week 6)	S	J	H'
Blank reference	7	0.73	1.42	Blank reference	76	0.91	3.93
Commercial reference	6	0.76	1.36	Commercial reference	14	0.82	2.17
Cu-Al LDH	79	0.94	4.13	Cu-Al LDH	20	0.88	2.63
CuPT	8	0.77	1.61	CuPT	15	0.88	2.38
CuPT@Zn-Al LDH	99	0.93	4.28	CuPT@Zn-Al LDH	22	0.88	2.72
Zn-Al LDH	4	0.89	1.23	Zn-Al LDH	71	0.91	3.86
Sea-Nine	7	0.74	1.44	Sea-Nine	11	0.78	1.87
Sea-Nine@SiNC	37	0.89	3.22	Sea-Nine@SiNC	69	0.90	3.79
SiNC	109	0.97	4.56	SiNC	67	0.92	3.87

3.3.2. Characterization of the microphytobenthos communities

Figure 25 shows the diatom abundance from the different coating over time. Abundance increased in all coatings since the first week of immersion. Diatoms abundance reached very high values in both controls and Zn-Al LDH related coatings at week 12 demonstrated a lower anti-microfouling efficacy comparing with the other AF modified coatings. There are significant differences in terms of abundance between the blank reference and the Sea-Nine coating at week 12. There are significant differences in terms of abundance between the commercial reference and Cu-Al LDH, CuPT, Sea-Nine and SiNC coatings.

Figure 25: Abundance of microphytobenthos adhered to tested coatings during 12 weeks of immersion in the field *: Blank reference vs. treatments; #: Commercial reference vs. treatments.

Figure 26 represents the biomass of the diatom community over time. There are significant differences in terms of biomass between the blank reference and: a) CuPT-based coatings (at week 3); b) Commercial reference, coatings with CuPT, Sea-Nine and CuPT@Zn-Al LDH (week 6); all coatings apart Zn-Al LDH (week 9) and all coatings apart Zn-Al LDH and the Commercial reference (week 12). Significant biomass differences are depicted between coatings containing Cu-Al LDH and CuPT and Sea-Nine (free) and its encapsulated form (Sea-Nine@SiNC) at week 6. The biomass from the commercial reference at week 12 was significantly different from CuPT and Sea-Nine-based coatings (free and encapsulated forms) and the coating with empty nanocapsules of silica (SiNC).

Figure 26: Biomass of microphytobenthos adhered to tested coatings during 12 weeks of immersion in the field. *: Blank reference vs. treatments; #: (Commercial reference vs. treatments; a: Cu-Al LDH vs CuPT; d: Sea-Nine vs Sea-Nine@SiNC.

Figure 27 represents the photosynthetic yield (proxy of fitness of the microphytobenthos community) over time. Photosynthetic yield increased over time, generally for all tested coatings. On week 9 were detected statistical differences between the blank reference and the coating containing Sea-Nine, between the commercial reference and the coating containing Cu-Al LDH and SiNC and lastly between Sea-Nine and its nanoform Sea-Nine@SiNC. Photosynthetic yield from coating with Sea-Nine remains low in week 12, with statistical differences between the blank reference and the commercial reference and the coating containing Sea- Nine. There were differences between the commercial reference and all the other coatings except CuPT and CuPT@Zn-Al LDH. The Cu-Al LDH coating and the one with the free biocide (CuPT), represented by the letter a, revealed statistical differences, as well as the one with the free biocide Sea-Nine and its encapsulated form (Sea-Nine@SiNC).
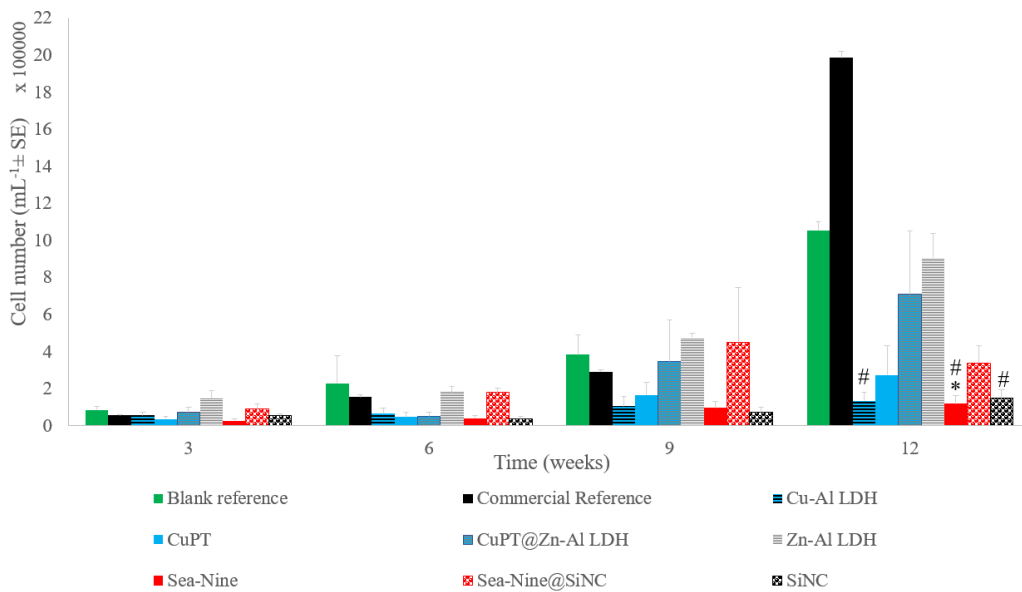

