



Evaluation of low copper content antifouling paints containing natural phenolic compounds as bioactive additives



Miriam Pérez^{a, b}, Mónica García^a, Guillermo Blustein^{a, c, *}

^a Centro de Investigación y Desarrollo en Tecnología de Pinturas (CIDEPINT), Av. 52 e/121 y 122, 1900 La Plata, Argentina

^b Facultad de Ciencias Naturales y Museo, Universidad Nacional de La Plata, 60 y 122, 1900 La Plata, Argentina

^c Facultad de Ciencias Agrarias y Forestales, Universidad Nacional de La Plata, 60 y 119, 1900 La Plata, Argentina

ARTICLE INFO

Article history:

Received 21 May 2015

Received in revised form

2 July 2015

Accepted 13 July 2015

Available online 17 July 2015

Keywords:

Low copper content

Thymol

Eugenol

Guaiacol

Antifouling paints

ABSTRACT

Cuprous oxide is the most commonly used biocide in antifouling paints. However, copper has harmful effects not only on the fouling community but also on non-target species. In the current study, we investigated the use of thymol, eugenol and guaiacol in this role combined with small quantities of copper. Phenolic compounds were tested for anti-settlement activity against cyprid larvae of the barnacle *Balanus amphitrite* and for their toxicity to nauplius larvae.

Thymol, eugenol and guaiacol were active for anti-settlement but guaiacol had the disadvantage of being toxic to nauplius larvae. However, all of them showed therapeutic ratio > 1.

Antifouling paints with thymol (low copper content/thymol, LCP/T), eugenol (low copper content/eugenol, LCP/E) and guaiacol (low copper content/guaiacol, LCP/G) combined with small copper content were formulated for field trials. After 12 months exposure in the sea, statistical analysis revealed that LCP/T and LCP/E paints were the most effective combinations and had similar performances to control paints with high copper content (traditional cuprous oxide based paints). In contrast, LCP/G paint was only partially effective in preventing and inhibiting biofouling and was colonized by some hard and soft foulers. However, this antifouling paint was effective against calcareous tubeworm *Hydroides elegans*.

In the light of various potential applications, thymol, eugenol and guaiacol have thus to be considered in future antifouling formulations.

© 2015 Elsevier Ltd. All rights reserved.

1. Introduction

Materials immersed in seawater are rapidly coated by a macromolecular film, which then favors colonization by prokaryotes (mostly bacteria), unicellular (microalgae, protozoans) and multicellular eukaryotes (barnacles, mussels, tubeworms, etc.). This phenomenon is called biofouling and can be defined as the accumulation of micro and macroorganisms on surfaces immersed in the sea. Biofouling represents a major nuisance for the maritime industries, especially for shipping, as biofouling on ship hulls increases the boat weight subsequently inducing over-consumption of fuel and increased maintenance costs (Schultz et al., 2011).

The most successful techniques to prevent fouling attachment have involved coating ship hulls with metal-containing antifouling

paints. These paints protect against fouling by continuously releasing toxic compounds, as copper, zinc and organotin, into the surrounding seawater.

The International Maritime Organization (IMO) announced the ban on the use of tributyltin (TBT) in 2003 when TBT-based products made up >80% of the global antifouling paint market (Scott, 1999). As a consequence, paints containing copper were coming into use again (Voulvoulis et al., 2002). They have now largely replaced to TBT-based coatings (Trentin et al., 2001) and present a much reduced risk compared to TBT (Hall and Anderson, 1999). Cuprous oxide was the first biocide developed for large scale industrial production of antifouling paints, and it is extensively used in TBT-free antifouling products today (Cima and Ballarin, 2012). However, observations of higher copper concentrations in water (cuprous and/or cupric cations; free and/or complexed forms), sediments and biological tissues close to marinas and harbours and increasing regulatory pressure on copper containing paints have caused intensified research into alternative, environmentally benign antifoulants (Claisse and Alzieu, 1993; Champ, 2000;

* Corresponding author. Centro de Investigación y Desarrollo en Tecnología de Pinturas (CIDEPINT), Av. 52 e/121 y 122, 1900 La Plata, Argentina.

E-mail address: antifouling@cidepint.gov.ar (G. Blustein).

Srinivasan and Swain, 2007). In spite of its high effectiveness, cuprous oxide alone is limited since it tends to work well against animal fouling but algal fouling is more resistant to it. As a result, over the years, chemists have spent much time and effort searching for additional biocides, called 'booster biocides', which can be added to cuprous oxide to boost its performance. Therefore, in antifouling paints present on the market, cuprous oxide is found within a polymeric matrix alone or, frequently, in combination with organic booster compounds, to increase the effectiveness and target spectrum of antifouling coatings. However, booster biocides can cause deleterious effects on ascidians embryos (Gallo and Tosti, 2015). Also, several studies have evaluated the toxicity of booster biocides on non-target species and have found most of them to be growth inhibitors for freshwater and marine autotrophs (Okamura et al., 2003) influencing key species, such as sea grasses (Chesworth et al., 2004) and corals (Owen et al., 2002). Therefore, there is a growing concern about the environmental fate and potential risks of these booster biocides and their use has been banned or restricted in many countries (Pérez et al., 2009; Guardiola et al., 2012).

Given that marine fouling is a multistep successional process, where colonization by bacteria precedes the establishment of macrofoulers, the incorporation of antimicrobial compounds could be viewed as an attempt to interrupt the fouling cascade at an early stage.

Plant essential oils and their components are known to exhibit antimicrobial activities and have applications in pharmacy and cosmetic industries. Thymol, eugenol and guaiacol are present in the essential oils from herbs and spices, as clove oil, thyme oil and cinnamon, and their antibacterial and antifungal properties are well known (Buchanan and Shepherd, 1981; Kim et al., 1995; Uttara et al., 1997; Cimanga et al., 2002). Thymol, one of the major components of thyme oil, is recognized for their pharmacological properties, including antimicrobial, antifungal, insecticidal, and antioxidant effects (Tepe et al., 2005; Braga et al., 2007; Preston et al., 2007; Dalleau et al., 2008; Sahaf and Moharrampour, 2008). Eugenol, a phenylpropanoid, is an allyl chain-substituted guaiacol, which is weakly acidic, slightly soluble in water and soluble in organic solvents. It is a clear to pale yellow liquid with a characteristic and pleasant odour of cloves, a spice pungent taste, antifungal properties (López-Malo et al., 2002; Kamatou et al., 2012) and is a quorum sensing inhibitor (Zhou et al., 2013). While commercial eugenol is almost exclusively derived from natural sources in a sustainable way, it can be also produced synthetically by allylation of guaiacol with allyl chloride (Kamatou et al., 2012). Conversely, the largest production of thymol and guaiacol is by organic synthesis (Fu et al., 2005; Amandi et al., 2005) and biotechnology processing (Witthuhn et al., 2012).

Guaiacol (o-methoxyphenol; methylcatechol) is a naturally occurring compound derived from the resin of *Guaiacum* trees (Parimal et al., 2011) and essential oil of many terrestrial plants (e.g. tobacco) (Rodgman and Perfetti, 2009).

