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Diatom-Cyanobacterial Biofilm Formation and Its Effects on Sediment Stability under Laboratory Conditions

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ABSTRACT

Coastal erosion in the Inner Gulf of Thailand is a major environmental issue. Algal biofilms represent one possible means of remediation. Cultures of six microalgae, three diatom species, and three cyanobacterial species, isolated from an eroded mudflat in Ban Khun Samut Chin were studied for their ability to produce exopolysaccharides (EPS) in xenic single species or mixed species cultures. Carbohydrates, a proxy for biofilm, were measured in the culture supernatants via the phenol/sulfuric acid procedure. EPS ranged from 6.57 to 11.78 mg/L, with diatoms having higher levels than cyanobacteria. EPS levels in mixed species cultures were either enhanced or reduced, ranging from 1.93 to 39.97 mg/L, depending on the algae tested and whether two, three, or four algae were present in the mixed community. Strong attachment of biofilm was detected in the mixed-species culture of a diatom, *Cylindrotheca closterium*, and a cyanobacterium, *Oscillatoria subbrevis*. The EPS level in this mixed culture was also one of the highest recorded, suggesting this pair could be especially beneficial for substrate stabilization. To test this hypothesis, this mixed culture was added to pre-cleaned sediment and allowed to grow for 15 days for surface biofilm accumulation. Turbulent flow conditions were generated using forces both in vertical and horizontal directions above sediment surfaces. Less suspended sediment was recorded in the water column overlaying the sediment with biofilm coverage in comparison to sediment without surface biofilm coverage. This indicated the potential of using microbial biofilms to increase sediment stability and hence mitigate coastal erosion.

Keywords: Biofilm; Mudflat; Exopolysaccharide; Microalgae; Sediment stability

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1. Introduction

Microalgae living as microphytobenthos secrete large quantities of exopolysaccharides (EPS) or biofilm. EPS is comprised of complex molecules, consisting mainly of polysaccharides [1]. A more detailed analysis of EPS reveals heteropolysaccharides with uronic acids and sulfate residues as well as other components including proteins, neutral sugars, pyruvates, acetates, lipids, carboxylic acids, lipopolysaccharides, and nucleic acids [2-3]. EPS constituents are, by definition, secreted from cells and form a three-dimensional structure within a locally charged gel-liked matrix in which the biofilm microorganisms are embedded [2]. EPS is produced by many diatoms such as *Skeletonema* sp., *Cylindrotheca* sp., *Thalassiosira* sp., *Navicula* sp., and *Amphora* sp. [2, 4-5]. The relationship between diatoms and cyanobacteria is recognized as symbiotic, as diatoms release dissolved organic matter for cyanobacteria; in return, cyanobacteria supply nitrogen, vitamins, and trace elements to promote diatom growth and biofilm formation [6]. The biofilms modify the surrounding micro-environment and vice versa; the diversity of micro-organisms also controls the properties of biofilms [3]. Diatoms and cyanobacteria are the focus of the work presented here, but it is important to point out the community within natural biofilms is very diverse, including heterotrophic bacteria, fungi, protozoans, and small invertebrates as well as viruses and/or phage.

In mudflat ecosystems, biofilm formation can frequently be found on surface sediments appearing as brown or yellowish patches [7]. Biofilms play many important ecological roles in mudflat communities. Mudflat organisms associated with, or living near, biofilms receive protection against high irradiance, UV exposure, pollution, and desiccation. Microbial biofilm comprised of autotrophic microorganisms may also be a food source for benthic animals [8]. Biofilms can trap fine grain sediment particles with its glue- like substance and binds sediment grains together, which leads to sediment deposition. This prevents the loss of sediment due to physical disturbances and increases sediment stability [3, 6, 9-10]. Previous studies revealed the ability of microbial biofilms on coarse substrates to increase stabilization of sediment and thus diminish coastal erosion [11-14]. However, coastal erosion problems in Thailand occur both in sandy beaches and muddy shores [15-16]. Thus, the aim of this study was to evaluate biofilm production from mono-species and mixed- species cultures of six microalgae isolated from mudflats of Ban Khunsamut Chin and to study the influence of biofilm produced by microalgae on the re-suspension of sediments in well- controlled chambers with vertically oscillating turbulence and horizontal current flow chambers.

