Comparative effects of indole derivatives as antifouling agents on the growth of two marine diatom species

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Comparative effects of indole derivatives as antifouling agents on the growth of two marine diatom species

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Antifouling agents, used to prevent biofouling, need to be assessed for their impacts on marine organisms and environment before the application. Diatoms are one of the main components of fouling biofilms, which play important roles in the formation of biofouling. Particularly, diatoms are also the important ingredients of primary production and present interest as ecotoxicological models in marine environment. In this study, two benthic diatoms \textit{Nitzschia closterium} \textit{f. minutissima} and \textit{Navicula climacospheniae}, widely distributed in fouling biofilm, were used as models for screening the activities of potential antifoulants. Nine indole derivatives were tested and CuSO\textsubscript{4} was used as a reference. Indole derivatives showed significant anti-algal activities and the EC\textsubscript{50} values of most indole derivatives were lower than that of CuSO\textsubscript{4}. Halogen substituent enhanced the anti-algal activities of compounds, and the most efficient compounds for \textit{N. closterium f. minutissima} were gramine and 7-chloroindole with the EC\textsubscript{50} values of 1.94 and 2.1 mg/L, while for \textit{N. climacospheniae}, 7-chloroindole and 6-bromoindole were the most efficient and the EC\textsubscript{50} values were 3.91 and 4.25 mg/L, respectively. In conclusion, indole derivatives would be one of the promising candidates as antifoulants and our results strengthened the need to perform antifouling activity assays and environment-friendly evaluations.

Keywords: indole derivatives; antifouling; diatom; EC\textsubscript{50}

Introduction

Biofouling results in serious problems for marine-related operations, such as aquaculture equipments, naval vessels and a diversity of industrial structures.[1,2] Antifouling agent is a kind of effective compound for inhibiting the attachment and growth of fouling organisms, some of which, for example, copper, lead and tributyltin (TBT), are not degraded in natural environment and make biological distortion.[3] Therefore, a total ban on the production of TBT-based coatings was implemented in January 2003.[4] Alternative biocide-based antifouling agents (Irgarol 1051, Sea-Nine 211, dichlofluanid, and zinc pyrithione) have been also found to be accumulated in waters and might induce toxic effects to marine organisms.[5,6] Therefore, searching for efficient, environmental friendly antifouling agent is widely focused on as a new field of antifouling technology.[7,8]
Based on the chemical defence of marine organisms that protect their body surfaces from biofouling, natural products and their analogues are preferred as potential antifouling agents, because these compounds show excellent inhibitory activities against microbes, algae and larvae of invertebrates as well as the fact that they are less toxic or non-toxic to the environment.[8,9] Natural products’ antifoulants consisted mainly of five kinds of compounds, such as terpenes, nitrogen-containing compounds, phenols, steroids and others.[10] Most of them were isolated from sponges, corals, mussels, seaweeds, microbes and terrestrial plants.[1,11–13] Indole derivatives belong to a class of nitrogen-containing compounds, such as indole derivatives 6-bromoindole-3-carbaldehyde and 2, 5, 6-tribromo-1-methyl-gramine (TBG), which were first isolated from marine organisms and showed excellent antifouling activities against barnacles and mussels.[14,15] Kon-Ya et al. [14] reported that the antifouling activity of TBG was higher than that of CuSO4 and its inhibitory activity was six times as strong as that of tributyltin oxide. Subsequently, more than 100 kinds of indole derivatives were synthesised by chemical methods, and some compounds showed high antifouling activities.[16] Indole derivatives as new natural antifouling agents have drawn wide attention because of their simple structures and good antifouling activities.

To develop new and highly effective antifouling agents applied in marine environment, we should pay attention to at least two aspects. One is their activities against fouling organisms, and the other is their toxicity against non-target organisms.[8] Diatom as a kind of main fouling organism and bacteria constitute biofilm, which is very important for the adhesion and growth of the following invertebrate larvae and macro-algal spores.[17–19] Screening the anti-algal activities of the substances would help us to find new and effective antifouling agents. Furthermore, diatoms are also important components of marine primary production and detrimental effects in these organisms may affect the entire food chain in marine ecosystem.[20] Study on the toxicity of substances to the algae would help us to better understand how the compounds affect non-target organisms, disrupt the food chain and cause imbalance in the entire ecosystem.[21,22] Therefore, the activities of indole derivatives against marine diatoms were evaluated in this study. Two marine benthic diatoms (Nitzschia closterium f. minutissima and Navicula climacospheniae) were used as models because of their wide distribution in fouling biofilm.[23,24] Moreover, the evaluation would help us to know the antifouling activities of indole derivatives and the relationships between the structure and activity. On the other hand, the evaluation would contribute to better understanding the toxic effects of indole derivatives on marine diatoms.

