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ORIGINAL ARTICLE

The Plant Alkaloid Camptothecin as a Novel Antifouling Compound for Marine Paints: Laboratory Bioassays and Field Trials

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Abstract

The extensive use of copper and booster biocides in antifouling (AF) paints has raised environmental concerns and the need to develop new AF agents. In the present study, 18 alkaloids derived from terrestrial plants were initially evaluated for AF activity using laboratory bioassays with the bryozoan *Bugula neritina* and the barnacle *Balanus albicostatus*. The results showed that 4 of the 18 alkaloids were effective in inhibiting larval settlement of *B. neritina*, with an EC_{50} range of 6.18 to 43.11 μM, and 15 of the 18 alkaloids inhibited larval settlement of *B. albicostatus*, with EC_{50} values ranging from 1.18 to 67.58 μM. Field trials that incorporated five alkaloids respectively into paints with 20% *w/w* indicated an in situ AF efficiency of evodiamine, strychnine, camptothecin (CPT), and cepharanthine, with the most potent compound being CPT, which also exhibited stronger AF efficiency than the commercial antifoulants cuprous oxide and zinc pyrithione in the field over a period of 12 months. Further field trials with different CPT concentrations (0.1 to 20% *w/w*) in the paints suggested a concentration-dependent AF performance in the natural environment, and the effective concentrations to significantly inhibit settlement of biofoulers in the field were ≥ 0.5% *w/w* (the efficiency of 0.5% *w/w* lasted for 2 months). Moreover, CPT toxicity against the crustacean *Artemia salina*, the planktonic microalgae *Phaeodactylum tricornutum* and *Isochrysis galbana*, was examined. The results showed that 24 h LC_{50} of CPT against *A. salina* was 20.75 μM, and 96 h EC_{50} (growth inhibition) values of CPT to *P. tricornutum* and *I. galbana* were 55.81 and 6.29 μM, respectively, indicating that CPT was comparatively less toxic than several commercial antifoulants previously reported. Our results suggest the novel potential application of CPT as an antifoulant.

Keywords Antifouling compound · Camptothecin · Alkaloid · Biofouling · Natural antifoulant

Introduction

Biofouling control is essential for maritime industries because settlement of marine fouling organisms on surfaces of artificial submerged structures causes serious economic problems (Yebra et al. 2004). Particularly for ships, fouling of hulls increases frictional drag, reduces ship speed, and leads to higher fuel cost (Townsin 2003). The widely used technology to control marine biofouling is antifouling (AF) paint that contains biocides. Organotin-based paints are highly effective, but have been banned due to their highly toxic effects on non-target organisms (IMO 2008; Yebra et al. 2004). Currently, AF paints containing copper and organic booster biocides (e.g., diuron, Irgarol 1051, Sea-Nine 211, dichlofluanid, and chlorothalonil) are widely applied as alternative. However, these antifoulants can also pose adverse impacts on the marine environment, which subsequently led to bans and regulations in various countries (Price and Readman 2013; Thomas and Brooks 2010). Consequently, increasing environmental concern and regulatory pressure has prompted the development of novel non- or less toxic AF agents.

Natural product antifoulants (NPAs) are considered as promising sources of environmentally friendly antifoulants (Qian et al. 2015). Although numerous compounds have been found to be AF active, there are obstacles for the commercial use of NPAs in marine antifouling paints. One major obstacle is the supply of NPAs. Most NPAs reported are derived from marine macroorganisms (including macroalgae, sponges, soft

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corals, bryozoans, and ascidians) (Fusetani 2011), usually with poor yield and complex structures (thereby making it difficult to synthesize at a reasonable cost). Finding NPAs from natural sources such as microorganisms and terrestrial plants, which could be cultured in large scale, may help solve this supply issue (Feng et al. 2009; Moodie et al. 2017a, b, 2018; Qian et al. 2010; Satheesh et al. 2016). Other major issues hindering the development of NPA-based paints include the time-consuming and costly government registration of new antifoulants. Searching NPAs from known compounds (such as pharmaceuticals) with detailed knowledge on their chemical properties, effects on living organisms, and/or mechanisms of action may facilitate registration (Dahlström et al. 2000; Dahlström and Elwing 2006; Rittschof et al. 2003). Furthermore, most studies on NPAs only employ laboratory bioassay(s) to test AF activity (Qian et al. 2015). To explore the potential of NPAs in commercial applications, these should be incorporated in paints and evaluated in the marine environment. Qian et al. (2010, 2015) and Maréchal and Hellio (2009) have previously presented details of the above-mentioned obstacles and suggestions in developing NPA-based paints.

Alkaloid compounds have been isolated in various terrestrial plants, marine organisms, and microorganisms. Previous studies have described the bioactivities of alkaloids, including antibacterial, antitumor, anti-inflammatory, antimalarial, antiviral, antiasthma, analgesic, and antioxidant (Cushnie et al. 2014; Farouk et al. 2008; Fernandez et al. 2009; Kittakoop et al. 2014; Shaheen et al. 2005; Souto et al. 2011). Various alkaloids are used as drugs or insecticides. Numerous marine-derived alkaloids have been found to be AF active, mainly using laboratory bioassay(s) (e.g., He et al. 2013; Hertiani et al. 2010; Pénez et al. 2011; Trepos et al. 2014). In contrast, our current understanding of AF activity in terrestrial derived alkaloids is limited (Huang et al. 2014). Among the very limited examples of terrestrial-derived alkaloids found with AF activity, the well-known one is capsaicin, which naturally occurs in chili peppers (Angarano et al. 2007; Xu et al. 2005).

In this study, 18 known alkaloids derived from terrestrial plants were initially evaluated for AF activity against barnacle and bryozoan. Barnacles and bryozoans both are major fouling organisms (Førde et al. 2016; Holm 2012), and these are frequently used as the test organisms in the AF laboratory bioassays (Briand 2009; Qi et al. 2008). Here, the barnacle *Balanus albicostatus* was used because it is a notorious fouler in East Asian waters (Chen 2006; Huang and Cai 1984; Lee and Kim 1991), and its cyprid larvae can be easily cultured in controlled laboratory conditions (Feng et al. 2009). The bryozoan *Bugula neritina*, one widely distributed fouler, was also used in the AF assays in this study. Five of the AF active alkaloids were then tested in field trials. Subsequently, one alkaloid with outstanding AF performance, camptothecin (CPT), was further subjected to another field trial at different concentrations in paints and toxic assays with three aquatic organisms, brine shrimp *Artemia salina*, planktonic microalgae *Phaeodactylum tricornutum* and *Isochrysis galbana*. *A. salina* lethality test was commonly used to examine the toxic effect of compounds (including antifoulants) on aquatic organisms (e.g., Koutsaftis and Aoyama 2007; Panagoula et al. 2002), and so was microalgal growth inhibition test (e.g., Martins et al. 2017; Mochida and Fujii 2009; Suratno et al. 2015; Zhang et al. 2008).

Materials and Methods

Test Alkaloids

Eighteen alkaloid compounds were examined for AF activity in this study. Their names, chemical properties, and plant sources are listed in Table 1. Their chemical structures are shown in Fig. 1. These were selected based on the fact that these are natural alkaloids from plants or derivatives of plant natural alkaloids and are commercially available. These alkaloids were purchased from the sources shown in Table 1, all with purities above 98%.

AF Bioassay with the Bryozoan *Bugula neritina* Larvae

Adult colonies of *B. neritina* were collected from a fish farm in Zhangzhou, Fujian Province, China. The larvae were obtained from the adults, and the AF assay with bryozoan larvae was performed as previously described by Feng et al. (2013) and Martín and Uriz (1993). Briefly, the adults of *B. neritina* placed in a glass tank filled with filtered (0.22 μm) seawater (FSW) were induced to release larvae through exposure to strong artificial light for approximately 30 min. The larvae were collected and immediately used in the AF assay. The 18 tested alkaloids were dissolved in ethyl acetate or methanol. Aliquots of the solution were applied to glass Petri dishes (6-cm diameter). After complete evaporation of the organic solvent at room temperature, 10 mL of FSW and 30 larvae were added to each dish. The concentrations ranged from 0.5 to 50 μg mL^{-1} for each alkaloid. There were three replicates for each concentration of each compound and the FSW control. The Petri dishes were incubated in the dark at 25 °C for 24 h, after which the number of settled larvae was counted under a stereomicroscope.