Phenolic compounds are characterized by the presence of a hydroxy (–OH) group, attached to a benzene ring or other complex aromatic ring structures, e.g., pyrogallol, catechol, or resorcinol. Natural phenolic substances are among the most antimicrobial active substances present in plant essential oils. In spite of their high antifungal, antibacterial and insecticidal efficacy, they show a very low toxic effect on homeothermic animals (Zabka and Pavela, 2013). Also, phenols were confirmed to possess strong antioxidant activity (Ruberto and Baratta, 2000). In particular, oxygenated monoterpenes as thymol are mainly responsible for the antioxidant potential of the plant oils (Laguori et al., 1993; Aeschbach et al., 1994; Baratta et al., 1998). Thymol, eugenol and guaiacol have that phenolic structure and therefore were chosen as promising

candidates for inhibiting marine biofouling.

There are few reports about the use of essential oils in the field of protective coatings. In particular, only a research article is referred to the use of eugenol in a formulation similar to an antifouling paint, actually is indeed a varnish with 10% of eugenol (Bhattarai et al., 2007).

However, these phytochemicals were used in another type of coatings with medical purposes and also in food industry. For instance, immobilized guaiacol in acrylamide-polymers (Liu et al., 2011) and polymeric derivatives of eugenol (Rojo et al., 2008) were employed in biomedical devices due to its anti-adhesion, anti-biofilm and antibacterial properties. Also, thymol was widely used as antimicrobial agent in coating applications for food packaging, cosmetic and pharmaceutical industries (Guarda et al., 2011; Wattanasatcha et al., 2012).

The goal of the present research work is to evaluate antifouling properties of thymol, eugenol and guaiacol in laboratory tests and then, formulate marine coatings with low copper content combined with these compounds as antifoulant additives.

2. Materials and methods

2.1. Chemicals

Analytical grade chemicals were used for laboratory and field-trials tests. Thymol (FLUKA ANALYTICAL), Guaiacol (SIGMA Life Science) and Eugenol (ALDRICH Chemistry) were purchased from SIGMA-ALDRICH Chem. Ltd., Argentina. Physicochemical parameters of these compounds were highlighted in Table 1.

Solubility of thymol, eugenol and guaiacol in ASW were experimentally determined by current procedures.

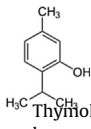
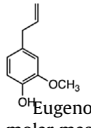

2.2. Laboratory assays

Bioassays were carried out using the cosmopolitan fouling barnacle *Balanus amphitrite*. Adults of this species were collected from Club de Motonáutica piers and rocks in the harbour of Mar del Plata (38°08' 17"S, 57°31' 18"W). In the laboratory, all organisms were conditioned in artificial seawater (ASTM D1141-D1198, 2013, pH 8.2) at 20 ± 1 °C with suitable aeration and natural light. Barnacles were fed with a daily diet of *Artemia salina* nauplii. Newly released *B. amphitrite* larvae (nauplii I) were transferred to a beaker containing filtered seawater; they molted and became nauplii II approximately 1 h after release. Some nauplii II actively swimming toward a light source were selected for each bioassay, while the remainder were put into a beaker containing seawater and fed with cultures of diatom *Skeletonema costatum*. In these conditions, 30–35% of the larvae metamorphosed to the cyprid stage, i.e., non-feeding stage whose role is to find a suitable place to settle, and were kept at 4 °C (Rittschof et al., 1992). Thirty nauplii II and twenty cyprids of *B. amphitrite* were used for the toxicity and settlement assays, respectively. Larvae were added using a Pasteur pipette to small crystallising dishes containing 30 mL of each solution.

To establish the baseline antilarval settlement potency of the compounds, screening tests using several dilutions of each compound were carried out. Then, concentrations for experiments were established, i.e., between 2.7 and 22 µM for thymol, 0.015 and 1.5 µM for eugenol and 0.091 and 9.1 mM for guaiacol. Artificial seawater without any compound added was used as negative control.

Observations were made under a stereomicroscope after 24 h. The inability of *B. amphitrite* nauplii II to stay in the water column and the loss of phototactic reaction were scored as toxic responses. Cyprids were scored as dead if they did not swim or move or close their valves. Three replicates were used in each concentration and

Table 1
Physicochemical parameters of thymol, eugenol and guaiacol.

Structural formula	Solubility			Pka (acidity)
	Oils	Water	Strong alkaline media	
 Thymol molar mass: 150.22 g/mol	soluble	0.85 g/l	highly soluble	10.59
 Eugenol molar mass: 164.20 g/mol	soluble	0.64 g/l	highly soluble	10.21
 Guaiacol molar mass: 124.14 g/mol	soluble	14.6 g/l	highly soluble	9.98

data collected from all concentrations were examined by Probit analysis to obtain a 24 h LC₅₀ (lethal concentration for 50% of nauplii) and EC₅₀ (effective concentration corresponding to the inhibition of 50% of settlement) with a 95% confidence interval (Finney, 1971).

Additionally, therapeutic ratio (TR) was also estimated. TR is defined as LC₅₀/EC₅₀ and indicates whether settlement inhibition is due to the toxicity of the compounds or related to other mechanisms (Vitalina et al., 1991; Rittschof et al., 1994). A comparison of LC₅₀ and EC₅₀ values provides insight to the possible working mechanisms of the compound tested. However, a thorough understanding of compound toxicity is given by recovery tests. To study the 'refreshing effect', larvae were removed from the test solutions and placed in vessels with artificial seawater. The refreshing effect was determined by observations of the organisms' recovery of swimming movements and ability to continue their development.

2.3. Antifouling paints-field trials

Two soluble matrix antifouling paints were prepared by dissolution of colophony (resin) and oleic acid (plasticizer) in a xylene/white spirit mixture (1:1) using a high-speed disperser. A laboratory scale ball mill was loaded with this mixture ('vehicle') and pigments (cuprous oxide and calcium carbonate), and dispersed for 24 h. One of them was formulated with high cuprous oxide content, 16%, similar to traditional cuprous oxide based paints (HCP, positive control) and the other with low cuprous oxide content, 1.6% (LCP). Then, LCP was fractionated in four portions, one of which was used as a control and the remaining as treatments. For treatments, thymol, eugenol and guaiacol were incorporated in LCP paint giving the following formulations: LCP/T, LCP/E and LCP/G, respectively. Finally, these paints were dispersed during 15 min (Table 2).

Sandblasted acrylic panels (8 × 12 cm), previously degreased with toluene, were painted for field trials. Also, uncoated acrylic panels were used as negative controls. Panels were hung from the marina at 50 cm below water line for 12 months. Field trials were carried out at Club de Motonáutica (Mar del Plata, Argentina). This sample station has around 2 m depth. Settlement of fouling organisms was measured as percentage cover on each panel using a dot-grid estimate method (Foster et al., 1991). All field tests were carried out in triplicate.

2.4. Statistical analysis

All statistical analyses were performed with *Statistica 7.0*. The normality assumption was verified with the Shapiro–Wilk's test (Shapiro and Wilk, 1965). The differences between treatment and control were determined by one-way analysis of variance (ANOVA) followed by Tukey post-hoc test. Differences were considered to be significant at $p < 0.05$.

3. Results

3.1. Solubility features of phenolic compounds

Phenolic compounds such as thymol, eugenol and guaiacol are partially soluble in both water and oil media. This amphipathic behaviour agrees with the structure of these molecules which have both a polar (hydroxyl group) and non-polar (benzene ring) moieties (Table 1).

