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# Antifouling Performances and Acute Toxicities of Eco-Friendly Biocides

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**Abstract**

There is a great need for developing of eco-friendly antifouling biocides since negative ecological effects have been proven for current antifouling biocides. In the present study acute toxicities of potassium sorbate (KS), tannic acid (TA), copper(II) sulphatepentahydrate (CS), *Caulerpa prolifera* extract (CPE) were investigated on the survival of *A.salina*. $LC_{50}$ values were found for KS, TA, CS and CPE as 19.96 g/L, 155.30 g/L, 10.47 g/L and 20.40%, respectively. In order to investigate the antifouling performances, the biocides were immobilized into phytagel and submerged into sea for one month in inner bay of İzmir. In conclusion, slightly dissolving versions of studied eco-friendly biocides could be replaced with copper based antifouling biocides.

## INTRODUCTION

Biofouling can be defined as attachment of micro and macro marine organisms onto a surface [1,2]. It begins with adsorption of organic compounds such as proteins, carbohydrates, lipids etc. to the surface which is also called as conditioning film formation [3,4] and this reversible process is driven by physical forces such as Brownian motion, electrostatic interactions and van der Waals forces [5,6]. Conditioning film formation is followed by settlement of bacteria. Settlement starts with adsorption process that is influenced by physical forces. It proceeds with irreversible adherence process due to the covalent bond formation between adhesive extracellular binding polymers secreted from bacteria and adsorbed molecules on the surface [1,4]. Increasing population of settled bacteria results in the formation of biofilms. This provides a good environment for the attachment of other micro and macro organisms such as diatoms, protozoa, barnacles and algal species onto artificial surface immersed in seawater. [4,7,8]. These organisms prefer accumulation since this behavior not only brings protection from predators and toxins but also obtaining nutrients easily by fouling onto a surface [6,9].

Biofouling is a major problem for the ships. As fouling organisms cover the hulls of the ships, they

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cause to increase of weight and reduction of speed and thus fouling leads to high fuel consumption, limitations in maneuverability and corrosion on the ship's hulls [10,11].

In the past some toxic substances were used in surface coatings of the ships for the prevention of biofouling. For this purpose, tributyltin (TBT) based compounds such as organotins and tin based polymers, booster biocides like irgarol and diuron, heavy metals such as copper oxide, zinc, arsenic and mercury oxide were successfully used as ingredients in antifouling paints [12]. Application of TBT based compounds in the antifouling paints formulations were banned after January 1, 2003 and the presence of such paints on the surface of the ships were completely restricted after January 1, 2008 [13]. Therefore, antifouling paint companies and scientists from universities and also research institutes have started to create novel eco-friendly approaches to get rid of the harmful effects of TBT based structures and also other harmful biocides.

Copper(I) oxide ($Cu_2O$) is still being used as an antifouling pigment in marine paints. Dissolution of copper(I) oxide involves formation of copper chloride complexes ($CuCl_2^-$, $CuCl_3^{2-}$) in two reversible reactions. In the presence of $O_2$(aq), copper complexes are oxidized to $Cu^{2+}$ ions which form coordination complexes with inorganic and organic compounds [14,15]. Variation between its species depends on pH, salinity and the presence of dissolved organic matter. It has shown that $Cu^{2+}$ ions are more toxic than $Cu^+$ and high concentrations of $Cu^{2+}$ ions in seawater have deleterious effects on marine ecosystem [16].

Diuron is a phenylurea and Irgarol 1051 is a hydrophobic triazine which are known as key booster biocides and both of them act on plants in similar ways, as inhibitors of photosynthesis by blocking electron binding sites at photosystem II [10,17,18]. Degradation product of Irgarol 1051 is M1 (2-methylthio-4-tertbutylamino-6-amino-s-triazine); on the other hand, Diuron degrades to DCPMU (1-(3,4-dichlorophenyl)-3-methylurea) and DCPU (3,4-dichlorophenylurea) which are relatively stable compared with the other organic booster biocides [19]. Due to their harmful effects on ecosystem, application of Diuron was totally banned and use of Irgarol on vessels longer than 25 m was restricted in UK since November 2002 [20,21].

So far, many attempts have been done to develop eco-friendly antifouling biocides compound in the literature. Among them, Perez et al [22] have recommended cupric tannate as an alternative biocide instead of $Cu_2O$ based paints. In another study, Stupak et al. have demonstrated that tannins have narcotic effects on nauplii of *Balanus amphitrite* [23].

