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Loading Effects of Aminoclays in Co-Culture of Two Cyanobacterial *Microcystis* and *Anabaena* Species as an Algicidal Role

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Abstract: In recent decades, harmful algal blooms (HABs) have been significantly affecting environments, aquatic ecosystems, and human health, as well as damaging economies, especially near rivers and lakes, and in coastal regions. *Microcystis* and *Anabaena* are two genera of harmful cyanobacteria that will often predominate during toxic microalgal blooms. In this study, we employ a method for control and mitigation of HABs by microalgal cell instability using different types of aminoclays (ACs). Allelopathic interactions between the two strains of algae are studied in mono-culture, co-culture, and filtrated cell-free medium in the presence of the ACs. The growth of the *Anabaena* strain is significantly reduced by the cyanobacterial strains in the co-culture media, and both are significantly affected by the Acs'-enhanced algicidal activity. *Anabaena* sp. KVSF7 shows higher sensitivity against the ACs than does *Microcystis* sp. KW. In this way, the algicidal activity of ACs is harnessed, the effects of which are in the order of aluminum aminoclay (AlAC) > magnesium aminoclay (MgAC) > calcium aminoclay (CaAC). The ammonium sites in the ACs carry positive charges to induce instability of HABs along with the electrostatic attraction between algal cells and AC. Therefore, the utilization of the algicidal activity of the ACs can effectively reduce HABs, especially on cyanobacterial blooms.

Keywords: aminoclays; cyanobacteria; co-culture; harmful algal blooms; allelopathic effects; algicidal activity

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1. Introduction

Over the last few decades, harmful algal blooms (HABs) formed by macroalgae and microalgae species have disrupted ecosystems and affected local organisms. "Tides" of cyanobacterial blooms disrupt ecosystems, often leading to the mass death of affected organisms. They produce toxins, reactive oxygen species (ROS), which compete with organisms by hindering their respiratory systems and consuming and depleting oxygen and nutrients [1–5]. HABs not only are harmful to organisms such as fish, birds, and aquatic animals, but also cause human illness and death in cases of high exposure. The problem posed by cyanobacterial blooms is becoming more frequent and urgent in rivers and lakes, especially in coastal areas. They cause enormous damage to countries' economies,

which were more than 1 billion dollars and 1 million dollars per year in Japan and Korea, respectively [1,6–9].

Therefore, controlling and eradicating HABs through the use of algicides is of interest. Using chemicals such as $CuSO_4$ or biological approaches such as algicidal activity of prodigiosin [10] and lactic acid bacteria [11] have been utilized to consume these blooming species [12–14]. In addition, the mechanical treatment of HABs with clay is an alternative method offering noticeable advantages such as non-toxicity and local alternative sources [15]. It creates flocculation by clay-algae "blocks" formation and deposition on the floor of an aquatic environment [6]. South Korea's coastal farms also use clay to treat HABs. Although this is considered to be successful, large HABs have escaped from clay dispersion [1,16–19]. This method also requires a large amount of clay and incurs high costs, and moreover, cell breakdown, is only moderate, the result being that it is not possible to entirely prevent HABs release, all of which factors in limiting the applicability [17,19,20].

In recent years, synthetic aminoclays (ACs), developed with unique properties to mimic natural clay, is a complex with nuclei consisting of metal cations (e.g., Ca^{2+}, Mg^{2+}, Al^{3+}, and Zn^{2+}) sandwiched between two tetrahedral structures with connections to amino functional groups (with a precursor of 3-aminopropyltriethoxysilane, APTES) through covalent bonds (Figure S1) [21,22]. The active sites ($-NH_3^+$) at the functional end sites create delaminated structures of clay sheets when dispersed in a water environment. Selected metal cation cores impact hydrodynamic size distribution in a one-pot sol-gel formation. AC was developed and applied to the formation of structures that encapsulate biological bodies, substances/materials such as enzymes, DNA, as well as other active organic substances [21–25]. A recent study identified and evaluated the antibacterial properties and low toxicity of AC to animal cells of magnesium aminoclay (MgAC) and calcium aminoclay (CaAC) [22]. Especially using MgAC, it has been investigated with selective algicidal activity of redtides in seawater [19] and both in increase of cell size and lipid contents in green microalgal culturing systems [26,27].

