On the inhibition of cyprid attachment by the combination of (-) menthol and *Omadine*® biocides

Sr. Avelin Mary[1], Craig Waldron[2], Jonathan R. Matias[3]

[1] Sacred Heart Marine Research Center, Beach Road, Tuticorin, India 628001 [2] Arch Chemicals, Inc., 350 Knotter Drive, Cheshire, CT 06410 USA [3] Poseidon Ocean Sciences Inc. 122 East 42nd Street, Suite 1700, New York, NY 10168 USA

ABSTRACT

(-)-Menthol has been demonstrated as an inhibitor of the settlement of the cyprids of the barnacle, Balanus amphitrite Darwin, and effective against hard fouling organisms in marine exposure tests. Copper Omadine biocide is as an effective compound against soft fouling marine organisms and moderately inhibitory to hard foulers. This study examines the effect of combining these two compounds in an effort to develop effective antifouling systems. In the standard cyprid assay in which test compounds were added into the seawater medium, copper Omadine biocide and (-) menthol inhibited cyprid settlement with an EC_{50} *of* $1x10^{-7}$ *mg/ml and* $4x10^{-3}$ *mg/ml, respectively. When (-) menthol was added simultaneously to seawater containing copper Omadine biocide, there was a significant increase in the inhibitory effect on the settlement of cyprid larvae. To further examine the effects of the combination of the two compounds, a modified assay was developed wherein the compounds were incorporated into an ultra-pure non-toxic VYHH resin at various concentrations and were compounds were incorporated into an ultra-pure non-toxic VYHH resin at various concentrations and were used to coat the bottom of petri dishes.*

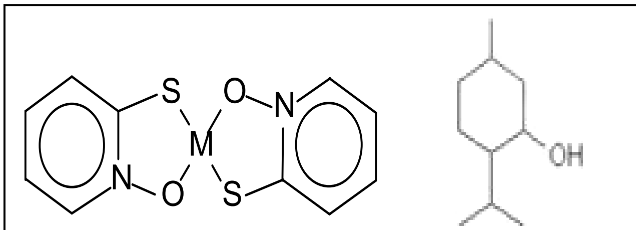
After drying the resin, the seawater containing the cyprids was added to the petri dishes and the total number of attached and metamorphosed larvae were counted 24 hours later. This new test system allowed the examination of the biological performance of antifoulants in a coating matrix as part of the screening method. When both compounds were incorporated into the VYHH resin, the results showed that the combination produced a greater degree of inhibition than either copper Omadine biocide or (-) menthol alone. Marine field exposure tests were conducted on resin/rosin coatings with menthol and Zinc Omadine. The data after 7 months of exposure in Tuticorin Harbor (India) showed significant antifouling effects of these compounds in an aggressive, year-round fouling environment.

Introduction

The Natural Bioproducts Screening Programme was established in 1992 to search for marine natural products that have practical pharmaceutical and/or industrial applications. One of the major targets of NBSP is to identify natural products that can be used as nontoxic replacement for toxic biocides in marine paints. By understanding the specific structural configurations required in producing an anti-settlement effect on hard fouling and through the study of structure-activity relationships, NBSP was able to identify a range of molecules, some of which are structurally related to menthol. The studies have shown that (-) menthol, the naturally occurring l-isomer found in peppermint, elicits a specific anti-settlement action against hard fouling organisms, specially in the barnacle, *Balanus amphitrite*.

This effect occurs in a reversible, nontoxic manner, possibly through a repellent mechanism. The barnacle larvae are prevented from attachment without toxic effects on the cyprids.

Menthol is a biologically active settlement inhibitor but has been observed to have weak effects against soft fouling organisms, such as algae. The marine paint industry already has various antifouling compounds in current use that are effective in controlling soft fouling. Among these antifoulants include Copper and Zinc *Omadines*, a broad-spectrum biocide against marine algae, bacterial slime, fungi and diatoms. The omadine biocide was first introduced in 1963 as an anti-dandruff agent and 30 years later as a co-biocide in marine paints.