Tannins, especially found in higher plants and brown algae, are phenolic compounds and they are able to form complexes with metals and also able to precipitate proteins. Due to their phenolic structure tannic acid derivatives show anti-corrosive, anti-bacterial and antifouling properties [22,24].

Potassium sorbate which is presently being used as food preservative, is another potential eco-friendly biocide. According to Blustein et al., potassium sorbate have reversible inhibitory effect on the settlement of nauplii and cyprids of *Balanus amphitrite* for a wide range of concentrations [25].

There are many algae species in sea ecosystems and they are also exposed to marine fouling. Some algae species have developed natural antifouling agents as a response to fouling organisms during their evoulutions. Most of algae species have many different types of secondary metabolites to get rid of the fouling organisms. Among marine algae, *Caulerpa* Genus is famous because of its two exotic-alien members, *Caulerpa racemosa* var.*cylindracea* (hereafter *C.racemosa*) and *Caulerpa taxifolia*. Although *C.taxifolia* is more famous compared to *C.racemosa*, the latter one has invaded 13 Mediterranean countries (Albania, Algeria, Croatia, Cyprus, France, Greece, Italy, Libya, Malta, Monaco (M.Verlaque, pers. commun.), Spain, Tunisia and Turkey [26]. *C.taxifolia* has been reported from 7 Mediterranean countries. Two species are well known and well studied invasive species in the Mediterranean Sea. Their successes can be associated with their joint secondary metabolite called caulerpenyne (CYN) [27-30]. CYN has sesquiterpene structure and shows antiproliferative and antiviral as well as antimicrobial properties [30-33]. Therefore, CPE which contains CYN, can be an ideal eco-friendly antifouling biocide.

Antifouling performances of eco-friendly biocides such as CT, TA and KS have been shown by Pérez et al. [22], Pérez et al. [24], Blustein et al. [25], respectively. However their antifouling performances have been detected at Mar del Plata harbour in Argentina. Therefore, in the present study, we also wanted to see antifouling performances of these eco-friendly biocides in the Mediterranean Sea by far from Argentina coastlines.

*A.salina* is a well accepted test animal because of its resistance to wide range of toxic substances, high hatching ability and commercial availability [34,35]. Use of *A.salina* in the toxicity test of antifouling agents is underlined by Koutsaftis and Aoyama (2007) [35].

Although potassium sorbate and tannic acids were recommended as eco-friendly biocides for antifouling paint technology [24,25], their toxicity effects were not tested on *A.salina*. Antifouling performance of another potential eco-friendly biocide, CPE, was also tested.

In this study it was aimed to compare antifouling performances of CPE against five potential eco-friendly biocides such as cupric tannate (CT), copper(I) oxide ($Cu_2O$), zinc(II) oxide (ZnO), potassium sorbate, tannic acid published in the literature. Acute toxicities of KS, TA, CS and CPE against *A.salina* were also determined dependent on time.

## MATERIALS AND METHODS

*A.salina* eggs were kindly provided by Ocean Life Aquarium Company in İzmir-Turkey. The eggs were placed into artificial seawater at 22-25$^{o}$C and conditioned for 3 days. Artificial seawater (AS) was prepared according to Grasshoff's method [36]. Actively swimming 10 individuals in 20 μL of AS were taken and placed into 96 wells microplate. Total volume of a microplate well was fixed to 220 μL by adding 200 μL biocide solution in different concentrations. Increasing concentrations of biocide solutions were prepared in AS by diluting from stock solutions. All experiments were repeated for three times. Survival percentage of *A.salina* in each microplate well was determined by counting actively swimming individuals after exposure to biocide solutions at corresponding time intervals. Differences between survival percentages of same and adjacent concentrations were statistically analysed by ttest method. $LC_{50}$ values were obtained from plotting survival percentage against different concentrations of biocides and reading corresponding concentration which is lethal to 50% of animals.