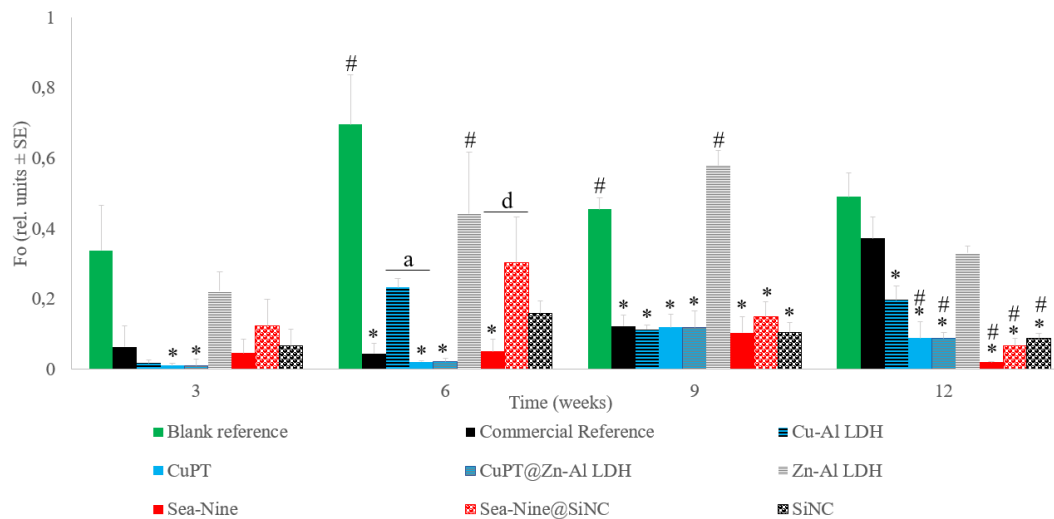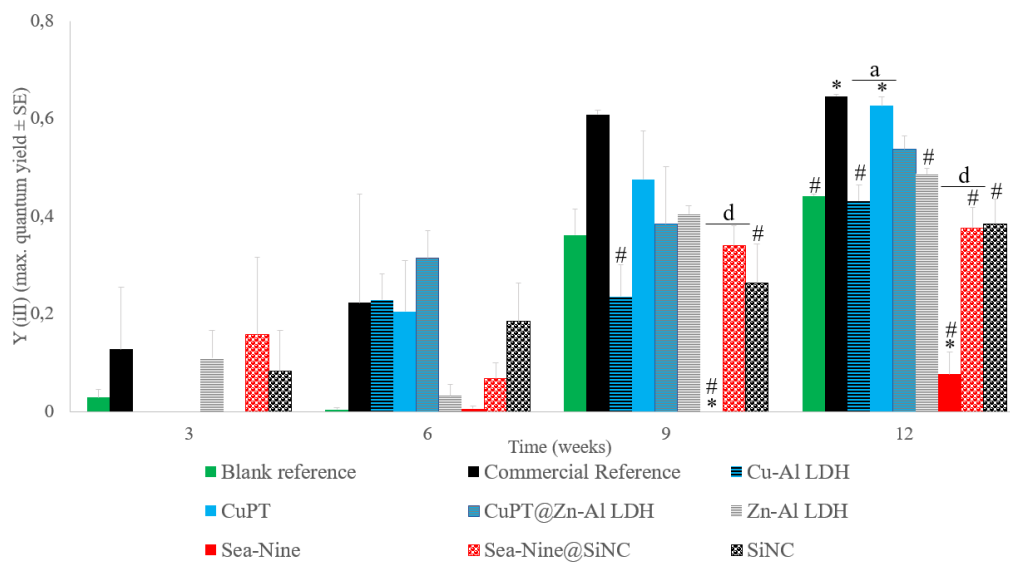*Figure 27: Photosynthetic yield of microphytobenthos adhered to tested coatings during 12 weeks of immersion in the field. *: Blank reference vs. treatments; #: Commercial reference vs. treatments; a: Cu-Al LDH vs CuPT; d: Sea-Nine vs Sea-Nine@SiNC*

