Effects of Currently Used Antifouling Booster Biocides on Green Fluorescent Proteins in *Anemonia Viridis*

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Research Article

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Abstract

Some of the antifouling booster biocides affects the marine ecosystem negatively. The booster biocides which are resistant to degradation are accumulated in the sediment of the oceans. One of the sedentary organisms in the Mediterranean Sea is *Anemonia viridis*. The aim of this study is to show the toxicities of common biocides such as irgarol, seanine-211, zinc omadine, and acticide on the fluorescence by GFPs of *A. viridis*. The decreases in the fluorescence intensities of the GFP were measured within different booster biocide concentrations. The results show that fluorescent intensities of GFP proteins decreased more than 50 percent when they are exposed to different concentrations of irgarol, zinc omadine, acticide. In conclusion, ecosystem health should be prioritized when new antifouling paint compositions are proposed. From the results, it seems that *A. viridis* can be considered as a vulnerable organism and also it is sensitive to booster biocides within self-polishing antifouling paint formulations.

Introduction

The surfaces of artificial objects immersed into seawater are covered by fouling organisms such as bacteria, diatom, algae, barnacle etc. This event is known as "marine biofouling". Although this event is a natural process occurred in nature, it affects marine transportation adversely. When fouling organisms settle and develop on a ship's hull, this causes increase in drag force, decrease in speed, increase in fuel consumption which results in increase in CO₂ emission and introduction of invasive species into new regions. In order to prevent the fouling organisms' settlement onto ships' hull, special coatings are used. These coatings are known as "antifouling paints". Antifouling (AF) paints contain very toxic chemicals such as copper (I) oxide (Cu₂O) [1]. Even though copper is an effective chemical to control growing of many marine organisms, some algae can tolerate its negative effect. Therefore, additional biocides into AF paint formulations are needed to get rid of the settlement of fouling organisms that are resistant to copper compounds in AF formulations [2]. Thus, antifouling paints also contain booster biocides such as diuron, irgarol 1051, zinc and copper pyrithione besides Cu₂O to be used against a wide range of fouling organisms. By this way, they efficiently protect ships' hull but they also damage the marine ecosystems [2]. Since these chemicals are toxic, they do not only kill the organisms on the ships' hull, but they also damage on the non-target marine organisms living in the same ecosystem [2]. Ecotoxicological impacts of these chemicals concern many researchers around the world and toxic effect of these chemicals on marine species has been under investigation. Diuron [1-(3, 4-dichlorophenyl)-3, 3-dimethylurea]] and Irgarol 1051 [2-methylthio-4-tertiary-butylamino-6-cyclopropylamino-s-triazine], are mostly used booster biocides in AF paints and they are algaecides that inhibits the photosystem II of photosynthetic organisms. The half-lives of these chemicals in seawater are too long (1 months–1 year for diuron and 100–350 d for irgarol) [2]. The effects of diuron and irgarol on meiofauna obtained from the sediments of São Sebastião Channel, Brazil were investigated by Gallucci et al., 2015[3]. The results demonstrated that both biocides caused significant decrease in meiofauna density and diversity. Zinc pyrithione [bis (1hydroxy-2(1H)-pyridethionato-O, S)-T-4zinc] is a well-known biocide which shows bactericidal, algicidal and fungicidal activities [3]. Individual and synergistic toxic effects of zinc pyrithione and copper on the

marine copepod *Tigriopus japonicas* were investigated by Bao et al. (2014) [4]. Mortality of adult copepods increased dramatically when they were exposed to both chemicals together while less mortality was observed when they were exposed to zinc pyrethrin and copper alone at the same concentrations.