2. Materials and Methods

2.1 Algal isolation

Surface sediment covered with microalgae biofilm were collected from mudflats of Ban Khunsamut Chin, Samut Prakarn province located along the northern part of the Inner Gulf of Thailand (13˚30′ 26. 20″ N and 100˚31′ 52. 35″ E). Sediment samples were transferred to polyethylene bags and transported under cool conditions in a closed container. Isolation of a single cell microalgal was achieved in two steps. First, the lens- tissue technique [17] was used to separate microalgal cells from sediment. Second, the pipette- method was used to isolate a single cell to start the culture.

2.2 Culture methods

Cultures of microalgae were maintained with f/2 medium [18] at a temperature of 27. 9±2. 0˚C and under light intensity of 135. 47±2. 00 μmol/ m^2/ s^1 with 12:12 hr light: dark cycles. All cultured algal strains were classified under a light microscope and/or a scanning electron

microscope following methodology from Round et al [19].

2.3 EPS quantification

Since carbohydrates are a major constituent of biofilm or EPS, most studies have used carbohydrate content as a proxy for biofilm or EPS production. Mono-species cultures of microalgae were grown with an initial cell density of 500 cells/L under the previously mentioned culture conditions and sampled at stationary phase to screen for EPS producing strains. The samples were centrifuged at 2000 rpm for 10 min and the supernatant was precipitated in 95% ethanol at a ratio of 1:1. After precipitation, the samples were centrifuged at 12,000 rpm, 4°C for 20 min [20] and analyzed for carbohydrate content using the phenol sulfuric acid method [21].

Four microalgal strains exhibited the highest carbohydrate production and were selected for further study in order to compare the ability of EPS production of each selected species together with combinations of two, three, and four mixed-species cultures. Each species was added to make up an initial cell density of 500 cells/L at the beginning of the experiment. Cultures yielding the highest EPS production were then selected and grown in a large volume to produce enough biomass for the analysis. The experiment was conducted under the same conditions as the previous experiment and sampled at stationary phase for the measurement of carbohydrate content by the phenol-sulfuric acid method [21].

2.4 Microalgal stabilization of sediments

To study the influence of microbial biofilm on sediment stability, two experiments, vertically oscillating shaker and flow flume, were performed.

2.4.1 Microalgal culture used in the experiment

Cultures with the highest EPS production (Section 2.3) were used for this experiment. Each species was cultured separately with the same medium and kept under the same conditions as done in previous studies, until reaching stationary growth phase.

2.4.2 Vertically Oscillating Shaker experiment

To evaluate the effect of biofilm on sediment resuspension due to vertically oscillating turbulence in water column, pre-sediment collected from mudflats of Ban Khunsamut Chin was first cleaned by UV radiation and filled into circular cell culture plates (CCPs) 12 cm in diameter and 1.8 cm in height. The CCPs were then placed in a glass aquarium filled to 5 cm f/2 seawater-medium. Stationary microalgal cultures from 2.4.1 were then added into aquarium to make up the equal densities of 500 cells/L. The cultures were kept at room temperature with illuminated light of 135.47±2.00 μmol/m^2/s^1 with 12:12 hr. light: dark cycle. All treatments were set up with at least 3 replications. A control treatment without the addition of biofilm producing microalgae was also set up. After 15 days of incubation, CCPs were placed in a cylindrical tube with a 15 cm diameter and 30 cm height equipped with a circularly rotating motorized blade to form turbulence (Fig. 1) with 10 cm depth of filtered seawater above the CCP. Then, the equipment was operated to form vertically oscillating turbulence in water column with wave power per unit wave crest length at approximately 47 Watts. This value was estimated from the following equation:

$$P = \frac{\rho g H^2 L}{16T}\left(1 + \frac{2kd}{\sinh(2kd)}\right),$$

where ρ is seawater density (1,029 kg/m^3), g is gravity acceleration (9.81 m/s^2), H is wave height (1 m), L is wavelength (3 cm), T is wave period (0.4 s), d is water depth (10 cm), and k is wave number $\left(\frac{2\pi}{L}\right)$ [22].