In the field tests, phytagels in petri plates which contained different concentrations of biocides were hanged between the period of February 9 - March 12, 2010 in inner bay of İzmir, Turkey. The geographical coordinates of the region are 38°24'27.63"K and 26°59'57.38"E. Stock solutions were prepared for each substance and later diluted to four different concentrations. The concentrations of stock solutions were 4 g/L for TA, 100 g/L for CS, 4.0 M for KS and 750 µL for CPE. Dilutions were made in the ratio of 100%, 75%, 50% and 25% (v/v). CT was prepared according to Pérez et al. [22]. Briefly, the tannin solution was prepared by dissolving 10 g of tannin in 500 mL of hot water. This solution was added simultaneously with 1.0 M 40 mL of cupric sulphate solution into a beaker containing 0.04 M 500 mL of sodium hydroxide by stirring constantly at 60$^oC$. In order to adjust pH of the solution, 40% (w/v) potassium hydroxide solution was added until the solution reaches to pH=5.5 and final solution was cooled in room temperature. Finally CT was obtained by filtration via Buchner funnel. CPE was prepared by homogenization of 5 g wet algae with 25 mL methanol. To prepare gel samples, 3.2 g of phytagel was dissolved in 100 mL of water by stirring for a few seconds and heated until it boils. Boiling solution was cooled to 45$^oC$ and transferred into petri plates. Then 5 mL from each test solution was added to phytagels and stirred strongly. Phytagels with cupric tannate, zinc(II) oxide, copper(I) oxide were prepared by adding 0.1, 0.2, 0.3, and 0.4 g from each biocide to phytagel solution at 45$^oC$. 25 phytagels were prepared, 24 of them were containing test solutions and one of them taken as control.

Figure 1. Control for the biocides submerged in İzmir inner bay.

## RESULTS

Antifouling performances of ZnO, $Cu_2O$, CT, KS, TA and CPE in phytagels were investigated in four different concentrations. Surface of control group was totally covered by microslime layer and seaweeds mainly *Ulva sp*. (Figure 1).

Antifouling effects of ZnO, $Cu_2O$, CT were higher than control group. It was very interesting to note that ZnO did not show any antifouling performance. The biofouled area of phytagel sample which contained 0.4 g ZnO was higher than that of sample that contained 0.1 g ZnO as can be seen at Figure 2.

Contrast to observations for ZnO, antifouling effects of $Cu_2O$ and CT were increased in order of increasing concentrations. Although $Cu_2O$ samples marked as A and C in Figure 3 were covered with mud not fouling organisms, it could be said that their antifouling performances were higher than that of

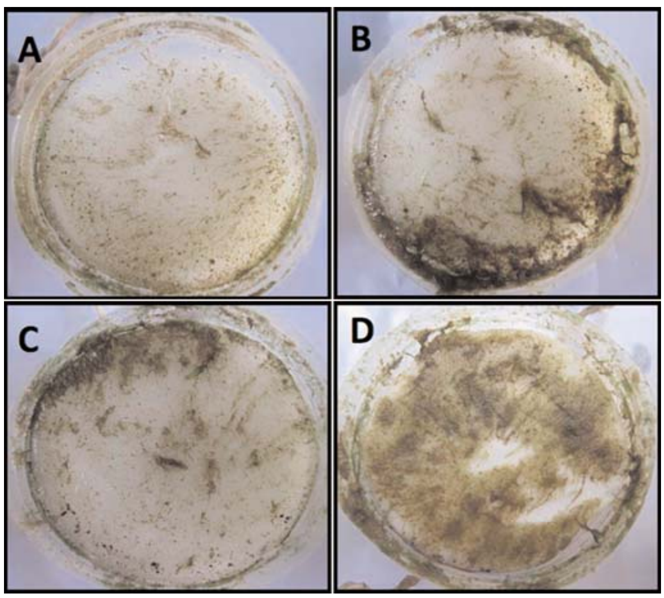


Figure 2. ZnO gels in order of increasing concentrations. (A) 0.1 g ZnO, (B) 0.2 g ZnO, (C) 0.3 g ZnO, (D) 0.4 g ZnO.

control. As can be seen from Figure 3B, a *Mytilus sp.* was settled on the phytagel sample which contained 0.2 g $Cu_2O$. On the other hand, no settlement was recorded for 0.4 g $Cu_2O$, 0.2 g CT and 0.4 g CT. Besides, there were muddy spots with no biofouling organisms on surface of 0.3 g CT can be seen at Figure 4C.

Antifouling performances of KS, CPE and TA were unexpectedly lower than that of control group. Surfaces of phytagels containing KS, TA and CPE were covered with seaweeds and microorganisms. Among them most fouled ones were TA samples (Figure 5). Antifouling performances of KS and CPE were similar to each other but greater than that of TA samples (Figures 6 and 7, respectively).

