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Coagulant Plus *Bacillus nitratireducens* Fermentation Broth Technique Provides a Rapid Algicidal Effect of Toxic Red Tide Dinoflagellate

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Abstract: When the toxic red tide alga *Gymnodinium catenatum* H.W. Graham accumulates in sediment through sexual reproduction, it provides the provenance of a periodic outbreak of red tide, a potential threat to the marine environment. In our study, the flocculation effects of four coagulants were compared. Bacteria fermentation (Ba3) broth and coagulant were combined with Ba3 to reduce the vegetative cells of *G. catenatum*, inhibit the cystic germination in the sediment, and control the red tide outbreak. To promote a more efficient and environmentally friendly algae suppression method, we studied these four coagulants combined with algae suppression bacteria for their effect on *G. catenatum*. The results show that polyaluminum chloride (PAC) is more efficient than other coagulants when used alone because it had a more substantial inhibitory effect. Ba3 broth also had a beneficial removal effect on the vegetative cells of *G. catenatum*. The inhibition efficiency of 2-day fermentation liquid was higher than that of 1-day and 3-day fermentation liquids. When combined, the PAC and Ba3 broth produced a pronounced algae inhibition effect that effectively hindered the germination of algae cysts. We conclude that this combination provides a scientific reference for the prevention and control of marine red tide. Our results suggest that designing environmentally friendly methods for the management of harmful algae is quite feasible.

Keywords: *Bacillus nitratireducens*; fermentation broth; polyaluminum chloride coagulation (PAC); *Gymnodinium catenatum*; cysts

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1. Introduction

At present, red tides have become one of the ocean's most catastrophic global disasters. The occurrence frequency, outbreak intensity, and impact range of red tides worldwide have been increasing and causing various degrees of harm to many countries' coastal regions. It is conservatively estimated that the annual loss of fishery and tourism caused by harmful algae blooms (HABs) in Europe is up to EUR 862 million, while the annual loss caused by HAB in the United States is up to USD 82 million. Between 1995 and 2004, an average of USD 1.31 million was lost annually in South Korea's fisheries due to harmful algal blooms. Red tide refers to the rapid proliferation or accumulation of various dinoflagellates and diatoms under external environmental conditions, mainly in marine environments such as coastlines or estuaries [1].The algal cell density reaches a certain level, causing water discoloration and affecting coastal areas and aquatic ecosystems, which, in turn, cause severe effects and hinder tourism [2]. The dinoflagellate *Gymnodinium catenatum* is a dominant harmful algae bloom (HAB)-forming species along coasts worldwide [3,4]. For example, *G. catenatum* is a bloom-forming species that forms HABs in China's Fujian

coastal waters almost every year [5]. In 2017, a red tide of *G. catenatum* broke out in the coastal waters near Quanzhou and the Zhangzhou Sea. The continuous eruption of *Gymnodinium* species and cystic settlements formed by sexual reproduction in sediments poses a significant threat to the aquaculture industry and people's health. Therefore, dinoflagellate cyst deposits in coastal areas, the distribution patterns, and the abundance of species are an urgent need to control the harmful effects generated by HABs [6].

Different methods have been developed to prevent and eliminate red tides, including physical, chemical, and biological methods in recent decades. These three methods have their advantages and disadvantages in red tide management [7]. Among these methods, physical methods will not harm the original ecosystem, but they are also inefficient, expensive, not suitable for large-scale red tides, and can only suppress red tides quickly. Chemical methods of algae removal are very efficient and can destroy algae cells. Regrettably, these methods are quite costly and may lead to secondary pollution [8–10]. Biological methods, however, are economical, effective, and environmentally friendly. In particular, microbial algae suppression methods offer many advantages such as simple operation, complete algae killing, and no secondary pollution to the environment [11]. The management of current red tide hotspot areas is of great significance, and the prospect of controlling HAB algae-killing microorganisms has also been in rapid development. In particular, these algaecide bacteria can lyse algae by directly or indirectly attacking cells [12–15]. Certain substances secreted by microorganisms will cause cell lysis and death by invading and contacting algae cells, although the action time is longer.

Nowadays, related research has discovered that the combined algae removal effect of multiple methods is more effective than that of a single method. However, the inhibitory effect of combined methods on coastal HAB species is much less studied. In the present study, chemical algae removal can reduce algae cells' density for a temporary period during a red tide outbreak. Among these particular methods, the coagulation method has relatively high safety [16]. Polyaluminum chloride (PAC), aluminum sulfate [$Al_2(SO_4)_3$], ferric sulfate [$Fe_2(SO_4)_3$], and ferric chloride ($FeCl_3$) can also increase HAB removal efficiency. Besides biological algae removal methods, algae suppression bacteria and fermentation broth algae suppression methods have become new research directions in recent years. Few studies have demonstrated that *Bacillus* sp. can suppress the growth of harmful algal bloom species [17]. However, the effect of the combined approach on micro-algae is little known. To the best of our knowledge, there have been no reports so far. In this study, we first compared four coagulants, namely PAC, $Al_2(SO_4)_3$, $Fe_2(SO_4)_3$, and $FeCl_3$, against *G. catenatum*. The coagulant we selected has the best flocculation effect and the best algae inhibition effect; on this basis, we explored the algaecide effect of *Bacillus* fermentation broth (Ba3) and further studied the inhibitory effect of the combination of coagulant and Ba3 on *G. catenatum*. Furthermore, we used this method to inhibit the germination of algae cysts in the sediments. We planned a systematic study of the comprehensive effect of the coagulant Ba3 on the red tide of algae and its germination as controlled from the source red tide outbreak, which may have crucial effective management strategies. The framework of the study design is shown schematically in Figure S1.

2. Material and Methods

2.1. Cultivation of the Dinoflagellate and Bacteria

Gymnodinium catenatum was obtained from the State Key Laboratory of Marine Environmental Science at Xiamen University, China. We maintained the axenic algal culture at 20 ± 2 °C in a sterile L1 medium prepared with natural seawater filtered to 0.45 µm and maintained under a 12:12 h light/dark cycle. We also counted cell numbers under a microscope. The algal culture was transferred once a week to a fresh, sterilized medium, which ensured that experiments were always conducted with cultures during the exponential growth phase.

We previously identified the algicidal bacteria preserved in the College of Life Sciences, Fujian Normal University, as *Bacillus nitratireducens*. The strain was initially cultivated

with a modified Bacillus Medium (Peptone 10 g/L, sodium chloride 5 g/L, beef paste 5 g/L, pH value 7.2~7.4, dissolved in deionized water) in a rotary shaker (30 °C, 180 rpm), and the resultant mixture kept in our laboratory was thoroughly stirred for subsequent experiments.

2.2. Cultivation of Gymnodinium catenatum Cysts

Dinoflagellate has a high cyst formation rate under low nitrogen and phosphorus environments [18]. Usually, the limitation of nutrients in the water phase is an effective way to induce vegetative cells to form cysts. Therefore, an L1 medium with low phosphate and nitrate was used to prepare *G. catenatum* cysts. The dilution ratio of phosphate and nitrate was 1:15 (named L15). In brief, we added 20 mL of 10^6 cells/L of *G. catenatum* in a 50-mL centrifuge at 3000 rpm for 10 min, discarded the supernatant, and then slowly added 20 mL of L15 medium. After adding the medium, the algae cells in the centrifuge tube were mixed with the medium and then transferred to a sterile Erlenmeyer flask for cultivation.