3.4 Multivariate data analysis

Figure 28 shows the PCO analysis of the ecotoxicity and field efficacy data. Axes 1 and 2 of the PCO account for 36% and 26% of the total variation, respectively. The axis 1 separates samples by immersion time: early stages with not relevant effects in the negative side of axis 1 from the others in the positive side where the release of chemicals influenced the observed toxicity and efficacy. The axis 2 splits leachates by toxicity: high toxicity in the negative part (DCOIT, CuPT and reference coatings, from the 3rd until the 12th week) and low toxicity in the positive part (all the others). Most correlated variables were DCOIT (with DCOIT coating), Cu, Zn, Al and nitrates content.
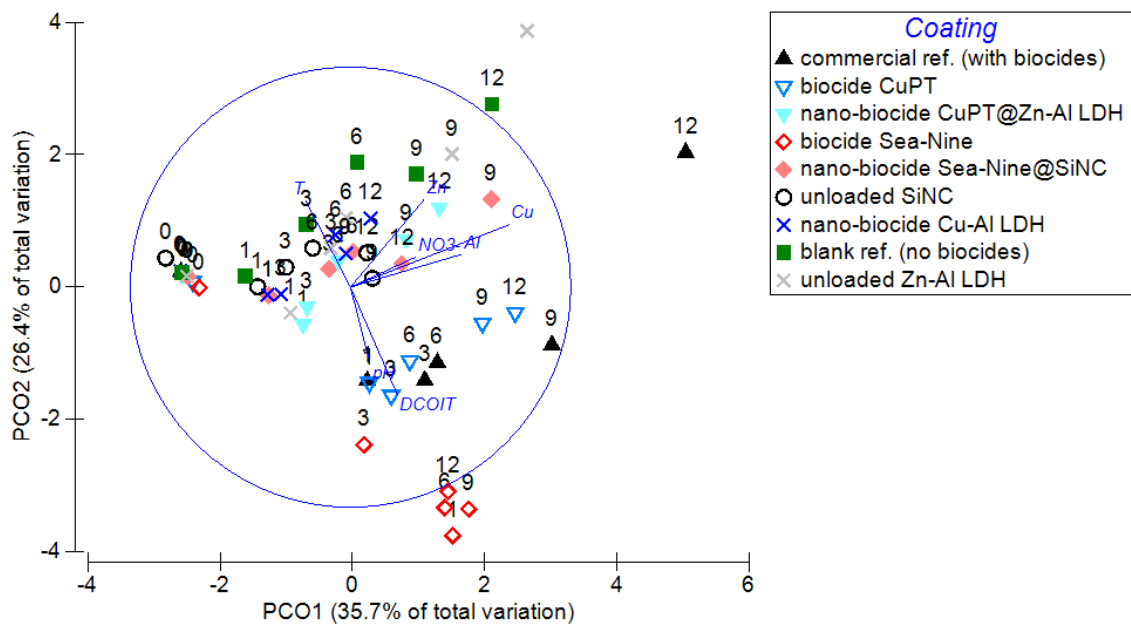
Figure 28: Ordination diagram (PCO) based on the leachates ecotoxicity and field AF efficacy of the tested coatings immersed for 3 months. Spearman (rho > 0.7) correlation vectors of environmental descriptors are provided as supplementary variables.

