Review

Biocide vs. Eco-Friendly Antifoulants: Role of the Antioxidative Defence and Settlement in *Mytilus galloprovincialis*

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Abstract: Antifoulant paints were developed to prevent and reduce biofouling on surfaces immersed in seawater. The widespread use of these substances over the years has led to a significant increase of their presence in the marine environment. These compounds were identified as environmental and human threats. As a result of an international ban, research in the last decade has focused on developing a new generation of benign antifoulant paints. This review outlines the detrimental effects associated with biocide versus eco-friendly antifoulants, highlighting what are effective antifoulants and why there is a need to monitor them. We examine the effects of biocide and eco-friendly antifoulants on the antioxidative defence mechanism and settlement in a higher sessile organism, specifically the Mediterranean mussel, *Mytilus galloprovincialis.* These antifoulants can indirectly assess the potential of these two parameters in order to outline implementation of sustainable antifoulants.

1. Introduction

Marine biofouling consists of the settlement of microorganisms, plants, and aquatic animals on artificial surfaces immersed in seawater (see for review [1]). It can cause several ecological, human, and economic concerns. In fact, it may introduce invasive species or can promote the settlement and spread of native species with subsequent modifications of biodiversity and/or alteration of the ecological process [2–5]; convey parasites that can affect native species and subsequently humans [6,7]; and induce high ship frictional resistance leading to an increase in fuel consumption and structural damage to ships over time due to corrosion and discoloration [8–10]. The most common antifoulant (AF) paints from the last century contain chemical compounds such as tributyltin oxide and tributyltin fluoride (TBTs), which were the most widely used since the 1950s marking a revolution in AF applications. These compounds are a class of organotin biocides. They are potent fungicides and completely inhibit the growth of most fouling organisms at a very low concentration [11] with oxidative stress phenomena (see for review [12]). Unfortunately, TBT systems adversely affect the environment, inducing endocrine disruption, loss of marine species biodiversity, and pose a human food security threat. The effects of this

substance have been known since the 1970s when it was identified as a hormone-like disease agent in an oyster farm in France [13]. Subsequently, the compound's activity has been detected in numerous marine organisms in which it accumulates, eventually posing serious risks to human health through the food chain as demonstrated by an increase in physiological antioxidative defence activity [14–16]. In response to the global ban of TBTs in 2008, the marine coatings industries have developed alternative AF biocides. New alternatives to AF paints were based on copper compounds such as copper oxide and copper thiocyanate with the incorporation of biocide boosters to control Cu-resistant fouling organisms [17,18]. These biocides were intended to be less harmful to the environment than organotin biocides. However, many of the booster biocides are also a threat to the marine environment [19,20]. Some of them can accumulate to high levels, despite claims of rapid degradation, and have a biocidal effect on non-target marine organisms' redox status [21,22]. With the growing concern for environmental protection, these biocides are expected to be phased out. In fact, these issues should be addressed as a priority under proper global governance and executed in line with the Sustainable Development Goals (SDGs) of the United Nations, specifically, to reach goals #3 (health and well-being) and #14 (Life Below Water) [23].

Therefore, the elimination of AF compounds from a wide range of biological organisms (e.g., algae, corals, sponges, and microbes) seems to be the preferred mitigation route, due in part to the high specificity of these compounds for fouling organisms [24,25]. While many commercial fouling release systems are already available on the market, the development of an efficient eco-friendly product entirely based on natural biocides seems still far away. Compounds such as terpenoids, steroids, carotenoids, phenolics, furanones, alkaloids, peptides and lactones all have AF activity [26]. Irritants extracted from land plants such as oleander and bell pepper are also important sources with AF action [1,27]. In nature, marine organisms have developed many biological strategies to interact with microorganisms for protection from pathogens or from being parasitized [28]. In particular, sponges and the associated microbiota produce compounds that interfere with marine biological fouling using quorum sensing (QS) mechanisms [29,30]. The use of marine biological secretions to interfere with bacterial QS can effectively inhibit bacterial biofilm formation, making it difficult for several species of Mytilus including *M. galloprovincialis* to adhere [31,32]. Understanding the molecular mechanism by which these eco-friendly AF compounds act can also help researchers to chemically enhance the functional groups and allow these compounds to achieve high specificity against targets without inducing overexpression of free radicals [4,33]. Their mechanisms include the inhibition of transmembrane transport [10], inhibition of quorum sensing [34] and neurotransmission blocking [35]. They can also inhibit enzymes involved in the production and/or release of adhesive molecules inhibiting cell growth [36]. Studies carried out on the effect of antifoulants are numerous and linked to specific bioindicator organisms. The model organism selected for this review is the Mediterranean mussel, *Mytilus galloprovincialis*, a filter feeder bivalve organism belonging to the phylum Mollusca. It attaches to a variety of surfaces due to adhesive proteins produced and stockpiled in its foot and then secreted into the byssal groove such as Mgfp-1, 2 and 3 (*Mytilus galloprovincialis* foot protein 1, 2 and 3). Collagen, cellulose, chitin, and mineral deposits also play an important role in the adhesive process. In addition, the enzymatic oxidation of specific polyphenolic proteins such as dihydroxyphenylalanine (DOPA) provides the distinctive resistance to moisture in mussel underwater adhesion. DOPA forms complexes with metal ions, oxides (Fe^{3+}, Mn^{3+}) and semimetals such as silicon, giving the mussel the ability to adhere to rocks and smooth surfaces [37]. This organism represents an important tool for biomonitoring environmental pollution; it is considered a sentinel organism, serving as a bio-indicator to evaluate chemical pollutants in marine environments. In fact, this species is capable of bio-accumulating relevant quantities of different xenobiotics (see for review: [38–40]). *M. galloprovincialis* has been used in several toxicological investigations due to its versatility, which makes it possible to work both on retrieved species and laboratory-grown individuals [41–45]. As part of mussels and filter