In order to see the environmental effect of chosen biocides, *A.salina* was selected as a model species for acute toxicity experiments. In these experimental procedures $Cu_2O$ and CT were not used because of their low solubilities. Instead of them CS and TA were used in eco-toxicity tests, respectively.

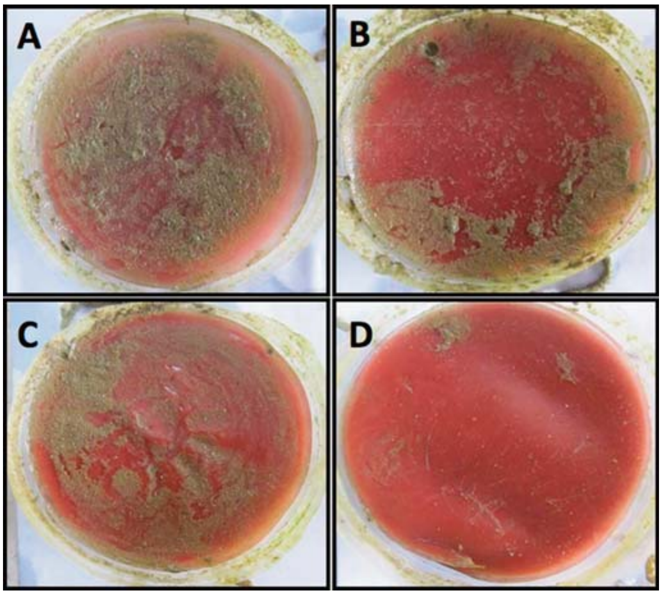


Figure 3. $Cu_2O$ gels in order of increasing concentrations. (A) 0.1 g $Cu_2O$, (B) 0.2 g $Cu_2O$, (C) 0.3 g $Cu_2O$, (D) 0.4 g $Cu_2O$.

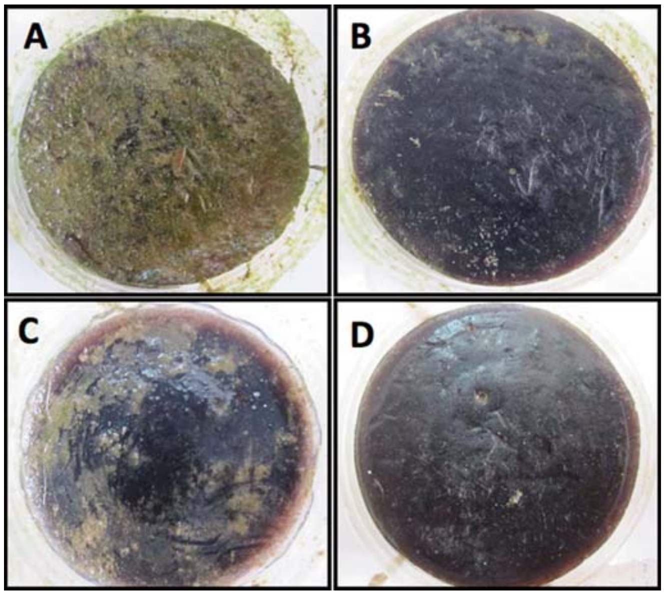


Figure 4. CT gels in order of increasing concentrations. (A) 0.1 g CT, (B) 0.2 g CT, (C) 0.3 g CT, (D) 0.4 g CT.