AF Bioassay with the Barnacle *Balanus albicostatus* Larvae

Adults of *B. albicostatus* were collected from the intertidal zone in Xiamen, Fujian Province, China. The adults released nauplii upon immersion in seawater. The nauplii of this species were reared to cyprids as described by Feng et al. (2009). The AF

Table 1 The eighteen alkaloids tested and their antifouling activity against the bryozoan *Bugula neritina* and the barnacle *Balanus albicostatus*

Compound	CAS number	Molecular weight (Da)	Common plant source	Bryozoan bioassay	Barnacle bioassay		
				EC_{50} (μM)	EC_{50} (μM)	LC_{50} (μM)	LC_{50}/ EC_{50}
Evodiamine[a]	518-17-2	303.4	*Evodia rutaecarpa*	NE	3.49	18.95	5.43
Rutaecarpine[a]	84-26-4	287.3	*Evodia rutaecarpa*	NE	NE	NE	–
Anisodamine[b]	55869-99-3	305.4	*Anisodus tanguticus*	NE	67.58	> 163.72	> 2.42
Scopolamine butylbromide[b]	149-64-4	440.4	Manufactured from scopolamine which occurs naturally in *Atropa belladonna*	NE	38.06	> 113.53	> 2.98
Scopolamine methobromide[b]	155-41-9	398.3	Manufactured from scopolamine which occurs naturally in *Atropa belladonna*	NE	8.99	> 125.53	> 13.96
Aconitine[a]	302-27-2	645.7	*Aconitum* sp.	NE	7.84	103.24	13.17
Hypaconitine[a]	6900-87-4	615.7	*Aconitum* sp.	NE	1.92	133.18	69.36
Mesaconitine[a]	2752-64-9	631.7	*Aconitum* sp.	NE	12.19	96.15	7.89
Camptothecin[a]	7689-03-4	348.4	*Camptotheca acuminata*	43.11	4.97	11.22	2.26
Sinomenine[c]	115-53-7	329.4	*Sinomenium acutum*	NE	10.32	> 151.79	> 14.71
Dicentrin[c]	517-66-8	339.4	*Dicentra spectabilis*	8.46	1.18	42.31	35.86
Ligustrazine hydrochloride[c]	76494-51-4	172.7	Manufactured from ligustrazine which occurs naturally in *Ligusticum chuanxiong*	NE	NE	NE	–
Cepharanthine[c]	481-49-2	606.7	*Stephania cepharantha*	8.22	3.54	11.80	3.33
Tetrandrine[c]	518-34-3	622.8	*Stephania tetrandra*	6.18	2.15	10.73	4.99
Berberine hydrochloride[a]	633-65-8	371.8	Manufactured from berberine which occurs naturally in *Berberis* sp.	NE	2.90	> 134.48	> 46.37
Palmatine hydrochloride[a]	10605-02-4	387.9	Manufactured from palmatine which occurs naturally in *Phellodendron amurense*	NE	63.68	> 128.90	> 2.02
Trigonelline[b]	535-83-1	137.1	*Trigonella foenum-graecum*	NE	NE	NE	–
Strychnine[c]	57-24-9	334.4	*Strychnos nux-vomica*	NE	1.32	> 149.52	> 113.27

NE no inhibitive effect or lethal effect at the concentrations tested, – not applicable

[a] Compounds purchased from Shanghai Winherb Medical Technology Co., Ltd., Shanghai, China

[b] Compounds purchased from National Institute for Food and Drug Control, Beijing, China

[c] Compounds purchased from Shenzhen Meihe Bioscience Co., Ltd., Shenzhen, China

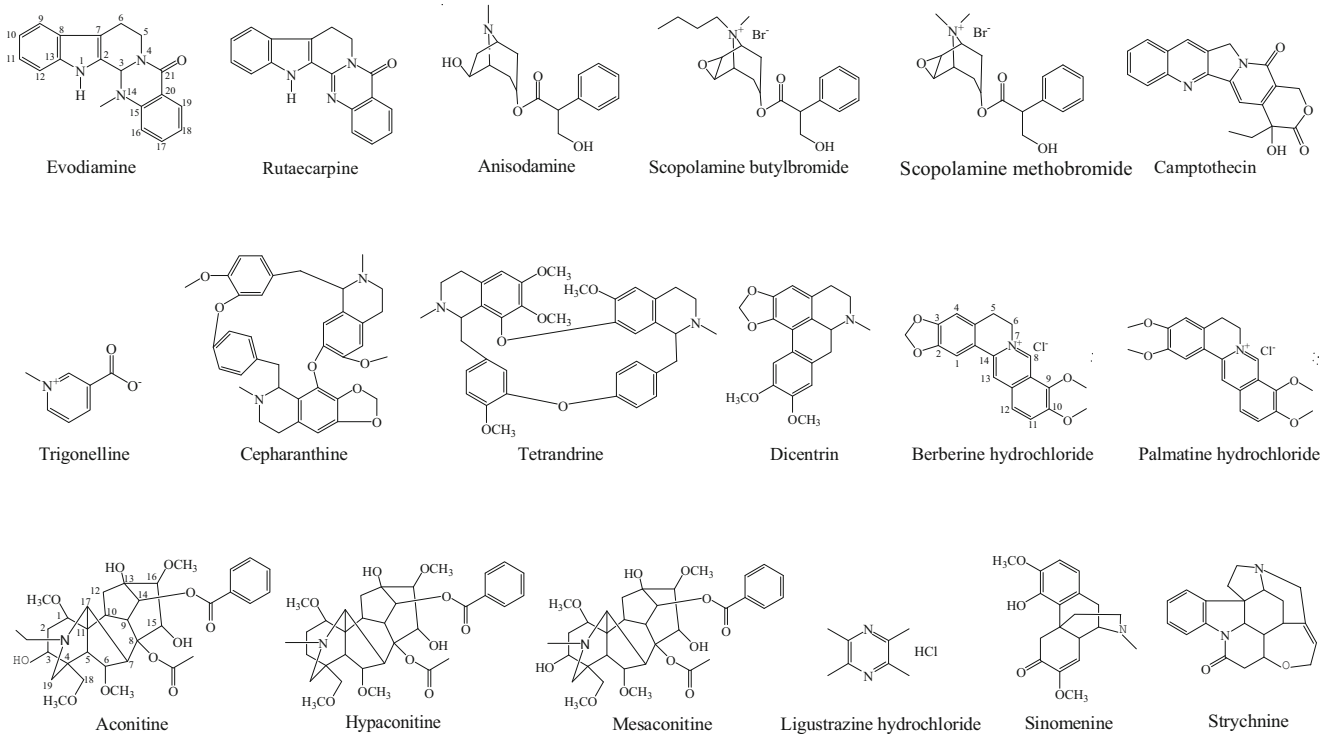

Fig. 1 Chemical structures of the tested alkaloids

assay with barnacle cyprids was conducted according to the methods described by Feng et al. (2009), Hellio et al. (2005), and Kitano et al. (2004). Briefly, the procedure for the AF assay with *B. albicostatus* was similar to that used with *B. neritina*, as described above. The 18 alkaloids were dissolved in ethyl acetate or methanol, and applied to Petri dishes. After complete evaporation of the organic solvent at room temperature, 10 mL of FSW and 30 cyprids were added to each dish. The concentrations of alkaloids, selected based on the results of a pilot study, ranged from 0.05 to 5 μg mL^{-1} for strychnine, from 0.5 to 100 μg mL^{-1} for aconitine, hypaconitine, and mesaconitine, and from 0.25 to 50 μg mL^{-1} for all of the other alkaloids. Three replicates were set up for each concentration of each compound and the FSW control. The Petri dishes were incubated in the dark at 25 °C for 48 h, after which the number of cyprids that had settled or died was counted under a stereomicroscope.