2.3. Selecting Coagulants and Preparing Concentration

Four agents, including PAC, $Al_2(SO_4)_3$, $Fe_2(SO_4)_3$, and $FeCl_3$, were used in the experiments. The coagulant concentration was set to 0, 10, 20, 30, 50, 70, and 90 mg/L; the first group (0 mg/L) was used as the control, and the following six were used as the treatment groups. Each treatment was established in triplicate.

2.4. Cell Inhibition Efficiency

2.4.1. First Experiment

The efficiency of the cell removal experiment was tested in 25-mL sterile test tubes. A 20-mL aliquot of the algal culture was placed in each tube with a density of 2×10^6~ 4×10^6 cells/L; then, 0, 10, 20, 30, 50, 70, or 90 mg/L of the four agents' stock solution was evenly added to the tube. After adding the agent, each tube was mixed thoroughly, and each concentration was tested in triplicate on the alga cells. After three periods (3, 24, and 48 h), three replicate samples from each group were pipetted from the upper-middle region of the collected liquid surface, and each sample's concentration of algal cell was determined under the microscope; photographs were taken for the bottom flocs of each treatment. In addition, the supernatant's pH value was measured to determine the total removal rate of zeta potential, turbidity, and UV_{254}; then the positive effects and the best dose of the coagulant were noted for further experiments.

2.4.2. Second Experiment

B. nitratireducens bacteria were inoculated into the culture medium and grown to the stationary phase (30 °C at 180 rpm for 24 h). The extraction of bacterial fermentation broth (Ba3) was collected using centrifugation (10,000, 15 min) over three days (1, 2, and 3 d). The supernatants were filtrated through 0.22-μm Millipore membrane filters and then used. Ba3 volume ratios of 0.3, 0.7, 1.0, and 2.0% were added to the 30-mL exponential phase *G. catenatum* algae. Each group was placed in a light incubator for culturing, shaken twice a day, and sampled once every two days until the end of the experiment. The group with no Ba3 served as a control for the experiments, and all experiments were repeated in triplicate.

2.4.3. Third Experiment

In the first and second experiments, a more effective dosage of mixed coagulant and Ba3 fermentation broth was selected. This experiment determined whether it was possible to improve the previous experiments results using combinations of more effective agents and Ba3. The above experiments' dosage of the various coagulants and the fermentation broth dosage for two days were 0.3, 0.7, 1.0, and 2.0%. The combined coagulants and fermentation broth were added to 20 mL *G. catenatum* alga in triplicate samples for each group for better cell inhibition efficiency. Each group was placed in a light incubator for

cultivation, and samples were assessed at 3, 24, 48, and 96 h for density determination to ensure that they had significant inhibition efficiency.

2.5. Calculation Method of Algae Removal Rate

The algae removal rate was monitored by estimating cell numbers utilizing a microscope and calculated according to the following formula.

$$RE\ (\%) = (1 - N_t/N_0) \times 100\%$$

where RE is the removal rate of algal cells; N_0: vegetative cell density before adding algae inhibitor; and N_t is the vegetative cell density after adding the algae inhibitor.

2.6. Analytical Methods

An optical microscope was used to observe the effect of fermentation broth on the morphological characteristics of algal cells. After the addition of algal inhibitor, 1 mL of the supernatant was removed, placed on a slide, and observed under a microscope. The size of the coagulant and the flocs as well as the compactness of the flocs were photographed for further characterization of the coagulation effect of different coagulants on the cells of dinoflagellates. UV_{254} was determined by using the spectrometer as an indicator to reflect the content of organic pollutants in water. The spectrometer reading does not represent a specific organic substance, but rather the total amount of multiple organic substances; therefore, the spectrometer reading refers to the many soluble fine particles and various inorganic compounds present in the experiment. In this experiment, after stirring, the supernatant was taken from 2 cm below the liquid level after standing for 24 h. The supernatant was filtered using a 0.45-μm acetate fiber membrane, and then, pure water was used as the reference solution to determine the results of each experiment group. A spectrophotometer analyzed turbidity with a wavelength of 660 nm. Zeta potential is one of the indexes that reflect the stability of suspended matter or colloid in water. The zeta potential was monitored using Zetasizer software. In this experiment, 2 mL of algal sedimentation floc was taken and injected into the sample pool with an injection needle, and the sample pool was put into the card slot to ensure that the algal sedimentation floc in the sample pool did not exceed the electrodes at both ends of the sample pool. Zetasizer software was used to measure and record the results. Dissolved inorganic phosphorus (DIP) and dissolved inorganic nitrogen (DIN) were determined using a spectrophotometer at a wavelength of 882 nm.

2.7. Provenance Control Experiment

This experiment was divided into two groups; one group was marine sediments—PAC + Ba3 + sediment (marked as I-1, I-2, and I-3)—and the other group was marine sediments + pure-breed *Gymnodinium* cysts 25 ± 2 cysts/g DW—PAC + Ba3 + sediments + pure-breed (marked as II-1, II-2, and II-3). The framework of the study design is shown schematically in Table S1. The dosages of the treatment used in the study were as follows: PAC 50 mg/L, and PAC + Ba3 50 mg/L + 0.3%.

2.8. A Calculation Method of Algae Cell Abundance

We calculated the algae cell abundance rate after germination in the sediment were calculated by visual observation method using a microscope and calculated according to the following formula.

$$N = (40/30) \times (V_0/V) \times n$$

where N is the abundance of algal cells after germination, with the unit of cells/mL; V is the count volume taken in each observation, with the unit of ml; V_0 is the volume of the sample after concentration, with the unit of mL; n is the number of algae in each sample.

2.9. Data Processing

The experimental data were analyzed using SPSS 22.0 software, and the difference between the data ($p < 0.05$) was significant ($p < 0.01$).

3. Results

3.1. The Effect of Different Coagulants on the Algae Inhibition

The four kinds of agents have noticeable removal effects on *G. catenatum*, but different degrees of the "back-dissolving" phenomenon appeared over time. Figure 1 shows that when the reagent dosage was low (10 mg/L) at 3 h, the PAC, $Fe_2(SO_4)_3$, $Al_2(SO_4)_3$, and $FeCl_3$ algae removal rates achieved were 80, 68.5, 77.5, and 80%, respectively. When the dosage was increased, the difference between PAC, $Fe_2(SO_4)_3$, $Al_2(SO_4)_3$, and $FeCl_3$ in algae removal efficiency rate gradually narrowed, while that of the $FeCl_3$ group was relatively low. The removal rate of *G. catenatum* in the PAC group increased when the dosage was increased. When the dosage was 50 mg/L, the removal rate reached more than 95.1%; when the dosage was 70 and 90 mg/L, the removal rate reached 100%.