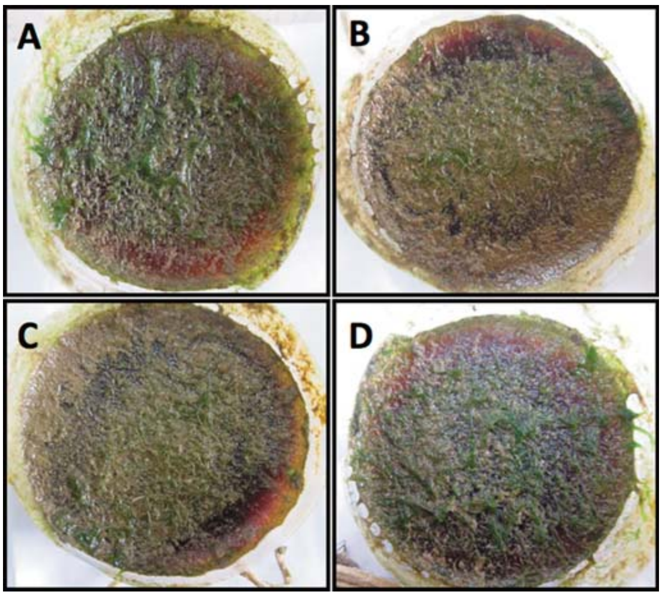


Figure 5. TA gels in order of increasing concentrations. (A) 16 g/L TA, (B) 32 g/L TA, (C) 64 g/L TA, (D) 128 g/L TA.

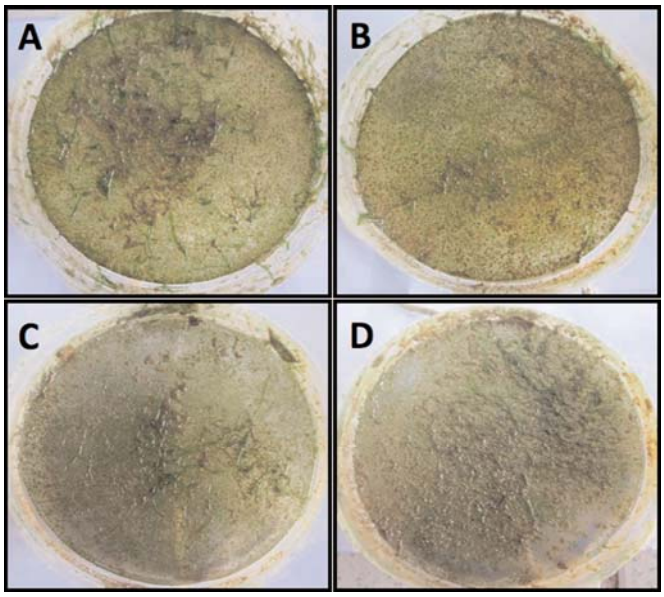


Figure 6. KS gels in order of increasing concentrations. (A) 0.5 M KS , (B) 1 M KS, (C) 2 M KS, (D) 4 M KS.

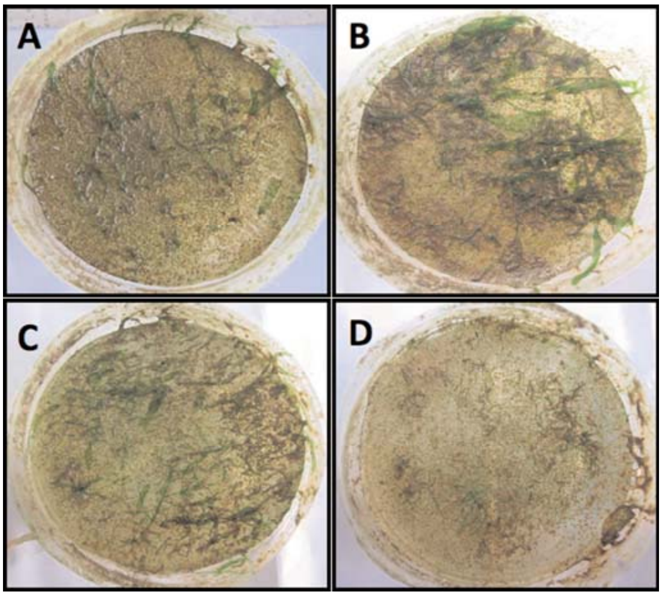


Figure 7. CPE gels in order of increasing concentrations. (A) 0.25 ml CPE, (B) 0.5 ml CPE, (C) 0.75 ml CPE, (D) 1 ml CPE.

The ranges of studied concentrations for determination of percentage survival of *A.salina* were obtained from previous experiences. There was no difference among survival percentages of *A.salina* exposed 10 g/L and 15 g/L of KS compared to control ($p>0.05$). Mortality of animals increased sharply at 25 g/L KS concentration after the 40 min of exposure. Survival percentage of *A.salina* were different ($p<0.05$) for 15 g/L and 25 g/L at 180th min. Although *A.salina* has resistance against toxins, no surviving animal was detected at 35.6 g/L KS concentration even at the earlier periods of experiment (Figure 8).