Anemonia viridis is one of the sedentary marine organisms in the Mediterranean Sea. Sea anemones are classified under Cnidaria phylum and Anthozoa class. *Anemonia viridis* has different phenotypic variations based on the colour of tentacles which caused by host pigments such as green fluorescent protein (GFP), red fluorescent proteins or pink non-fluorescent chromoproteins [5]. Green tentacles including GFPs, with pink tips, due to the expression of the chromoproteins, are specific to *Anemonia viridis* [5]. In addition to these features, *Anemonia viridis* is an example for endosymbiotic life form between marine invertebrates belongs to *Cnidaria phylum*, and unicellular photosynthetic algae commonly named zooxanthellae [6]. Zooxanthellae are protected from its predator within the host animal. Also, cnidarian supplies inorganic compounds for photosynthetic activity of microalgae and host animal utilizes the organic carbon products of photosynthesis [6]. However, under stressful conditions such as high sea temperatures, cold shock, infections, reduced salinity, etc. host animal expels zooxanthellae and/or zooxanthellae lose their pigments which results in decolorization of host animal [7]. This phenomenon is known as 'bleaching' which causes an important ecological problem. Observation of bleached *Anemonia viridis* samples within ports can be explained with the exposure of antifouling booster biocides within the water bodies which are detectable levels in Turkish coastlines [8]. Since *Anemonia viridis* are vulnerable and also sensitive living organisms in the Mediterranean Sea, possible reasons of the bleaching events in anemone samples should carefully be investigated. The present paper aims to research the hypothesis related to negative effects of booster biocides within AF paints formulations. To the best of our knowledge, this is the first scientific research on the effects of booster biocides on *Anemonia viridis*.

Materials And Methods

Collection of biological material

In Turkey, an official permission is needed for the collection of biological materials from sea ecosystems. A written permission from General Directorate for Agricultural Research and Policies in Turkish Ministry of Agriculture was taken before the collection of biological materials. *Anemonia viridis* was collected from Tuzla-Istanbul, Turkey. The geographical coordinates (Google Earth) are 40°48'56.41"N and 29°17'36.28"E. The temperature of seawater when specimens were collected was between 10.5°C and 12.3°C. The organisms were transferred to the laboratory within a plastic box in one hour after collection. The samples used in this paper were *Anemonia sulcata* var. *smaragdina* and *Anemonia rustica*. While the first species is characterized with the pink tips in their tentacles, *Anemonia rustica* has brown tentacles. Although both species were initially recognised as the color morphotypes of *Anemonia sulcata*, Bulnheim and Sauer (1984) separated these two species based on alloenzyme frequencies [7, 9]. According to a

very recent paper by Mallien et al (2017), the both species can be classified under *Anemonia viridis* [5]. Therefore, we used *Anemonia viridis* to express the collected samples.

Preparation of green fluorescent protein-rich supernatants

Anatomical structure (cross section) of *Anemonia viridis* was given in Figure 1. The tentacles were cut from their bases and then they stored at -14°C until used. The tissue concentration in supernatants was 0.017 g wet tissue / mL.

Figure 1 Anatomical section of *Anemonia viridis*

The tentacles are divided into three sub-zones as green, pink and green/pink zone as given in Figure 2. The tentacle samples were ground by glass rode within an eppendorf tube.

Figure 2 Zones of *Anemonia viridis* tentacle

The homogenised solution was mixed vigorously before centrifugation at 4000 rpm for 7 minutes. The supernatants were taken for further measurements in spectrofluorometer (Perkin Elmer LS 55). Shimadzu UV-1800 spectrophotometer was used to take the UV-VIS spectra of the supernatants.

Biocide based experiments

For all biocide based experiments, 950 µl GFP supernatant was used. The amount of biocide volume in 1 mL quartz cell was 50 µl with various concentrations. Distilled water was used as a solvent for acticide (Thor) and acetone for irgarol. Xylene was used as a solvent for seanine 211N-(DOW-Turkey). Ethanol was used as a solvent of zinc omadine (Lara, Atlanta, GA). Irgarol (46105, purity: 98.4%) standard was purchased from Fluka.

Determination of protein in the GFP-rich supernatants

Bradford method was used to determine the protein concentrations in the GFP-rich supernatants [10]. From Bradford protein analysis, protein value in GFP-rich supernatant was found as 1.3 µg/ml. The experiments show that when tentacles are frozen before the homogenisation process amount of GFP extracted is increased. Also storage conditions are tested and the results show that when GFP extract solution is frozen at -14° C no significant change was observed at its fluorescence intensity (data not shown).