Field Experiments of Different Alkaloids

Field experiments were performed to evaluate the in situ performance of AF compounds. Five alkaloids, namely evodiamine, CPT, sinomenine, cepharanthine, and strychnine, were selected for the field experiments. The five alkaloids were selected because these showed relatively high AF activity against the settlement of barnacles (Table 1), the most important group of organisms responsible for fouling (Briand 2009), and could readily be purchased in markets at sufficient quantities for the field experiment. The alkaloids were incorporated into a matrix paint. The composition of the matrix paint, expressed as weight percentage, was as follows: 14.8% rosin, 65.8% solution A (i.e., 50% polyacrylic resin in dimethylbenzene), 7.7% Fe_2O_3, 1.4% bentonite, and 10.3% mixed solvent (coal-tar/n-butanol/dimethyllbenzene = 14:3:13). Each alkaloid was added into the above mixture at 20% (*w*/*w*) and dispersed using a high-speed disperser. Paints were prepared to a fineness of grind of ca 80 μm [which was measured using a Fineness of Grind Gauge, Tianjin Hongjuli Test Equipment Factory (Tianjin, China)]. Matrix paint without any AF compounds was used as negative control. AF paints respectively containing 20% of capsaicin, cuprous oxide, and zinc pyrithione, prepared as described above, were used as positive controls. Capsaicin was selected in this experiment because it is a plant natural alkaloid compound and that it shows AF activity (Xu et al. 2005). Cuprous oxide and zinc pyrithione were selected based on the fact that these are commonly used antifoulants in commercial AF paints.

The prepared paints were brushed onto sandblasted epoxy panels, with the painted area of 25 cm × 9 cm for each paint. Three layers of paint were applied and allowed to dry in the dark between each application, finally generating a dry film with a thickness of approximately 150 μm. The panels were tested over two time periods and different sites. The panels treated with control paints and the paints containing evodiamine and strychnine were hung on a floating raft near Gulangyu Islet (24°46′N, 118°07′E) of Xiamen Bay, China, on September 22, 2009. The panels were submerged at a depth of 1 m in seawater, with all panels in one bay (1 m × 1.8 m) of the floating raft (it is made of structural steel parts and its central portion is divided into 18 bays). There were six

replicates for each tested paint and each control paint. They were submerged for 12 months and photographed at several time points during submersion. For each image, the percentage of the surface colonized by the fouling organisms on each panel was calculated using Adobe Photoshop 7.0. On the other hand, the panels coated with control paints and the paints containing CPT, cepharanthine, and sinomenine were hung in a bay of the floating raft at a depth of 1 m in seawater near Dalipuyu Islet (24°56′N, 118°16′E) of Xiamen Bay, China, on June 6, 2010 (the test site was changed because the floating raft was moved from Gulangyu Islet to Dalipuyu Islet). There were six replicates for each tested paint and each control paint. They were submerged for 12 months and photographed at various time points after submersion, and the percentage of the surface colonized by the fouling organisms on each panel was calculated using Adobe Photoshop 7.0. Both study sites were located in Xiamen Bay, where the tide belongs to regular semidiurnal tide (Luo et al. 2012). The seawater temperature ranges between 13 and 33 °C in Xiamen Bay. There is Jiulong River Estuary located in the Xiamen Bay, and Gulangyu Islet is nearer Jiulong River than Dalipuyu Islet. The salinity of seawater surrounding Gulangyu Islet ranges between 25 and 30 and the salinity of seawater surrounding Dalipuyu Islet ranges between 28 and 33 (provided by Dr. Zhi bin Luo, College of Ocean and Earth Sciences, Xiamen University).

Field Experiment of Different CPT Concentrations

Because CPT exhibited remarkable in situ AF efficiency at a concentration of 20% (*w*/*w*) (Figs. 4 and 5), further investigation was performed to ascertain its effective concentration range in the field. In this experiment, CPT was incorporated into the matrix paint at six concentrations [0.1, 0.5, 1, 5, 10, and 20% (*w*/*w*), respectively]. The paints containing different concentrations of CPT were prepared and applied to panels as earlier described. Matrix paint without any AF compounds was used as negative control. A commercial AF paint [L40-32 (813-1) AF paint, Shanghai Kailin Paint Factory (Shanghai, China)], which contains cuprous oxide, was used as positive control. The panels were hung in a bay of the floating raft at a depth of 1 m in seawater near Dalipuyu Islet of Xiamen Bay on April 25, 2012. There were six replicates for each tested paint and each control paint. They were submerged for 11 months and photographed at various time points after submersion. The percentage of the surface colonized by the fouling organisms on each panel was calculated using Adobe Photoshop 7.0.

Toxic Bioassay with the Brine Shrimp *Artemia salina* for CPT

The toxicity of CPT was tested against *A. salina* using the Artoxkit M procedure (Artoxkit 1990). *Artemia* cysts were purchased from Wudi Aijia Pet Aquarium Co., Ltd. (Binzhou, China). These were incubated in FSW (salinity 27, pH 8.13) with continuous illumination (3000–4000 lx) and aeration at 25 °C for 24 h. The hatched larvae were transferred to fresh FSW and cultured for another 24 h. Then *Artemia* nauplii at instar stages II–III were collected and used in the bioassay. The bioassay was performed using 24-well plates. CPT was dissolved in dimethyl sulfoxide (DMSO). A volume of 10 μL of compound solution, 1.99 mL FSW, and 10 nauplii were added into each well. Wells containing 0.5% DMSO in FSW (*v*/*v*) were used as control. The concentrations of the testing solutions were 0 (0.5% DMSO control), 0.5, 1, 2, 5, 10, 20, 50, and 100 μg mL^{-1} (i.e., 0, 1.44, 2.87, 5.74, 14.35, 28.70, 57.41, 143.51, and 287.03 μM). The pilot study showed there was no significant effect of 0.5% DMSO on *A. salina* survival. This assay was performed in six replicates. The plates were incubated in the dark at 25 °C. The dead nauplii in each well were counted after 24 h of incubation with the aid of a stereomicroscope. Nauplii that had appendages showing no movement within 10 s were counted as dead (Zulkifli et al. 2014).

Toxic Bioassay with Microalgae *Phaeodactylum tricornutum* and *Isochrysis galbana* for CPT

The marine planktonic microalgae *Phaeodactylum tricornutum* and *Isochrysis galbana* were obtained from the Center for Collections of Marine Algae, State Key Laboratory of Marine Environmental Science, Xiamen University. Algae were cultured in filtered (0.22 μm) and autoclaved f/2 medium (Guillard and Ryther 1962) at 24 ± 1 °C under illumination of 3000–4000 lx (12 h light/12 h dark photoperiod). Cultures were shaken twice a day. Algae were sampled at the exponential phase for the bioassay. The 96-h algal growth inhibition test was carried out by following of Zhang et al. (2008). Glass flasks of 250 mL were sterilized at 121 °C for 30 min before use. CPT was dissolved in DMSO. A volume of 0.5 mL of compound solution and 99.5 mL f/2 medium containing algae were added into each flask. The initial cell concentration of algae was 1.94×10^5 cells mL^{-1} for *P. tricornutum* and 2.50×10^5 cells mL^{-1} for *I. galbana*. The concentrations of CPT were 0 (0.5% DMSO control), 0.625, 1.25, 2.5, 5, 10, and 20 μg mL^{-1} (i.e., 0, 1.79, 3.59, 7.18, 14.35, 28.70, and 57.41 μM) for *P. tricornutum* and 0 μg mL^{-1} (0.5% DMSO control), 1, 1.5, 2, 2.5, and 5 μg mL^{-1} (i.e., 0, 2.87, 4.31, 5.74, 7.18, and 14.35 μM) for *I. galbana*. The concentrations were obtained from preliminary range-finding, and 0.5% DMSO had no significant effect on the algal growth. There were three replicates for each treatment. Test flasks were kept at 24 ± 1 °C under illumination of 3000–4000 lx (12 h light/12 h dark photoperiod) and shaken twice a day. After 96 h of culture, cell density was measured using a hemocytometer for each treatment. Growth inhibition was calculated as follows:

Inhibition (%) = [(C − T) / C] × 100%, where T is the cell density in treatment, and C is the cell density of the control.

Statistical Analysis

In the bioassays using bryozoans, barnacles, brine shrimp, or microalgae, the EC_{50} (the concentration that inhibited larval settlement or algal growth by 50% relative to the control) and LC_{50} (the concentration that resulted in 50% mortality) of compounds were calculated using the Spearman-Karber method (Hamilton et al. 1977, 1978; Reichelt-Brushett and Michalek-Wagner 2005). In the field experiments, differences in macrofouling coverage between panels were analyzed by one-way ANOVA followed by a Tukey post-hoc test. The significance level was defined as $P < 0.05$.