Experimental solubility of thymol, eugenol and guaiacol in ASW was similar to those reported in distilled water (Table 1).

When a weak acid is dissolved in water, dissociation equilibrium according to equation (1) is established. The dissociated/undissociated relationship for weak acids as a function of pH and pKa values is given by Henderson-Hasselbalch's equation (2).



$$pH = pKa + \log \frac{[Ph - O^-]}{[Ph - OH]} \quad (2)$$

where $[Ph - O^-]$ = concentration of dissociated phenols and

Table 2
Paint composition expressed as volume percentage.

Components	Paints					
		HCP	LCP	LCP/T	LCP/E	LCP/G
Pigments	Cuprous oxide	16.0	1.6	1.6	1.6	1.6
	Calcium carbonate	11.0	25.4	23.4	23.4	23.4
	Thymol	–	–	2.0	–	–
	Eugenol	–	–	–	2.0	–
	Guaiacol	–	–	–	–	2.0
Vehicle	Colophony (resin): 27.0 + oleic acid (plasticizer): 6.0					
Solvents	Xylene/white spirit mixture (4:1)					

$[Ph-OH]$ = concentration of undissociated phenols

According to (2), it is expected that thymol, eugenol and guaiacol have a high solubility in strongly alkaline media as shown in Table 1. At high pH values, phenols are predominantly in their dissociated form (phenoxides). The phenoxide anions, for its highly polar nature, are more soluble than the same undissociated molecules. By contrast, in ASW (pH 8.2–8.4) and considering the pKa values of Table 1, the dissociation degree of the studied compounds is low (~1%), and in these conditions the predominant form corresponds to the undissociated molecule. This explains the solubility values for phenols in ASW close to those found in distilled water (pH 7).

On the other hand, calcium carbonate inside the paint plays a significant role as controller of the phenol-compound solubility in order to maintain an adequate concentration to prevent the settlement of organisms in the paint/seawater interface.

3.2. Laboratory assays

When healthy larvae (nauplii II) of *B. amphitrite* were exposed to either thymol, eugenol and guaiacol they fell to the bottom of the vessels so that the number could readily be counted. *B. amphitrite* larvae were susceptible to thymol, eugenol and guaiacol. Toxicity and settlement inhibition assays revealed that these compounds varied in effectiveness to a great extent, even though they are structurally similar compounds.

Significant differences between treatments and control were observed ($p < 0.05$). Thymol and eugenol affected naupliar activity from low concentrations. Nauplii lost phototactic response and swimming movements. This effect was observed above 3.00 μM for thymol solutions and above 0.048 μM for eugenol solutions. Particularly, LC_{50} was determined at 4.41 μM for thymol and 0.059 μM for eugenol.

In spite of guaiacol shown strong inhibition on larvae, the threshold concentration necessary to reach inactivity was determined at 0.364 mM and LC_{50} at 0.53 mM, that is to say, three magnitude orders larger than thymol and eugenol.

Naupliar toxicity assays were performed and results for all compounds are shown in Fig. 1. The antissettlement activity of thymol, eugenol and guaiacol was examined by exposure of cyprids for 24 h. Cyprid behaviour was affected by exposure to the compound, swimming cyprids responded with a rapid immobilization and of closing their valves. Cyprid settlement was also affected by

all three compounds. Results of settlement inhibition are shown in Fig. 2.

It is important to remark that, in all cases, larvae responded to the compounds in a dose-dependent manner.

In Table 3, therapeutic ratio values calculated both for nauplii and cyprids are shown.

3.3. Refreshing test

After 24 h exposition, nauplii and cyprids were transferred to fresh artificial seawater and the percentage recovery was estimated (data not shown).

Larvae exposed to all concentrations of thymol and eugenol solutions could recover; they metamorphosed and settled in high percentages (100% and 90%, respectively). Therefore, these compounds affected larvae temporary. However, low recovery percentages were observed for larvae exposed to guaiacol solutions (<30%).

3.4. Field trials

As expected, a strong recruitment of fouling organisms was observed in unpainted acrylic panels (Figs. 3 and 4).

Significant differences ($p < 0.05$) in cover percentages were observed between treatments and controls (HCP and LCP) after 12 months exposure in the sea (Figs. 3 and 4).

4. Discussion

The present study combined laboratory and field experiments to test whether thymol, eugenol and guaiacol have potential to be used for antifouling protection.

Based on the results of the present work, all these compounds inhibited barnacle settlement in a non-toxic way in the laboratory. Also, these phenolic compounds combined with 1.6% cuprous oxide maintained a broad-spectrum antifouling activity in the sea for twelve months. Particularly, thymol and eugenol had better performances. Dambolena et al. (2012) conducted antifungal tests using natural phenolic compounds and determined a similar response, they concluded that this activity was attributable to the difference in lipophilicity of thymol and eugenol in relation to guaiacol.

To be selected as a promising new antifouling formulation,

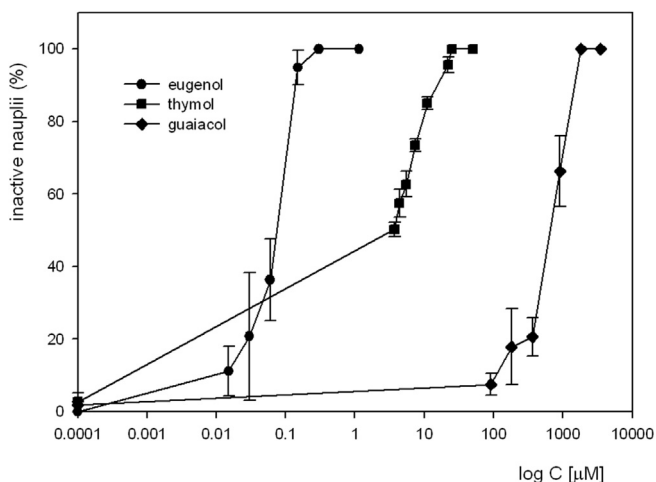


Fig. 1. Naupliar inactivity percentage vs. phenolic compounds concentration. Bars = mean \pm SE.

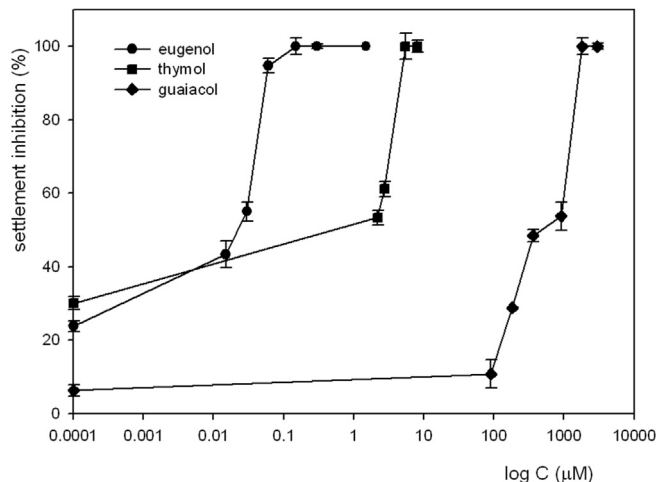


Fig. 2. Settlement inhibition percentage vs. phenolic compounds concentration. Bars = mean \pm SE.