Results And Discussion

The effects of antifouling booster biocides on the GFP-rich supernatants were investigated in this study. *Anemonia viridis* contains different GFP molecules on their tentacles. The colour difference can be seen in the tentacles under day light and also UV-light easily (Figure 2, the photo was taken under daylight). In our research, there are 3 different regions in the tentacles of *Anemonia viridis* that were collected from Tuzla region. These regions are green zone (approximately 2 cm from the base) green/pink zone and pink zone (approximately 3 cm from the base). The comparison of different zones' emission values of

Anemonia viridis was given in Figure 3. The samples taken from green zone of the tentacles show the highest fluorescence intensity whereas the samples taken from pedal disk of *Anemonia viridis* has the lowest fluorescence intensity.

Figure 3 Comparison of different zone's emission values of fresh *Anemonia viridis* ($\lambda_{\text{ex}} = 425\text{nm}$; slit: 5 nm)

The changes of emission spectra of *Anemonia viridis* collected from different days (1st, 2nd, and 17th days) are shown in Figure 4. When the spectral properties of *Anemonia viridis* are concerned, these changes fit well the photoprotection of GFPs. Because it is possible by photosynthesis action which realizes between the waveband in the maximum of its spectrum and the waveband where photosynthetic pigments absorb [11].

Figure 4 Intra-species comparison of different fresh *Anemonia viridis* emission values ($\lambda_{\text{ex}} = 425\text{nm}$; slit 5 nm for sample 1(1st day) and sample 2(2nd day); slit 2.5nm for *Anemonia viridis* sample 3-6 (collected on the 17th day)).

On the other hand, although the emission bands of some GFP are not the waveband of photosynthesis action, the energy transfers from green to green-pink region's pigments can be occurred. Therefore, results suggest that fluorescent coupling is carried out the photoprotection function of green fluorescent pigments [11].

The range of irgarol concentration studied was 0.001-0.01 g/L. The effects of irgarol concentrations on the fluorescence intensity were studied and the results were given in Figure 5.

Figure 5 Effects of irgarol concentration on the relative GFP fluorescent intensity (% , I_{max}).

A remarkable relationship was observed between increased irgarol concentration and decreased fluorescence intensity in our samples. The experimental results show that fluorescence intensity decreased by more than 50% after 0.01 g/L irgarol concentration. GFP is very sensitive to irgarol when relative intensity begins to decrease even at low concentration between 0.001 and 0.01 g/L. The effects of irgarol on the inhibition in cell number and decrease in photosynthetic activity of marine organisms are discussed by Amara et al., 2018 [12]. Based on the published reports on the irgarol [13- 18], it could be said that irgarol can mainly be associated with the disruption of photosynthetic electron transport chain and also it may interact with other important supramolecules in living organisms. Fernandez-Alba et al (2002) reported the range of EC_{50} for individual and mixture of booster biocides as 0.001 to 28.9 mg/L [16]. They studied *Vibrio fischeri* and *Daphnia magna* as the model organisms. The toxicity of well used booster biocides, Irgarol 1051 and Sea-Nine 211, on marine alga *Fucus serratus* were studied by Braithwaite and Fletcher (2005) [17]. No observable effect concentrations as 8 $\mu\text{g/L}$ for both Irgarol 1051 and Sea-Nine 211 were given by the authors. Arrhenius et al (2006) studied the algal reproduction and periphyton photosynthesis in the conditions of well-known booster biocides such as TBT, diuron and irgarol [18]. They reported the NOEC values for periphyton photosynthesis to be 32 nM, 1.8 nM and 178