General structure of the Omadines | Structure of menthol

ANTI-SETTLEMENT STUDIES USING CYPRID BIOASSAYS

The cyprid settlement assay described by Rittschof, et al (*J Chem Ecol, 11:551-563, 1985*) was the screening method used for the determination of the antifouling activity of the Omadine biocide and (-) menthol. The barnacle, *Balanus amphitrite Darwin*, used in the experiments were collected from Tuticorin Bay (India). The barnacles were crushed and the nauplius stage larvae were collected for culture to the cyprid stage following the method of Rittschof et al (*J Exp Mar Biol Ecol, 82:131-146, 1984*). Briefly, Falcon 50 x 9 mm plastic petri dishes were filled with filtered seawater at salinity of 33 – 35 ppt and into which 3-day old cyprid stage larvae were added.

The test compounds were introduced at various concentrations into the dishes containing the seawater. Controls represented those dishes in which no test compounds were added. After overnight incubation at 28^{o}C, the dishes were examined under a dissecting microscope for mortality. The larvae were then killed with 10% formalin and the attached, unattached and metamorphosed larvae were counted.

ANTI-SETTLEMENT STUDIES ON RESIN COATED PETRI DISHES

In this procedure, the test compounds at various concentrations were dissolved in methyl isobutyl ketone (MIBK) along with VYHH resin. The solution was then added to the bottom of glass petri dishes and the MIBK was allowed to evaporate at room temperature. Preliminary studies (not shown here) indicated that the resulting film produced by the resin was nontoxic and the cyprids are able to normally attach to the surface. The cyprids and seawater were added and the number of settled, unattached and metamorphosed larvae was counted 16 hours later. The total surface area covered by the resin film was 45.4 cm^2 and the total dry weight of the film was 5.5 mg per cm^2.

FIELD STUDIES

A resin/rosin type paint system was prepared at Arch Chemicals. Zinc *Omadine,* menthol or combinations of the two chemicals were added to the paint and coated on the PVC rods (8mm wide by 11 cm long). The test rods were mounted securely on a meshed net and immersed into the sea at a depth of 1.5 feet from the surface. The marine exposure platform used for this study was located in Karrapad Creek, Tuticorin, India. This site was selected because of year-round aggressive fouling of submerged surfaces. At monthly intervals, the rods were taken out of the seawater, examined for fouling and photographed.

CHEMICALS

The menthol used for these studies were obtained from Sigma-Aldrich (St. Louis, IL). Zinc and Cu *Omadines* were obtained from Arch Chemicals (Cheshire, CT). VYHH resin was a gift from Du Pont.

Laboratory Studies

The data shown in Fig. 1 and Fig. 2 demonstrate the biological potential of the Omadine biocide and menthol in preventing the attachment of cyprids. The EC_{50} for menthol is 4 x 10^{-3} while the EC_{50} for Cu *Omadine* is approximately 10^{-7} mg/ml. Although the standard cyprid settlement assay is suitable for the fast, convenient screening of biological activity, it does not provide sufficient information on the behavior of the active chemical when impregnated in a film or coating. When incorporated into a paint formulation, the compositions that make up the paint tend to be toxic to cyprids especially in a confined space of the petri dish wherein seawater exchanges in large enough volume is not practical. In order to bridge this constraint, we have devised a modification of the screening assay by incorporating the active ingredient in ultrapure VYHH resin and using this resin to create a film containing the active ingredient at the bottom surface of the petri dish. This alternative method is diagrammatically presented in Figure 3. The coated dish assay enabled the opportunity to look at the performance of active ingredients in a coated film by studying the movement or diffusion of active ingredient.

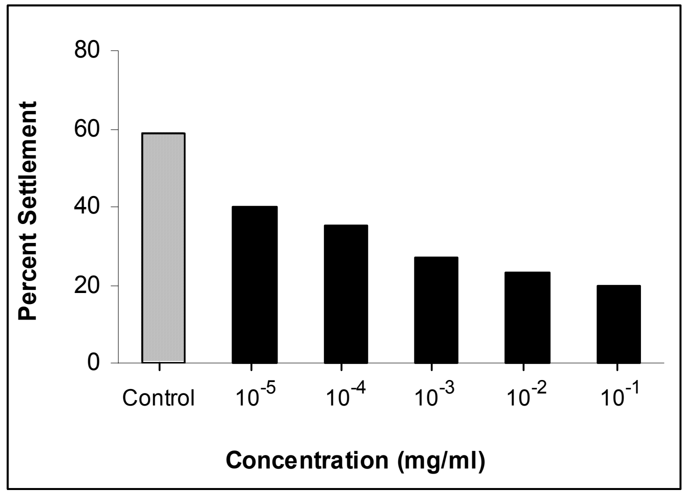


Figure 1. The dose dependent inhibition of the settlement of the cyprids of the barnacle, *Balanus amphitrite*, in the presence of (-) menthol. Each data point represents the mean of triplicate tests.