Results

AF Activity of Alkaloid Compounds Against Bryozoan and Barnacle

Table 1 shows the AF activity of 18 alkaloids against the bryozoan *B. neritina* and the barnacle *B. albicostatus*. Among the 18 tested compounds, only four compounds (CPT, dicentrin, cepharanthine, and tetrandrine) imparted inhibitory effects against larval settlement of *B. neritina* at the concentrations tested here, with EC_{50} values ranging from 6.18 to 43.11 μM. More alkaloids were found to be AF active against barnacle settlement. Except for rutaecarpine, ligustrazine hydrochloride, and trigonelline, the other 15 compounds displayed inhibitory effects against larval settlement of *B. albicostatus*. The most effective anti-settlement activity was observed for dicentrin (EC_{50} = 1.18 μM), followed by strychnine (EC_{50} = 1.32 μM). Ten compounds (evodiamine, scopolamine methobromide, aconitine, hypaconitine, CPT, dicentrin, cepharanthine, tetrandrine, berberine hydrochloride, and strychnine) were within the EC_{50} range of 1 to 10 μM against barnacle settlement, and relatively higher EC_{50} values (10 μM < EC_{50} < 100 μM) were found in anisodamine, scopolamine butylbromide, mesaconitine, sinomenine, and palmatine hydrochloride. In the assay using *B. albicostatus*, each of the 15 AF compounds yielded an LC_{50} value greater than its EC_{50} value, particularly hypaconitine, dicentrin, berberine hydrochloride, and strychnine, which had high LC_{50}/EC_{50} ratios (i.e., the therapeutic ratio, 69.36, 35.86, > 46.37, and > 113.27, respectively).

Field Experiments of Alkaloid Compounds

Five alkaloid compounds, namely, evodiamine, CPT, cepharanthine, sinomenine, and strychnine, were incorporated into paints with 20% *w*/*w* and submerged in sea. Figures 2 and 3 show the results of the field experiment

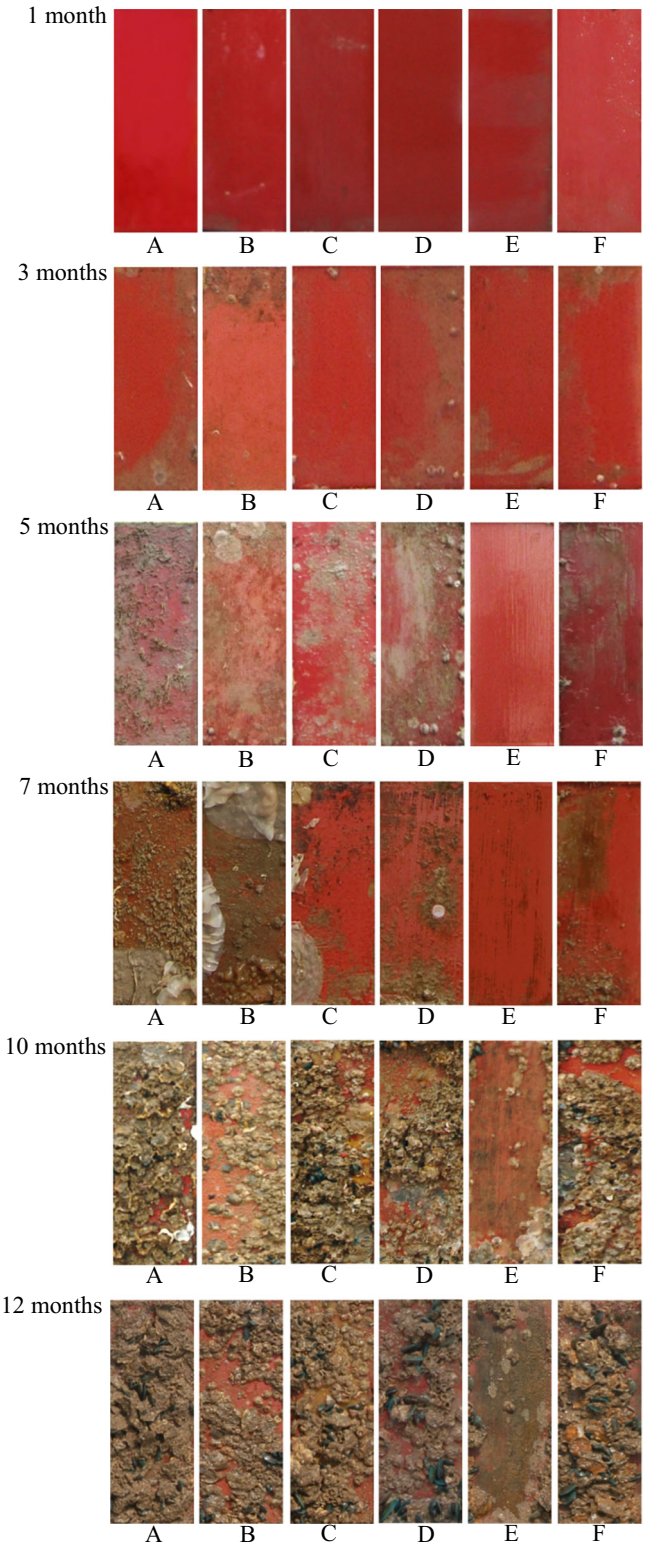

Fig. 2 Test panels after immersion in seawater near Gulangyu Islet of Xiamen Bay at different time points. A, coated with the matrix paint only (negative control paint); B, coated with the paint containing evodiamine; C, coated with the paint containing strychnine; D, coated with the positive control paint containing capsaicin; E, coated with the positive control paint containing cuprous oxide; F, coated with the positive control paint containing zinc pyrithione. Each panel was randomly selected from six replicates of every treatment for every immersion time

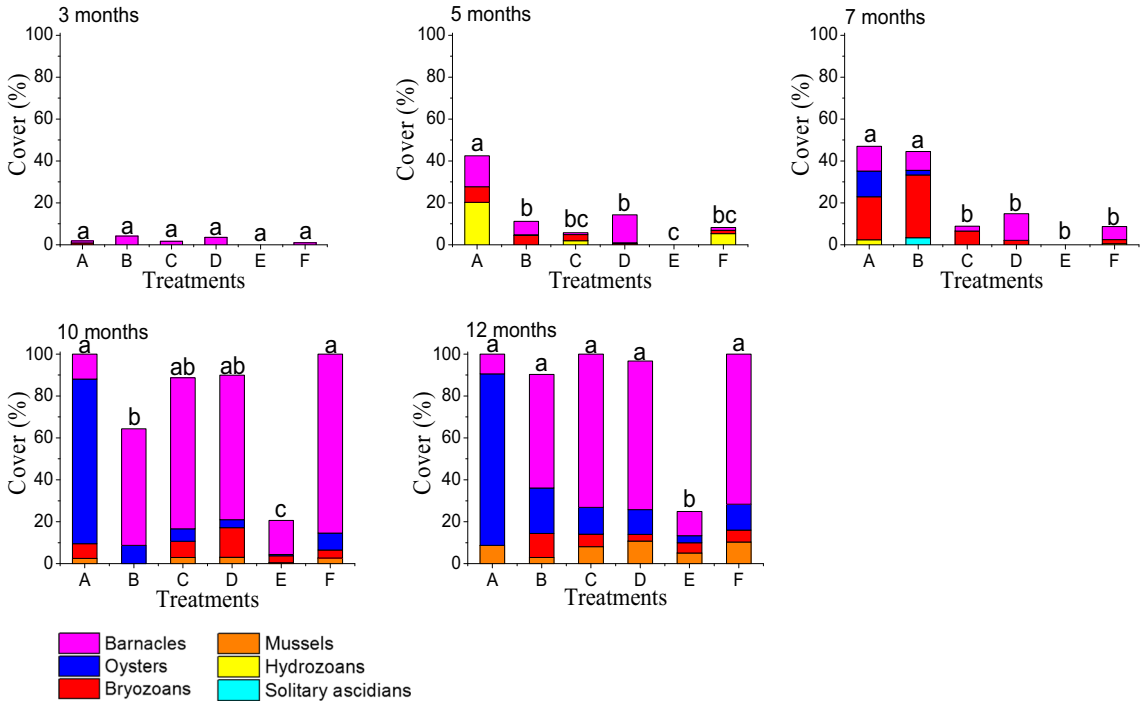

Fig. 3 Macrofouling percentage cover on the test panels in Fig. 2 after different immersion times. Details for treatments of A, B, C, D, E, and F are shown as in the caption of Fig. 2. Data shown are mean of six replicates. No data was shown for the immersion time of 1 month because no macrofouling organisms on all panels were observed at that time point. Different letters above columns indicate significant differences in macrofouling coverage among various panels ($P < 0.05$)

involving evodiamine and strychnine. Figures 4 and 5 present the findings of the field experiment involving CPT, cepharanthine, and sinomenine.