nM for TBT, Irgarol and Sea Nine 211, respectively. On the other hand, NOEC values related to algal reproduction for TBT, Irgarol and Sea Nine 211 were given to be 133 nM, 2 nM and 96 nM, respectively. These low values reveal that photosynthetic organisms are remarkably affected by the booster biocides. In our research, we found the minimum concentration as 10 mg/L to decrease the fluorescent intensity as 55%. This value is well in line with the published reports discussed above. It is very important to note that the supernatant including GFPs were studied in this report compared to the published reports in which live organisms used in toxicity assays. The toxicity of booster biocides was also tested on some vertebrates. Okamura et al (2002) studied suspension-cultured fish (*Oncorhynchus tshawytscha*) cell line to show the toxicity of booster biocides released from antifouling paints[14]. The authors reported low toxicity of sea nine and irgarol compared to the compounds such as copper pyrithione and zinc pyrithione in their study. As can be known from the biology of sea anemones, the anemones are consisted of symbiotic life form of zooxanthellae and anemones. Zooxanthellae settles inside the tentacles of anemones. Therefore, exposure of these photosynthetic algae to the booster biocides released from self-polishing antifouling paints may harm the population of zooxanthellae. Decreased intensity of zooxanthellae in seawater may be one of the explanations for the bleaching of anemones. However, the zooxanthellae density in seawater where the bleaching of anemones observed must be studied to explain this phenomenon.

Figure 6 Effects of seanine concentration on the relative GFP fluorescent intensity (% I_{max}).

To study the effect of sea nine 211(N), the concentration ranges between 0.05-5.00 (% v/v) was interacted with the GFP extract obtained from *Anemonia viridis*. According to Figure 6, no significant decrease was observed for sea nine 211(N), the structure of sea nine 211(N), contains a n-octyl, a carbonyl and two chloride groups. Although we did not observe remarkable negative effects on the fluorescence intensity of GFP, this does not mean that this chemical is safe for marine ecosystems. Many scientific papers report negative effects such as endocrine disruption and reproductive impairment [19], early developmental stages of the sea urchin *Paracentrotus lividus* [20–21], and inhibition of the detoxification system in *Oryzias melastigma* [22]. A new regulation, a possible ban, on this chemical must be discussed under the lights of the scientific papers related to the effects of this chemical on marine ecosystem.

Zinc omadine, one of the well-known antifouling agents, was also studied to understand its effect on the GFP from *Anemonia viridis*. According to Figure 7, zinc omadine decreased the fluorescence intensity significantly.

Figure 7 Effects of zinc omadine concentration on the relative GFP fluorescent intensity (% I_{max}).

Metabolic effect of the chemical was explained many years ago. Chandler and Segel (1978) reported its membrane disturbing effect [23]. After banning of TBT based paints in the antifouling paint market, zinc omadin was used widely as one of the alternative compounds of TBT because of its easy degradation. However, many papers mentioned the resistant degradation of zinc omadin such as mentioned by

Marcheselli et al (2010) [24]. Active zinc atom in the structure might have been interacted with the 3-D structure of GFP. Reeder et al (2011) showed that zinc pyrithione shows antifungal effect by increasing cellular copper level. This causes collapsing of iron-sulphur clusters in the proteins [25]. The toxic effects of zinc omadine on the three marine microalgae *Tisochrysis lutea*, *Skeletonema marinoi* and *Tetraselmis suecica* were studied by Dupraz et al (2018) [26]. When they tested the toxicities of irgarol, zinc pyrithione and copper pyrithione individually, they found the toxicity of irgarol remarkable compared to zinc pyrithione and copper pyrithione. The authors also reported the increased efficiency in binary mixtures with copper pyrithione. Our results partially support the values of Dupraz et al (2018) [26]. Single chemical such as zinc pyrithione may reveal different effects on the different microalgal species. Marcheselli et al (2011) studied the effects of zinc pyrithione on the marine mussel *Mytilus galloprovincialis* [27]. The authors reported the bioaccumulation of zinc pyrithione in the tissues of *Mytilus galloprovincialis*. They also reported HSP over-expression and DNA damage in the important tissues of this marine mussel even if the samples were exposed to the non-lethal concentrations. Although we did not measure the level of zinc pyrithione in the tissues of sea anemones, it is clear that a possible zinc pyrithione bioaccumulation may be expected in the living organisms in the harbours.