Subsequently, 60 mL of seawater was collected from the water column at the

midpoint of the cylindrical tube at 0, 1, 2, 3, 4, 5, and 6 hr. for analysis of total suspended solids following the 2540D analytical method [23]. Both treatments were set up with 3 replications.

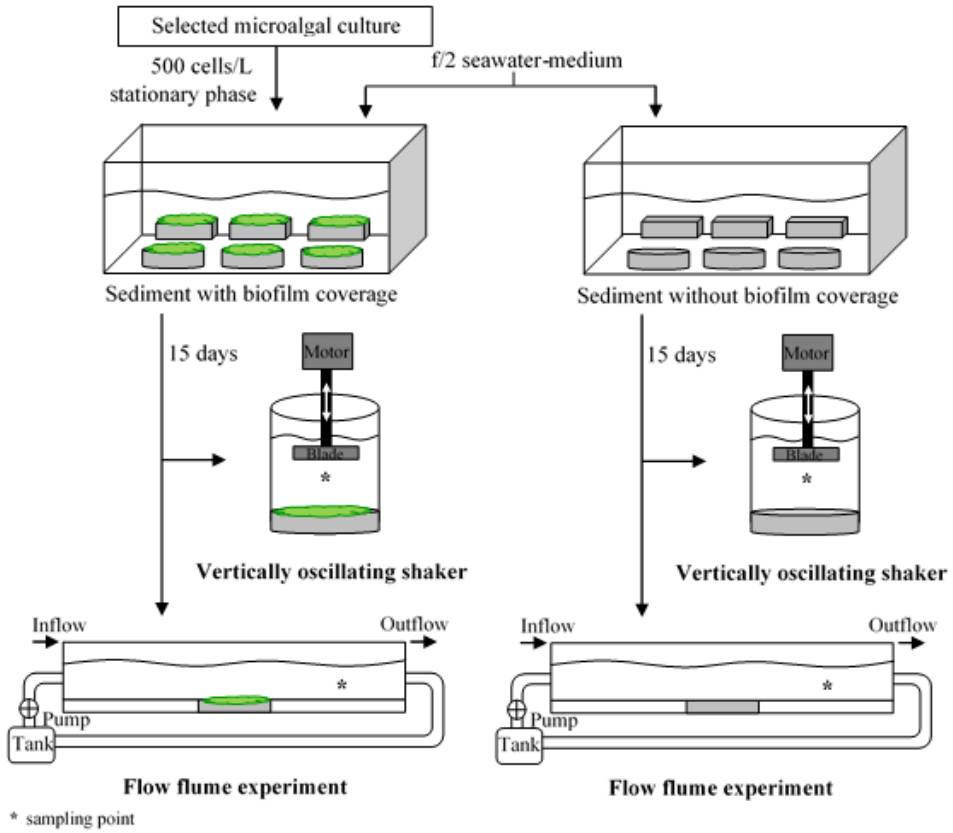

Fig. 1. Experimental setup for study of sediment re-suspension in vertically oscillating shaker experiment and flow flume experiment.

2.4.3 Flow flume experiment

This experiment was set up to evaluate the effect of horizontal current flow on sediments covered with biofilm. A straight flume 1.20 m long and 8 cm wide with a space for a square cell culture plate (SCCP) in the middle of flume bed also connecting pipes of inflow and outflow was used (Fig. 1). A pump was installed with one outlet connecting to an inflow pipe and another outlet connecting to an outflow pipe to create a water flow of 0.3 m/s. Sediment was filled into the SCCPs, which were 7 cm long, 5.8 cm wide, and 1.8 cm tall. Sediment samples, either with microalgal biofilms or without biofilm, were prepared in the same fashion as previous experiments. All treatments were set up with at least 3 replications. After 15 days, a SCCP was placed in the space of the flume bed, cleaned seawater was then filled carefully, and the water pump was turned on to initiate the water flow. Subsequently, 60 mL of seawater was collected from the water column at the midpoint between SCCP and outflow at 0, 1, 2, 3, 4, 5, and 6 hr. after the flow started, to determine the total suspended solids [23]. To determine the differences in the carbohydrate content of the monoculture and mixed-species cultures of microalgae, and the total suspended solids under conditions of water turbulence and current flow, the Kruskal-Wallis test, T-test, and Mann Whitney test were used to test for significance.