In the first field experiment (Figs. 2 and 3), no macrofoulers were observed on the panels after immersion for 1 month in the autumn. After 5 months in sea, significant differences in percentage cover were observed among treatments [$F\ (5, 30) = 37.579$, $P < 0.001$]. Paints containing evodiamine and strychnine showed significantly lower cover of macrofoulers than the control of matrix paint (fouled by barnacles, bryozoans, and hydrozoans), indicating their AF efficiency in the field. After 7 months, the paint containing evodiamine lost AF efficiency. In terms of the paint containing strychnine, it maintained its AF effect, with fouling cover (8.82%) still significantly lower than the negative control (47.04%). After 10 months, except for the positive control of cuprous oxide, the other panels all showed high percentage cover (64.38–100%), with oysters, barnacles, and bryozoans as main settlers. Strychnine treatment lost AF efficiency. After 12 months of immersion, fouling on treatments was similar to that at the 10-month submersion time point, with only the positive control of cuprous oxide retaining AF efficiency.

In the second field experiment (Figs. 4 and 5), after immersion for 1 month, significant differences in percentage cover were observed among treatments [$F\ (6, 35) = 12.241$, $P < 0.001$]. Both treatments of CPT and cepharanthine inhibited settlement of barnacles and bryozoans compared with the negative control (particularly for CPT treatment, wherein no macrofouling organisms were observed). In contrast, sinomenine treatment showed similar cover to that of the negative control. Microfouling was observed on the positive control of cuprous oxide (Fig. 4). After 3 months, the cover for the treatment of cepharanthine was > 50%, but the CPT treatment remained free from macrofouling, showing settlement inhibition of mainly mussels, oysters, barnacles, and bryozoans. At 5 months of submersion, the paint containing CPT still exhibited outstanding AF performance, with only 1.75% cover, which was significantly lower than all the other treatments including the three positive controls of capsaicin, cuprous oxide, and zinc pyrithione. At 7 to 12 months after submersion, the paint containing CPT maintained AF efficiency, whereas all the other tested panels exhibited severe fouling of mainly bryozoans, ascidians, and barnacles. During the field experiment, microfouling was observed on the paint containing CPT, especially after 3 months of submersion.

These results indicate that among the five tested compounds in the field, CPT showed superior AF performance. Further evaluation of this compound is warranted.

Field Experiment of Different CPT Concentrations

Here, six concentrations ranging from 0.1 to 20% *w*/*w* CPT in paints were tested in terms of in situ AF efficiency (Figs. 6 and 7). The inhibition of biofouler settlement using CPT in paints

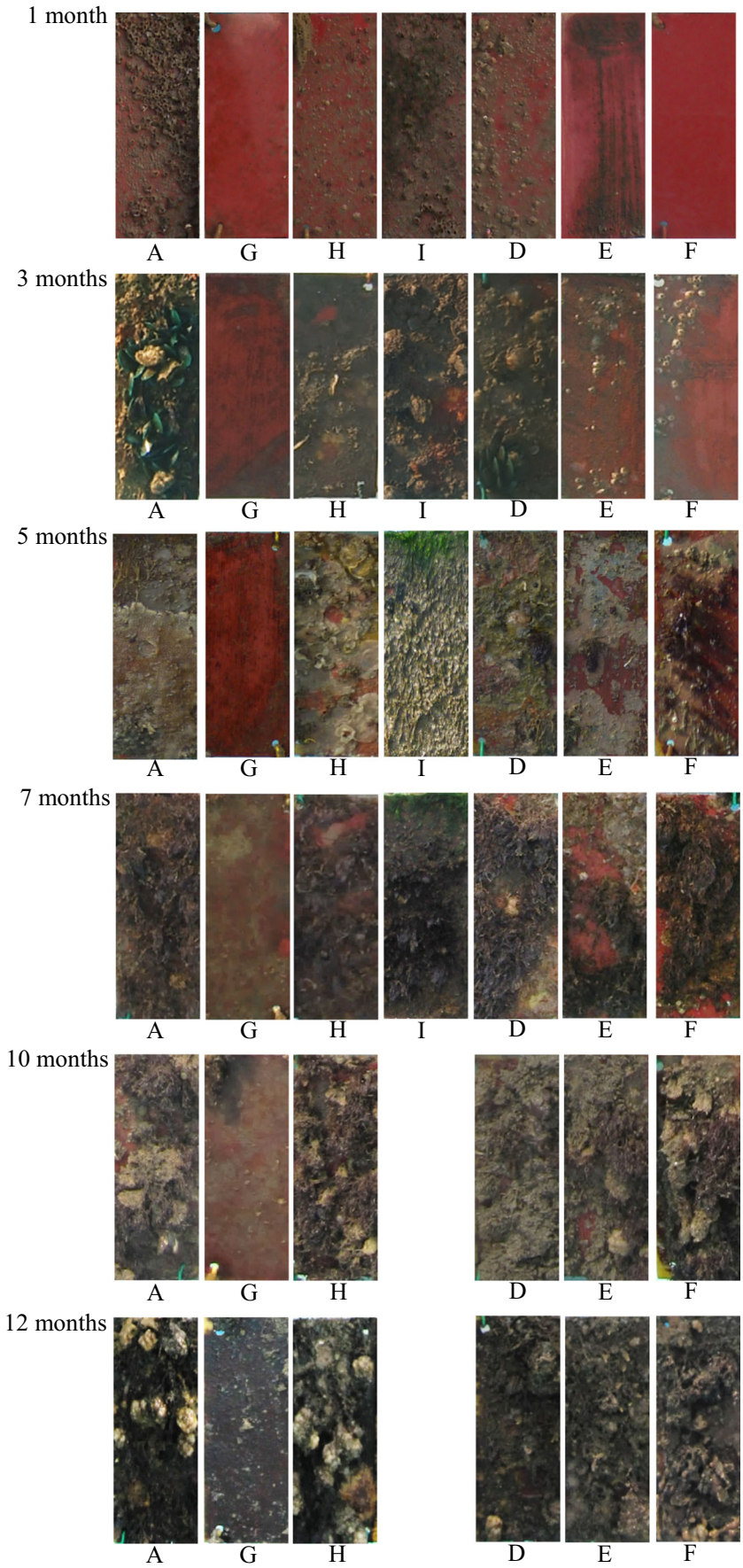

Fig. 4 Test panels after immersion in seawater near Dalipuyu Islet of Xiamen Bay at different time points. A, coated with the matrix paint only (negative control paint); G, coated with the paint containing camptothecin; H, coated with the paint containing cepharanthine; I, coated with the paint containing sinomenine; D, coated with the positive control paint containing capsaicin; E, coated with the positive control paint containing cuprous oxide; F, coated with the positive control paint containing zinc pyrithione. Each panel was randomly selected from six replicates of every treatment for every immersion time