Materials and methods

Test organisms and culture conditions

The marine diatom N. closterium f. minutissima was provided by Ocean University of China and N. climacospheniae was purchased from State Key Laboratory of Marine Environmental Science, Xia Men University. The two diatoms were cultured with f/2 medium in light incubator, at light intensity of 48 µmol photons / (m2s) and 20 with a 12:12 h light: dark cycle. The diatoms were cultured to the exponential phase before inoculation in the following experiments.[25]

Detergent used

Indole, 5-chloroindole, 6-chloroindole, 7-chloroindole, 5-bromoindole, 6-bromoindole, 7-bromoindole, 6-chlorooxindole and gramine were purchased from Alfa Aesar Company,
Tianjing. The structures of indole derivatives used are shown in the online Supplementary material (Table 1S). The commercial biocidal agent CuSO$_4$ was tested as a ref. [10]. All other chemicals were of analytical or higher grades.

**Effects of indole derivatives on the growth of diatoms**

The inhibitory effects of indole derivatives on the growth of *N. closterium* f. *minutissima* and *N. climacospheniae* were determined in 96-well microplates, each well containing 200 µL of f/2 medium inoculated with algal cells at $2 \times 10^6$ cells/mL and the cells were exposed to a range of final concentrations of indole derivatives (0, 1, 2, 5, 10, 20, 30 and 50 mg/L). The optical density of 680 nm was measured by using muti-well plate reader (Infinite M2000, Tecan) to represent algal growth for 3 days. All the tests were performed in six replicates. The growth rate ($\mu$) was calculated according to the equation as follows:

$$\mu = \frac{\ln N_2 - \ln N_1}{t_2 - t_1},$$

where $N_1$ and $N_2$ are the optical densities of the beginning and the end of certain growth phase; $t_2 - t_1$ is the time interval in days. The growth rate of control group is expressed by 100%, and the treatment groups are expressed by relative growth rate (%). This was used to determine EC$_{50}$ of indole derivatives on diatoms.[26]

**Data analysis**

Data were analysed by Microsoft Word Excel to calculate the means and standard deviation. According to the per cent growth rate and the logarithm of tested concentrations, the EC$_{50}$ values which represent the inhibitory effects of indole derivatives on the growth of algal cells were calculated by using a logistic/sigmoid model (SigmaPlot 10.0).[27]

**Results**

**Effects of indole derivatives on the growth of *N. closterium* f. *minutissima***

Figure 1 shows the inhibitory effects of indole derivatives and CuSO$_4$ on the growth of *N. closterium* f. *minutissima*. Among these compounds, only the EC$_{50}$ values of indole and 7-bromoindole were higher than that of CuSO$_4$ (5.06 mg/L), and the values were 10.51 and 6.03 mg/L, respectively. The anti-algal activities of all other indole derivatives against *N. closterium* f. *minutissima* were better than that of CuSO$_4$. The EC$_{50}$ value of gramine was the lowest (1.94 mg/L), followed by that of 7-chloroindole (2.1 mg/L) and 6-chloroindole (2.49 mg/L). The EC$_{50}$ values of these compounds against *N. closterium* f. *minutissima* were orderly indole > 7-bromoindole > CuSO$_4$ > 5-chloroindole > 5-bromoindole > 6-bromoindole > 6-chloroindole > 6-chloroindole > 7-chloroindole > gramine.

**Effects of indole derivatives on the growth of *N. climacospheniae***

The Effects of indole derivatives and CuSO$_4$ on the growth of *N. climacospheniae* are shown in Figure 2. The EC$_{50}$ values of 6-chloroindole, 7-chloroindole, 5-bromoindole, 6-bromoindole and gramine against *N. climacospheniae* were 4.44, 3.91, 4.49, 4.25 and 4.86 mg/L, respectively, which were lower than that of CuSO$_4$ (5.29 mg/L). Among these EC$_{50}$ values, the value of 7-chloroindole was the lowest, followed by 6-bromoindole and 6-chloroindole. The EC$_{50}$ values of these compounds against *N. climacospheniae* were orderly 7-bromoindole > 6-chloroindole > indole > 5-chloroindole > CuSO$_4$ > gramine > 5-bromoindole > 6-chloroindole > 6-bromoindole > 7-chloroindole.