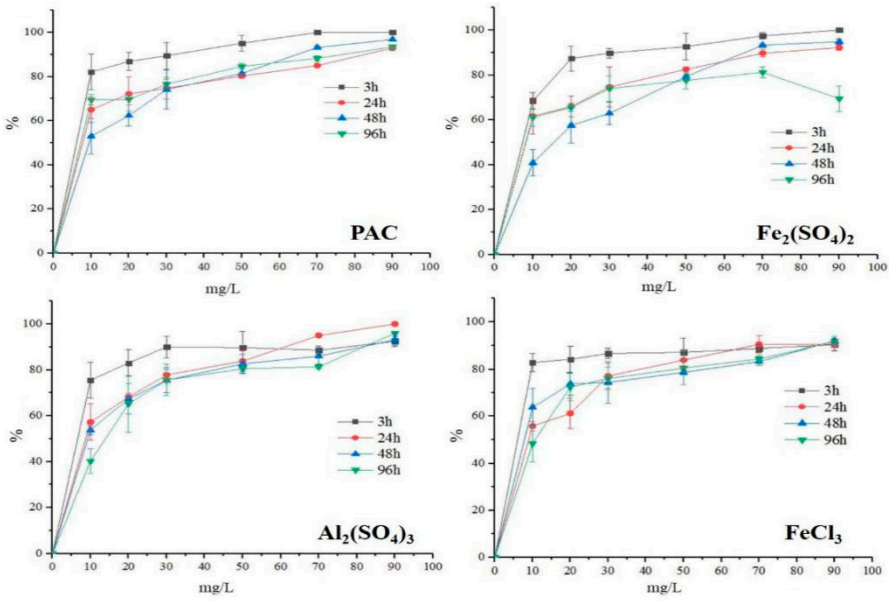

Figure 1. Removal effect of four different coagulants on *G. catenatum.*

After 24 h of dosing, the removal rates of different dosing amounts were between 65.0 and 92.9%. When the dosage was 50 mg/L, the removal rate of *G. catenatum* decreased to 80.3%. After 48 h of dosing, the resolution phenomenon was more marked when the dosage was 10 mg/L, which reduced the removal rate to 52.9%. After 96 h of dosing, the removal effect of *G. catenatum* increased compared with that at 48 h, and the removal rate was 69.4% at 10 mg/L. However, when the dosage was greater than 50 mg/L, the removal rate of *G. catenatum* was still higher than 80% over time. After 3 h of dosing, the different dosages' removal rates differed significantly from those between 24 and 96 h ($p < 0.05$). When the dosage of $Fe_2(SO_4)_3$ was higher than 20 mg/L, the removal rate was higher than 85%. After 48 h of administration, the removal rate decreased; after 96 h of administration, the removal rates of the 10, 20, and 30 mg/L groups increased, but when the dosage of ferric sulfate increased to 90 mg/L, the removal rate decreased to 69.4%. The removal rate was not altered between 24 and 96 h. When the $Al_2(SO_4)_3$ dosage was 30 mg/L, the removal rate reached 85% or more; above this dosage, the removal rate did not change significantly ($p > 0.05$). When the dosage was 90 mg/L, the removal rate of *G. catenatum* between 24 and 96 h was slightly higher than that after 3 h. The $FeCl_3$ group activity was relatively low with the increase in the dosage, and the removal rate did not increase significantly ($p > 0.05$); when the dosage was 90 mg/L, the removal rate was 90.2%. With

time, the removal rate decreased significantly at a low dosage (10~50 mg/L) ($p < 0.05$). When the dosage was 70 mg/L, the removal rate was stable (Figure 1). After adding different dosages, the removal rate did not change significantly between 24 and 96 h.

Figure 2A shows the four coagulants' influence the pH range, and the control group's pH value was 8.1. With the addition of coagulants, the pH values of the four groups showed a downward trend. $FeCl_3$ had the smallest effect on pH at 90 mg/L, and the pH value was the lowest, which was 7.6. The pH range of the $Al_2(SO_4)_3$ group was 8.1~6.8. The influence trend of the PAC and $Fe_2(SO_4)_3$ groups affected *G. catenatum* in almost the same way, and the variation range was 8.1~6.8. The pH range between 6.8 and 8.1 exhibited a good algal removal effect at both a higher dosage of coagulant and a lower dosage of coagulant, a pH decline, and the efficiency was slightly less reactive (Figure 2A).

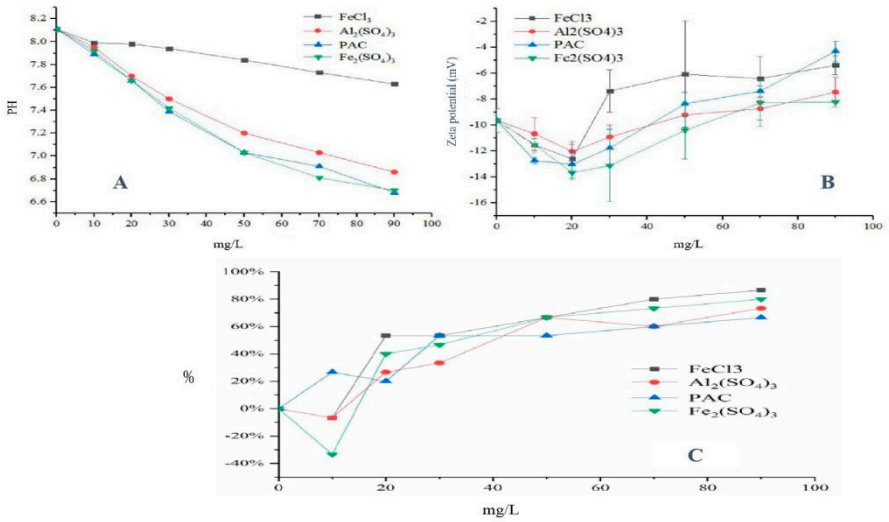

Figure 2. (**A**) Effect of coagulants on pH; (**B**) changes in zeta potential; (**C**) removal effects of coagulants on turbidity.

Zeta potential is one of the evaluation indices of the coagulation method's water treatment effect, showing the stability of colloids or suspended solids in a solution system. Coagulation and algae removal usually use the positively charged aluminum or iron hydrolyzed cations formed by the coagulant in water and the negative charge on the algae cells' surface to attract each other. Figure 2B shows the potential change in the surface of *G. catenatum* with different coagulants added. The potential of the four coagulants decreased first and then increased with the increase in the dosage. The lowest point of the potential appeared when the dosage was 20 mg/L. Among them, the rising trend of $FeCl_3$ was the most obvious. When the dosage was 50 mg/L, the potential was the highest at −6.1 mV, but the fluctuation range was also higher. The $Al_2(SO_4)_3$ group fluctuated slowly with the dosage increase, and the dosage potential at 20 mg/L was higher than other coagulant groups. The $Fe_2(SO_4)_3$ group's potential decreased the most, and at a high dose (90 mg/L), the potential exceeded the other coagulant groups. In the PAC group, after the dosage was higher than 20 mg/L, and with the increase in the dosage, the surface potential of the algae cells increased rapidly. The highest point appeared at 90 mg/L, with a potential of −5.3 mV (Figure 2B). The turbidity of the system reflects the sedimentation effect of algae cells, and different dosages of different coagulants have different effects on the removal of turbidity.

Figure 2C illustrates the dosage at 10 mg/L. The turbidity of the four retardants showed significant differences, among which the turbidity of $Fe_2(SO_4)_3$ was the lowest, followed by the turbidity of $Al_2(SO_4)_3$, $FeCl_3$, and PAC. That said, the removal rate was higher except for PAC; the other three coagulant groups' turbidity removal rates were all negative, thus increasing the system's turbidity. When the dosage was greater than 30 mg/L, the turbidity's changing trend was stable (Figure 2C). Comprehensive analysis of

the removal effect of the density, turbidity, and organic matter of *G. catenatum* when using the four coagulants PAC, $FeCl_3$, $Fe_2(SO_4)_3$, and $Al_2(SO_4)_3$ showed that PAC had the most robust ability to remove algae on *G. catenatum* and had a low re-solubilization rate. Large flocs made of a large number of flocculated algal cells were formed, and PAC proved to have a good removal effect on their turbidity and organic matter.