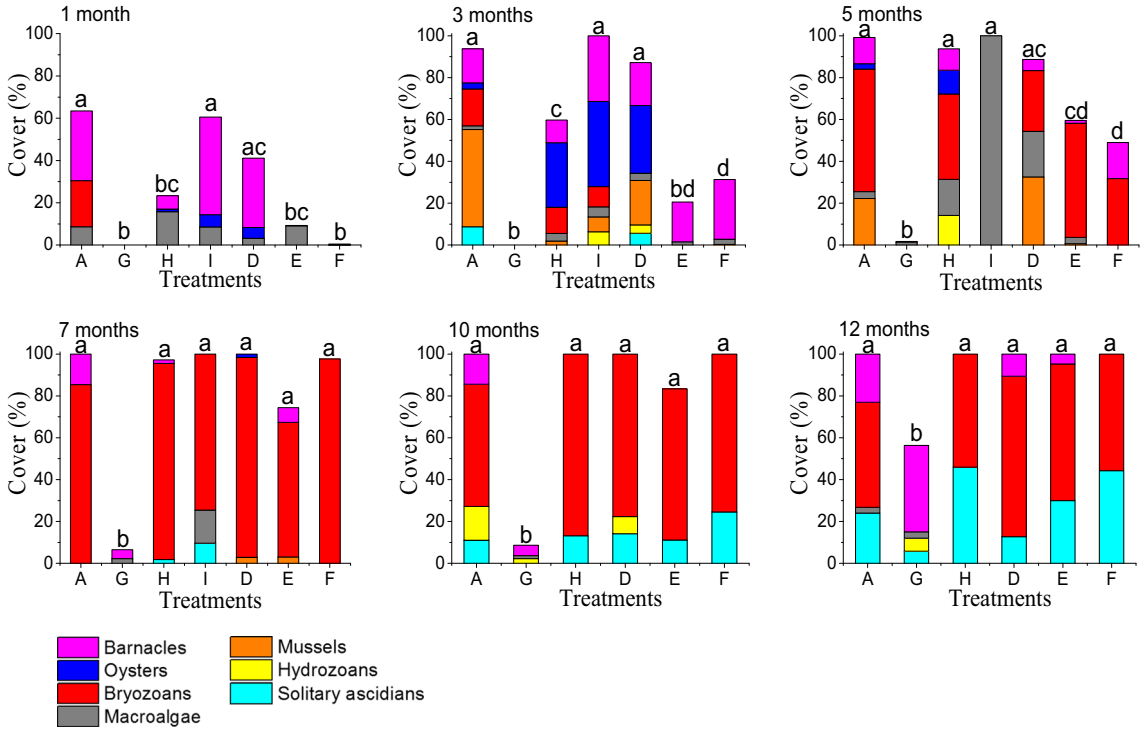

Fig. 5 Macrofouling percentage cover on the test panels in Fig. 4 after different immersion times. Details for treatments of A, G, H, I, D, E, and F are shown as in the caption of Fig. 4. Data shown are mean of six replicates. The panels with the treatment of I fell off and were lost to sea after 7 months of immersion. Different letters above columns indicate significant differences in macrofouling coverage among various panels ($P < 0.05$)

in the field was concentration-dependent. No significant AF effect was noted for the treatment of 0.1% CPT throughout the testing period. However, 0.5 and 1% CPT showed significantly lower cover than the negative control during the first 2 months of submersion. At 2 months, the treatments of 0.5 and 1% CPT had cover of 50.39 and 46.01%, respectively, whereas the negative control had cover of 76.09%. After 3 months of submersion, these two concentrations exhibited similar cover as the negative control, indicating loss of AF efficiency. The treatment of 5% CPT retained significantly lower cover than the negative control for 6 months ($P = 0.043$), suggesting better AF performance than 0.5 and 1% CPT. As the concentration increased to 10%, CPT showed higher AF efficiency, which was retained for 9 months after submersion. At 9 months, bryozoans appeared to be the main macrofoulers. The positive control, i.e., the commercial copper-based paint, lost its AF efficiency (with cover similar to the negative control) after 9 months of submersion, but 10% CPT still had significantly lower cover than the negative control ($P < 0.001$). Superior AF performance was observed with 20% CPT, which lasted for 11 months. During this experiment, the concentrations of 10 and 20% (especially 20%) showed better AF performance than the commercial copper-based paint. Microfouling was found on 10 and 20% CPT treatments as well as on the positive control, particularly after 3 months of submersion.

Toxicity of CPT Against Three Aquatic Organisms

The effect of exposure to CPT at different concentrations on survival of *A. salina* is shown in Fig. 8. The 24-h LC_{50} value of CPT against this crustacean species was 20.75 μM (Table 2). It was observed that CPT also inhibited the growth of two microalgal species (*P. tricornutum* and *I. galbana*) in a concentration-dependent manner (Fig. 9). The 96-h EC_{50} values of CPT to *P. tricornutum* and *I. galbana* were 55.81 and 6.29 μM, respectively (Table 2). As indicated by the comparison of 96 h EC_{50} values, *P. tricornutum* was less sensitive to CPT than *I. galbana*.

Discussion

Recent concern over the negative environmental impacts of AF paints based on Cu_2O and booster biocides has highlighted the importance of developing new, eco-friendly AF agents. In the present study, 18 alkaloids derived from plants were evaluated for AF activity using bioassays with two different fouling organisms. Four alkaloids were found to be active against the bryozoan *B. neritina*, whereas 15 alkaloids were active against the barnacle *B. albicostatus*, indicating the species-specific effects of the compounds. The EC_{50} values of these active compounds were all lower than 25 μg/mL, which is the standard requirement

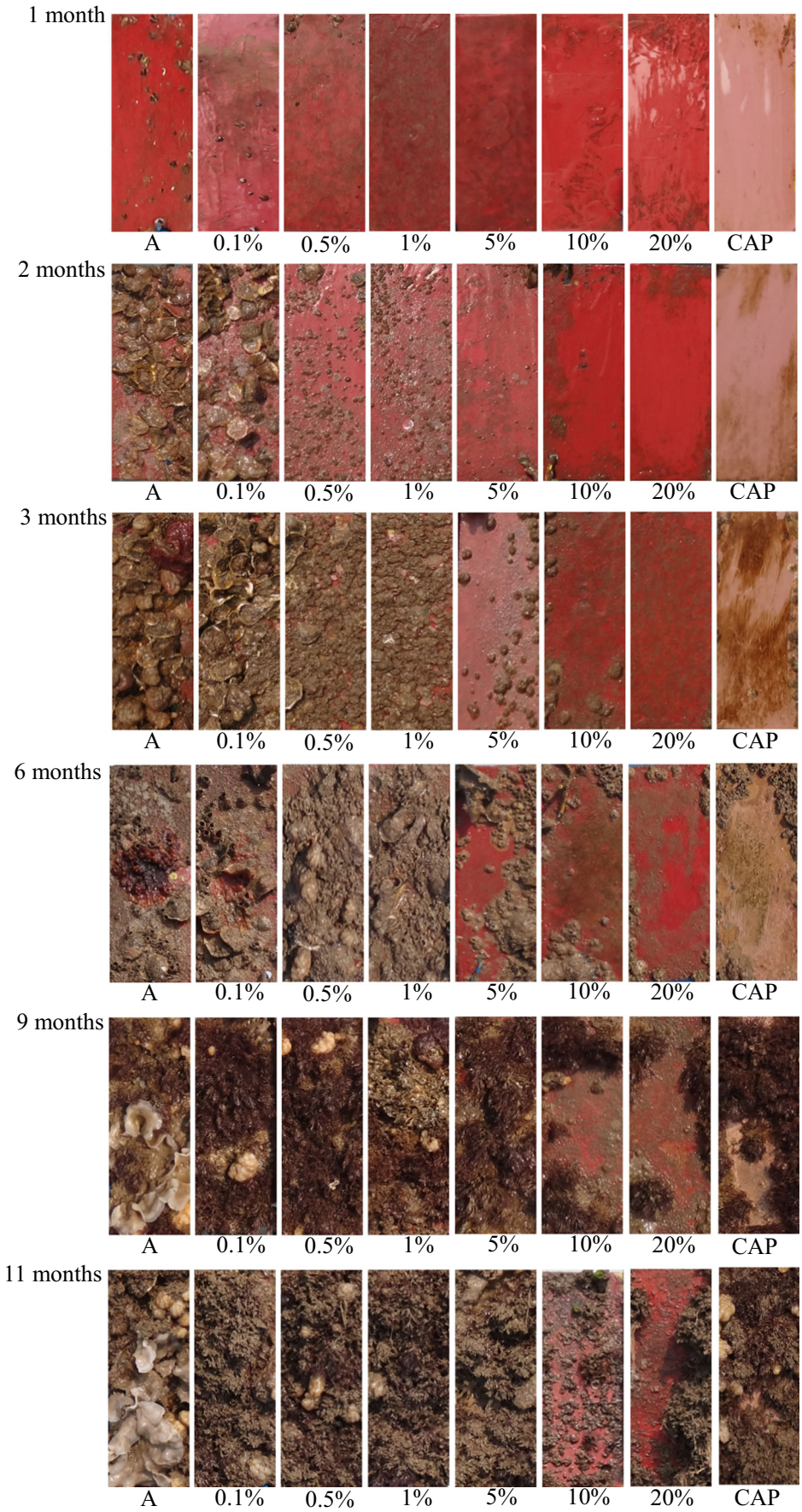

Fig. 6 Test panels after immersion in seawater near Dalipuyu Islet of Xiamen Bay at different time points. A, coated with the matrix paint only (negative control paint); 0.1%, coated with the paint containing 0.1% camptothecin (CPT); 0.5%, coated with the paint containing 0.5% CPT; 1%, coated with the paint containing 1% CPT; 5%, coated with the paint containing 5% CPT; 10%, coated with the paint containing 10% CPT; 20%, coated with the paint containing 20% CPT; CAP, coated with commercial antifouling paint (L40-32 (813-1) AF paint, Shanghai Kailin Paint Factory). Each panel was randomly selected from six replicates of every treatment for every immersion time