feeders, they are exposed to a wide range of natural stressors that result in biochemical and physiological changes [46]. Mussels are also known to exhibit signs of oxidative stress by overexpression of free radicals when exposed to toxic compounds, which are harmful to cells because they cause disruption of cell membrane fluidity by peroxidation, as well as DNA damage in the form of degradation, base deletions and mutations [46–48]. Indeed, antioxidant enzymes which counteract oxidative stress are used on this biofouling model as contaminant/stress-related biomarkers [49–52]. Thus, the primary objective of the present review is to summarise and collect information available in the literature on the effect of eco-friendly and biocide AFs and their role in the antioxidative defence and settlement in *M. galloprovincialis* as a biofouling model organism.

2. Biocide Antifoulants and Their Effect on *Mytilus galloprovincialis*

Biocides in antifoulants are active substances designed to destroy, deter, control or prevent the action of any harmful organism which is considered a detrimental and unwanted biological presence for humans, animals and the environment [1,53,54]. Antifouling efficacy is affected by many factors, including physical, chemical, the type of biocide present in the coating, as well as other factors. These in turn determine the polishing behavior of the coating and its ability to control the biocide [1,53–56]. It has been shown in the literature how these substances can cause antioxidant enzymatic alterations, through cellular reactive oxygen species (ROS) in *M. galloprovincialis*, compromising larval metamorphosis [57] and causing an inhibition in reaching a well-developed veliger larval stage [58] and/or inhibiting the settlement process [59].

Copper (Cu) and copper-based derivatives have long been used as biocidal agents and have found use in AF paints to prevent colonisation of ship hulls by fouling organisms [60]. It is a naturally occurring trace metal that is essential for the proper functioning of biological systems, but it is toxic when present in excessive concentrations, as demonstrated by numerous ecotoxicological studies reporting disruption of many biological functions in marine organisms [20,61–63].

Cima and Varello [20] assessed the potential disruptive effects of copper-based (Cu_2O) AF paints on the biodiversity of coastal macro-fouling communities, including *M. galloprovincialis* (Table 1). Wooden and steel panels were coated with four copper(I)-based AFs; paint A, a mix of TBT compounds (Cu_2O, TBT methacrylate and antifouling agent tributyltin (TBTO)); paint B, copper(I) (Cu_2O); paint C, copper(I) as cuprous thiosyante (CuSCN) mixed with dichlofluanid; paint D, Cu_2O mixed with s-triazine herbicide (Irgarol 1051)/organochlorine fungicide (chlorothalonil). In particular, paint B was the only one based on contact-leaching technology containing only copper (I) oxide without boosters. Besides having lower AF efficiency on the macro-fouling communities, paint B had a significant inhibitory effect on *M. galloprovincialis* on the wooden panels. Paint D, had the higher biocidal power and also prevented development of mollusks on the wood panels. Thus, they found that the co-presence of organic booster biocides and the type of polymeric matrix can significantly increase the performance of the AF. Their results showed how copper-based paints have different effects on the settlement and growth of key macro-fouling species.

Table 1. Summarized results and data references for biocide antifoulants occurring in *Mytilus galloprovincialis*.