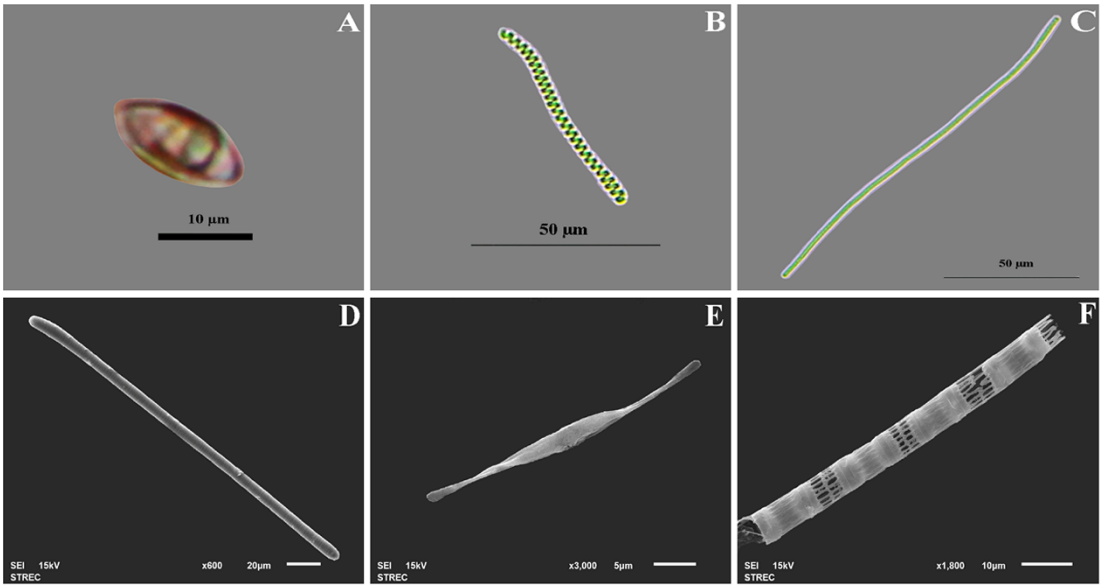

Fig. 2. Image of six mono-clonal cultures used in this study (A) *Navicula* sp. (B) *Spirulina* sp. (C) *Oscillatoria* sp. (D) *Oscillatoria subbrevis* (E) *Cylindrotheca closterium* and (F) *Skeletonema costatum.*

3. Results and Discussion

3.1 EPS in single species culture

The diatom and cyanobacteria strains in this study were previously reported as dominant microalgae species in the mudflat community of Ban Khunsamut Chin during the last decade [24-25]. Mono-clonal cultures of *Navicula* sp., *Spirulina* sp., *Oscillatoria* sp., *Oscillatoria subbrevis*, *Cylindrotheca Closterium,* and *Skeletonema costatum* (Fig. 2) at the age of 15 days produced considerable amounts of EPS (measured as carbohydrate) from 6.57 mg/L to 11.78 mg/L (Fig. 3). A test of significance revealed that the amount of carbohydrate produced by mono-clonal cultures were significantly different ($p < 0.05$). The diatom species *C. closterium* produced the highest amount of carbohydrate, about double that of other species, while the amount of carbohydrate produced by the cyanobacterium *Spirulina* sp. was lower than the other species tested.