In the present study, the algicidal activity of AC was assessed on the basis of monocultures and co-cultures of two common toxic cyanobacterial blooms *Microcystis* and *Anabaena*, as well as on cell-free media. The allelopathic interactions between the above microalgal strains and AC and with the cell-free medium also were observed. Therefrom, additional observations and assessments of the three AC types-utility for HAB control efficacies were derived.

2. Materials and Methods

2.1. Materials

Magnesium chloride hexahydrate ($MgCl_2 \bullet 6H_2O$; 203.30 g/mol, 98%) and calcium chloride dihydrate ($CaCl_2 \bullet 2H_2O$; 147.01 g/mol, 99%) were received from Junsei Chemical Co., Ltd. (Tokyo, Japan). Aluminum chloride hexahydrate ($AlCl_3 \bullet 6H_2O$; 241.43 g/mol, 99%), 3-aminopropyltriethoxysilane (APTES; 221.37 g/mol, ≥98%), dimethyl sulfoxide (DMSO; 78.13 g/mol, ≥99.9%), and 2′, 7′-dichlorodihydrofluorescein diacetate (DCFDA; 487.29 g/mol, ≥97%) were obtained from Merck KGaA (Darmstadt, Germany). Hexane (86.18 g/mol, ≥95%) and methanol (32.04 g/mol, ≥99.9%) were obtained from Junsei Chemical Co. Ltd. (ACS reagent, Tokyo, Japan). Ethanol (46.07 g/mol, ≥94.5%) was acquired from Samchun Pure Chemicals (Pyungtack, Korea). Unless otherwise mentioned, all of the chemicals were used without further treatment. Double-distilled deionized water (DI water; resistance > 18 mΩ) was also employed in all of the experiments.

2.2. Synthesis of Aminoclays (ACs)

The aminoclays (ACs) were synthesized, according to the standard protocol in the literature [28]. Specifically, APTES (13 mL) was poured into ethanol solutions (200 mL), each containing one metal chloride ($MgCl_2 \bullet 6H_2O$; 8.4 g, or $CaCl_2 \bullet 2H_2O$; 8.4 g or $AlCl_3 \bullet 6H_2O$; 8.4 g) and stirred for over 6 h. The products, obtained by centrifugation at 3134 G-force for 10 min, were washed once with bulk ethanol (100 mL), and the solvents finally were

evaporated solvents under 50 °C ambient temperature in an oven. Each prepared ACs, according to the cationic metal forming the backbone and APTES, was labeled as follows: MgAC, CaAC, and AlAC, respectively. The molar ratio of APTES to cationic metal was set at approximately 2.0. The detailed physiochemical characteristics of the AC are available in the literature [21,29]. Figure S1 shows the ideal AC structure.

2.3. Mono-Culture Experiments on Cyanobacterial Strains in Presence of Aminoclay (AC)

Microcystis sp. KW (denoted as M.S.) and *Anabaena* sp. KVSF7 (denoted as A.S.) were obtained from the Korea Research Institute of Bioscience and Biotechnology (KRIBB, Daejeon, Korea). Two cyanobacterial seeds were cultured with 300 mL of a BG-11 medium (original recipe) in a 500 mL glass flask, along with 2.0% CO_2 (v/v) bubbling (flow rate, 150 mL/min; light intensity, 100 µmol/($m^2 \times s$), and 125 rpm mixing). For MgAC-treated cultivation, M.S. and A.S. cultures (with a cell density of 5×10^5 cells/mL) were grown in a BG-11 medium and subjected to the above conditions, but with the addition of AC in different concentrations (0, 0.01, 0.02, 0.05, 0.1, 0.5, and 1.0 g/L). The supernatant in samples was taken periodically to monitor the optical density (OD) values. The sampling was selected from the middle site along with the glass flask.