An optical microscope was used to observe the settled flocs to observe coagulant and dinoflagellate coagulation effects. Figure 3a–d show the coagulation effects of the four groups of coagulants on *G. catenatum*. The micrograph suggests that the algae cells in the flocs, formed by the coagulation of aluminum sulfate and algae cells, were not tight enough, and there were few algae cells fixed in the flocs; however, the cell morphology of *G. catenatum* was also observed, and the cell shape was still active. The floc's micrograph that settled on the bottom after the $FeCl_3$ group was added for 96 h (Figure 3b). It is also evident that ferric chloride has a good coagulation effect on *G. catenatum* because the flocs were large, and few algal cells were swimming outside the flocs. The algae cells in the flocs were many and dense, but the flocs were not tight. The swimming algae cells could easily break away from the flocs and return to the water body. The morphology of algae cells in the flocs was almost unchanged at 400×. A micrograph of the floc that settled to the bottom after the $Fe_2(SO_4)_3$ group, which was added for 96 h, is shown in Figure 3c.

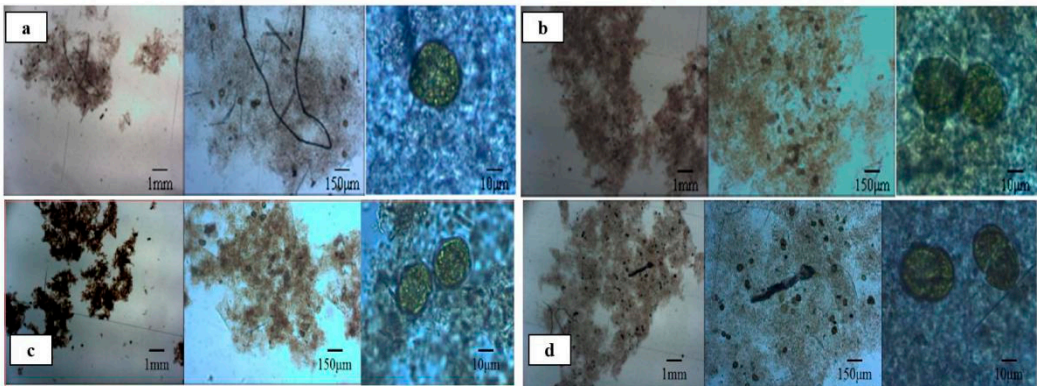

Figure 3. (**a**–**d**) Micrographs of flocs after sedimentation of (**a**) $Al_2(SO_4)_3$, (**b**) $FeCl_3$, (**c**) $Fe_2(SO_4)_3$, and (**d**) polyaluminum chloride (PAC) group for 96 h.

Clearly, the flocs were small and scattered, and algae cells were swimming outside the flocs. As the floc is small, it distributes the algae cells on the edge of the floc. As the algae cells swim, the algal cells may break free from the flocs, causing a "back-dissolution" phenomenon. A micrograph of a floc that settled to the bottom after PAC administration for 96 h is shown in Figure 3d. At 40×, we could see that the flocs formed by the flocculation and *G. catenatum* cells were more extensive, and there were fewer algal cells outside the flocs. Under the 100× microscope, we observed that the algae cells in the flocs were relatively dense, there were few algae cells at the edges of the flocs, and the coagulation effect was better. PAC has a minor effect on the cell morphology of *G. catenatum*, and the four coagulants have different effects on the removal of algae. Although the surface potential of the algal cells increased after PAC addition, the fluctuations were larger.

3.2. The Effect of Bacterial Fermentation Broth (Ba3) on the Algae Inhibition

Ba3 fermented broth from 1, 2, and 3 d was added to an algal concentration of 1200~1500 cells/mL at four dosage levels of 0.3, 0.7, 1.0, and 2.0% (*v/v*). The experimental results are displayed in Figure 4A. With the increase in the action time, the concentration of *G. catenatum* continued to decrease. After the second day of dosing, the concentration of algal cells decreased to below 500 cells/mL. The removal rates of all groups were at least 68.1%, and the highest one was 92.9%. On days 2–6, the concentration was stable. After adding Ba3 bacteria fermentation broth, compared with the control group, all concentrations of fermentation broth had a more obvious removal effect. From the results of adding

Ba3 bacteria 1-d fermentation broth, we observed that with the increase in the addition, the concentration of *G. catenatum* showed a downward trend. When the addition was 2.0%, the cell number reached the lowest value. The removal rate was as high as 82.1%. Ba3 2-d bacteria fermentation broth and 3-d fermentation broth also increased the algaecide effect with the dosage. The fermentation broth usage at different fermentation times also has a certain impact on the algaecide effect. The figure clearly shows that the effect of Ba3 bacteria 2-d fermentation broth was higher than that of the 1-d fermentation broth and 3-d fermentation broth but was not affected by the dosage or influence of time. Figure 4B shows the effect of removing algae from fermentation broth on turbidity. We designed this experiment with two groups—with and without algae—to observe the turbidity changes by adding bacteria fermentation liquid. The turbidity of the algal and algal-free groups increased with the addition of the fermentation broth. Among them, the turbidity of each dosage of Ba3 bacteria 2-d fermentation broth was slightly lower than that of the 1-d and 3-d fermentation broths. Except for the control group, the algae-containing group's turbidity and the non-algae-containing group in each experimental group differed, showing that after the bacterial fermentation broth destroyed the algal cells, a large amount of content was released, leading to a significant increase in turbidity.

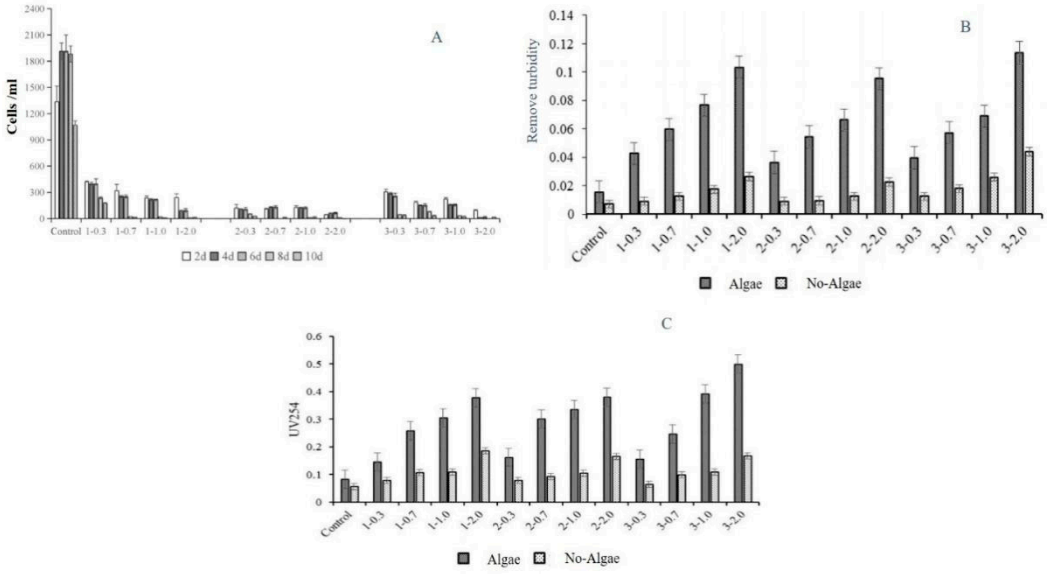

Figure 4. (**A**) Effect of fermentation broth on the growth of *G. catenatum;* (**B**) effect of algal removal from fermentation broth on turbidity; (**C**) effect of algal removal from fermentation broth on UV_{254}.