Biocide Antifoulant	Exposure Time	Toxicity Index/Concentration	Sample	Effect on Antioxidative Defence and Settlement	Reference
Paint B	10 months	Cu_2O (41 wt.%) Cu leaching rate: 25.53 µg cm^{-2}/d	Total body	Settlements inhibition	Cima and Varello [20]
Paint D	10 months	Cu_2O (42 wt.%) Chlorothalonil (7 wt.%)	Total body	Settlements inhibition	
A-3	45 days	20 wt.% Cu_2O: 17.9 µg/L			
A-4	45 days	30 wt.% Cu_2O: 21.2 µg/L	Byssus	Byssus production inhibition as %Cu_2O increase	Kojima et al. [59]
A-5	45 days	40 wt.% Cu_2O: 22.4–42.2 µg/L			
Cu^{2+}	15 days	10 µg/L	Digestive gland/Gills	SOD↑, CAT↑, GPX↑	Gomes et al. [64,65]
CuO NPs	15 days	10 µg/L	Digestive gland/Gills	SOD↑, CAT↑, GPX↑	
Cu	4 days 4 days	5 µg/L 15 µg/L	Gills	GST↑	Perić and Burić [66]
Cu + Chp	4 days 4 days	Cu 5 µg/L, Chp 0.05 µg/L Cu 15 µg/L, Chp 0.05 µg/L	Gills	GST↑	
B[a]P	7 days	10 µg/L	Gills Gills	CAT↑, tGPx↑, GST↑ SOD↓	
Cu	7 days	5, 10, 25 µg/L	Digestive gland Digestive gland	GPx↓, GR↓, CAT↓, tGPx↓, GSH↓, SOD↓ GR↓, CAT↓, tGPx↓, SOD↓, GST↑	Maria and Bebianno [67]
B[a]P + Cu	7 days	B[a]P 10 µg/L, Cu 5, 10, 25 µg/L	Gills	SOD↑, CAT↑, GR↑	
ZnPT	96 h	20 and 40 µg/L	Digestive gland/Gills	SOD↑, GSH↓	Katalay et al. [68]
Mexel 432®	14 days	0.5, 1, 2 mg/L	Digestive gland/Gills	GST↑, CAT↓	
NaClO	14 days	0.1, 0.2, 0.5 mg/L	Digestive gland Gills	GST↑, CAT↓ GST↑, CAT↑	López-Galindo et al. [69]
NaClO + Mexel 432®	14 days	NaClO: 0.1, 0.2, 0.5 mg/L, Mexel 432®: 0.5, 1, 2 mg/L	Gills	GST↑, CAT↑	
ZnO NPs	14 days	100 µg/L	Digestive gland/Gills	GST↑	
TiO$_2$ NPs	14 days	100 µg/L	Digestive gland	SOD↑, CAT↑	Bouzidi et al. [70]
Diuron	14 days	50 and 100 µg/L	Digestive gland/Gills	SOD↑, CAT↑, GST↑	
ZnONP + Diuron	14 days	100 µg/L	Digestive gland/Gills	SOD↑, CAT↑, GST↑	
Antifoulants containing: Cu_2O, aromatic hydrocarbons, zinc oxide, rosin, zineb	12 months	n.d.	Gonads	Settlements inhibition, GST↑, *gpx*4↑	Madonna et al. [4]

Abbreviations: Cu^{2+}, cupric ion or copper ions; CuO NPs Copper oxide nanoparticles; Cu, copper; Chp, Chlorpyrifos; B[a]P, Benzo(a)pyrene; ZnPT, Zinc Pyrithione; NaClO, Sodium hypochlorite; ZnO NPs, Zinc Oxide Nanoparticle; TiO$_2$ NPs, Titanium dioxide nanoparticles; Cu_2O, Copper(I) oxide; wt.%, weight percent; n.d., not determined, (i.e., no information reported in manufacturer's datasheet or literature); SOD, superoxide dismutase; ↑ increase; ↓ decrease activity; CAT, catalase; GPX, glutathione peroxidase; GST, glutathione S-transferase; tGPX, total glutathione peroxidase; GR, glutathione reductase; GSH, glutathione; *gpx*4, glutathione peroxidase 4.

Moreover, six different AFs containing 0, 5, 10, 20, 30 and 40 wt.% of Cu_2O were tested by Kojima and colleagues [59] on the formation of filaments produced by the bissal apparatus in *M. galloprovincialis* (Table 1). This apparatus is composed of several secretory glands: the collagen gland produces the bissal collagen core, the accessory gland is responsible for the protein cortex, the phenolic glands produce the protein adhesive, and another glandular system is known to produce a sulfur-rich mucopolysaccharide [71]. Each paint formulation was coated on one surface for 45 days. A behavioural test was then conducted using mussels *M. galloprovincialis* that were glued to the coated surface of each aged test plate. The number of byssus threads produced by each mussel generally decreased as the Cu_2O content of the coating increased. In fact, a positive correlation was observed between the Cu_2O content in the paints and the inhibition of byssus threads production showing significant AF efficacy at greater than 20 wt.% of Cu_2O. Thus, the authors showed that AF containing a minimum threshold of 20 wt.% of Cu_2O have a repellent effect on the mussels settlement.

Copper oxide nanoparticles (CuO NP) are often used for their antimicrobial properties in AF paints. Moreover, given the widespread use of copper nanoparticles in various industrial and commercial applications, they will inevitably end up in the aquatic environment.

In this regard, Gomes and colleagues [64,65] investigated the effects of copper nanoparticles in the digestive gland and gills of *M. galloprovincialis* and assessed oxidative stress and level of metal bioaccumulation (Table 1). Mussels of *M. galloprovincialis* were exposed to Cu as nanoparticles (CuO NPs) and Cu^{2+} for 15 days. In the digestive gland of mussels exposed to CuO NPs, they showed an increase in superoxide dismutase (SOD), Catalase (CAT), Glutathione peroxidase (GPX) activity and lipid peroxide (LPO) levels after 7 days of exposure while metallothionein (MT) levels increased linearly with time. In the gills exposed to CuO NPs, SOD activity increased linearly in the first 7 days, CAT was only induced after 3 days while GPX was induced after one week. LPO and MT increased linearly with time of exposure, while inhibition of acetylcholinesterase (AChE) was observed only on the last exposure day (at the end of the experiment, day 15). Regarding Cu^{2+} exposure in the digestive gland, the mussels exhibited an increase in SOD activity after the first three days and remained unchanged throughout the exposure period while CAT activity increased with exposure time. Significant increases in GPX activity and LPO levels were detected in the digestive gland from day 7 of exposure while MT was observed only at the end of the exposure period (day 15). In the gills, SOD activity was observed during the whole experiment, while CAT and GPX activity only increased after 3 days of exposure. MT levels in the gills also increased in the first week of exposure while LPO increased linearly throughout the entire exposure period. Inhibition of AChE was observed only on the last exposure day. Results of antioxidant enzyme activities showed responses in the digestive gland and gills of exposed mussels demonstrating a susceptibility to CuO NP toxicity and Cu^{2+}.