Table 3

LC₅₀, EC₅₀ and therapeutic ratio values for larvae exposed to thymol, eugenol and guaiacol. In parenthesis: 95% lower and upper fiducial limits are shown.

Compound	LC ₅₀	EC ₅₀	TR
Thymol	4.41 μM (3.94–4.89)	2.260 μM (1.16–2.70)	1.95
Eugenol	0.059 μM (0.048–0.072)	0.024 μM (0.023–0.026)	2.46
Guaiacol	0.53 mM (0.425–0.679)	0.419 mM (0.314–0.565)	1.26

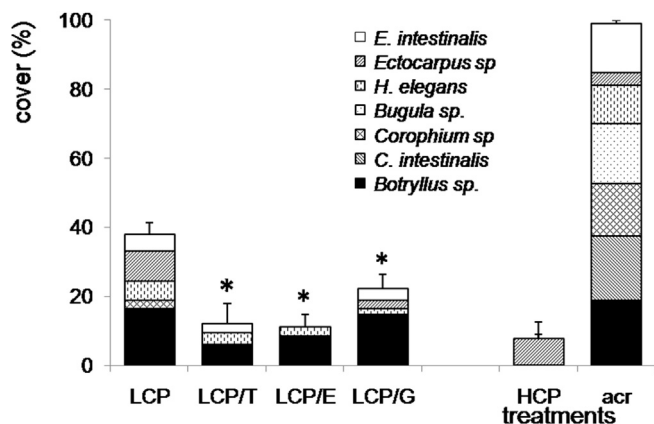


Fig. 3. Fouling cover percentage on panels exposed at Mar del Plata harbour (12 months immersion). LCP: 1.6% copper paint; LCP/T: 1.6% copper + thymol; LCP/E: 1.6% copper + eugenol; LCP/G: 1.6% copper + guaiacol; HCP: 16% copper paint; acr: acrylic. *significant differences from controls (HCP and LCP). Bars = mean ± SE.

compounds need to have an effective concentration (EC₅₀) lower than the lethal concentration (LC₅₀) (Dhams and Hellio, 2009). Therapeutic ratio (TR) obtained in these experiments were, in all

cases, higher than 1. Recent trends consider as non toxic anti-foulants candidates to those compounds with LC₅₀/EC₅₀ ratio >15 (Qian et al., 2010). Although molecules studied in this paper had TR values between 1.26 and 2.46, the non toxic effect was indeed confirmed by recovery tests, i.e., thymol and eugenol affected larvae in a reversible way. However, larval recovery percentage after exposure to guaiacol was low, and this result indicate that TR should be confirmed by recovery tests in order to determine toxicity degree of a compound.

These molecules are small and were effective at low concentrations. It is worth noting that these compounds could be degraded in the environment by photolysis, mineralization, as well as by aerobic and anaerobic microorganisms. (Chamberlain and Dagley, 1968; Tadasa, 1977; González et al., 1993; Rabenhorst, 1996; Peiró et al., 2001; Pracht et al., 2001; Overhage et al., 2002; Amat et al., 2005; Ashengroph et al., 2011; Samet et al., 2011; Hahn et al., 2013; Mishra et al., 2013).

Field experiments were the next step in assessing the anti-fouling performance. The main biofouling organisms observed were encrusting species such as tubeworms, bryozoans, ascidians, and algae. These organisms grew and developed quickly on the surface of the acrylic panels from the first month of immersion and covered the entire surface after immersion for twelve months. LCP was invaded by several species demonstrating that cuprous oxide content was not enough to inhibit fouling settlement. The paints studied contain 10 times less cuprous oxide than traditional ones and were combined with thymol, eugenol or guaiacol. LCP/T and LCP/E paints were the most effective combinations and had similar performances to HCP. Because of these formulations have the same cuprous oxide content as LCP, the successful antifouling activity is attributable to the addition of thymol or eugenol. In contrast, LCP/G paint was only partially effective in preventing and inhibiting biofouling and were colonized by some hard and soft foulers

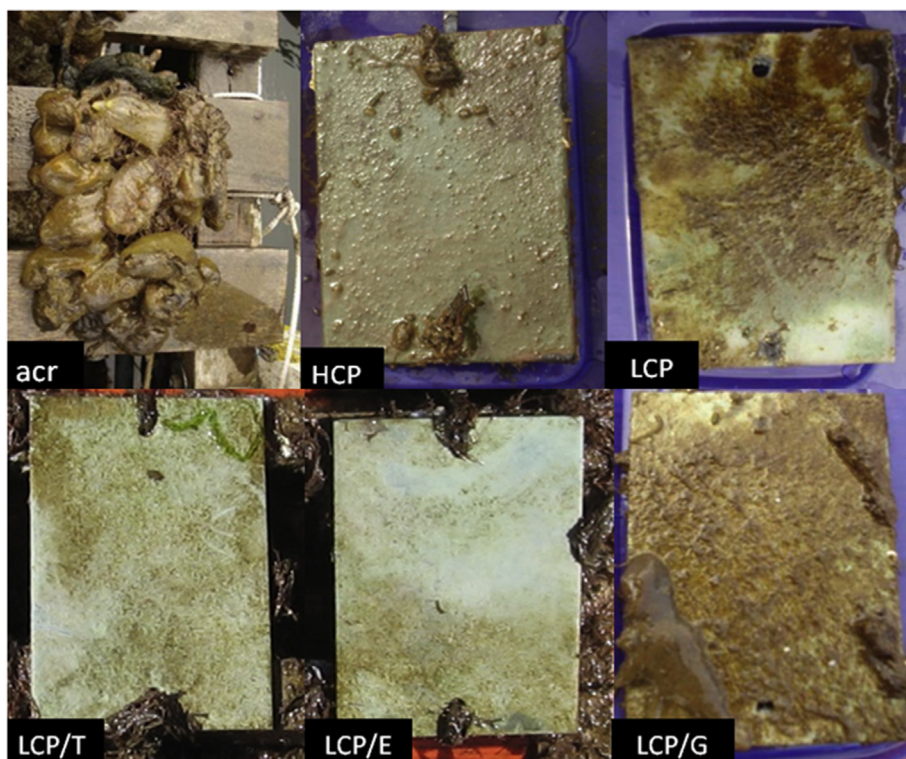


Fig. 4. Panels exposed at Mar del Plata harbour (12 months immersion). LCP: 1.6% copper paint; LCP/T: 1.6% copper + thymol; LCP/E: 1.6% copper + eugenol; LCP/G: 1.6% copper + guaiacol; HCP: 16% copper paint; acr: acrylic.

($p < 0.05$). As stated above, the attachment of some algal species is not affected by traditional cuprous oxide based paints. The results of this study confirmed the settlement of *Ectocarpus* sp. on both, HCP and LCP panels. In contrast, combinations of LCP/T and LCP/E completely inhibited the settlement of this alga. In spite of LCP/G paint was not as effective as previous combinations, it is clear that significantly reduced *Ectocarpus* sp. attachment and had better performance than HCP and LCP. Additionally, LCP/E paint prevents the settlement of the green algae *Enteromorpha intestinalis* in a similar way as HCP.