The bacterial fermentation broth consisted of a variety of active products secreted by the bacterial body. Therefore, the various organic substances present in the bacterial fermentation broth not only increased the turbidity of the water body but also increased the UV_{254} value of the water body. The algae cells ruptured, and the organics inside the cells flowed out. In short, the fermentation broth can destroy the integrity of algal cells, but the action time is longer. After the algae cells dissolve, the body's organic matter will be released, and the bacterial fermentation broth will maintain certain turbidity. As a result, the water phase's turbidity and organic content will increase after processing the bacterial fermentation broth (Figure 4C).

Figure 5 shows the influence of algal cell morphology. The surface of the algae cells of *G. catenatum* was smooth and complete before the bacterial fermentation broth treatment, with prominent horizontal grooves and nuclei (Figure 5A). After 12 h of Ba3 treatment (Figure 5B), the upper shell of algal cells had become transparent, and the nucleus was visible. The cell wall showed damage to a certain extent, but the cell membrane was still intact and no content flowed out. Over 24 h (Figure 5C), the algae cells' surfaces were loose and ruptured, and the cell membrane was damaged. At 36 h (Figure 5D), the cell

membrane was revealed to be severely damaged. Many granular materials of different sizes appeared around the cells. It is possible that after the cell membrane of the cell wall is ruptured, the contents of the cell overflow, and the algal cell's morphological structure becomes relatively blurred. After 96 h of observation, *G. catenatum* had lost its morphology entirely, and the algal cells gradually decomposed and ruptured into small particles that were almost unrecognizable (Figure 5E,F). From the analysis, the algicidal effect of Ba3 on *G. catenatum* is triggered via indirect algaecide activity. The bacteria secrete some active substances for algae cells to lyse the algae cells, achieving the algae-killing effect.

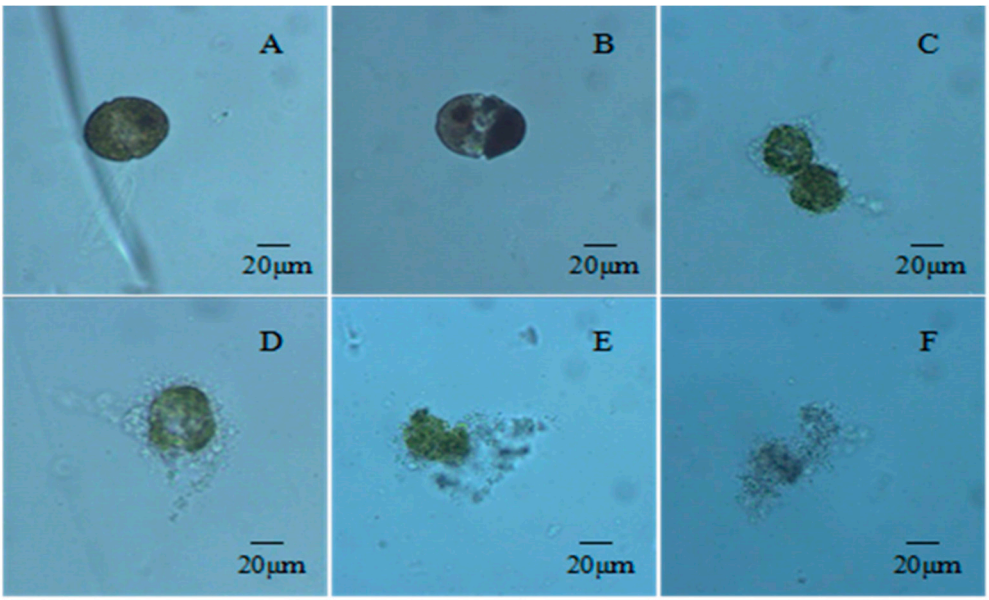

Figure 5. Effect of fermentation broth on *G. catenatum* ((**A**)—0 h; (**B**)—12 h; (**C**)—24 h; (**D**)—36 h; (**E**) and (**F**)—96 h).

3.3. The Effect of Combined Coagulant and Ba3 on the Algae Inhibition

In this study, the coagulant and Ba3 fermentation broth were combined to eliminate algal cells in order to increase the removal effects on *G. catenatum*. As shown in Figure 6A, the removal effect of the four coagulants combined with four concentrations of Ba3 broth on *G. catenatum* occurred at different times. The PAC and Ba3 fermented broth group had the best algae removal effect. The removal rate reached 100%, and the number of algal cells in the overlying water did not increase over time. The effect of other coagulants and algae cells combined with the algae inhibition method was lower than that of the PAC and Ba3 fermented broth group. Figure 6B shows that the group of $Al_2(SO_4)_3$, combined with the Ba3 fermented broth, caused the algae density to reach the highest value at 24 h; the removal rate of *G. catenatum* was 82.3%. Figure 6C shows that with the $Fe_2(SO_4)_3$ and Ba3 broth group, the removal of algal cells reached the highest rate at 80.7%. Figure 6D shows the $FeCl_3$ and Ba3 fermented broth group and the changing trend; all combinations reach the highest value at 24 h. Overall, the combination of PAC and Ba3 broth has the best algae inhibition effect, and when the volume ratio of bacterial fermentation broth is 0.3%, the removal rate can reach 100%. The combination method of algae suppression can improve algae suppression efficiency quickly and reduce the effect of algae cell re-dissolution. This method can be combined with the addition of the coagulant to act on the algae cells to form flocs and precipitate to the bottom; the bacterial fermentation broth also acts on the algae cells, which are gradually lysed under the stimulation of the active substance, and the exercise ability gradually decreases until the cells rupture.

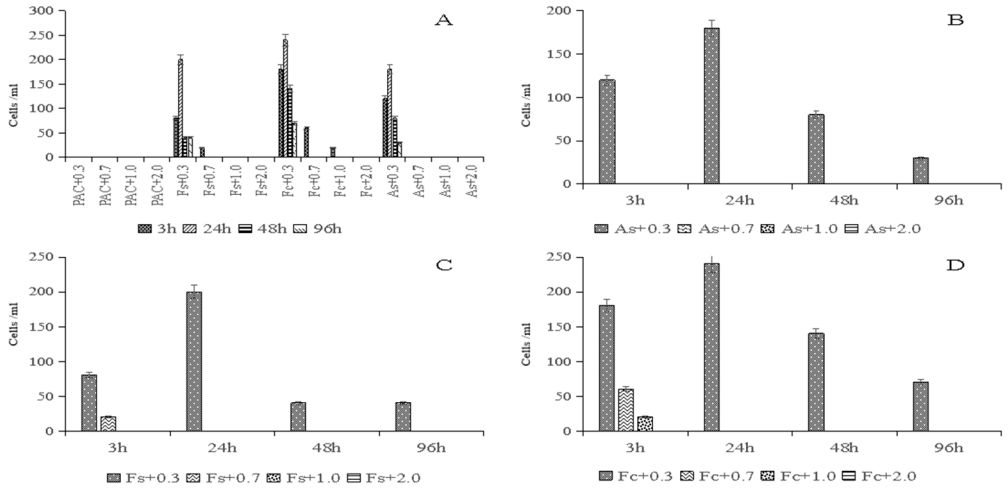

Figure 6. Effects of coagulant and fermentation broth on growth of *G. catenatum*. (**A**) Combination of four coagulants and fermentation broth to inhibit algae; (**B**) $Al_2(SO_4)_3$ + Ba3 broth; (**C**) $Fe_2(SO_4)_3$ + Ba3 broth; and (**D**) $FeCl_3$ + Ba3 broth.