Further studies were carried out on compounds in combination with copper. Perić and Burić [66] studied the combinatory effect of Cu and the organophosphorus pesticide chlorpyrifos (Chp) in *M. galloprovincialis* after short-term exposure to sublethal concentrations (Table 1). Chp is a common organophosphorus pesticide, a controversial compound due to its wide consumption and environmental implications [72]. Previous studies have reported that Chp is capable of mediating cytotoxicity through various mechanisms [73] such as oxidative damage further inducing systemic diseases such as inflammation and immune response [74]. Thus, the mussels were exposed for 4 days to Cu, and Chp both separately as well as to their binary mixtures at different concentrations. AChE, GST activities, MT content and LPO levels were used as biomarkers of the oxidative response using digestive gland and gill tissue. Exposure to Cu or Chp alone did not induce changes in AChE activity, whereas Cu alone caused a significant increase in GST activity and LPO level. Exposure to lower and higher concentrations of Chp alone resulted in an increase in the MT content and in the LPO level, respectively. The mixture of a lower concentration of Chp when combined with Cu significantly increased GST activity, while a higher concentration of

Cu in combination with Chp, resulted in a significant decrease of LPO. Higher concentrations of either Cu or Chp alone both decreased AChE activity. Thus, the authors in this study showed that low and environmentally relevant concentrations of Cu and Chp can alter the biological response in *M. galloprovincialis* showing that the activity of GST was the most sensitive biomarker that revealed oxidative damage imposed by both the single compounds and their mixtures. Subsequent studies on combined toxicological effects using copper together with benzo[a]pyrene (B[a]P) were performed. This latter, is a carcinogenic polynuclear aromatic hydrocarbon belonging to a class of compounds produced by any incomplete combustion of organic material and are therefore present all over the world due to anthropogenic activity [75].

Maria and Bebianno [67] studied the effects of single and binary mixtures of B[a]P and copper (Cu) on the antioxidant system and the lipid peroxidative response in the gills and digestive system of *M. galloprovincialis* (Table 1). They observed that different antioxidant sets are altered in gills and digestive system in relation to the dose-response to mixtures used. In gills, the B[a]P exposure induces an increase in CAT, total Glutathione Peroxidase (tGPx), GST, while Cu exposure induces an increase in MR and LPO and a decrease in SOD. In the digestive system, the B[a]P exposure induces a decrease of SOD, glutathione reductase (GR) and MT, while Cu exposure induces a decrease of GPx, GR, CAT, tGPx, glutathione (GSH) and SOD. In addition, the authors also tested the combined effect of these two compounds on the same tissues. In fact, in gills, the effect of B[a]P + Cu compounds significantly increased SOD, CAT and GR, while there were no significant changes in other antioxidants. However, increasing the Cu concentration altered LPO tGPx and GST showing a mixture-dependent increase. In the digestive gland, the enzymatic activity of GR, CAT and tGPx decreases significantly and the activity of LPO increases, favouring the inhibition of SOD, while GST activity increases. Thus, the authors showed that mussels *M. galloprovincialis* exposed to a combination of B[a]P and Cu were characterised by the strongest antioxidant response. They observed a better pro-oxidant power, while Cu concentration is higher with a B[a]P/Cu mixture suggesting a lowering of the mussel's immune defence to the copper contaminant.

Zinc pyrithione, 2-mercapto-pyridine-1-oxide complex (ZnPT), is one of the most widely used alternative biocides in AF paints, toxic to a wide range of marine organisms, including bivalves. It was introduced to the market as a replacement for organotin biocides (TBT) by Arch Chemicals (Norwalk, CT, USA) in 1991, and is currently considered the best candidate to replace TBT in AF paints [76]. There is little information on the toxicity of ZnPT in bivalves. The effect of ZnPT is also known to be toxic at the embryonic development stages in other organism belonging to the same genus such us the *Mytilus edulis* or other organisms such as the sea urchin (*Paracentrotus lividus*). In fact, Bellas and colleagues [77] demonstrated through the results of the embryo-larval toxicity test with *M. edulis* that ZnPT significantly influenced normal embryonic development with dose-dependent effect.

Katalay and colleagues [68] tested the effect after acute exposure to ZnPT using hepatopancreas and gill of *M. galloprovincialis* (Table 1). They observed an increase in SOD enzyme activity in both tissues treated by ZnPT with a significant GSH inhibition in gills. Furthermore, the authors observed loss of tubular epithelial integrity of digestive tubules, ciliate fractures, fusions, erosions and loss of epithelial cells in gill filaments. Histological changes in the tissues increased with increasing ZnPT concentration. ZnPT also caused a dose-dependent increase in cells undergoing apoptosis, particularly in the hepatopancreas and gill tissues. Thus, the authors observed that this specific type of biocide activates the antioxidative response in *M. galloprovincialis*. More precisely, an increase in SOD and a decrease in GSH was found in the gills, which are the first tissues in contact with the external environment, and a decrease in SOD in the hepatopancreatic tissues, which is the site of metabolic activity, demonstrating how this biocide can disturb the organs responsible for filtration and storage/bioaccumulation. In line with these results, Marcheselli and colleagues [78] have previously demonstrated how zinc pyrithione and its main secondary products, Zn and ionized pyrithione (PT−), rapidly accumulated in the

tissues of exposed mussels, identifying the gills and digestive gland as important targets in the biological pathway of contaminants.