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Figure 1. Effects of indole derivatives and CuSO₄ on the growth of *N. closterium f. minutissima*. All error bars indicate SD of the six replicates. The EC50 values are shown in the corresponding figures.
Figure 2. Effects of indole derivatives and CuSO₄ on the growth of *N. climacospheniae*. All error bars indicate SD of the six replicates. The EC50 values are shown in the corresponding figures.
Discussion

The discovery of effective and environment-friendly antifouling agents from natural sources (plants, animals and microorganisms) has been a hot topic and the ultimate goal of many researchers over recent decades.[11] Many compounds were isolated and reported for their high activities against fouling organisms. Marine microorganism is also a kind of important source of screening active substances. 1-hydroxymyristic acid and 9-Z-oleic acid which were isolated from the marine bacterium *Shewanella oneidensis* totally inhibited the germination of spores of *Ulva pertusa* at 10 mg/L exposure. More importantly, antifouling coatings containing 10% 9-Z-oleic acid were able to reduce the attachment of micro- and macro-foulers for about 1.5 years.[28,29] Alkylated butenolides were also a class of metabolites from marine bacteria *Streptomyces* sp., which showed potent antifouling activity against the larvae of fouling organisms with very weak toxicity. The study of structure–activity relationships revealed that the 2-furanone ring was essential for the activity and lipophilicity obviously influenced their antifouling activities.[30] In addition, there were many active substances isolated from seaweeds and aquatic plants. For example, meroditerpenoids isolated from the brown alga *Halidrys siliquosa* significantly inhibited the settlement of cyprids of *Amphibalanus (=Balanus) amphitrite* (B. amphitrite) with EC_{50} values from 1 to 5 mg/L.[31] Floridoside isolated from the red alga *Grateloupia turuturu* showed anti-barnacle activity with EC_{50} value of below 1 mg/L.[32] Particularly, many active compounds were isolated from marine invertebrates. Bromotyrosine derivatives, polybrominated diphenyl ethers, subergoric acid and so on were all isolated from sponges, which were promising sources of antifoulants.[33–35] Li et al. [36] reported that 49 secondary metabolites, including diterpenoids, steroids and polyketides, were isolated from soft corals, gorgonians, brown algae and fungi. Twenty of the compounds were found to inhibit significantly the settlement of barnacle larvae at a concentration of 25 mg/L. Two briarane diterpenoids with EC_{50} values less than 0.13 mg/L were the most promising anti-larval settlement candidates.

Natural indole antifoulants mainly included indole-3-carbaldehyde, 6-bromoindole-3-carbaldehyde isolated from the ascidian *Stomozoa murrayi* and epiphytic bacteria, and TBG isolated from marine bryozoan *Zoobotryon pellucidum*. All of them exhibited good antifouling activities against barnacles and mussels. The EC_{50} values of 6-bromoindole-3-carbaldehyde and TBG on the barnacle larvae were only 5 and 1 mg/L, respectively.[14,15] Because the contents of these natural indole derivatives are very low in marine organisms or marine environment, chemical synthesis for these derivatives becomes a key method for their application. Kon-ya et al. [16] reported that 93 kinds of indole derivatives could be chemically synthesised by conventional methods and these compounds prevented the attachment of the larvae of *B. amphitrite* at different levels. One hundred and fifty-five kinds of gramine compounds were also synthesised and reported. Of these compounds, 5, 6-dichlorogramine, 5-chloro-2-methygramine and 5, 6-dichrolo-1-methygramine (DCMG) showed high antifouling activities for inhibiting the attachment of barnacle larvae. Additionally, DCMG was selected as the antifouling paint ingredient and a silicone-based antifouling paint containing 5–10% of DCMG made the surfaces remain almost barnacle-free for 1.5 years by testing in the field.[37] These results indicated that indole derivatives could be candidates as efficient antifouling agents and deserved to be further studied.