It can be said that TA did not show any significant effect on *A.salina* until 40th min. No difference was appeared in first two concentrations during 180 min ($p>0.05$). At the 180th min more than half of animals were affected in 128 g/L TA. At the 390th min mortality was similar between the 64 g/L and 128 g/L as shown in Figure 9. Percentage survival of *A.salina* was suddenly decreased after 40 min and no living animals were recorded at 390th min.

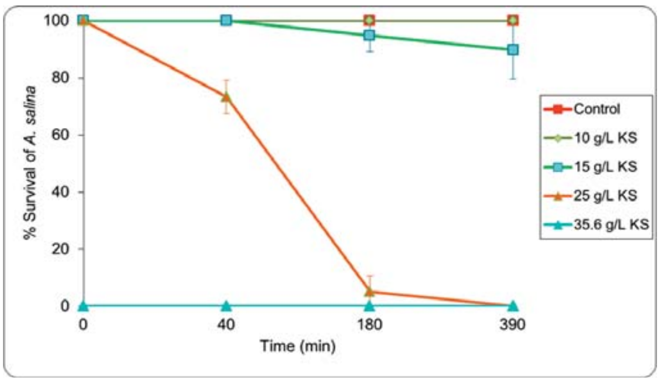


Figure 8. Survival percentage of *Artemia salina* exposured to different concentrations of potassium sorbate (KS).

All concentrations of CPE showed the same effect during first 30 min. After the 150th min a few *A.salina* stayed motionless in 15% CPE and most of animals were dead in 25% CPE. 50% CPE has shown high inhibitory effect on *A.salina* after the 150th min (Figure 10).

CS solutions in all concentrations were caused an orderly decreasing curve as expected. Although the values of intermediate concentrations were close to each other at the 180th min survival of *A. salina* had lower value at the last concentration of CS (Figure 11).

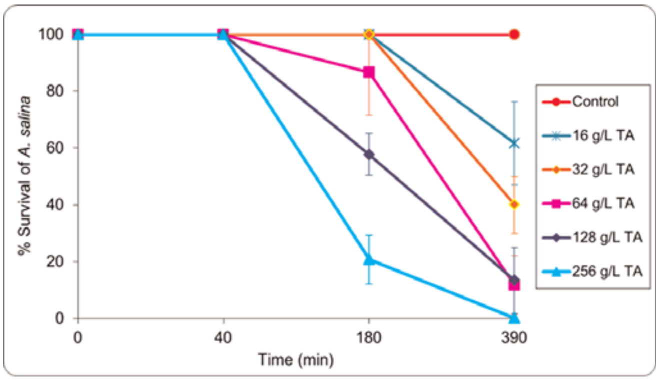


Figure 9. Survival percentage of *Artemia salina* exposured to different concentrations of tannic acid (TA).

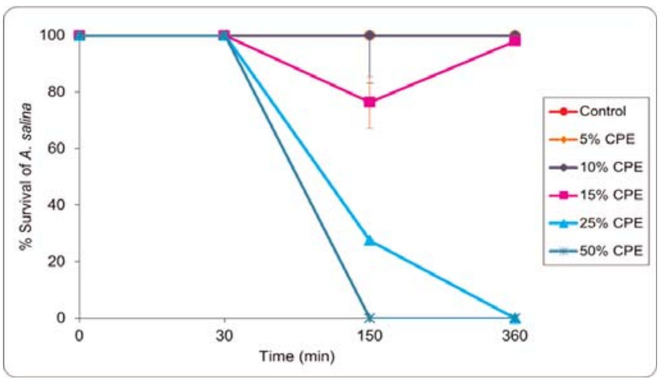


Figure 10. Survival percentage of *Artemia salina* exposured to different concentrations of *Caulerpa prolifera* extract (CPE).

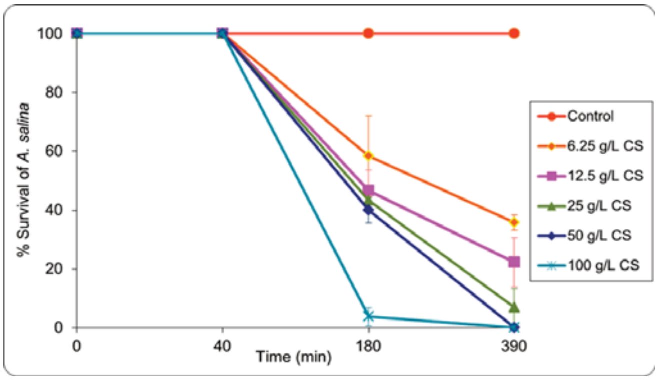


Figure 11. Survival percentage of *Artemia salina* exposured to different concentrations of copper(II) sulfate pentahydrate (CS).