López and colleagues [69] tested two AFs: sodium hypochlorite (NaClO) and an alkylamine surfactant (Mexel 432®) that is used as a biocide in the cooling systems of power plants to reduce biofouling (Table 1). Cooling water from power plants is usually not treated to remove the biocide before discharge into the sea, which can have negative consequences for nearby aquatic life [79]. Sodium hypochlorite (NaClO) is a derivative of chlorine and one of the most widely used disinfecting agents, due to its great ability to inhibit the proliferation of microorganisms in general. Using the common mussel *M. galloprovincialis* as the study organism, the authors assessed enzyme activity and responses to oxidative stress. Mussels were exposed to different concentrations of NaClO and alkylamine surfactant. The digestive gland and gills of each mussel were dissected after 1, 3, 7 and 14 days of exposure. Both AFs caused a response of the enzymes CST, CAT AChE and LPO. NaClO initiated the oxidation process in the gills, increasing LPO levels, GST and CAT activity and decreasing AChE activity. In the digestive gland, GST activity also increased, while CAT activity was decreased but there were no other signs of damage (such as increased LPO levels). No clear trends in antioxidant enzymes (GR and GPX activity) were observed in the digestive gland, despite an initial inhibition of GR and an increase in GPX. Mussels exposed to Mexel 432 experienced increased GST activity in the digestive gland as well as in the gill, while inhibiting CAT; LPO levels were slightly elevated in the digestive gland. Thus, the authors showed how the overall exposure to NaClO and Mexel 432 reflected in a toxic response mainly in the gills of *M. galloprovincialis*, causing a strong impact on oxidative response.

The herbicide Diuron is widely used for weed control in many types of crops. It reaches water bodies through various pathways and can negatively threaten non-target organisms. Ons Bacha and colleagues [80] studied this type of biocide to assess the antioxidant activity on the mollusc *M. galloprovincialis* during seven days of exposure (Table 1). Biomarkers of oxidative stress were assessed in the gills and digestive gland of the mollusc by observing changes in the activities of enzymes such as CAT, GST, AChE and MDA. The results obtained showed that Diuron altered biomarkers of oxidative stress in both the gills and digestive gland.

Combinatory effects using Diuron with nanoparticle NPs (ZnO NPs and TiO2 NPs) at sub-lethal doses were assessed on the marine mussel *M. galloprovincialis*. Bouzidi and colleagues [70] tested these two known antifouling biocides on digestive glands and gills (Table 1). SOD, CAT and GST were used as toxicological biomarkers. Upon Diuron exposure, SOD, CAT, GST, and LPO activity increased both in the digestive gland and gills directly with the compound concentration. At higher exposure, TiO_2 and ZnO NPs increased SOD, CAT, GST and LPO activity in the digestive gland while in the gills only GST and LPO activity increased. The combined effects of Diuron with the two NPs in different redox markers showed concentration-dependent effects. The mixture of higher concentration of Diuron with ZnO NPs (ZnONP2 + Di2) increased significantly the activities of SOD, CAT and GST and level of LPO in the digestive gland and gills. Thus, the authors showed how the antioxidant defense mechanisms can be complex in response to herbicide and nanoparticle compounds used in AFs.

3. Eco-Friendly Antifoulants and Their Effect on *Mytilus galloprovincialis*

Over the years the application of antifoulant paints has been criticised due to the damage they cause to the environment [81]. As people become more environmentally aware and regulations become more stringent, eco-friendly antifoulant systems have become the focus of current research [82]. The development of alternative AF solutions and emerging non-toxic strategies are generating a new class of protective coatings (Table 2), which follow two main approaches:

I.   Applying non-biocidal strategy which is the most environmentally friendly and well represented by dirt-release coatings.

II.   Applying biocidal strategy based on the controlled release of harmless bioactive agents and on chemical immobilisation strategy avoiding the release of biocidal agents.

Non-stick coatings are a non-toxic alternative due to many factors including low surface free energy, low glass transition temperature, low micro-rugosity, and a high mobility of polymer chains. Therefore, fouling species cannot safely attach to the surface and can be easily removed hydro-dynamically [84].