Figure 7 shows the effect of the combination method of coagulant and Ba3 fermented broth on UV_{254}. With the increase in Ba3 broth dosage, each group's organic removal effect showed a downward trend. In the PAC-combined Ba3 broth group, when the dosage of the bacterial fermentation broth was 0.3% (*v*/*v*), the maximum removal rate was 41.0%, and the lowest removal rate was 1.0%. This removal rate confirms that when the dosage of Ba3 broth is higher than 1.0%, the content of organic matter in water is higher than the flocculation effect of PAC. The removal rate of $Al_2(SO_4)_3$-combined Ba3 broth group was negative when the dosage was higher than 0.3%, demonstrating that the removal effect of $Al_2(SO_4)_3$ on the organic matter is lower than that of PAC. In the fermented $Fe_2(SO_4)_3$- and $FeCl_3$-combined Ba3 broth groups, the two groups' removal rates were negative for different bacterial fermentation broth dosages. Therefore, the effects of the four coagulants combined with different concentrations of Ba3 broth on the removal of organic matter followed the order of PAC + Ba3 fermented broth > $Al_2(SO_4)_3$ + Ba3 broth > $Fe_2(SO_4)_3$ + Ba3 broth > $FeCl_3$ + Ba3 fermented broth.

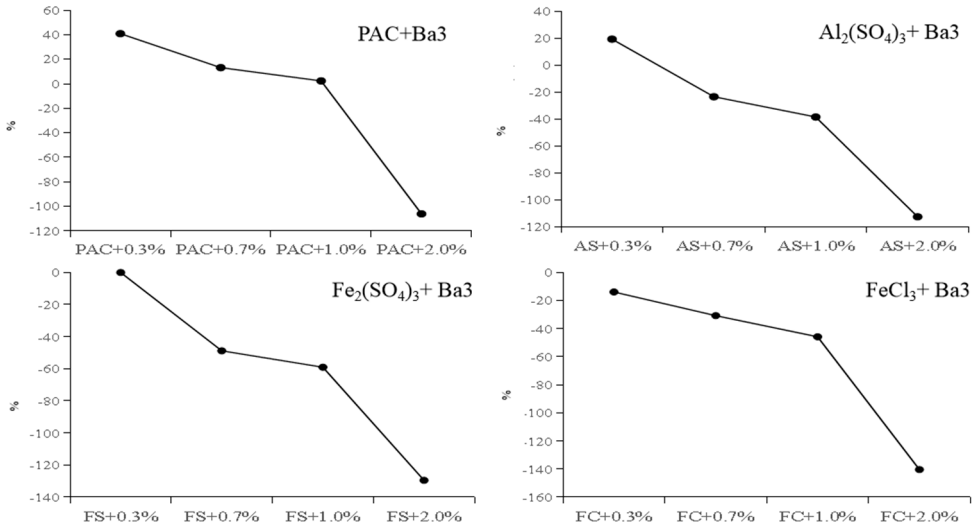

Figure 7. Removal effects on UV_{254} by the combination of coagulants and fermentation broth.

Figure 8 shows the effect of the combination method of coagulant and Ba3 broth on turbidity. Each group's turbidity removal effect showed a downward trend with the increase in bacterial fermentation broth dosage. Each group had the highest removal rate

when the bacterial fermentation broth was added at 0.3%. The removal rates of turbidity in the fermentation broth group of PAC combination Ba3 broth were between 46.3 and 89.1%; the removal rates of turbidity in the fermentation broth group of $Al_2(SO_4)_3$ combination bacteria were between 55.8 and 83.7%; with $Fe_2(SO_4)_3$, the turbidity removal rate of the combined Ba3 broth group was between 58.2 and 89.1%; the turbidity removal rates of the $FeCl_3$-combined Ba3 broth group were between 57.8 and 88.4%.

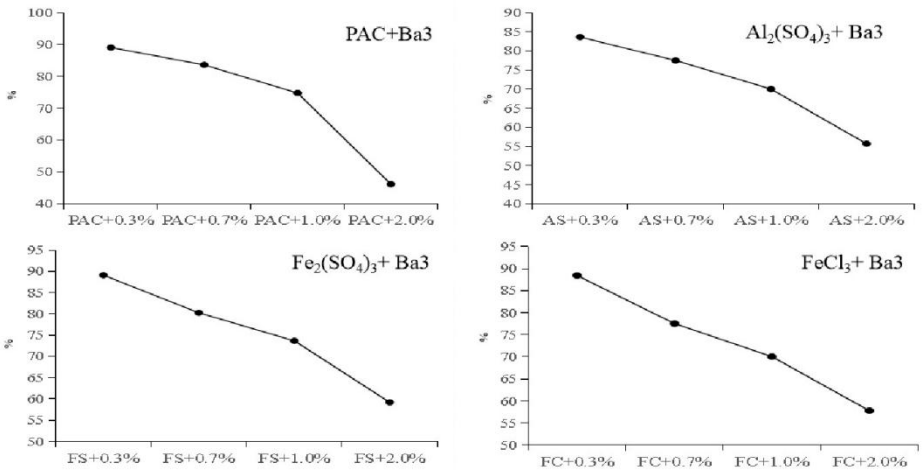

Figure 8. Removal effects on turbidity by the combination of coagulants and fermentation broth.

3.4. The Effect of Combined Coagulant and Ba3 on Cyst Germination Inhibition

The sediment used for algae germination in the simulated sediment was from the Quanzhou section where the red tide of *G. catenatum* had occurred, and the total abundance of phytoplankton in the sediment was 8.04×10^2 cells/g, mainly diatoms and dinoflagellate cysts. The in situ experiment sediment samples were taken at 5, 10, and 15 d to observe the phytoplankton species and abundance in the water phase after germination (Figure 9). The sediment's overlying water was L1 medium with no algae, and the proportion of primary algae diatoms germinated in the three tests was higher. After five days of culture, the abundance of algae in the overlying water of the in situ sediment group (I-1) was approximately 20.3 cells/mL, in which the diatoms reached 17.2 cells/mL. Only an insignificant amount of dinoflagellates germinated. In the group (I-2) with the PAC group, the algae's germination rate was low; only diatoms emerged, and the inhibition rate reached over 70%. However, the phytoplankton abundance of the PAC group (I-2) was 32.5 cells/mL, indicating that mainly diatoms and dinoflagellates still existed. The inhibition rate of this group decreased to 57.2%. After 15 days of culture, the algal abundance of the in situ sediments group was slightly lower than that of the 10-d culture, and the inhibition rate was 61.3%. In the whole experiment period, no algal cell germination was observed in the group of PAC and Ba3 fermented broth (I-3).

In the in situ sediment with added *G. catenatum* cyst (II-1) group culture, diatoms were dominant in the overlying water and we detected only a few dinoflagellates. After 10 d of cultivation, the total abundance of germinated phytoplankton reached 90.5 cells/mL, and the density of *G. catenatum* was significantly higher than that in group I-1, which reached 18.0 cells/mL; the addition of PAC (II-2), compared with the II-1 group, has a particular inhibitory effect on algal cell germination, especially diatoms. After 15 days of cultivation, the algae density in the overlying water was slightly lower than that after 10 days of cultivation, which may be related to the content of nutrients in the water. With the increase in cultivation time, the water's nutrients were gradually consumed, which ultimately decreased the water's nutrients. The PAC-combined Ba3 fermented broth group (II-3) was only detected on the 10th day with an insignificant amount of diatoms appearing, with a removal rate of over 98% (Figure 9). The PAC had a better inhibitory effect on germination in the sediment; there was almost no algae germination. This lack of germination may be

because the active substances in the bacterial fermentation broth have a good destruction effect on the algae cells, the experimental water body has a weak exchange flow ability, and the concentration of the bacterial fermentation broth in the water body remains almost unchanged; therefore, the provenance in the sediment will likely be affected by the bacteria to a certain extent; the destruction of active substances will not occur.