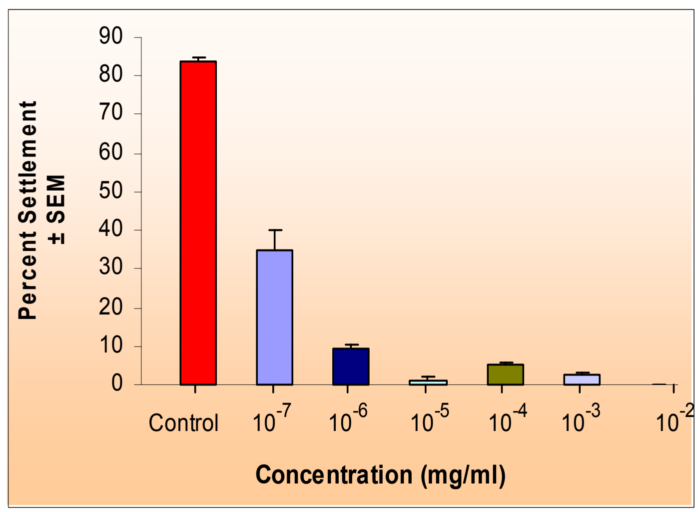


Figure 2. Inhibition of cyprid settlement in the presence various concentration of Copper *Omadine*.

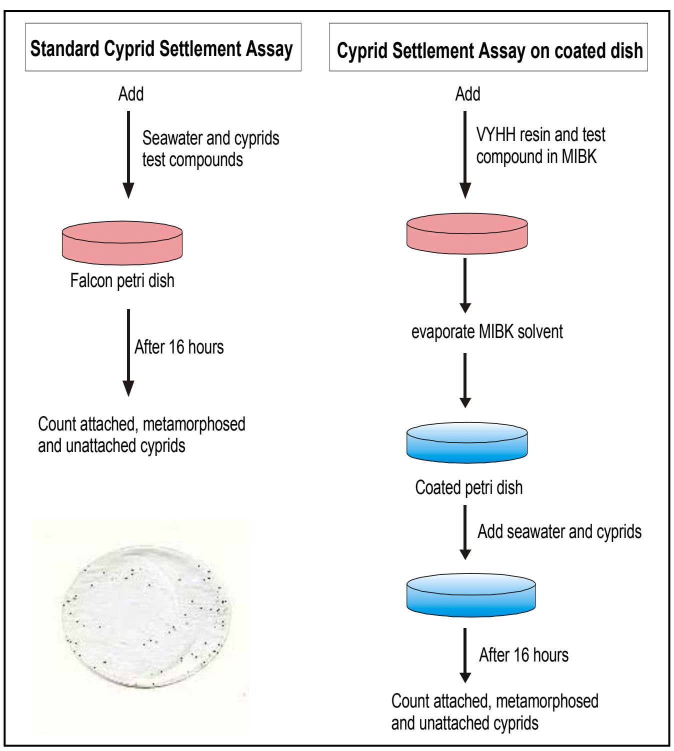


Figure 3. Diagrammatic description of the steps involved in the standard cyprid settlement assay and the coated petri dish assay. Photo on the left shows the VYHH film after 24 hours of adding the cyprids. The black dots represent each individual metamorphosed cyprid.

The data shown in Figure 4 describe the anti-settlement effect of menthol when incorporated in VYHH resin. In the control, the settlement was determined as 68% and this is comparable to the degree of settlement normally observed in uncoated petri dishes. When menthol was added to VYHH resin at a concentration of 10% (w/w), we observed a significant inhibition of settlement ($P<0.001$, Students t-test). There was a possibility that the effect observed might have been due to the fast release of menthol from the resin so that the menthol that might be present in the seawater may in fact cause the inhibition. We tested this possibility by taking the seawater media and adding fresh cyprids. The data in Fig. 4 showed that the old seawater from the menthol test possessed no inhibitory effect, suggesting that the effect observed was due to the menthol present on the surface of the VYHH resin.