In this context, Madonna and colleagues [4] assessed the biological effects of AFs containing biocide-free paint and metal biocide using *M. galloprovincialis* gonads (Table 2). They painted submerged panels with eco-friendly AFs based on silicone and hydrogel which are fouling release coatings. In parallel, another set of panels were painted with well-known AF biocides such as copper oxide, aromatic hydrocarbons, zinc oxide, rosin and zineb. They observed an alteration in antioxidant defence in relation to the type of AF used. The GST enzymatic activity and the *gpx4* transcriptional activity were higher in the *M. galloprovincialis* gonads treated with biocidal AFs than in eco-friendly treated organisms. The results obtained indicate that the levels of *gpx4* and GST in *M. galloprovincialis* increased with exposure to paint containing the biocides copper and zinc proving that these two elements are promoters of oxidative stress while eco-friendly AFs did not activate the antioxidant response. In fact, GST enzyme activity and *gpx4* transcriptional activity are involved in detoxification response and apoptosis regulation respectively, so their alteration in biocide-treated *Mytilus* may indicate an acceleration of these processes. As antioxidant increases are mainly observed in the gonads, this study showed how the use of AFs containing biocides such as copper or zinc affects the reproductive state of the organism. In addition, the authors also assessed the AF effectiveness on the settlement of the benthic community. They identified, in total, 20 species belonging to 8 different phyla: Arthropoda, Mollusca, Porifera, Anellidae, Bryozoa, Cnidaria, Cordati, and Chlorophyta. They found lower diversity and richness values in panels treated with AF paint containing metal biocide, confirming their effectiveness as well as the level of toxicity that these types of compounds exert on the fouling community. This is in line with findings by Miller and colleagues [85] stating that coatings containing a mixture of these two biocides significantly reduce the settlement reported as abundance and biodiversity. The work carried out by Madonna and colleagues [4] allowed them to define the biodiversity of the submerged benthic community as related to the biofouling observed in their sampling zone and also allowed an understanding of how organisms such as *M. galloprovincialis* respond to the stresses induced by two types of antifoulants, i.e. eco-friendly and biocide AFs. They showed that an eco-friendly AF is ecologically more sustainable and does not cause oxidative stress as caused by toxic agents.

Natural products are suggested as an alternative to toxic biocides in AF paints for biofouling control [86]. Recent advances in AF research on natural products obtained from both marine and terrestrial sources have highlighted their biofouling inhibitory activities on marine microorganisms [87].

Bioactive compounds are synthesised by organisms in small and complex mixtures making their extraction and purification processes very laborious [88,89]. In addition, in order to derive bioactive compounds from marine organisms, a large number of organisms would need to be harvested from the sea. This may be of concern from a biodiversity conservation point of view [90].

Table 2. Summarized results and data references for eco-friendly antifoulants occurring in *Mytilus galloprovincialis*.

Eco-Friendly Antifoulant	Exposure Time	Toxicity Index/Concentration	Sample	Effect on Antioxidative Defence and Settlement	Reference
Silicone and hydrogel-based	12 months	nd.	Gonads	Settlement inhibition	Madonna et al. [4]
Napyradiomycin A1	15 h	EC_{50} = 0.655 [0.300; 0.906] µg/mL	Total body	Settlement inhibition	
18-Hydroxynapyradiomycin A1	15 h	EC_{50} = 1.999 [1.581; 2.547] µg/mL LC_{50} > 12 µg/mL	Total body	Settlement inhibition	
Napyradiomycins SF2415B3	15 h	EC_{50} = 1.092 [0.225; 2.933] µg/mL LC_{50} > 12 µg/mL	Total body	Settlement inhibition	
Napyradiomycin A2	15 h	$EC50$ = 6.339 [5.602; 7.181] µg/mL LC_{50} > 12 µg/mL	Total body	Settlement inhibition	
16-Oxonapyradiomycin A2	15 h	EC_{50} = 4.331 [2.911; 7.091] µg/mL LC_{50} > 12 µg/mL	Total body	Settlement inhibition	
4-Dehydro-4a-dechloro-napyradiomycin A2	15 h	EC_{50} = 4.331 [2.911; 7.091] µg/mL LC_{50}>12 µg/mL	Total body	Settlement inhibition	Pereira et al. [83]
4-Dehydro-4a-dechloronapyradiomycin SF2415B3	15 h	EC_{50} = 1.092 [0.225; 2.933] µg/mL LC_{50} = >12 µg/mL	Total body	Settlement inhibition	
Napyradiomycin B3	15 h	EC_{50} = 0.727 [0.065; 1.406] µg/mL LC_{50} > 12 µg/mL	Total body	Settlement inhibition	
Napyradiomycin a80915A	15 h	EC_{50} = 0.947 [0.586; 1.473] µg/mL LC_{50} > 12 µg/mL	Total body	Settlement inhibition	
Napyradiomycin a80915C	15 h	EC_{50} = 0.102 [0.072; 0.140] µg/mL LC_{50} > 12 µg/mL	Total body	Settlement inhibition	
4-Dehydro-4a dechloronapyradiomycin B3	15 h	EC_{50} = 0.727 [0.065; 1.406] µg/mL LC_{50} > 12 µg/mL	Total body	Settlement inhibition	
4-Dehydro-4a-dechloronapyradiomycin A80915A	15 h	EC_{50} = 0.947 [0.586; 1.473] µg/mL LC_{50} > 12 µg/mL	Total body	Settlement inhibition	
Rutin 2″,2‴,3′,3″,3‴,4′,4″,4‴,7-nonasulfate	Concentration- response analysis	EC_{50} = 22.59 µM; 36.84 µg/mL	Total body	Settlement inhibition	
3,6-bis (β-D-glucopyranosyl)xanthone persulfate	Concentration- response analysis	EC_{50} = 23.19 µM; 31.74 µg/mL	Total body	Settlement inhibition	Almeida et al. [33]
gallic acid persulfate	concentration- response analysis	EC_{50} = 17.65 µM; 8.4 µg/mL	Total body	Settlement inhibition	

Abbreviations: n.d., not determined, (i.e., no information reported in manufacturer's datasheet or literature); EC_{50}, Half maximal effective concentration, median [interquartile range]; LC_{50}, Lethal Concentration at 50% of the tested population.