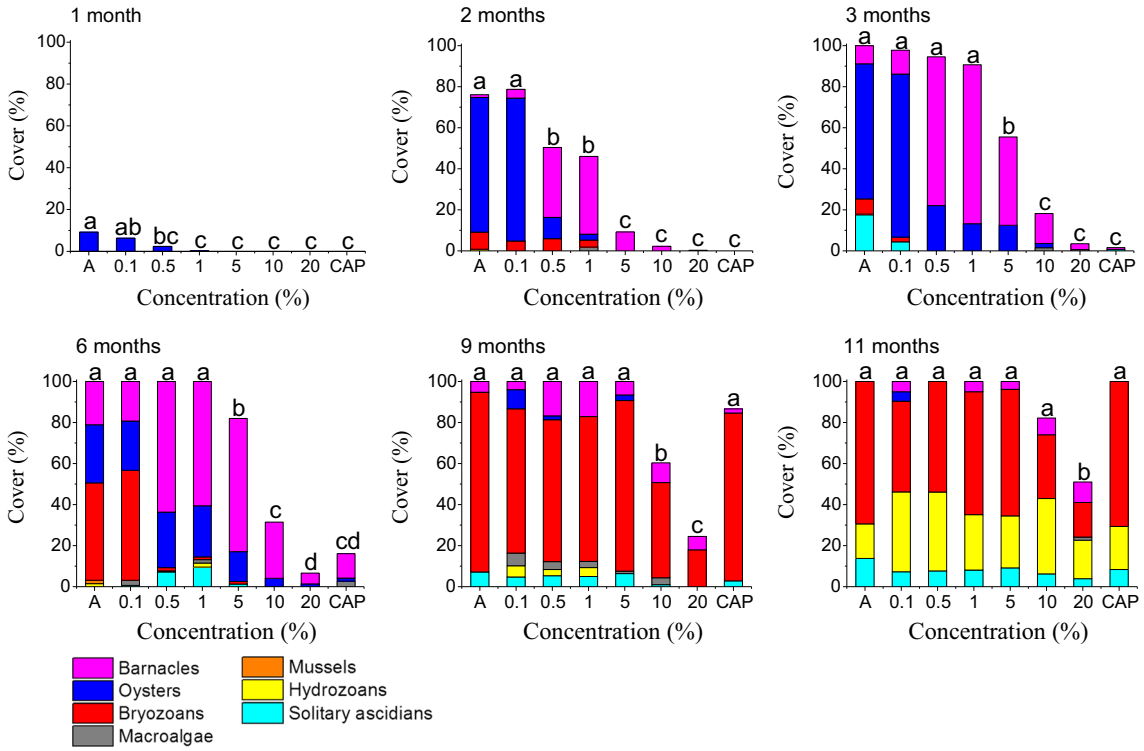

Fig. 7 Macrofouling percentage cover on the test panels in Fig. 6 after different immersion times. A, the matrix paint; CPT, camptothecin; CAP, commercial antifouling paint. Data shown are mean of six replicates. Different letters above columns indicate significant differences in macrofouling coverage among various panels ($P < 0.05$)

established by the US Navy program as a potency criterion for natural antifoulants (Rittschof 2001). The AF activities of these tested alkaloids were reported for the first time in this study, expanding the list of NPAs. Furthermore, the NPAs found in this study may be potentially utilized as structural scaffolds for future chemical synthesis or modifications in generating highly active antifoulants.

Although evodiamine and rutaecarpine have very similar structures (Fig. 1), their antifouling activities against the barnacle *B. albicostatus* are remarkably different (Table 1).

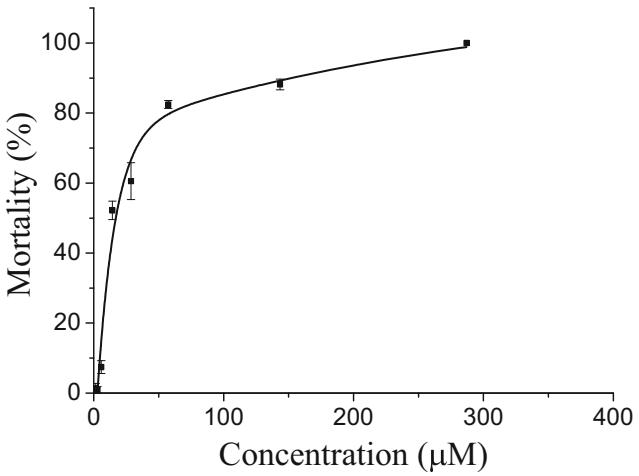

Fig. 8 Toxic effects of camptothecin on *Artemia salina* after exposure of 24 h. Data are expressed as the mean ± SE ($n = 6$)

Evodiamine inhibited settlement of *B. albicostatus* with EC_{50} of 3.49 μM, but rutaecarpine showed no inhibitive effect on *B. albicostatus* settlement at the tested concentrations of 0.25 to 50 μg mL^{-1} (0.87 to 174.03 μM). The higher activity of evodiamine than rutaecarpine could be due to the presence of a methyl group at N-14 position and that of a hydrogen at C-3 position in evodiamine, which are absent in rutaecarpine. Furthermore, as revealed by comparison of the EC_{50} values of scopolamine butylbromide and scopolamine methobromide, the antifouling activity against barnacle exhibited by scopolamine butylbromide is lower than that of scopolamine methobromide (in which the butyl group attached to the nitrogen atom in scopolamine butylbromide is substituted by the methyl group), suggesting that substituting the butyl group in scopolamine butylbromide by the methyl group enhances antifouling activity against barnacle. In addition, the structure of palmatine hydrochloride differs from that of berberine hydrochloride only in the substitution of a methylenedioxy group for two methoxy groups at C-2 and C-3 positions, but the EC_{50} of palmatine hydrochloride against barnacle is much higher than that of berberine hydrochloride, indicating that the replacement of the methylenedioxy group at C-2 and C-3 positions by two methoxy groups reduces the antifouling activity against barnacle. Some information about the structure-activity relationship could also be obtained by considering the similar structures of aconitine, hypaconitine, and mesaconitine. For aconitine and mesaconitine, their structures

Table 2 Comparison of toxicity of camptothecin and commonly used antifouling agents to three aquatic organisms

Species	Endpoint	Compound	LC_{50} or EC_{50} (μM)	Reference
Artemia saline	24 h LC_{50}	Camptothecin	20.75	This study
		Tributyltin[a]	1.27	Panagoula et al. (2002)
		Irgarol[a]	6.39	Panagoula et al. (2002)
		Copper pyrithione[a]	2.63	Koutsaftis and Aoyama (2007)
		Zinc pyrithione[a]	9.98	Koutsaftis and Aoyama (2007)
		Diuron[a]	51.52	Koutsaftis and Aoyama (2007)
		Chlorothalonil[a]	3.76	Koutsaftis and Aoyama (2007)
Phaeodactylum tricornutum	96 h EC_{50}	Camptothecin	55.81	This study
		Diuron[a]	0.043	Mayer (1987)
Isochrysis galbana	96 h EC_{50}	Camptothecin	6.29	This study
		Diuron[a]	0.043	Mayer (1987)
		Copper[a]	0.005	Suratno et al. (2015)

[a] LC_{50} or EC_{50} values expressed in miligram per liter, microgram per liter, or nanogram per liter in the references were transformed in micromolar here

differ only in the substitution of an ethyl group (in aconitine) for a methyl group (in mesaconitine) attached to the nitrogen atom, which does not influence antifouling activity against barnacle remarkably since there is only slight difference in EC_{50} values of these two compounds against barnacle. For hypaconitine and mesaconitine, the only structural difference between them is the presence of the hydroxyl group at C-3 position in mesaconitine. As revealed by the higher EC_{50} of mesaconitine than hypaconitine in the barnacle bioassay, the hydroxyl group at C-3 position in mesaconitine reduces its antifouling activity against barnacle.