Table 1. $LC_{50}$ values of KS, TA, CPE, and CS for *A.salina*.

| Biocides | KS, g/L | TA, g/L | CPE, % | CS, g/L |
|---|---|---|---|---|
| $LC_{50}$ | 19.96 | 155.30 | 20.40 | 10.47 |

$LC_{50}$ values for KS, TA, CS and CPE solutions have illustrated in Table 1. Lethal concentrations of KS, TA, CS and CPE on *A. salina* were found as 19.96 g/L, 155.30 g/L, 10.47 g/L and 20.40%, respectively (Table 1).

## DISCUSSION

In this study, acute toxicities of some eco-friendly biocides were investigated on *A.salina*. In the laboratory experiments, *A.salina* was exposed to different concentrations of KS, TA, CS, and CPE solutions in microplate wells. TA and CS have shown their toxic effects after 40 min of exposure and same effects have observed for CPE after 30 min of exposure. In contrast 35.6 g/L KS have shown its acute toxicity at first min of exposure.

In several studies it have shown that potassium sorbate have growth inhibitory effect on organisms involved in diverse mechanisms such as inhibition of enzyme activity and amino acid transport [25,37,38] and cytoplasm acidification during weak-acid stress in both yeasts and moulds [25,38,39]. It has also shown that sorbic acid and potassium sorbate are rapidly metabolized by the same pathways as other fatty acids [25,40]. Degradation of sorbic acid in some organisms is driven by enzymatic decarboxylation reaction [25,39,41,42].

Tannic acid is a water soluble, polyphenolic compound which is capable of precipitating proteins and have antimicrobial properties [24]. Stupak et al., have reported that exposure of *B. amphitrite* to tannate solutions at increasing concentrations caused loss of phototactic response and reduction in appendage activity. In the same study it was also reported that nauplii were able to swim and continue their development when taken into fresh water [43]. In order to evaluate antifouling effect, paints prepared with tannates were tested and these paints demonstrated a rapid larval inhibition [43].

Copper is an important element for animals but also it has toxic effects on marine invertebrates [23,44]. Copper can be regulated in the bodies of crustaceans and required for hemocyanin synthesis [45,46]. Hassall and Dangerfield, 1990, have reported that copper have an essential role in functioning of copper-dependent enzymes [46]. In several studies it have emphasised that high concentrations of copper have adverse affects on crustaceans. For instance, exposure to high amount of copper caused disruption of respiration [46,47] and adversely affected the osmoregulation in *Carcinus maenas* [46,48-50].

In field tests, antifouling performances of ZnO, $Cu_2O$, KS, TA, CT, and CPE in phytagels were investigated for one month immersed in sea. In spite of its muddy surface, best antifouling performance was observed at $Cu_2O$ gels. Its performance followed by CT and ZnO gels. Fouling was observed on 0.2, 0.3, and 0.4 g ZnO gels at mild level. The only fouled gel among other CT gels was 0.1 CT.

Better antifouling performances of $Cu_2O$, ZnO and CT can be explained by their slightly dissolving nature in seawater. As a result of their low solubility they could be remained on surface of gels much longer. On the other hand, surfaces of KS, TA, and CPE gels were heavily fouled by microslime layer and seaweeds due to their fast leaching rates in seawater.

Antifouling performances of eco-friendly biocides such as CT, TA and KS have been shown by Pérez et al. [22,24], Blustein et al. [25], respectively, in Argentina coastlines [25]. In this study, CT has shown antifouling performance, but same results was not detected for KS and TA in Mediterranean Sea as in previous studies performed in Argentina. This result can be explained as different nature of the Mediterranean Sea and Argentina coastlines. Also, $Cu_2O$ and ZnO were used in this study for comparison of antifouling performances of the other studied biocides.

In conclusion, slightly dissolving versions of studied eco-friendly biocides could be replaced with copper based antifouling biocides. The present study proposes to use *A.salina* to evaluate environmental effects and investigate antifouling performances of newly developed biocides in the field of antifouling paint technology.

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