Among the various natural products used as AFs are napiradiomycins derivatives. Napiradiomycins are an interesting group of natural halogenated secondary metabolites produced mainly by bacteria of the Streptomycetaceae family. Almost 47 different napiradiomycins have currently been described [91–94]. They belong to a class of hybrid isoprenoids and/or meroterpenoids previously known for their antimicrobial and antitumor activities [92–94]. In fact, in silico–toxicity predictions of napiradiomycins suggested similar toxicity to marketed drugs and AF biocides with a low bioaccumulation factor and no mutagenicity [83]. These compounds had already been shown to be effective at concentrations below 25 μg/mL [95].

Lacret and colleagues [96] showed that a novel napiradiomycin isolated from the genus *Streptomyces*, exhibited potent antimicrobial activity against microorganisms. Pereira and colleagues [83] attempted to identify new compounds with inhibitory activity against biofouling (Table 2). They isolated and tested 12 compounds from napiradiomycins. The authors assessed inhibitory capacity of napiradiomycins isolated from *Streptomyces aculeolatus* against micro- and macro-fouling species. They isolated five molecules from PTM-029 *S. aculeolatus* strains having a methyl group in the core structure at position 7 and seven molecules from PTM-420 *S. aculeolatus* strains containing hydrogen atom at position 7 having a hydrogen atom in that position of their structure. In order to assess the AF activity, the authors tested these group compounds on different organisms, including higher sessile organisms. Larval settlement tests conducted on *M. galloprovincialis* showed that both napyradomycins groups were effective in preventing their settlement with values ranging from 0.10 to 6.34 μg/mL. They found that the most promising AF affecting larvae were those with EC50 below 1 μg/mL (EC$_{50}$, half maximal effective concentration). In the same study, the authors also investigated the same napiradiomycin groups on lower organisms and tested their inhibitory activity on bacterial propagation and bacterial biofilm formation confirming their activity as AFs. Thus, Pereira and colleagues have shown napiradiomycins as potential AF agents. Given their potent antibacterial, anti-biofilm, and anti-sediment activities, they revealed the ability to prevent the settlement of higher organisms.

Nature has implemented several metabolic strategies, such as sulfation, to prevent toxic action during physiological and pathological processes. It has been observed that some sulphate secondary metabolites from marine organisms such as flavonoids, coumarins, cinnamic acids and sulphate sterols, have an AF effect while also being safe for the environment [97–102]. Since it is difficult and unsustainable to obtain quantities for commercial use directly from nature, the synthesis of non-natural small sulphate molecules could be an effective alternative for new and relevant non-toxic AF agents. These polysaccharide compounds are highly hydrophilic and thus able to form hydrogels that incorporate water. As shown by many studies, coatings with amphiphilic properties have a high potential for their use as inert surface coatings. This can be achieved by chemically modifying polysaccharide structures with hydrophobic molecules [85].

Almeida and colleagues [33] synthesised sulphate polyphenols and tested them for AF potential as assessed by anti-settlement activity in *M. galloprovincialis* plantigrade post-larvae (Table 2). From this class of polyphenols they isolated the following three compounds: rutin persulfate 3,6-bis(β-D-glucopyranosyl), xanthone persulfate and gallic acid persulfate. These compounds exhibited therapeutic ratios (LC 50/EC 50) greater than 15, which is consistent with the standard requirement for the level of efficacy of natural AF agents as established by the U.S. Navy Program [86]. They first showed that none of these isolated compounds were lethal on *M. galloprovincialis* at any of the concentrations tested compared to a biocide AF (commercial AF agent ECONEA®).

The authors found that gallic acid persulfate resulted in the most effective anti-settling activity on the *M. galloprovincialis* thus presenting the highest potential as an effective AF agent. When comparing the structures of the above three tested compounds, the presence of the benzoic acid scaffold in the gallic acid persulfate compared to the other two compounds led to the hypothesis that it is related to a more interesting AF activity, as already observed [103] on other organisms such as *Escherichia coli*. In addition, this

compound has a similar structure to a naturally occurring AF, zosteric acid, which is a metabolite of the marine herb *Zostera marina*. Zosteric acid has been found to have antibacterial [104–107] and antifungal [108,109] activity and prevents attachment of higher order organisms [110] at non-toxic concentrations. Its AF activity has been attributed primarily to the sulfate–ester group [106]. Recently, it has been shown that the anti-biofilm activity of zosteric acid against *E. coli* is related to the cinnamic acid scaffold [103,111].

In addition, these natural compounds were tested to quantify oxidative damage as reflected by acetylcholinesterase and tyrosinase which are involved in the settlement mechanism of *M. galloprovincialis* [112,113]. Adhesive plaques of DOPA-containing mussels are also produced by tyrosinase [114]. No effect was observed in the activity of these enzymes indicating that at least these two enzymatic processes seemed to remain unchanged after exposure to these AFs. Therefore, the authors found that the same compounds effective on mussels were less effective on biofouling bacteria. This led to the conclusion that the anti-settling action of sulphate compounds appears to be organism specific, more related to the metabolic pathways of *M. galloprovincialis* than to the biofouling succession cascade that begins with biofilm colonisation [115], while not altering the antioxidant response of the mussels. In addition, this study advanced the importance of the synthesis of small, non-natural sulphated molecules to generate a new class of non-toxic AF agents.