An estuarine diatom, *Cylindrotheca Closterium,* produced the highest amount of carbohydrate, maybe due to the fact that it has a much greater rate of growth than many other diatom species [26-27]. The biofilm produced by microalgae is species dependent and depends on various other factors including light, nutrient availability, temperature, and pH [28-29]. Light intensity, as well as photoperiod type and nutrient limitations affected growth rate and EPS production in *C. closterium*. [5, 30]. Increased EPS production by *Dunaliella salina* was reported to positively correlate with salinity [31]. Biopolymer produced by *Spirulina* sp. decreased when the temperature increased [32]. An increased EPS production by *Anabaena* sp. was observed when temperatures switched from 30-35° C to 40-45° C [33].

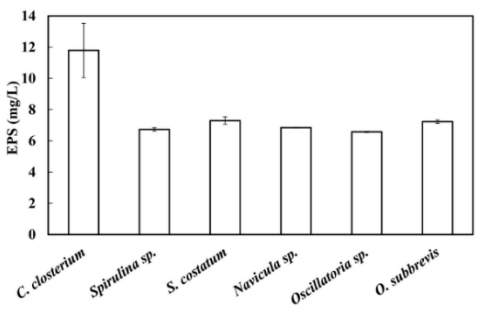

Fig. 3. EPS (measured as carbohydrate) produced by mono-species of microalgae (mean±SE, n=3).

3.2 EPS in mixed-species culture

The four microalgal species that produced high carbohydrate content, *C. closterium, S. costatum, Navicula* sp., and *O. subbrevis*, were used for culture combinations to evaluate EPS production in mixed-species cultures (Fig.4). The results revealed the carbohydrate contents of 2, 3, and 4 species cultures containing *C. closterium*, *S. costatum, Navicula sp*., and *O. subbrevis* varied from 1.93 mg/L-39.97 mg/L. The lowest value of EPS was seen in the mixed-species culture of *S. costatum* and *O. subbrevis*, while the highest value of EPS was produced by the mixed-species culture of *C. closterium* with *Navicula* sp., followed by the mixed-species culture of *C. closterium* with *S. costatum, C. closterium* with *S. costatum,* and *C. closterium* with *O. subbrevis.* The amounts of carbohydrate produced by 2 species-combination cultures containing *C. closterium* (* in Fig. 4) were significantly different ($p < 0.05$) from the other two combinations (** and *** in Fig. 4). Pairwise comparisons were performed for the differences in carbohydrate production of the three highest algal culture combinations (mixed cultures of *C. closterium* with *Navicula* sp., *C. closterium* with *S. costatum,* and *C. closterium* with *O. subbrevis*) but indicated no significant difference in EPS content ($p > 0.05$).

Since there were no significant differences in EPS production by the mixed cultures, we employed a different strategy to identify mixed cultures that may be better to aid substrate stabilization. Visual inspection of the growth containers used for these experiments revealed clear, rapidly apparent, differences in the degree to which different cultures became attached to the sides of containers. The mixed- species culture of *C. closterium* with *O. subbrevis* was chosen for further studies as shown in Fig. 5. Filamentous cyanobacteria enhance diatom attachment by creating a cohesive net and the embeding of diatoms.

The increase in carbohydrate production in mixed- species cultures in comparison to that of

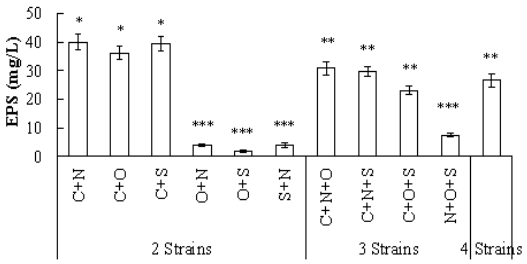

Fig. 4. Carbohydrate produced by mixed-species of microalgae (mean±SE, n=4). C: *Cylindrotheca closterium*; N: *Navicula* sp. ; O: *Oscillatoria subbrevis*; S: *Skeletonema costatum* (*, **, *** indicated the significant differences of carbohydrate contents among culture combinations).

mono-species cultures in our study may have been due to a combination of biotic and abiotic factors. Co-culture species had higher biomass and EPS production than the mono-species did [34-36]. However, mixed species in a biofilm exhibited an antagonistic effect on biofilm formation caused by the more adhesive species removing the less adhesive species from the community. Furthermore, a shifting in diatom species may change the relationship with bacteria flora and hence affect diatom growth [37]. Abiotic factors, such as nutrient availability may be related to biofilm formation. Carbohydrate content increased with sufficient levels of nutrients and when nutrient levels were insufficient, there was a decrease in growth rate and an effect on EPS composition, which may lead to a decrease in biofilm formation [1].