The bioactive mixtures are also used in the antifouling paint manufacturing. The composition of different bioactive agents show better antifouling performance compared to the individual compounds. One of the recommended commercial products, acticide produced by Thor, was also tested in this study. The active agents in acticide are isothiazolinones, iodopropynyl butyl carbamate and a range of quaternary ammonium compounds reported by the manufacturer (<https://www.thor.com/biocides.html>).

Figure 8 Effects of acticide concentration on the relative GFP fluorescent intensity (% $I_{\max}$).

The range of acticide concentration studied was 0.25-3% (v/v). In order to show the relationship between acticide concentration and fluorescence intensity, Figure 8 was plotted. From Figure 8, it could be said that increased concentration of acticide decreased the fluorescence intensity from *Anemonia viridis* extracts. According to the literature, it has fungistatic and bacteriostatic properties. This product is generally used to prevent the microbial colonisation in the outer surfaces of the historical buildings for restoration purposes [28]. There has been no published item in the scientific literature related to its antifouling performance of this product. Since we did not study each of the ingredients separately, each chemical in the content might have showed different effects on the GFP fluorescence intensity. Further studies can be carried out for understanding the responsible chemical in the acticide on the decreased fluorescence intensity of GFP from *Anemonia viridis*.

Conclusion

Antifouling paints are of great importance for maritime transportations. These paints directly affect the cost of transported materials in ships. Up to 2003, the TBT-based paints showed excellent success and provided fouling free ships' hull. On the other hand, environmental impacts of the TBT based paints created a big gap in the antifouling paint industry. Currently, self-polishing antifouling paints in which

rosin is used as binder are commonly used in the antifouling paint industry because of their low-cost prices. Self-polishing antifouling paints release toxic compounds such as copper (I) oxide to marine ecosystems. Therefore, eco-friendly versions of the antifouling paints should be developed. In the present study, we investigated the effects of common biocides and mixtures used in the self-polishing paints on a sedentary animal in marine ecosystem, *Anemonia viridis*. The results show that some of the antifouling agents investigated in this study decreased the intensity of GFP of *Anemonia viridis*. Since GFP is considered as a bioindicator molecule for the anemones in the marine ecosystems, a new biotest based on GFP can be developed beside the current toxicity test animals. In conclusion, the list of affected marine organisms from commonly used antifouling paints/biocides must be prepared so that manufacturers can take care of their productions. The booster biocides which can be easily degraded via marine microorganisms and sun light should be selected to produce antifouling paints. Moreover, green label can be assigned for new antifouling paint that includes no harmful booster biocides to the marine ecosystems. This will increase not only sale rates but also it will create an awareness on the environmental protection.

Abbreviations

GFP Green Fluorescent Protein

AF Antifouling

UV Ultra-Violet

EC₅₀ Half maximal effective concentration

TBT Tributyltin

HSP Heat Shock Protein

DNA Deoxyribonucleic acid

NOEC No observed effect concentration

Declarations

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Authors’ contributions All authors contributed to the study conception and design. Material preparation, data collection and analysis were performed by all authors. The first draft of the manuscript was written by [Batuhan Ünver]. The manuscript was reviewed and edited by [Levent Çavaş], and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

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Figures

Figure 1

Figure 2

Zones of Anemonia viridis tentacle

Figure 3

Comparison of different zone's emission values of fresh Anemonia viridis ($\lambda_{ex}=425\text{nm}$; slt: 5 nm)

Figure 4

Intra-species comparison of different fresh *Anemonia viridis* emission values ($\lambda_{ex}=425\text{nm}$; slit 5 nm for sample 1(1st day) and sample 2(2nd day); slit 2.5nm for *Anemonia viridis* sample 3-6 (collected on the 17th day)).

Figure 5

Effects of irgarol concentration on the relative GFP fluorescent intensity (% , I_{max}).

Figure 6

Effects of seanine concentration on the relative GFP fluorescent intensity (% , I_{max}).

Figure 7

Effects of zinc omadine concentration on the relative GFP fluorescent intensity (% , I_{max}).

Figure 8

Effects of acticide concentration on the relative GFP fluorescent intensity (% , I_{max}).