In nature, there are found not only compounds derived from bacteria with AF potential, but also AF compounds derived from algae (among other organisms). Benthic marine organisms are subject to intense competition for space [116]. It is assumed that benthic microalgae produce natural products showing AF activity. A natural product derived from benthic microalgae was recently discovered: the cyclic imin protamine. It was randomly identified during extraction of pinnatoxins from *Vulcanodinium rugosum* dinoflagellate [117]. Cyclic imines are a class of polycyclic ethers with a spirocyclic imine ring and are found only in microalgae [118]. They are generally fast-acting bioactives which inhibit neuromuscular transmission via blocking of nicotinic acetylcholine receptors and exhibit pro-apoptotic activity in cells [119]. Apoptosis plays a key role in the metamorphic processes of many biofouling organisms [120]. Given the high biocidal potential of portimin, its defensive activity against other benthic organisms may also be plausible [121].

In this regard, Brooke and colleagues [121] evaluated the AF activity of protamine against *M. galloprovincialis* (Table 2). Furthermore, a distinct AF action was seen by three related compounds: gymnodimine-A, 13-desmethyl spirolide C and pinnatoxin-F.

Larval development and metamorphosis were selected as endpoints of the bioassay because apoptosis is an integral part of these processes in marine invertebrates [122–125].

In fact, using *M. galloprovincialis* they found that the effect, as observed visually, of portamin on embryo/larvae morphology was concentration dependent. At a concentration lower than 0.15 ng/mL of protamine the organisms were intact but with incomplete embryogenesis. At higher concentrations (0.3 ng/mL) the organisms were disaggregated/disintegrated. Furthermore, none of the other three protamine related compounds were effective against *M. galloprovincialis*. Brooke and colleagues also tested inhibitory concentrations (EC50) of protamine against other marine macrofouling organisms, such as *Ciona savignyi*, *Spirobranchus cariniferus* and *Amphibalanus improvisus*, showing its potential as an AF. Thus, the authors found an impressive high potency of protamine against the settlement of *M. galloprovincialis* embryos/larvae which were absent at the end of the bioassay assuming that this compound can induce a massive apoptosis event to disintegrate and/or disaggregate *M. galloprovincialis* embryos.

4. Conclusions

Antifoulant paints were developed to prevent and reduce biofouling of surfaces in seawater. They were used as a surface treatment since ancient times. By the second half of the 20th century, TBT-based compounds were detected to be harmful to the ecosystem. Thus, as a substitute for highly toxic compounds, less damaging copper-based compounds were developed. Although less toxic, they are biocides designed to eliminate and reduce

organisms' settlement on surfaces. The widespread use of these substances over the years has led to a significant increase of their presence in the marine environment.

Due to an increasing number of toxicological studies, these compounds were identified as environmental and human threats. Thus, they were subject to regulation as in the case of organotin compounds which were completely banned by the International Marine Organization - Committee for the Protection of the Marine Environment (IMO-MEPC) in 1998, and subsequently by the Ordinance no. 782/2003, 14 April 2003, of the European Commission. Subsequently, new, naturally occurring compounds with much lower toxicity have been developed to replace the use of these toxic agents. They are designed to have the same efficiency as biocides without having a significant impact on the environment.

In this data collection, we have highlighted that biocide paints can cause antioxidant enzymatic alterations through reactive oxygen cell species and they can interfere with the formation of byssus in *M. galloprovincialis* leading to anti-settlement activity. We also discussed a dose-response relationship, i.e. different antioxidative responses caused by increasing biocide exposure in different tissues. In fact, antioxidative defence activation was found in gills and in the digestive gland, demonstrating how these biocides can disrupt organs responsible for filtration, storage/bioaccumulation and sustainability of reproductive processes/resources. In addition, some natural products derived from bacteria and algae have proved to be a valid ecological alternative to the canonical antifoulants on the market. They showed an inhibitory capacity against micro- and macro-fouling, with antibacterial, anti-biofilm and anti-settlement activity. In some cases, they exhibited similar toxicity to marketed drugs and antifouling biocides with a low bioaccumulation factor and no mutagenicity. Furthermore, through the analysis of biomarkers implicated in the antioxidative response in *Mytilus galloprovincialis*, it was found that they did not induce any oxidative damage. Taken together, the data obtained by these investigations emphasizes the discrepancy between biocide and eco-friendly antifoulants while also acknowledging that they both achieve the primary goal of inhibiting settlement. Moreover, using antioxidative markers we are able to detect the degree of environmental impact of antifoulants and their potential damage to biodiversity and human food security. Accordingly, to this end, transcriptomic antioxidative biomarker investigations as well as enzymatic investigations may provide useful indicators as to which future toxicological studies to focus on in order to evaluate environmentally friendly compounds used as antifoulants.

Since antifoulant paints have global economic and environmental impacts, the implementation of effective eco-friendly strategies is needed. In fact, the term antifoulant is no longer synonymous with biocidal. Bio-inspired chemical strategies are the most promising for developing a new generation of antifoulants.

Collaborations among governments, scientists, biologists, chemists, engineers and many others from various fields have motivated advancements in eco-friendly strategies for sustainable development to improve health and well-being (SDG 3), climate action (SDG 13), and Life Below Water (SDG 14).

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