The study in Figure 5 was designed to examine the effect of the combination of menthol and *Omadine* biocide on the settlement of cyprids. The concentrations selected for this study was purposely chosen to have only a small effect biologically when used individually. This allowed one to see if there was enhancement of the effect when added together. The combination of the two compounds showed a significant reduction of settlement compared to Cu *Omadine* alone ($P<0.01$) and menthol ($P<0.001$). This effect of the combination appeared to be an additive rather than a synergistic one.

Field Studies

The POSEIDON-SHMRC Marine Exposure Center. Poseidon maintains an exclusive collaborative research agreement with Sacred Heart Marine Research Center (SHMRC), a marine science group located in the port city of Tuticorin in the southern tip of India. This location is an optimum site for marine fouling research because of the high diversity of fouling community in Tuticorin Harbor and the year-round presence of fouling organisms. Barnacles and sponges are the most dominant representatives of the fouling community. Sea anemones, hydroids, polychaetes, bivalves and ascidians have significant presence in the waters of Tuticorin Harbor area.

More information about SHMRC can be found in the poster presentation in the same session of the 11th ICMCF entitled "Poseidon-SHMRC Marine Biofouling Research Programme."

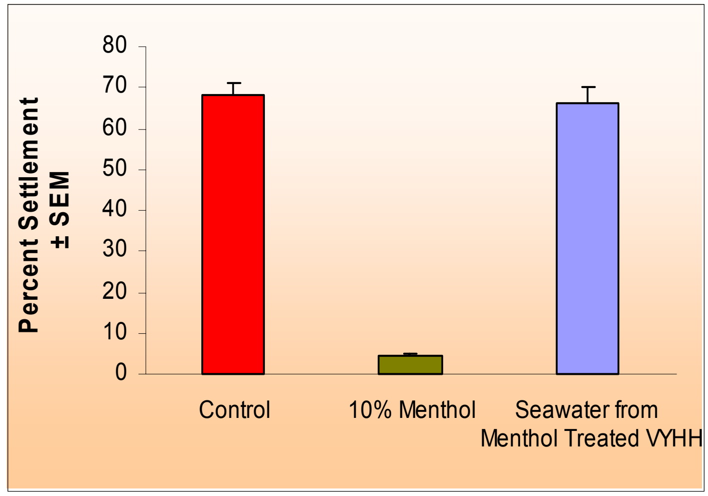


Figure 4. The inhibition of cyprid settlement by the addition of (-) menthol in VYHH resin.

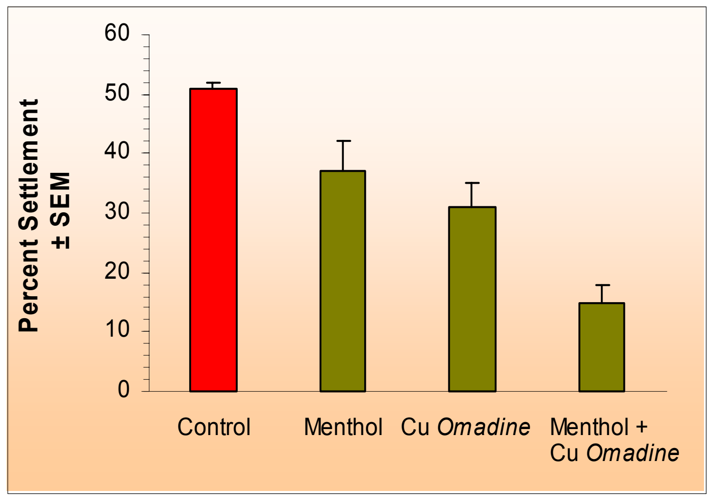


Figure 5. The inhibition of cyprid settlement by the combination of Menthol and Cu Omadine biocide using the coated petri dish assay. Each bar represents mean data for 10 tests. The concentrations were: menthol 1% in VYHH resin (w/w), Cu *Omadine* 1% (w/w).

Results of the marine exposure of coated rods. As shown in Figure 6, the coated rods were mounted on a mesh net and submerged in the sea off Tuticorin Harbor. The tests were done in duplicate and the fouling conditions for the duplicates were in agreement throughout the study period. The representative photographs of the rods after 7 months of exposure are shown in Figure 7. Both the blank and vehicle controls were completely fouled primarily by barnacles, which have grown on top of each other. In contrast, menthol and *Omadine* treated rods were relatively free of barnacle fouling, although slime and algal fouling appeared to be present.