In this study, we tested the anti-algal activities of nine compounds with simple indole ring and halogenated substituent against two diatom species *N. closterium* f. *minutissima* and *N. clima-cospheniae* for exploring their activities and structure–activity relationships. Our results showed that indole derivatives significantly inhibited the growth of two diatoms. With the increase of exposure concentrations, the growth rates of diatoms decreased. Qian et al. [8] suggested that only small molecules with EC_{50} < 5 mg/L against both micro- and macro-foulers should be considered as antifouling agents. In this study, all the EC_{50} values of indole derivatives were...
below 5 mg/L except indole, 7-bromoindole against *N. closterium f. minutissima*. While for *N. climacospheniae*, it showed different inhibitory effects, and the EC$_{50}$ values of indole, 5-chloroindole, 6-chlorooxindole and 7-bromoindole were all higher than 5 mg/L. Both the EC$_{50}$ values of copper sulphate against two diatoms were slightly higher than 5 mg/L. Based on our results, it was found that the anti-algal activities of indole derivatives varied with structural differences. Halogenated substituents strengthened the anti-algal activities which depended on the position of halogen in the indole ring. Most of halogenated indole derivatives showed lower values of EC$_{50}$ than that of indole. For two diatoms, the activity of indole compounds of chlorine substituent in position 7 was better than that in positions 6 and 5, while the activity of bromine substituent in position 6 is better than that in positions 5 and 7. The activity of compounds that halogenated substituents (chlorine or bromine) in the same position against *N. closterium f. minutissima* was superior to that against *N. climacospheniae*. Similarly, gramine and 6-chlorooxindole showed higher activities on the growth of *N. closterium f. minutissima* than that of *N. climacospheniae*. Therefore, diatom species also determined the sensitivity of various indole derivatives. Overall, all these results showed that halogenated indole derivatives had efficient anti-algal activities and they could be developed as potent antifouling agents. Kawamata et al. [37] reported that gramine derivatives substituted with an alkyl, an alkoxy, a halogen, a carboxyl, a carbonyl, or an acetoxyl moiety in indole skeleton, did not enhance the anti-settlement activity to cypris larvae of barnacle, except for 2,3-dimetylindole. However, in our study, simple indole derivatives with a halogen substituent strengthened the anti-algal activities compared with that of indole, except the activity of 7-bromoindole on *N. climacospheniae*. From these results, it was found that the aminomethyl at the position 3 of gramine could influence the activity of halogenated substituent in indole ring. Isabelle et al. [38] also reported that bromination increased the cytotoxicity of the indole-3-carbaldehyde derivatives against sea urchin eggs, which indicated that halogen substituents might be useful for improving the activities of compounds as antifouling agents.

Many natural products had been isolated and found to have excellent antifouling activities, and by chemical synthesis, many active substances were produced for further using in marine antifouling.[6,9] However, the poor knowledge of action mode or mechanism studies of antifouling compounds against micro- and macro-foulers might lead to their short lifespans because of technology or environmental safety.[8] Indole derivatives, originated from marine animals, were found to have good activities as promising antifouling agents. In our study, all simple halogenated indole derivatives showed efficient anti-algal activities. We had also studied the action mechanism of 6-chloroindole on the diatom *Cylindrotheca* sp. and found that it induced algal cellular Ca$^{2+}$ efflux.[39] It was reported that the action mode of indole derivatives on the organisms was closely related to calcium ion. Indole derivatives 5, 6-dibromo-1, 2-dimethylgramine (DBG) evoked Ca$^{2+}$ release from skeletal muscle *Sarcoplasmic reticulum* through ryanodine receptors, which indicated that gramine derivatives were useful tools for the investigation of Ca$^{2+}$ release from *S. reticulum*.[40] Iwata et al. [41] also reported that TBG and DBG decreased cytosolic Ca$^{2+}$ level by inhibiting the transient increase in isolated rat aorta. These indole derivatives reduced intracellular Ca$^{2+}$ abundance in animal cells, which might contribute to the inhibitory effects on the growth and attachment of biofouling organisms as antifouling agents. In addition, indole derivatives 2, 5, 6-tribromoindole-3-carbaldehyde significantly inhibited DNA replication and protein synthesis during the first mitotic cycle of fertilised sea urchin eggs (*Stomoza murrayi*).[38] These might be the preliminary mechanism studies about indole derivatives against fouling organisms and many things remained unknown. Therefore, the antifouling activities of indole derivatives against various fouling organisms, the antifouling mechanisms and the environmental safety are worthy of being further studied for the commercial application in the marine environment.
In conclusion, the present study showed the antifouling activities of halogenated indole derivatives on two diatom species, which played important roles in biofouling formation and primary production in marine environment. Halogenated substituents enhanced the antifouling activities of indole derivatives and the structure–activity relationships revealed that the substituted position of halogen in indole ring significantly influenced the antifouling activities. At the same time, we clearly confirmed that diatom species displayed different sensitivities to the diverse indole derivatives. Therefore, the evaluation of the efficiency of compounds should be performed on different diatoms or other organisms presenting various antifouling activity profiles. In addition, the study extended our knowledge of the toxicological effects of indole derivatives on the growth of diatoms because these chemical compounds might result in environmental risk for primary producers in marine ecosystems.

Disclosure statement

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Supplementary material

Supplemental data for this article can be accessed at 10.1080/02757540.2015.1022536/description of location.

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