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A new diatom growth inhibition assay using the XTT colorimetric method

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ABSTRACT

Marine biofouling, which leads to significant operational stress and economic damage on marine infrastructures, is a major problem in marine related industries. Currently, the most common way to avoid marine biofouling involves the use of biocidal products in surface coatings. However, the need for environmentally friendly antibiofouling compounds has increased rapidly with the recent global prohibition of harmful antifoulants, such as tributyltin (TBT). In particular, periphytic diatoms have been shown to contribute significantly to biofilms, which play an important role in biofouling. Therefore, inhibiting the proliferation of fouling diatoms is a very important step in the prevention of marine biofouling. In this study, we developed a new, rapid, accurate, and convenient growth inhibition assay using the XTT colorimetric method to prevent the growth of the fouling periphytic diatom, *Nitzschia amabilis* Hidek. Suzuki (replaced synonym, *Nitzschia laevis* Hustedt). The feasibility of this method was verified by determining the growth inhibition activities of two standard photosynthetic inhibitors, DCMU and $CuSO_4$. However, neither inhibitor had any cytotoxic activities at the range of concentrations tested. Moreover, this method was applied by screening and purification of herbicidic but non-cytotoxic compounds from cyanobacteria extracts. Our results demonstrate the utility of this newly established growth inhibition assay for the identification of marine anti-biofouling compounds.

1. Introduction

Marine biological fouling, also referred to as "marine biofouling", is a major problem on marine infrastructures including ship hulls, fishing gear, and cooling systems for thermal power plants (Matsunaga et al., 1998; Yebra et al., 2004; Rosenhahn et al., 2008, 2010). In particular, the issues caused by the biofouling in the shipping industry are well known (Townsin, 2003). The rough texture generated by marine biofouling on ship bottoms leads to frictional resistance, increasing the ship weight, reducing navigation speed and increasing the economic cost of shipping due to subsequently higher fuel consumption (Rascio, 2000; Townsin, 2003; Yebra et al., 2004). In the case of fishing gear, changing and cleaning nets incur a major cost to the industry, and also risk damage or loss of material (Hodson et al., 1997; Fitridge et al., 2012). Moreover, biofouling increases the net weight, which encumbers the cage structure and also causes detrimental effects on fish health by leading to a low flow-through of water and reduced dissolved oxygen availability, which may inhibit fish growth rates (Hodson et al., 2000; Swift et al., 2006; Fitridge et al., 2012). In addition, since thermal power plants are usually located in coastal areas in order to conveniently obtain water for cooling systems, biofouling caused by seawater is a universal problem (Rajagopal et al., 1991; Nair, 1999). Biofouling in cooling water circuits leads to a reduction in water flow, which dramatically inhibits the cooling effect. Furthermore, the submergence of cooling pumps can occur due to the mass accumulation of mussels and barnacles (Nair, 1999).

Biofouling usually begins with the development of a conditioning film consisting of organic molecules and matter from the marine environment within few minutes of exposure to seawater. Subsequently, marine bacteria, diatoms, and fungi form an initial biofilm (slime), after which more complex organisms, including soft and hard macrofoulers such as barnacles, mussels, and tubeworms finally populate equipment surfaces (Lewis, 1998; Maréchal and Hellio, 2009; Rosenhahn et al., 2010). Additionally, among the causative components of marine biofouling, periphytic diatoms have been identified as a major contributor to microbial slimes that develop on artificial surfaces placed in the marine environment (Schultz, 2004; Molino and Wetherbee, 2008). Therefore, inhibition of biofilm (slime) formation, as well as the proliferation of fouling diatoms is an important task for the preservation of marine infrastructures. Although there are an estimated at least 30,000 and probably *ca.* 100,000 diatom species on earth (Mann and Vanormelingen, 2013), only those belonging to a few genera including *Navicula*, *Amphora*, *Nitzschia*, *Pleurosigma* and *Thalassionema*, have commonly been reported to dominate biofilms (Patil and Anil, 2005; Molino and Wetherbee, 2008; de Messano et al., 2009; Zargiel and Swain, 2014).

Currently, the use of biocides has become a common method to inhibit marine biofouling, which are products in surface coatings that kill colonizing organisms (Chambers et al., 2006). As one of the most successful antifoulants, tributyltin (TBT) was widely employed in

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antifouling paints. However, this compound was reported to affect the diversity of marine invertebrates (Fent, 1996; Negri et al., 2004). This discovery led to the adoption of a convention to "Control the use of harmful anti-fouling systems on ships" by the International Maritime Organization (IMO), which banned the use of TBT globally in order to protect the marine environment (Champ, 2003). Therefore, there is a need for the development of environmentally friendly anti-fouling compounds (Maréchal and Hellio, 2009), which should be effective, non-toxic, and last for at least five years (Ralston and Swain, 2009). Furthermore, bioactive marine natural products have been reported to be the best sources for antifoulants (Fusetani, 2004; Dobretsov et al., 2006; Maréchal and Hellio, 2009).

Moreover, the inhibition of diatom growth by an allelopathic effect of cyanobacteria living in the same lake was observed (Keating, 1978; Suikkanen et al., 2005), which is a chemical interaction frequently observed between marine algal species, leading to stimulation or inhibition of growth (Molisch, 1937; Chan et al., 1980; Graneli et al., 2008). In this study, it was hypothesized that the use of allelopathic compounds from cyanobacteria might inhibit biofouling on ship hulls or fishing gear. Thus, it is necessary to establish a simple but effective method for growth inhibition of the epiphytic diatom that accelerates the colonization of fouling marine invertebrates.