It is important to remark that all formulations (HCP, LCP, LCP/T, LCP/E and LCP/G) were capable of inhibiting the settlement of *Bugula* sp. and *Ciona intestinalis*. A particular effect was observed for the amphipod *Corophium* sp. because this species was completely inhibited in all treatments except for LCP in which some sandtubes were registered.

The calcareous tubeworm *Hydroides elegans* was strongly inhibited by LCP/G combination and only a few tubes were registered on these panels. This performance was very similar to positive control HCP which completely inhibited tubeworm attachment. On the other hand, LCP/T and LCP/E combinations also reduced the settlement of *H. elegans* but in relation to LCP. Additionally, the colonial ascidian *Botryllus* sp. was markedly affected in settlement by LCP/T and LCP/E.

Both, LCP/T and LCP/E had broad spectrum activity. Although LCP/G paint did not show significant performance was the most effective against tubeworm species. Then, it would be necessary formulate new coatings employing new combinations of these phenolic compounds.

Several studies indicate that copper released from a coating in the marine environment has been shown to be strongly associated with organic matter (ligands) (Campos and van den Berg, 1994; Voulvoulis et al., 1999), and these copper-ligand complexes are less labile and therefore less toxic than the uncomplexed form (Cu^{2+} , referred herein as free copper). Thus, high concentrations of organic copper-binding ligands in coastal estuaries have been shown to effectively buffer copper toxicity even at relatively high copper loadings (Buck and Bruland, 2005; Rivera-Duarte et al., 2005). Copper concentrations that exceed the binding capacity of the natural ligands can lead to potentially toxic copper conditions (Rivera-Duarte et al., 2005). In contrast, other studies indicate that copper could also form lipophilic complexes with organic compounds, e.g. dithiocarbamates, increasing their toxic effect by means of synergistic effect (Bonnamain and Dive, 1990).

Other synergistic interactions between copper and organic compounds such as thymol, eugenol and guaiacol could be possible. In this sense, some plant extracts are currently being used to synthesize metal nanoparticles because they contain organic compounds of high reducing power (Irvani, 2011; Abdelmonem and Amin, 2014; Makarov et al., 2014). Green synthesis of metallic nanoparticles such as copper, silver and gold were carried out using pure compounds present in different plant essential oils, e.g. eugenol and guaiacol (Singh et al., 2010; Milczarek and Ciszewski, 2012; Subhankari and Nayac, 2013; Yin et al., 2014). Eugenol and guaiacol act as powerful reducing agents and probably would delay the oxidation process and dissolution of cuprous oxide at paint-film/seawater interface. Furthermore, part of the dissolved copper (Cu^{+2}) in seawater from the cuprous oxide of the paint, may be reduced to metallic copper nanoparticles in the presence of eugenol or guaiacol. The bactericidal effect of metal nanoparticles has been attributed to their small size and high surface to volume ratio, which allows them to interact closely with microbial membranes and is not merely due to the release of metal ions in solution (Kon and Rai, 2013).

Reducing the content of copper in antifouling paints is a

research topic that has been taking place since more 20 years ago, and remains in force until today (Vetere et al., 1997; Pérez et al., 2003, 2006; Peres et al., 2014). In this regard various antifouling pigments with low copper content have been synthesized but none of them has reached the market yet. Whilst in such cases the reduction of the copper content was greater, preparing these antifouling pigments at industrial scale implies a financial investment. By contrast, our research is aimed at obtaining new antifouling paint formulations with cheaper raw materials available on the market, such as thymol, eugenol and guaiacol.

Bactericidal activity of thymol is mostly attributed to the action of its phenolic structure (Juven et al., 1994; Ultee et al., 1998; Lambert et al., 2001; Friedman et al., 2002). Due to their hydrophobic nature, thymol interacts with the lipid bilayer of cytoplasmic membranes causing loss of integrity and leakage of cellular material such as ions, ATP and nucleic acid (Helander et al., 1998; Ultee et al., 1999; Lambert et al., 2001; Trombetta et al., 2005). Thymol and eugenol, known to be lipophilic, can enter between the fatty acyl chains making up membrane lipid bilayers, altering the fluidity and permeability of cell membranes (Sikkema et al., 1995; Pina Vaz et al., 2004; Di Pasqua et al., 2007). Also, the mechanism of phenolic toxicity towards fungi is based on the inhibition of fungal enzymes, which contain –SH groups in their active sites (Cowan, 1999; Celimene et al., 1999). As stated above, eugenol is a member of the phenylpropanoid class of plant secondary metabolites. Aromatic amino acids, specifically phenylalanine, are precursors of eugenol in the phenylpropanoid biosynthesis pathway (Louie et al., 2007). The molecular structure of eugenol is very similar to these aromatic amino acid precursors and because of these structural similarities eugenol may interfere with active sites of acid permeases in the cytoplasmic membrane of yeast (Darvishi et al., 2013).

The main fouling organisms at Mar del Plata harbour exhibited higher sensitivity to thymol and eugenol than guaiacol. Trace of copper at the cellular level are often involved in oxidative stress, which results in the production of reactive oxygen species (ROS). ROS includes the superoxide radical, hydrogen peroxide and the hydroxyl radical, all of which affect mainly lipids, proteins, carbohydrates, and nucleic acids (Rajkumar and Milton, 2011). The importance of antioxidant enzymes is generally emphasized in the prevention of oxidative stresses by scavenging of ROS. Antioxidant system comprises several enzymes such as superoxide dismutase (SOD), catalase (CAT), and guaiacol peroxidase (GPx). Under this oxidative stress, many organisms significantly increase its antioxidant enzymes, e.g. guaiacol peroxidase (Pinto et al., 2003; Li et al., 2012; Sharma et al., 2012). In this context, we can infer that copper could increase the activity of GPx and consequently decrease the concentration of guaiacol (via polymerization) and therefore its antifouling effect (Doerge et al., 1997). Further research is needed to clarify this hypothesis.

In conclusion, these experiments highlight the potential of thymol, eugenol and guaiacol as active additives in antifouling coatings. The incorporation of thymol, eugenol and guaiacol into marine paints show clear evidence of antifouling activity at concentrations tested. Within the context of worldwide pressure for legislation limiting the use of biocides, these compounds in combination with small quantities of copper are considered the most promising alternative environmentally friendly for antifouling technology.

Acknowledgements

We thank to Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Comisión de Investigaciones Científicas de la provincia de Buenos Aires (CICPBA) and Universidad Nacional de La

Plata for their financial support. We also wish to thank the Club de Motonáutica of Mar del Plata for permission to use their marine testing site.