4. Discussion

Biocides are a key component of current maritime AF coatings (e.g. Almeida *et. al.*, 2007). State-of-the-art AF coatings usually have Cu-based biocides (e.g. cooper oxide Cu_2O) as the main biocide and a co-biocide to improve their performance. The commercial reference used in the present study (Sea Quantum Ultra S) has Cu_2O as the main active ingredient, combined with copper pyrithione, a biocide with very high efficacy against foulers (e.g. Price *et. al.,* 2012; Gutner-Hoch *et. al.*, 2018). An ideal AF coating should be effective in the impediment of the fixation of fouler species, associating low toxicity to non-target species, low maintenance and long durability.

Karlsson *et. al.* (2010) state that current regulations do not include antifouling coatings with physical mode of action (only the chemical and biological). So, it is important to understand the toxicity, AF efficacy and behavior of the biocidal additives not only in solution (Figueiredo *et. al.*, 2019), but also in the formulations of the chemical type of AF coatings used in this study. As far as we know, studies about the ecotoxicity and efficacy of this type of antifouling coatings are scarce considering the total amount of coatings available in the market (cf. Table 2).

The AF efficacy assessment of the present study was run in a marina influenced by tides, in static and full immersion conditions, being physicochemical variables regarded as normal. The present study demonstrated different performances in terms of anti-microfouling and anti-macrofouling efficacy of the tested coatings shown, respectively by the diversity and composition of the microbiological and microphytobenthos communities since the first week of immersion and the macroalgae and macroinvertebrates coverage which greatly varied between coatings. Painted plates presented different surface roughness due to the additives incorporation. Surface roughness, among other variables, play a key role in the settlement of flora and fauna, particularly relevant during the biofilm formation. Despite no relevant AF differences were detected over a year of immersion, except the blank reference which was heavily fouled by macroalgae, improvement of nanoadditives incorporation and compatibility with the coating matrix is highly recommended for a future commercial application. In opposition, Karlsson *et.al.* (2010) showed that the coating without any recognized AF booster biocide was the most toxic of the tested coatings. This coating, besides a different mode of action, had zinc pyrithione (ZnPT) as a preservative, which is indeed currently

regarded as an AF biocide. Recent studies showed that ZnPT is a toxic biocide towards several marine invertebrates (e.g. Avelelas *et. al.*, 2017; Gutner-Hoch *et. al.*, 2018, 2019a).

Bacterial diversity increased in biocides-free coatings as well as in the coatings with regular biocides from the first and sixth week, while decreased in Cu-Al LDH, CuPT@Zn-Al LDH and SiNC-based coatings. Briand *et al.*, (2012), using the DGGE technique in samples collected from the coatings' surface immersed for 15 days, showed that all of them had bacteria. Coatings without biocides and with ZnPT had high bacterial richness, unlike the one with CuPT, which revealed low bacterial richness. In the present study, the microbiological samples analysis also revealed bacterial presence in all samples, varying in the richness of communities in each type of coating, between the first and sixth week of immersion. The blank reference (without biocides) and coatings with Zn-Al LDH, or SiNC had the highest richness values in the sixth week of immersion. Copper and Sea-Nine based additives (both free or nanostructured) exhibited a low bacterial richness after 6 weeks of immersion demonstrating a great potential as anti-microfouling biocides. Briand *et. al.*, (2012) also demonstrated that coatings with Sea-Nine or CuPT promote a decrease of bacterial abundance. Cu is an essential metal that become toxic in certain doses to different types of organisms (Gledhill *et. al.*, 1997), while Sea-Nine is a widely-used booster biocide which may explain the AF success of these coatings. In fact, McElroy *et. al.*, (2017) revealed that copper limits the photosynthetic growth.