From this study, the mixed-species culture of *C. closterium* with *O. subbrevis* showed a strong adhesion of biofilm on the substrate. Typically, *Oscillatoria* is a filamentous cyanobacterium with a rapid colonization ability, thus having a high binding efficiency and the ability to stabilize non-cohesive sediment [38]. The adhesive property of *O. subbrevis* together with the high EPS producing ability of *C. closterium*

may support the binding and stabilizing of biofilm on a substrate. Substrate covered with EPS produced from the networking of *O. subbrevis* and *C. closterium* can have a higher resistance to erosion than a substrate

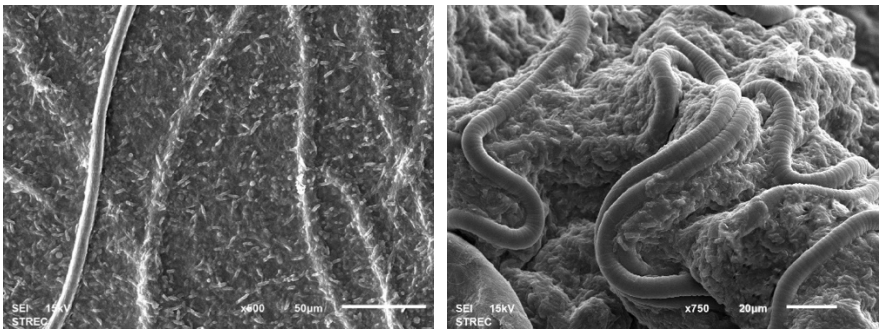

Fig. 5. Scanning electron micrographs of *Oscillatoria subbrevis* filament binding *Cylindrotheca closterium* in mixed species culture biofilm at different magnifications of x500 (left) and x750 (right).

without biofilm coverage. Thus, EPS produced by *C. closterium* and filaments of *O. subbrevis* can bind cohesive and non-cohesive particles together to form a thin mat that affects substrate stability. Angelis et al. [34] reported that algal mats formed by filamentous *Ulva intestinalis* can slightly reduce the fluctuation velocity influenced by waves, effectively reducing the bed shear stress of moving grains over the sediment bed. Changes in critically eroded velocity of sediment with colonized biofilms were reported to enhance the stability of sediment due to the mat-like shape and filamentous structure of biofilm penetrating into the bed. These structures play the role of a protective shield from the overlying flow and intensify adhesive forces [12]. This may enhance the properties of the biofilm produced by the diatom *C. closterium* with the cyanobacterium *O. subbrevis* more so than the other combinations of microalgae tested in this study.

3.3 Microalgal stabilization of sediments

Sediment samples either with selected microalgal cultures or without selected microalgal cultures were prepared under laboratory conditions to study the influence of microbial biofilm on sediment stability. The influence of biofilm produced by microalgae on the re-suspension of sediment under the effects of a vertically oscillating shaker and flow flume are presented in Fig. 6. The amount of total suspended solid (TSS) in the experiment unit of sediment with biofilm was lower than the amount in control unit for both the vertically oscillating shaker experiment and flow flume experiment. Vertically oscillating turbulence in the water column caused resuspended sediment in the amount of 16.66 mg/L up to 326.66 mg/L from the sediment surface without biofilm (control unit) while only 1.66 mg/L to 13.33 mg/L of sediment resuspended from

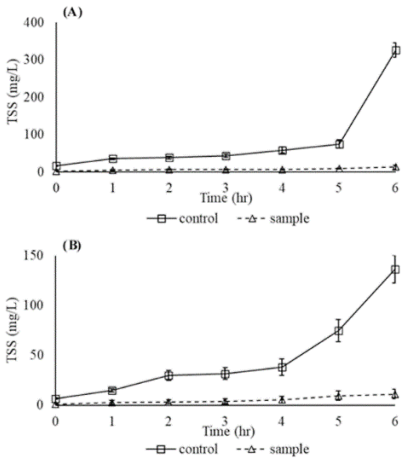

Fig. 6. Total suspended solid (mg/L) under (A) vertically oscillating shaker and (B) flow flume experiment.