References

- Abdelmonem, A., Amin, R., 2014. Rapid green synthesis of metal nanoparticles using pomegranate polyphenols. *IJSBAR* 15 (1), 57–65.
- Aeschbach, R., Loliger, J., Scott, B., Murcia, A., Butler, J., Halliwell, B., Aruoma, O., 1994. Antioxidant actions of thymol, carvacrol, 6-gingerol, zingerone and hydroxytyrosol. *Food Chem. Toxicol.* 32, 31–36.
- Amandi, R., Hyde, J., Ross, S., Lotz, T., Poliakoff, M., 2005. Continuous reactions in supercritical fluids; a cleaner, more selective synthesis of thymol in supercritical CO₂. *Green Chem.* 7, 288–293.
- Amat, A., Arques, A., López, F., Miranda, M., 2005. Solar photo-catalysis to remove paper mill wastewater pollutants. *Sol. Energy* 79, 393–401.
- Ashengroph, M., Nahvi, I., Zarkesh-Esfahani, H., Momenbeik, F., 2011. *Pseudomonas resinovorans* SPR1, a newly isolated strain with potential of transforming eugenol to vanillin and vanillic acid. *N. Biotechnol.* 28, 656–664.
- ASTM D1141–98, 2013. Standard Practice for the Preparation of Substitute Ocean Water. ASTM International, West Conshohocken, PA.
- Baratta, M., Dorman, H., Deans, S., Figueiredo, A., Barroso, J., Ruberto, G., 1998. Antimicrobial and antioxidant properties of some commercial essential oils. *Flavour Frag. J.* 13, 235–244.
- Bhattarai, H., Paudel, B., Park, N., Lee, K., Shin, H., 2007. Evaluation of antifouling activity of eight commercially available organic chemicals against the early foulers marine bacteria and *Ulva* spores. *JEB* 28, 857–863.
- Bonnemain, H., Dive, D., 1990. Studies on synergistic toxic effects of copper and dithiocarbamate pesticides with the ciliate protozoan *Colpidium campylum* (Stokes). *Ecotoxicol. Environ. Saf.* 19, 320–326.
- Braga, P., Alfieri, M., Culici, M., Dal Sasso, M., 2007. Inhibitory activity of thymol against formation and viability of *Candida albicans* hyphae. *Mycoses* 50, 502–506.
- Buchanan, R., Shepherd, A., 1981. Inhibition of *Aspergillus parasiticus* by thymol. *J. Food Sci.* 46, 976–977.
- Buck, K., Bruland, K., 2005. Copper speciation in San Francisco Bay: a novel approach using multiple analytical windows. *Mar. Chem.* 96, 185–198.
- Campos, M., van den Berg, C., 1994. Determination of copper complexation in seawater by cathodic stripping voltammetry and ligand competition with salicylaldehyde. *Anal. Chim. Acta* 284, 481–496.
- Celimene, C., Micales, J., Ferge, L., Young, R., 1999. Efficacy of pinosylvins against white-rot and brown-rot fungi. *Holzforchung* 53, 49–497.
- Chamberlain, E., Dagley, S., 1968. The metabolism of thymol by a *Pseudomonas*. *Biochem. J.* 110, 755–763.
- Champ, M., 2000. A review of organotin regulatory strategies, pending actions, related costs and benefits. *Sci. Total Environ.* 258, 21–71.
- Chesworth, J., Donkin, M., Brown, M., 2004. The interactive effects of the antifouling herbicides Irgarol 1051 and Diuron on the seagrass *Zostera marina* (L.). *Aquat. Toxicol.* 66, 293–305.
- Cima, F., Ballarin, L., 2012. Immunotoxicity in ascidians: antifouling compounds alternative to organotins III. The case of copper(I) and Irgarol 105. *Chemosphere* 89, 19–29.
- Cimanga, K., Kambu, K., Tona, L., Apers, S., Bruyne, T., Hermans, N., Totte, J., Pieters, L., Vlietinck, A., 2002. Correlation between chemical composition and antibacterial activity of essential oils of some aromatic medicinal plants growing in the Democratic Republic of Congo. *J. Ethnopharm.* 79, 213–220.
- Claisse, D., Alzieu, C., 1993. Copper contamination as a result of antifouling paint regulations. *Mar. Pollut. Bull.* 26, 395–397.
- Cowan, M., 1999. Plant products as antimicrobial agents. *CMR* 12, 564–582.
- Dalleau, S., Cateau, E., Bergès, T., Berjeaud, J., Imbert, C., 2008. In vitro activity of terpenes against *Candida* biofilms. *Int. J. Antimicrob. Ag.* 6, 572–576.
- Dambolena, J., López, A., Meriles, J., Rubinstein, H., Zygadlo, J., 2012. Inhibitory effect of 10 natural phenolic compounds on *Fusarium verticillioides*. A structure-property-activity relationship study. *Food Control* 28, 163–170.
- Darvishi, E., Omid, M., Bushehri, A., Golshani, A., Smith, M., 2013. The antifungal eugenol perturbs dual aromatic and branched-chain amino acid permeases in the cytoplasmic membrane of yeast. *PLoS One* 8, e76028.
- Dhams, H., Hellio, C., 2009. Laboratory bioassays for screening marine antifouling compounds. In: Hellio, C., Yebra, D. (Eds.), *Advances in Marine Antifouling Coatings and Technologies*. Woodhead Publishing, Cambridge, UK, pp. 275–307.
- Di Pasqua, R., Betts, G., Hoskins, N., Edwards, M., Ercolini, D., Mauriello, G., 2007. Membrane toxicity of antimicrobial compounds from essential oils. *J. Agric. Food Chem.* 55, 4863–4870.
- Doerge, D., Divi, R., Churchwell, M., 1997. Identification of the colored guaiacol oxidation product produced by peroxidases. *Anal. Biochem.* 250, 10–17.
- Finney, D., 1971. *Probit Analysis*, third ed. Cambridge University Press, Cambridge.
- Foster, M., Harrold, C., Hardin, D., 1991. Points versus photo quadrat estimates of the cover of sessile marine organisms. *J. Exp. Mar. Biol. Ecol.* 146, 193–203.
- Friedman, M., Henika, P., Mandrell, R., 2002. Bactericidal activities of plant essential oils and some of their isolated constituents control, against *Campylobacter jejuni*, *Escherichia coli*, *Listeria monocytogenes*, and *Salmonella enterica*. *J. Food Prot.* 65, 1545–1560.
- Fu, Z., Yu, Y., Yin, D., Xu, Y., Liu, H., Liao, H., Xu, Q., Tan, F., Wang, J., 2005. Vapor phase highly selective O-methylation of catechol with methanol over ZnCl₂ modified γ -Al₂O₃ catalysts. *J. Mol. Catal. A* 232, 69–75.