Figure 6. Typical method of hanging the painted rods and placement of the test series on the exposure platform in Karrapad, India.

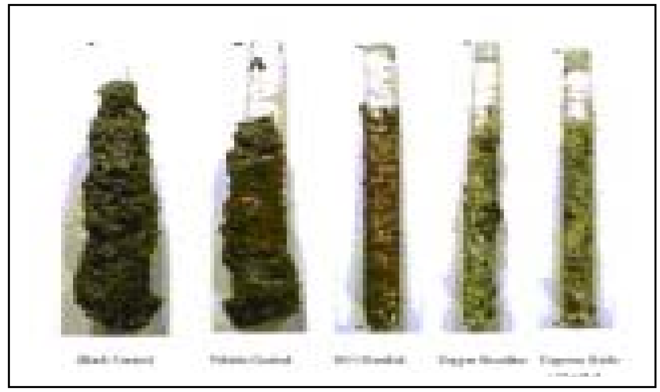


Figure 7. Comparison of fouling conditions of test rods after 7 months under total immersion in Tuticorin Harbor, India.

When examined under the dissecting microscope, the fouling was composed of diatoms, algae and sponges. These fouling organisms were lightly attached and can be removed with jet spray. The differences between the combination and the individual antifoulants were not apparent at 7 months of exposure and it will require longer exposure times to validate these differences. However, preliminary data on the measurement of the menthol content of the coatings after 7 months of exposure showed already a major depletion of the menthol.

In these studies, we have demonstrated the anti-settlement activity of both menthol and *Omadine* biocide on the standard cyprid assay test and also through a modified coated dish assay. Biological performance under field conditions was also demonstrated for both compounds. Because menthol is a small molecule, it is expected to leach out of a resin/rosin system at a faster rate compared to *Omadine* biocide that is a highly insoluble and larger molecule. During the forthcoming session in this conference, more detailed information regarding the safety and biological activity of menthol and menthol analogues would be presented. This will not be discussed in detail here. It is suffice to mention that menthol has been in human and animal use for over 100 years and has been proven to be a safe compound in terrestrial and marine organisms.

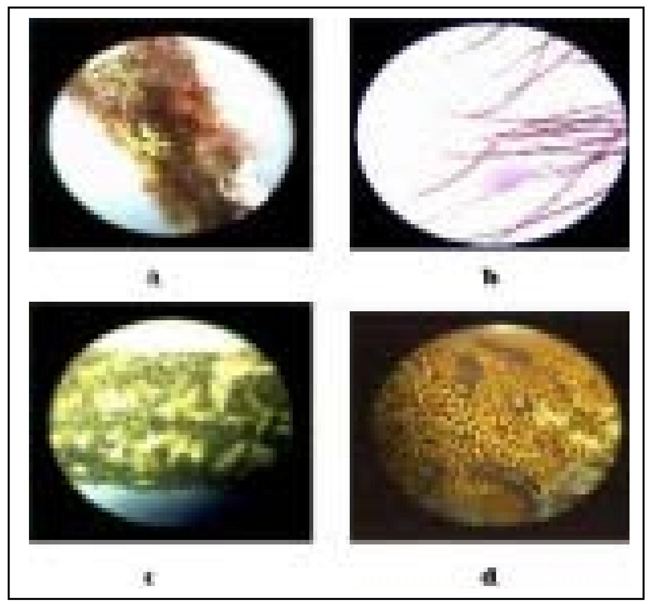


Figure 8. Close-up microscopic examination of the fouling attachments found in the rods coated with paints containing menthol, Zn Omadine and combination of the two compounds. (a) algae and diatoms, (b) close up of the fouling strands in a, (c) green algae, (d) sponges.

The investment of R&D effort on menthol as a potential antifoulant for hard fouling organisms is warranted not only because of its nontoxic nature, but also because of the reasonable cost of manufacturing and the availability of thousands of tons of material for industrial use. The major challenges are: (1) to develop a novel delivery system that can control the leach rate of this chemical through the various paints systems; (2) to develop a modification of the menthol molecule itself to make it more insoluble; and (3) to develop the appropriate paint compositions to design totally metal-free system. These challenges are actively being pursued at Poseidon Sciences.