Field verification of AF activity of NPAs is essential in the development of novel antifoulants (Qian et al. 2015; Wang et al. 2015). Although numerous NPAs have been evaluated by laboratory bioassays, only a few have been subjected to field tests (Qian et al. 2015). Here, five alkaloids were tested in the field trial. Except for sinomenine, the other tested compounds (evodiamine, strychnine, CPT, and cepharanthine) exhibited variable AF efficiencies in the natural environment, which is indicative of their potential use in marine AF paints. In the two field experiments at different locations and seasons during panel immersion, fouling pressure markedly differed, which was indicated by fouling on the controls. In the first field experiment (Fig. 2), no distinct signs of fouling were observed on the control matrix paint until after 5 months of exposure at sea, whereas in the second field experiment (Fig. 4), macrofouling cover of the control matrix paint reached 63.4% within 1 month of immersion. CPT displayed the highest AF activity relative to zinc pyrithione and cuprous oxide, which are two commonly used commercial antifoulants, thereby suggesting that CPT may be potentially used as a new antifoulant. The AF efficiency of CPT was further proven by the field experiment of different CPT concentrations, which demonstrated AF potency at low concentrations. Combining usage of water-soluble binder (rosin) and other polymeric ingredients (such as polyacrylic resin) is common in preparing AF paints (Almeida et al. 2007; Yebra et al. 2004), which we used here to prepare the paints. However, there is possibility that the paint formula in the present work may be not very good for the effective release of CPT. Therefore, AF performance of low-concentration CPT may be further improved by modifying paint formulations such as using more suitable polymer(s) or microencapsulation of the active compound to better control its leaching rate (Kristensen et al. 2010; Ma et al. 2017; Price et al. 1992). Furthermore, as shown

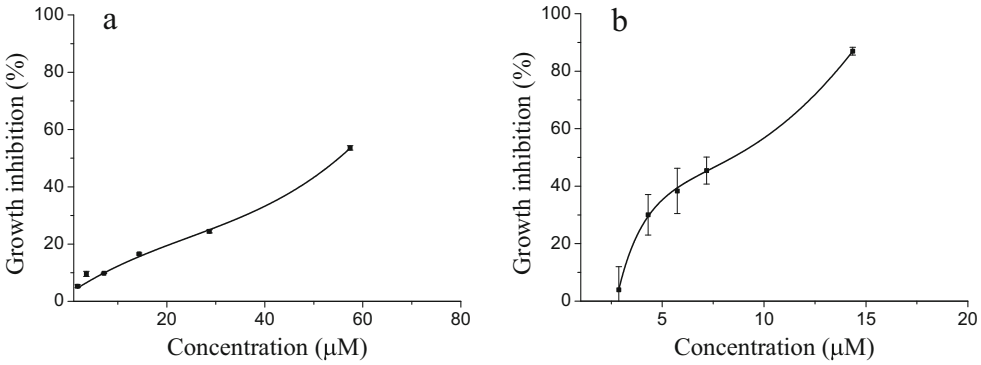

Fig. 9 Growth inhibition of *Phaeodactylum tricornutum* (**a**) and *Isochrysis galbana* (**b**) after 96 h exposure to camptothecin. Data are expressed as the mean ± SE ($n = 3$)

by the field results, CPT did not show outstanding efficiency against microfouling. Mixture with substance(s) against microfouling may also further improve the AF efficiency of paints containing CPT.

CPT was first isolated by Wall et al. in 1966 from the stem wood of the tree *Camptotheca acuminata*. It has also been found in other plants, including *Nothapodytes nimmoniana* (Govindachari and Viswanathan 1972), *Eravatamia heyneana* (Gunasekera et al. 1979), *Merrilliodendron megacarpum* (Arisawa et al. 1981), *Mostuea brunonis* (Dai et al. 1999), *Pyrenacantha klaineana* (Zhou et al. 2000), *P. volubilis* (Suma et al. 2014), *Chonemorpha fragrance* (Kulkarni et al. 2010), *Dysoxylum binectariferum* (Jain et al. 2014), and some species of the genus *Ophiorrhiza* (Aimi et al. 1990; Sriram et al. 2005). CPT has been reported to possess antitumor (Ulukan and Swaan 2002; Wall et al. 1966), anti-HIV (Priel et al. 1991), and antiprotozoal activities (Bodley et al. 1998). Its antitumor mechanism of action has been suggested to involve the inhibition of topoisomerase I (topo I), a nuclear enzyme that reduces the torsional stress of supercoiled DNA (Garcia-Carbonero and Supko 2002). CPT binds to the DNA-topo I cleavage complex and stabilizes it, which inhibits DNA relegation, thereby leading to apoptosis (Ulukan and Swaan 2002; Garcia-Carbonero and Supko 2002; Svejstrup et al. 1991). The mechanism of pharmaceutical action of CPT may provide useful information in understanding its AF mechanism against biofoulers. Furthermore, due to poor solubility and adverse effects, CPT is prohibited for clinical use, but many CPT analogues have been developed based on the findings from structure-activity relationship (SAR) studies (Ulukan and Swaan 2002). Two water-soluble analogues, topotecan and irinotecan, have already approved by the Food and Drug Administration (FDA) in the USA and are used as anti-cancer drugs (Sriram et al. 2005). Various synthesized CPT analogues and their SAR studies may facilitate studies on the structural features that are responsible for their AF activity, and the development of analogues with higher AF potency.

Here, the toxicity of CPT to the crustacean *A. salina* and the microalgae *P. tricornutum* and *I. galbana* was tested. The 24-h LC_{50} value of CPT against *A. salina* was compared to those of six commercial antifoulants (Table 2), all obtained using the standard Artoxkit M procedure for *Artemia* bioassay (Artoxkit 1990). It was indicated that CPT is less toxic to *A. salina* than tributyltin, irgarol, copper pyrithione, zinc pyrithione, and chlorothalonil. Furthermore, as shown in Table 2, CPT is much less toxic to *P. tricornutum* than the commercial antifoulant diuron. With regard to the toxicity to *I. galbana*, CPT also showed obviously less toxic than diuron and copper.

The future application of CPT in AF practice may result in an increase in its demand. CPT production currently relies on its extraction from source plants (Isah and Mujib 2015). The total chemical synthesis of CPT has been successfully achieved (Corey et al. 1975), although currently this approach is not economically feasible compared to extraction from plants. Different synthetic strategies for this compound have been explored (e.g., Ciufolini and Roschangar 1997; Li et al. 2016), and it may be possible to find an economically practical synthetic route in the future. Furthermore, a few endophytic fungi isolated from CPT-yielding plant species have been determined to also produce CPT (Amna et al. 2006; Bhalkar et al. 2016), which provides a promising alternative source of CPT since microbes can be cultured on a large scale by fermentation (Aqueveque et al. 2017; Kebede et al. 2017). Plant biotechnology such as plant tissue culture techniques and cultivation techniques of source plants have also been explored to improve CPT yield (Isah and Mujib 2015; Zeng et al. 2009). CPT production at a larger scale may possibly contribute to lower price for this compound. In China, CPT is sold as a phytochemical at a price of approximately USD 4500 per kg (Tong and Feng 2016), which makes it difficult to be widely used in antifouling paints, especially as compared with cuprous oxide which is sold at about USD 4800–8000 per ton (price of cuprous oxide provided by Hongze Century Metal Products Co., Ltd., Jiangsu, China).

In conclusion, 15 alkaloids were found to be AF active by laboratory bioassays. Furthermore, four alkaloids displayed AF efficiency in the sea, with CPT showing the highest potency. Further field trials of different CPT concentrations in paints indicated the concentration-dependent in situ AF activity of this compound, and that lowest concentration for CPT to significantly inhibit settlement of biofoulers in the field was 0.5% (*w*/*w*). Comparison of toxicity to three aquatic organisms revealed that CPT was less toxic than several commonly used commercial antifoulants. It is suggested here that CPT can be considered as a promising AF candidate. Future work on increasing its yield and lowering its price is needed for its application in AF practice.

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Compliance with Ethical Standards

Conflict of Interest On behalf of all authors, the corresponding author states that there is no conflict of interest.

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