the biofilm-covered sediment in the experiment units. The results of the TSS under flow flume experiment were similar to the vertically oscillating shaker experiments. The TSS in bare sediment (no biofilm) was in the range of 6.66-136.66 mg/L, whereas the amount of TSS was 1.11 mg/L to 11.11 mg/L from units of biofilm covered sediments. Statistical comparison between the control units (without biofilm) and experimental units (biofilm-covered sediment) at different time points in the vertically oscillating shaker experiment and flow flume experiment were evaluated. There were significant differences between control units and experimental units in both the vertically oscillating shaker experiment and the flow flume experiment ($p < 0.05$). One possible extrapolation of these results is that biofilm may play a vital role in increasing sediment stability. The secretion of EPS and formation of biofilm by microalgae on the sediment surface helps to reduce sediment resuspension, more so than what is seen in sediment without biofilm.

Previous studies on sediment transport focused on sediment from reservoirs and rivers that had different sediment size fractions from sediment from mudflat areas [13-14]. From this study, bottom sediment with biofilm released less suspended sediment when disturbed with both vertically oscillating turbulence in the water column, and horizontal current flow. This result is in line with the findings of Fang et al. [13] and Fang et al. [14] studying suspended sediments in the water columns of flumes containing sediment with and without biofilm coverage. Fang et al. [13] reported the study of suspended sediment concentrations in clean sediments and sediments covered with biofilms at different cultivation periods (5, 10 and 15 days). The results showed the highest suspended sediment concentration was found in clean sediments followed by 5, 10, and 15 days of biofilm-growth sediments with decreases of 1.5%, 78%, and 94.5% suspended sediment compared to clean sediment, respectively. An increase in sediment size as an effect of biofilm growth was also reported. Moreover, increasing shear stress of sediment covered with biofilm was observed concurrent with the increase in cultivation periods. Fang et al. [14] reported that sediment covered with biofilms showed increased erosion thresholds, but decreased flow resistance as well as the presence of turbulent kinetic energy at the sediment surface. High concentrations of suspended sediments were also recorded in the water column overlaying sediments without biofilm coverage, more so than sediments with biofilm. The results of sediment transportation in sediments covered with biofilm revealed the deposition of sediment accumulating near the sediment bed, whereas sediments without biofilm were deposited far away from the sediment bed and were more easily resuspended after deposition. Therefore, these results indicate that sediments covered by biofilm affected sediment transportation and deposition and, subsequently, that sediment will become more resistant to erosion and have increased stability.

4. Conclusion

This study has shown that biofilms produced by the mixed-species culture of *Cylindrotheca closterium* and *Oscillatoria subbrevis* (CO culture) help to stabilize sediments exposed to both horizontal and vertically oscillating turbulent shearing under short periods of time under well defined, but limited, conditions. We predict these biofilms would provide sediment stabilization in natural intertidal mudflats, such as those of Ban Khunsamut Chin, Samut Prakarn Province. We look forward to conducting long-term studies, including additional environmental parameters (e.g., exposure to periodic flooding and drying, similar conditions in the natural environment), exposure to high light and/or UV light (as expected during periods of low tides), to determine the effects, if any, on EPS and biofilm production and, ultimately,

sediment stability. We also look forward to determining the chemical composition of EPS and biofilms in these CO cultures to ascertain which biochemical constituents are responsible for enhanced sediment stabilization; once identified, the cultures or culture conditions that enhance the accumulation of these " sediment cohesive biochemicals" (SCBs) could be more easily identified. It would also be interesting to conduct "transplant" experiments in the field (e. g. , cores taken from mudflat areas that appear to be undergoing sediment stabilization being transplanted to areas without obvious sediment accumulation to parse environmental factors from species factors, similar to work conducted in Georgia tidal flats [39].

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