Alga growth inhibition tests are usually performed according to standard methods (OECD, 2011) in which test vessels are normally glass flasks and cell counts are most often used to determine growth rates and yield throughout the entire test duration. However, for some periphytic diatoms that tend to clump or attach to the flask surface, measurements based on cell counts are impractical (Swanson et al., 1991). Moreover, it is difficult to simultaneously complete the experiments efficiently with multiple samples since it would require an extensive amount of labor. Previously, a 96-microplate was utilized in the algal toxicity assay, and U-bottom shape microplates were required when an electronic particle reader or manual enumeration was used (Environment Canada, 2007). Instead, the photometric method using a flat bottom microplate was employed for the growth inhibition test against periphytic diatoms (Ishihara et al., 2006). Furthermore, while the classic tests generally required conical glass flasks and an algal culture volume of 1 L (Joubert, 1980), methods using microplates require considerably smaller sample volumes (Blaise et al., 1986). There are many advantages to use microplates to conduct algal experiments. Some of the advantages are as follows: The material needed is inexpensive, the assays are simple to perform (Heldal et al., 1978), and the assays do not require large amounts of laboratory space (Thellen et al., 1989; Blaise and Vasseur, 2005). In addition, measurements using photometric method have been proven by Eisentraeger et al. (2003) to be highly sensitive. Recently, Nagai et al. (2013) utilized the 96-well microplate assay to measure the *in vivo* fluorescence of chlorophyll *a*; however, the test requires approximately 72–96 h, which is a relatively long operation time.

XTT (2, 3-bis (2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide inner sodium salt) is a yellow tetrazolium salt (Paull et al., 1988) and its colorimetric reaction is widely utilized in cell proliferation as well as drug sensitivity tests with highly accurate and reproducible results (Scudiero et al., 1988; Hawser et al., 1998). During the colorimetric assay, XTT is reduced by mitochondrial dehydrogenases in metabolically viable cells and transformed to an orange, water-soluble XTT formazan product (Roehm et al., 1991; Hawser, 1996; Wang et al., 2011). This reagent allows for direct absorbance readings, shortening the duration of the micro-culture growth assay (Scudiero et al., 1988). However, a test for algae growth inhibition using the XTT assay has not been developed. Therefore, a new rapid, accurate, and convenient growth inhibition assay with the XTT colorimetric reaction was designed in this study using species of the fouling periphytic diatom genus *Nitzschia* species. In this study, we developed a new method to assay the sensitivity of diatom cells against herbicides, as well as screen and purify herbicidic compounds from cyanobacterial extracts.

2. Materials and methods

2.1. Diatom species

The fouling periphytic marine diatom, *Nitzschia amabilis* Hidek. Suzuki (replaced synonym, *Nitzschia laevis* Hustedt) (Suzuki et al., 2009, 2010) responsible for biofouling was chosen as the test organism for this study due to its ease of handling, cultivation, and high rate of proliferation. The diatom was obtained originally from the surface of *Caloglossa ogasawaraensis* Okamura (red algae) collected from Keihin Canal, the estuary of Meguro River, Konan, Minato-ku, Tokyo.

2.2. Culture medium

While Provasoli's enriched sea water (PES) is often used as diatom culture medium (Provasoli, 1968), we used KW21 (Daiichi Seimo Co., Ltd., Kumamoto, Japan), which enabled us to maintain a stable proliferation rate of diatom (Nagao et al., 2007; Matsuda et al., 2009). The KW21 culture medium consisted of a mixture of 0.01% KW21 in artificial seawater, 45 μg/mL sodium silicate, and a penicillin–streptomycin solution (penicillin, 100 units/mL; streptomycin, 100 mg/L). The solution was filter sterilized with a membrane filter with pore size of 0.45 μm (HLC-DISK13, Kanto Chemical, Tokyo, Japan).

2.3. Diatom subculture

Five milliliters of diatom cells were cultured in φ 50 mm plastic Petri dishes with 0.01% KW21 medium for 3 or 4 days at 20 °C under a 14:10 h light/dark cycle in an illuminating incubator with a fluorescent light intensity of 10 μmol m^{-2} s^{-1}. Subcultures of *N. amabilis* were performed twice per week. After removing 4 mL of culture medium from the Petri dish, diatom cells were completely dissociated by pipetting up and down gently at least 10 times with a 3 mL pipette. Cell counting was performed with a hemocytometer under a microscope (Olympus, CK2, Optical Co., Tokyo, Japan), and the density of diatom cells was then adjusted to 1×10^4 cells/mL. Five milliliters of prepared *N. amabilis* cells (5×10^4 cells) were placed in a new plastic Petri dish and incubated for an additional 3 or 4 days at 20 °C in an illuminating incubator.

2.4. Effect of initial cell density and KW21 medium concentration on diatom proliferation

To confirm the diatom culture condition, cell culture experiments were performed with the initial densities of 1×10^4, 1×10^5, and 1×10^6 cells/mL respectively culturing in φ 50 mm plastic Petri dishes. In addition, to determine the suitable concentration of KW21 culture medium, the tests were carried out with 0.01% KW21, 0.10% KW21, and PES, all prepared with artificial seawater. An experiment using only artificial seawater was used as the control. Sodium silicate was also added to artificial seawater in each test at a concentration of 45 μg/mL. The cell density in each experiment was calculated after t ($t = 1$–5) days of cultivation.

2.5. The relationship between diatom cell density and XTT colorimetric reaction

To confirm the relationship between cell density identified by eye and the XTT colorimetric reaction, both tests were carried out simultaneously as follows. The density of *N. amabilis* cells, cultivated in φ 50 mm plastic Petri dishes, was counted and adjusted to 1×10^4 cells/mL. We then cultured 100 μL of diatom in a 96-well microplate for t ($t = 0$–5) days, at 20 °C under a 14:10 h light/dark cycle in an illuminating incubator. In one test, the density of diatom

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