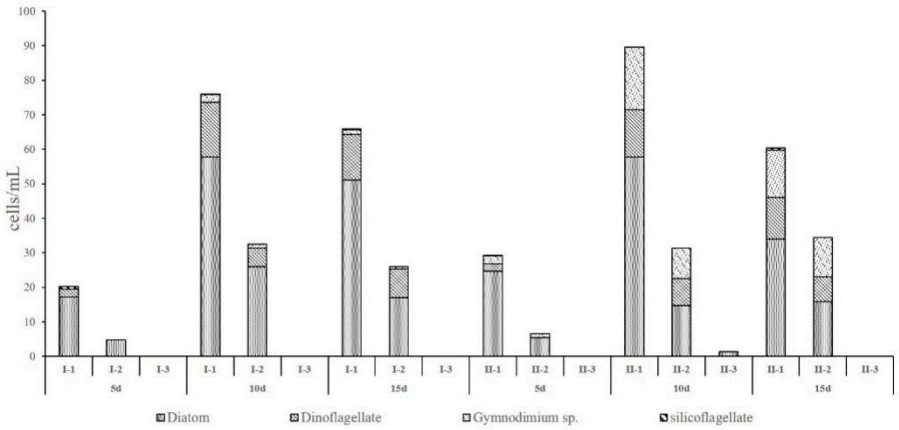

Figure 9. Effect of different algal inhibition method on microcapsule germination in sediment (I) and In situ sediment added with *G. catenatum* cyst (II).

After the L1 medium was added to the sediment, the nitrogen and phosphorus elements in water were essential for the phytoplankton's growth. The initial DIP concentration was 0.13 mg/L, the DIN concentration was 5.333 mg/L, of which the nitrite concentration was 0.042 mg/L, and the ammonium salt concentration was 0.007 mg/L of nitrate. The concentration was 5.283 mg/L. Figure 10 displays the effects of different algae suppression methods on algae cyst germination in the in situ sediments. After five days of PAC administration and PAC-combined Ba3 fermented broth, they dissolved inorganic phosphorus in the water body. The removal effect of the PAC group (I-2) on DIP reached 86.4%. On the 10th day, the removal rate was 87.3%; with the extension of the cultivation time, the removal effect showed a tendency to decrease. On the 15th day, the DIP concentration was 0.011 mg/L and the removal rate dropped to 77.4%. The PAC-combined Ba3 group (I-3) also had a beneficial removal effect on DIP, and the removal effect was slightly higher than that of the PAC group; there was no reduction in removal rate on the 15th day (Figure 10A). Each control group's removal affected the in situ sediment + *G. catenatum* cyst (group II) germination group. Within 5-10 days of the PAC group's control, the removal rate was higher than 85% on the 5th day; on the 15th day, the DIP concentration was 0.009 mg/L, and the removal rate dropped to 81.3%. Compared with group I-2, the DIP content was lower in group II-2, which may consume DIP due to the higher abundance of algae. PAC's removal rate in the combined Ba3 group broth group (II-3) reached more than 87% (Figure 10B).

Figure 10C, D show the effects of different algae inhibitors on dissolved inorganic nitrogen in water and in the in situ sediment germination group. The dissolved inorganic nitrogen in both groups showed a downward trend with time. The cultivation did not transform in each group until the 5th day, but the I-3 group (PAC + Ba3 broth) inorganic nitrogen concentration is higher than that of group I-2 (PAC); when cultured to day 10, group I-3 shows a higher value than those of groups I-2 and I-1. On the 15th day of cultivation, the difference between the groups was more prominent. The dissolved inorganic nitrogen concentration in group I-3 was 2.30 mg/L, while the dissolved inorganic nitrogen concentration in group I-1 was 1.34 mg/L. The lower of the two groups was 0.79 mg/L. Compared with the initial inorganic nitrogen concentration, the decrease rate of inorganic nitrogen was 59.0~85.2% in the entire cultivation cycle under the control of PAC. Figure 10D shows, in the germination group of in situ sediments + *G. catenatum* cysts, that the three groups all

have the same tendency of change, and the concentration of dissolved inorganic nitrogen shows a tendency to decrease over time. The concentration of dissolved inorganic nitrogen in the three groups at different incubation times was in the order II-1 > II-3 > II-2 during the entire cultivation cycle. Regarding the decrease rate of group II-2 compared with the initial inorganic nitrogen concentration, the range was between 61.6 and 80.8%, and the removal rate of group II-3 was between 56.6 and 77.8%. Using PAC and PAC combined with Ba3 broth had no noticeable effect on removing dissolved inorganic nitrogen in the water. Compared with group II-2, the addition of bacterial fermentation broth in II-3 increased the content of dissolved inorganic nitrogen in the water body, which was mainly related to the nitrogen-containing compounds in the bacteria's fermentation product. The PAC-combined Ba3 broth group had a beneficial removal effect on the soluble inorganic phosphorus, and the removal rate reached over 87%. The active bacteria substance has nitrogen-containing compounds; therefore, this group's soluble inorganic nitrogen content is higher than that of PAC.

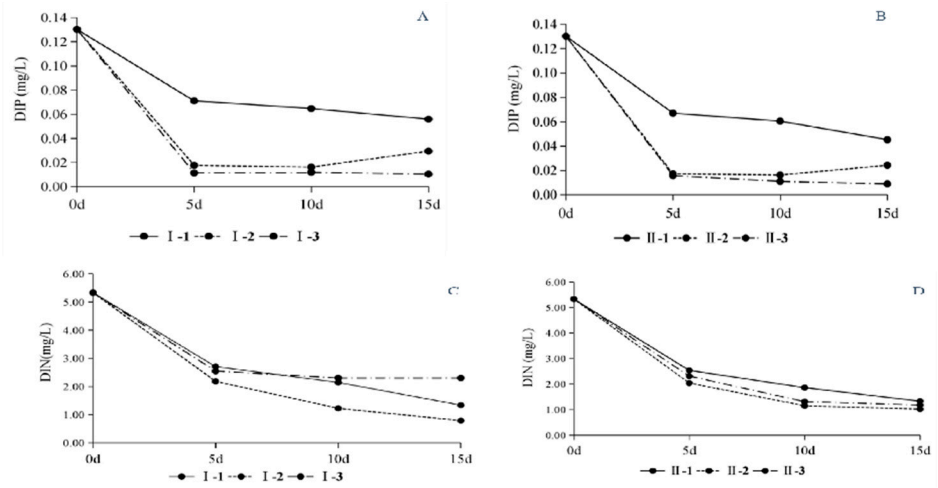

Figure 10. Effect of different algal inhibition methods on (**A**) dissolved inorganic phosphorus (DIP) and (**C**) dissolved inorganic nitrogen (DIN) in sediment; and effect of different algal inhibition methods on (**B**) DIP and (**D**) DIN in sediment (added *G. catenatum* cyst).