The evolution of the diatom's community in the plates of the present study, either measured in terms of abundance, biomass or yield, was generally lower for the coating containing Sea-Nine. The coating with free CuPT, the abundance and biomass also had low values until week 12 but the yield revealed higher values. Commercial reference (containing two Cu-based biocides) showed a poorer performance, with the highest values for the three considered parameters. In particular, the coating containing Sea-Nine has the lowest diatoms biomass, relatively stable abundance and low yield revealing a good AF efficacy.

Karlsson *et. al.* (2004) tested commercially available coatings with macroalgae and crustaceans, and after 14 days of leaching, concluded the high toxic effects of four of the tested paints. In the present study it was also verified a rapid leaching in the coatings

containing the free biocides (commercial reference and the two modified coatings with Sea-Nine and CuPT), even after one week of immersion, with significant deleterious effects in the tested species, while leachates from coatings with the nanostructured biocides caused no/low toxicity in the tested non-target species. The observed pattern supports preliminary studies in solution that showed a decrease of the biocides toxicity when encapsulated/immobilized (Figueiredo *et. al.*, 2019).

Physicochemical parameters and particles size in the aquaria water were weekly measured, via DLS, and had a negligible variation over time relatively to the only ASW samples. In the other hand, chemicals leaching rate seemed to have a great effect on the observed toxicity patterns. DCOIT (the active ingredient of Sea-Nine^{TM}) was only detected in leachates from the coating with the free form of the biocide, at levels higher than in the marine environment, justifying the extreme toxic effects of these leachates and the relevance of the present study. In water samples collected in a Danish Harbor, Sea-Nine^{TM} was found in values between 30 and 70 ng/L (Steen *et. al.*, 2004). The absence of detectable levels of Sea-Nine in the leachates from coatings with the nanostructured biocide prove that the encapsulation promotes a controlled release with environmental benefits. Copper is a common compound on natural seawater, however several studies highlighted that the release of this compound from commercial AF paints, which is usual in similar coatings, can harm the marine biota, depending on the concentration (Blossom, 2015). In the leachates of the commercial reference, copper levels were high in all sampling times, which is in concordance with its biocidal composition (Cu_2O and CuPT). The leachates chemical analysis showed a soft variation of Zn concentration; the highest Zn concentration was found in leachates from the coating containing CuPT@Zn-Al LDH which can be assigned to partial Zn dissolution from the nanoclays. The presence of Fe was detected since the early phases of plates immersion which may be due to the natural presence of the compound in the water. The ICP-OES revealed normal values in the measurements of the samples from week 6.

A clear separation of the coatings in terms of toxic effects over the immersion time can be depicted from the PCO analysis. Thus, as an overview of this study, coatings with free biocides and the commercial reference stand out as highly toxic in opposition to the nanostructured-biocides based formulations; both coating types showed good efficacy. The encapsulation/ immobilization via engineered nanomaterials appears to be a promising path for future applications.

5. Conclusions

This study allowed to have a more holistic view of the efficacy and toxicity of AF coatings containing state-of-the-art and novel nano-based additives, as well as a corroboration of the ecotoxicological data of those compounds previously assessed in solution. As coatings are complex mixtures of chemical compounds, their toxicity can be very different from the main active ingredient. Here, it was possible to confirm that the biocides absence (as in the blank reference) resulted in a low AF performance and toxicity. Free biocides coatings had a high field efficacy and laboratorial toxicity towards non-target microalgae and crustaceans, contrasting with the coatings with the promising novel nanostructured biocides which generally exhibited low toxicity and high AF efficacy.

Although there are antifouling coatings with different forms of action, the investment in the biocide field will allow a greater knowledge of the compounds when used for the AF purpose.

Preserving the environment must always be the priority number one, whatever the field of study. So, smart and "green" solutions will allow the improvement of the use of some compounds, including choosing the most appropriate and complete techniques to archive the outlined objectives of each study without disregarding the importance of the ecosystems health and wellness.

6. References

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