- Gallo, E., Tosti, E., 2015. Reprotoxicity of the antifouling chlorothalonil in ascidians: an ecological risk assessment. *PLoS One* 10, 1–14.
- González, B., Acevedo, C., Brezny, R., Joyce, T., 1993. Metabolism of chlorinated guaiacols by a guaiacol-degrading *Acinetobacter junii* strain. *Appl. Environ. Microbiol.* 59, 3424–3429.
- Guarda, A., Rubilar, J., Miltz, J., Galotto, M., 2011. The antimicrobial activity of microencapsulated thymol and carvacrol. *Int. J. Food Microbiol.* 146, 144–150.
- Guardiola, F., Cuesta, A., Meseguer, J., Esteban, M., 2012. Risks of using antifouling biocides in aquaculture. *Int. J. Mol. Sci.* 13, 1541–1560.
- Hahn, V., Sünwoldt, K., Mikolasch, A., Schauer, F., 2013. Two different primary oxidation mechanisms during biotransformation of thymol by gram-positive bacteria of the genera *Nocardia* and *Mycobacterium*. *Appl. Microbiol. Biotechnol.* 97, 1289–1297.
- Hall, L., Anderson, R., 1999. A deterministic ecological risk assessment for copper in European saltwater environments. *Mar. Pollut. Bull.* 38, 207–218.
- Helander, I., Alakomi, H., Latva-Kala, K., Mattila-Sandholm, T., Pol, I., Smid, E., Gorris, L., Von Wright, A., 1998. Characterization of the action of selected essential oil components on Gram-negative bacteria. *J. Agric. Food Chem.* 46, 3590–3595.
- Iravani, S., 2011. Green synthesis of metal nanoparticles using plants. *Green Chem.* 13, 2638–2650.
- Juven, B., Kanner, J., Schved, F., Weisslowicz, H., 1994. Factors that interact with the antibacterial action of thyme essential oil and its active constituents. *J. Appl. Bacteriol.* 76, 626–631.
- Kamatou, G., Vermaak, I., Viljoen, A., 2012. Eugenol—from the remote Maluku Islands to the international market place: a review of a remarkable and versatile molecule. *Molecules* 17, 6953–6981.
- Kim, J., Marshall, M., Wei, C., 1995. Antibacterial activity of some essential oil components against five foodborne pathogens. *J. Agric. Food Chem.* 43, 2839–2845.
- Kon, K., Rai, M., 2013. Metallic nanoparticles: mechanism of antibacterial action and influencing factors. *J. Comp. Clin. Path. Res.* 2/1, 160–174.
- Laguori, V., Blekas, G., Tsimidou, M., Kokkini, S., Boskou, D., 1993. Composition and antioxidant activity of essential oils from oregano plants grown wild in Greece. *Z. Leb. Unters. Forchs.* 197, 20–23.
- Lambert, R., Skandamis, P., Coote, P., Nychas, G., 2001. A study of the minimum inhibitory concentration and mode of action of oregano essential oil, thymol and carvacrol. *J. Appl. Microbiol.* 91, 453–462.
- Li, L., Huang, X., Devajit, B., Ni, H., 2012. Photosynthetic activity and antioxidative response of seagrass *Thalassia hemprichii* to trace metal stress. *Acta Oceanol. Sin.* 31, 98–108.
- Liu, H., Lepoittevin, B., Roddier, C., Guérineau, V., Bech, L., Herry, J., Bellon-Fontaine, M., Roger, P., 2011. Facile synthesis and promising antibacterial properties of a new guaiacol-based polymer. *Polymer* 52, 1908–1916.
- López-Malo, A., Alzamora, S., Palou, E., 2002. *Aspergillus flavus* dose-response curves to selected natural and synthetic antimicrobials. *Int. J. Food Microbiol.* 73, 213–218.
- Louie, G., Baiga, T., Bowman, M., Koeduka, T., Taylor, J., Spassova, S., Pichersky, E., Noel, J., 2007. Structure and reaction mechanism of basil eugenol synthase. *PLoS One* 2, e993.
- Makarov, V., Love, A., Sinitsyna, O., Makarova, S., Yaminsky, I., Taliansky, M., Kalinina, N., 2014. “Green” nanotechnologies: synthesis of metal nanoparticles using plants. *Acta Naturae* 6, 35–44.
- Milczarek, G., Ciszewski, A., 2012. Functionalized gold nanoparticles and films stabilized by in situ formed polyeugenol. *Colloids Surf. B* 90, 53–57.
- Mishra, S., Sachan, A., Sachan, S., 2013. Production of natural value-added compounds: an insight into the eugenol biotransformation pathway. *J. Ind. Microbiol. Biotechnol.* 40, 545–550.
- Okamura, H., Nishida, T., Ono, Y., Shim, W., 2003. Phytotoxic effects of antifouling compounds on nontarget plant species. *Bull. Environ. Contam. Toxicol.* 71, 881–886.
- Outtara, B., Simard, R., Holley, R., Piette, G., Bégin, A., 1997. Antibacterial activity of selected fatty acids and essential oils against six meat spoilage organisms. *Int. J. Food Microbiol.* 37, 155–162.
- Overhage, J., Steinbüchel, A., Priefert, H., 2002. Biotransformation of eugenol to ferulic acid by a recombinant strain of *Ralstonia eutropha* H16. *Appl. Environ. Microbiol.* 9, 4315–4321.
- Owen, R., Knap, A., Toasperm, M., Carbery, K., 2002. Inhibition of coral photosynthesis by the antifouling herbicide Irgarol 1051. *Mar. Pollut. Bull.* 44, 623–632.
- Parimal, K., Khale, A., Pramod, K., 2011. Resins from herbal origin and a focus on their applications. *JPSR* 2, 1077–1085.
- Peiró, A., Ayllón, J., Peral, J., Doménech, X., 2001. TiO₂-photocatalyzed degradation of phenol and ortho-substituted phenolic compounds. *Appl. Catal. B. Environ.* 30, 359–373.
- Peres, R., Baldissera, A., Armelin, E., Alemán, C., Ferreira, C., 2014. Marine-friendly antifouling coating based on the use of a fatty acid derivative as a pigment. *Mater. Res.* 17, 720–727.
- Pérez, M., García, M., del Amo, B., Blustein, G., Stupak, M., 2003. Core-shell pigments in antifouling paints. *Surf. Coat. Int. Part B-Coat. Trans.* 86, 259–262.
- Pérez, M., Blustein, G., García, M., del Amo, B., Stupak, M., 2006. Cupric tannate: a low copper content antifouling pigment. *Prog. Org. Coat.* 55, 311–315.
- Pérez, M., Stupak, M., Blustein, G., García, M., Martensson Linblad, L., 2009. Organic alternatives to copper in the control of marine biofouling. In: Hellio, C., Yebra, D.