4. Discussion

Our experimental study results revealed that all four coagulant chemicals, PAC, $Al_2(SO_4)_3$, $Fe_2(SO_4)_3$, and $FeCl_3$, exhibited a high removal efficiency against *G. catenatum*, with some differences. However, PAC has the best removal effect, with a low re-solubilization rate, large floc formation, and the most considerable amount of flocculated algal cells. It also has a beneficial removal effect on turbidity and organic matter. Recent studies showed that the high removal efficiencies with five kinds of coagulants are comparable to the results of Liu Lijuan et al. for the control of a lake containing algae bloom [16]. They concluded that PAC has a more impressive algae removal effect by changing the dosage and pH conditions. The algae removal mechanism of polyaluminum salts showed that PAC and algae cells in the water first underwent adsorption and an electrical neutralization reaction, and a bridging network was trapped, so that the flocs were more extensive and, therefore, more prone to settle [19]. Moreover, a pilot test of enhanced coagulation of raw water in the Yangtze River with PAC showed that PAC had a beneficial coagulation effect on the Yangtze River water, forming large flocs and a rapid settling speed [20].

Previous studies have shown that specific bacterial populations can inhibit HAB species' growth through microbial algae suppression into direct algae suppression and indirect algae suppression [21,22]. In one of these studies, Ba3 (*Bacillus* fermentation broth) had a high algae inhibition effect, reaching over 90%. To date, several publications have proven that *Bacillus* sp. can inhibit the growth of harmful algal blooms [17,23]. Compared with other algaecide bacteria isolated from aquatic water, *Bacillus* showed similar or more

potent algicidal activity against algae [24]. Zhao et al. [25] isolated four algicidal metabolites from a fermentation broth of *Bacillus* B1 strain against *Phaeocystis globosa*, and all metabolites had a strong alga dissolving effect. In this study, a Ba3 *Bacillus* strain of fermentation broth was used to influence algal growth, and the results showed that the 2-d fermentation broth had the best removal effect on *G catenatum*. Under the microscope, we observed that the cell wall gradually loses its ability to move under the action of the Ba3 broth, the algae cells gradually become transparent, and the contents of the cell overflow due to rupture, though there is no complete algal cell morphology. In terms of their biological safety, anti-compounds are biodegradable. Moreover, when used to control blooms, they appear to be harmless to the environment [26]. These results strongly bolster the claim that Ba3 has potential applications in controlling algae outbreaks.

The coagulant removes algae quickly, and it can coagulate with algae cells to form flocs and settle to the bottom immediately after addition, but the coagulant algae suppression will have a certain back-dissolving phenomenon. The algae killing effect of the fermentation broth is thorough, but the action time is relatively long. However, previous research revealed that PAC combined with algae-lysing bacteria has a high inhibitory effect on *Microcytis aeruginosa* and nutrients in the water [27]. Furthermore, Wang et al. [28] studied the inhibition of the growth of *Scrippsiella trochoidea* by combining algae-suppressing bacteria with two modified clays. The results show that the combined algae suppression method can improve the algae suppression effect, increase the algae suppression time, and reduce algae cells' rebound effect. In this study, the combination of coagulants and Ba3 fermented broth was used to suppress algae and remove algae cells in a short time while killing algae cells. The results of the four coagulants combined with *Bacillus* Ba3 fermentation broth, when combined with algae inhibition, showed that the combination of PAC and Ba3 fermented broth had the best effect on inhibiting *G. catenatum* with an inhibition rate of 100% and no rebound. The bacterial fermentation broth is indeed yellow and contains many organic substances. An increased dosage of bacterial fermentation broth leads to increased water turbidity and organic matter content; when the Ba3 broth dosage in the PAC-combined bacterial fermentation broth group was 0.3%, it had the highest removal rate of turbidity and organic matter in water.

Although the red tide community's increase may be significant and alarming, this issue should not be linked to on-site production. Researchers have also proposed other HABs from resting cysts germinating from the bottom sediments, which seriously affect many aspects of the red tide phenomenon [29]. Cysts are also particularly efficacious for community spread. They enable species to survive under adverse conditions, and because their development generally involves sexuality, they promote genetic recombination [30]. In addition, the high abundance of cysts in the sediment may reflect the recent blooming of these species in the area. According to previous studies, many resting cysts will be produced by the end of the blooming cycle. The formation of cysts is one of the predominant factors in bloom termination [31,32]. Several researchers believe that the vegetative cell inoculum size from cyst germination is critical to the beginning of blooming. However, the final bloom size cannot measure the effect of altering cell growth or the aggregation of complex natural conditions because unpredictable variables control these effects. Therefore, it is necessary to control the sediment cyst before germination. Nevertheless, there have only been a few studies on the coagulant effect with combine bacterial fermented broth on cyst formation and germination to control HAB species.

We divided the germination experiment into two groups: in situ sediments and in situ sediments after adding *G. catenatum* cysts. In this study, treatment with the PAC-combined Ba3 fermented broth group significantly removed over 90% of the *G. catenatum* vegetative cells. Recent research has also found that nutrient changes caused by modifying clay can be re-released from the algae matrix into the water, which may contribute to the formation of resting cysts [33]. These results offer a contrast to this study where the PAC-combined Ba3 broth group had a good removal effect on the soluble inorganic phosphorus, and the removal rate reached over 87%. The active bacteria substance has nitrogen-containing

compounds; therefore, the content of soluble inorganic nitrogen in this group was higher than that of the PAC group. These treatments not only were effective for removing the vegetative cells but also lowered the nutrients level.

5. Conclusions

In conclusion, our results show that the feasibility of using appropriate concentrations of PAC combined with Ba3 fermented broth is potentially useful for controlling *G. catenatum* blooms. The effect of bacterial fermentation broth on killing algae is complete, but the time needed for effective action is longer. The active substances in the fermentation broth act on the algae cells to lyse them. The four coagulants combined with the bacterial fermentation broth group had an obvious inhibitory effect on *G. catenatum*. Through the comprehensive analysis of the removal effects of algal cells, turbidity, and organic matter, it was found that after the action of the polyaluminum chloride (50 mg/L) combined with the bacterial fermentation broth (0.3%, *v/v*) for 3 h, the algal inhibition rate was 100%, and the algal cells did not rebound. The inhibition rate of the bacterial fermentation broth and polyaluminum chloride group was more than 98%. Our findings will integrate well into the future studies of controlling toxic dinoflagellate cysts in the benthic environment. Our research also shows that dinoflagellate organisms use nitrogen and phosphorus cues to determine when vegetative growth occurs. Understanding the environmental cues related to algae dormancy is essential for the understanding and management of HABs in aquatic ecosystems.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/jmse9040395/s1, Figure S1—A schematic design of the study on the proliferation regulation of *Bacillus* sp. Fermentation broth combined with coagulant on *G. catenatum*, Table S1—A schematic design of the study on the control of cyst germination of *Gymnodinium catenum* by the combination of coagulant and fermentation broth

Author Contributions: Conceptualization, Y.S. and B.P.B.; methodology, B.P.B. and W.Z.; validation, Y.S.; formal analysis, B.P.B. and W.Z.; investigation, Y.S.; data curation, B.P.B., H.L. and W.Z; writing—original draft preparation, B.P.B. and W.Y.; writing—review and editing, Y.S., B.P.B. and H.Y.; supervision, Y.S.; project administration, Y.S.; funding acquisition, Y.S. All authors have read and agreed to the published version of the manuscript.

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