- (Eds.), *Advances in Marine Antifouling Coatings and Technologies*. Woodhead Publishing Limited, Cambridge, UK, pp. 554–571.
- Pina Vaz, C., Rodrigues, A., Pinto, E., Costa-de-Oliveira, S., Tavares, C., Salgueiro, L., Cavaleiro, C., Gonçalves, M., Martínez-de-Oliveira, J., 2004. Antifungal activity of *Thymus* oils and their major compounds. *J. Eur. Acad. Dermatol.* 18, 73–78.
- Pinto, E., Sigaud-kutner, T., Leitão, M., Okamoto, O., Morse, D., Colepicolo, P., 2003. Heavy metal-induced oxidative stress in algae. *J. Phycol.* 39, 1008–1018.
- Pracht, J., Boenigk, J., Isenbeck-Schröter, M., Keppler, F., Schöler, H., 2001. Abiotic Fe(III) induced mineralization of phenolic substances. *Chemosphere* 44, 613–619.
- Preston, K., Higham, S., Smith, P., 2007. The efficacy of techniques for the disinfection of artificial sub-surface dental caries lesions and their effect on demineralization and remineralization in vitro. *J. Dent.* 35, 490–495.
- Qian, P., Xu, Y., Fusetani, N., 2010. Natural products as antifouling compounds: recent progress and future perspectives. *Biofouling* 26, 223–234.
- Rabenhorst, J., 1996. Production of methoxyphenol-type natural aroma chemicals by biotransformation of eugenol with a new *Pseudomonas* sp. *Appl. Microbiol. Biotechnol.* 46, 470–474.
- Rajkumar, J., Milton, M., 2011. Biochemical changes induced by cadmium, copper, lead and zinc exposure to *Perna viridis* under longterm toxicity test. *IJPBS* 2, 50–59.
- Rittschof, D., Clare, A., Gerhart, D., Mary, S., Bonaventura, J., 1992. Barnacle in vitro assays for biologically active substances: toxicity and settlement inhibition assays using mass cultured *Balanus amphitrite amphitrite* Darwin. *Biofouling* 6, 115–122.
- Rittschof, D., Sasikumar, N., Murlless, D., Clare, A., Gerhart, C., Bonaventura, J., 1994. Mixture interactions of lactones and furans and a commercial biocide: toxicity and antibarnacle settlement activity. In: Thompson, M., Nagabhushanam, R., Sarojini, R., Fingerman, M. (Eds.), *Recent Developments in Biofouling Control*, pp. 269–274. Rotterdam.
- Rivera-Duarte, I., Rosen, G., Lapota, D., Chadwick, D., Kear-Padilla, L., Zirino, A., 2005. Copper toxicity to larval stages of three marine invertebrates and copper complexation capacity in San Diego Bay, California. *Environ. Sci. Technol.* 39, 1542–1546.
- Rodgman, A., Perfetti, T., 2009. Phenols and Quinones. In: *The Chemical Components of Tobacco and Tobacco Smoke*. CRC Press Taylor and Francis Group, Boca Raton, USA, pp. 487–554.
- Rojo, L., Barcenilla, J., Vázquez, B., González, R., San Román, J., 2008. Intrinsically antibacterial materials based on polymeric derivatives of eugenol for biomedical applications. *Biomacromolecules* 9, 2530–2535.
- Ruberto, G., Baratta, M., 2000. Antioxidant activity of selected essential oil components in two lipid model systems. *Food Chem.* 69, 167–174.
- Sahaf, B., Moharrampour, S., 2008. Fumigant toxicity of *Carum copticum* and *Vitex pseudo-negundo* essential oils against eggs, larvae and adults of *Callosobruchus maculatus*. *J. Pest Sci.* 81, 213–220.
- Samet, Y., Wali, I., Abdelhédi, R., 2011. Kinetic degradation of the pollutant guaiacol by dark Fenton and solar photo-Fenton processes. *Environ. Sci. Pollut. Res.* 18, 1497–1507.
- Schultz, M., Bendick, J., Holm, E., Hertel, W., 2011. Economic impact of biofouling on a naval surface ship. *Biofouling* 27, 87–98.
- Scott, A., 1999. Biocides-IMO agrees on tributyl tin ban. *Chem. Week* 161, 24.
- Shapiro, S., Wilk, M., 1965. An analysis of variance test for normality (complete samples). *Biometrika* 52, 591–611.
- Sharma, P., Jha, A., Dubey, R., Pessarakli, M., 2012. Reactive oxygen species, oxidative damage, and antioxidative defense mechanism in plants under stressful conditions. *J. Bot.* 2012, 1–26.
- Sikkema, J., de Bont, J., Poolman, B., 1995. Mechanisms of membrane toxicity of hydrocarbons. *Microbiol. Rev.* 59, 201–222.
- Singh, A., Talat, M., Singh, D., Srivastava, O., 2010. Biosynthesis of gold and silver nanoparticles by natural precursor clove and their functionalization with amine group. *J. Nanopart. Res.* 12, 1667–1675.
- Srinivasan, M., Swain, G., 2007. Managing the use of copper based antifouling paints. *Environ. Manage* 39, 423–441.
- Subhankari, I., Nayak, P., 2013. Synthesis of copper nanoparticles using *Syzygium aromaticum* (cloves) aqueous extract by using green chemistry. *WJNST* 2, 14–17.
- Tadasa, K., 1977. Degradation of eugenol by a microorganisms. *Agric. Biol. Chem.* 41, 925–929.
- Tepe, B., Sokmen, M., Askin Akpulat, H., Daferera, D., Polissiou, M., Sokmen, A., 2005. Antioxidative activity of the essential oils of *Thymus sipyleus* subsp. *sipyleus* and *Thymus sipyleus* subsp. *sipyleus* var. *rosulans*. *J. Food Eng.* 66, 474–454.
- Trentin, I., Romairone, V., Marcenaro, G., De Carolis, G., 2001. Quick test methods for marine antifouling paints. *Prog. Org. Coat.* 42, 15–19.
- Trombetta, D., Castelli, F., Sarpietro, M., Venuti, V., Cristani, M., Daniele, C., Saija, A., Mozzanti, G., Bisignano, G., 2005. Mechanisms of antibacterial action of three monoterpenes. *Antimicrob. Agents Chemother.* 49, 2474–2478.
- Ultee, A., Gorris, L., Smid, E., 1998. Bactericidal activity of carvacrol towards the food-borne pathogen *Bacillus cereus*. *J. Appl. Microbiol.* 85, 211–218.
- Ultee, A., Kets, E., Smid, J., 1999. Mechanisms of action of carvacrol on the food-borne pathogen *Bacillus cereus*. *Appl. Environ. Microbiol.* 65, 4606–4610.
- Vetere, V., Pérez, M., Romagnoli, R., Stupak, M., del Amo, B., 1997. Solubility and toxic effect of the cuprous thiocyanate antifouling pigment on barnacle larvae. *J. Coat. Technol.* 69, 39–45.
- Vitalina, S., Avelin, S., Rittschof, D., Sarojini, R., Nagabhushanam, R., 1991. Compounds from octocorals that inhibit barnacle settlement; isolation and biological potency. In: Thompson, M., Sarojini, R., Nagabhushanam, R. (Eds.), *Bioactive Compounds from Marine Organisms*, pp. 331–339. Rotterdam.
- Voulvoulis, N., Scrimshaw, D., Lester, J., 1999. Alternative antifouling biocides. *Appl. Organometal. Chem.* 13, 135–143.
- Voulvoulis, N., Scrimshaw, D., Lester, J., 2002. Comparative environmental assessment of biocides used in antifouling paints. *Chemosphere* 47, 789–795.
- Wattanasatcha, A., Rengpipat, S., Wanichwecharungruang, S., 2012. Thymol nanoparticles as an effective anti-bacterial agent. *Int. J. Pharm.* 434, 360–365.
- Witthuhn, R., van der Merwe, E., Venter, P., Cameron, M., 2012. Guaiacol production from ferulic acid, vanillin and vanillic acid by *Alicyclobacillus acidoterrestris*. *Int. J. Food Microbiol.* 157, 113–117.
- Yin, G., Huo, Z., Zeng, X., Yao, G., Jing, Z., Jin, F., 2014. Reduction of CuO into Cu with guaiacol as a model compound of lignin with a homogeneous catalyst of NaOH. *Ind. Eng. Chem. Res.* 53, 7856–7865.
- Zabka, M., Pavela, R., 2013. Antifungal efficacy of some natural phenolic compounds against significant pathogenic and toxinogenic filamentous fungi. *Chemosphere* 93, 1051–1056.
- Zhou, L., Zheng, H., Tang, Y., Yu, W., Gong, Q., 2013. Eugenol inhibits quorum sensing at sub-inhibitory concentrations. *Biotechnol. Lett.* 35, 631–637.