2.4. Co-Culture Experiments on Cyanobacterial Strains in Presence of Aminoclay (AC)

M.S. was co-cultured with A.S. in 500 mL of BG-11 culture medium contained in 1 L Erlenmeyer flasks for 6 days. For AC-treated cultivation, the co-cultures were subjected to the above conditions, with addition of each type of AC (MgAC, CaAC, or AlAC) over a defined concentration range (0.01, 0.05, 0.1, 0.5, and 1.0 g/L) (Table S1) to find aggregation, inhibition or death of microalgal cells. Previous studies have suggested that growth dominance is influenced by the initial cell density, which would mean that species with higher cell densities are advantageous [30,31]. Therefore, we used a 1:1 biovolume ratio when co-cultivating the two cyanobacterial strains, with each species at 2.0×10^6 µm^3/mL. Cultures containing only one cyanobacterial strain were treated and observed as control samples. Samples are taken daily for cell-growth measurement and other tests.

2.5. Effect of Microcystis Cell (M.S.)-Free Medium Containing Aminoclay (AC) on Anabaena sp. KVSF7 (A.S.)

The cell-free medium was adjusted for nutrients (N and P) to balance it with the fresh BG-11 medium in order to ensure that the algae were not nutritionally restricted. A total of 300 mL of M.S.-free medium was prepared and added to a 500 mL flask with an initial A.S. cell density of 5 × 105 cells/mL (Table S2). CaAC was added to the above culture medium in increasing doses (0.01, 0.05, 0.1, 0.25, and 0.5 g/L). A control sample from the BG-11 culture medium was taken as well. The culture experiments were conducted over the course of 6 days. Samples were collected daily for subsequent tests.

2.6. Data Collection

The OD values were monitored at 680 nm by UV-VIS spectrophotometry (Optizen 2120 UV, Mecasys Co., Daejeon, Korea). A pH meter (Thermo Fisher Scientific Korea Ltd., Seoul, Korea) was used to measure pH. For cell counting of the cultures, *Microcystis* sp. KW was stabilized with Lugol's solution and counted using the Hausser Scientific hemocytometer counting chamber [32]. Meanwhile, counting of *Anabaena* filaments was performed with the Sedgwick-Rafter counting chamber, which allows for accurate quantification of fibrous cell density without cell/fiber layering. Sonication was employed to separate the *Anabaena* and *Microcystis* cells preparatory to counting. The measurement of this round and fibrous cell size and biovolume was performed according to Hillebrand et al. (1999). All of the test samples are itemized to at least 200 cells. Cell imaging was conducted by bright optical microscopy (Leica Microsystems, Heerbrugg, Switzerland). Reactive oxygen species (ROS) assays were performed following the method prescribed in the literature [33]. The zeta potentials were measured by a Zetasizer Nano-ZS particle analyzer (Malvern Panalytical, Malvern, UK). The structural confirmation of the ACs also followed the literature [21].

2.7. Statistical Tests

All of the experiments were conducted in triplicate. The results were calculated based on the average value of three independent iterations. The standard errors were expressed by an error bar for each condition. To assess the difference between the sample means, one-way ANOVA was used in the analysis of variance. A $p < 0.05$ value was considered to represent significant difference.

3. Results

3.1. Cell Growth of Mono-Culture of Cyanobacterial Strains in Presence of Aminoclay (AC)

M.S. and A.S. were mono-cultured separately under the conditions specified in Section 2.3 and in the presence of increasing concentrations of MgAC (from 0.01 to 1.0 g/L for 14 days). Two cyanobacterial cell growths in terms of optical density (OD) at A_{680}, under different AC concentrations, were plotted (Figure 1). The OD values of the M.S. culture increased with the increase in utilized AC concentrations. Cells cultured at low concentrations (at 0.01—0.02 g/L) showed similar or slightly higher numbers relative to the control case. Cell growth in the case of MgAC addition at 0.05 g/L was the highest. Overall, low dosages (0.01–0.1 g/L) of MgAC resulted in higher cell growth rates and OD values than the controlled culture grown without MgAC (Figure 1). However, at the beginning of day 4, microalgal growth was obstructed by the presence of MgAC at high concentrations (0.5–1.0 g/L). The inhibition effects of MgAC on the growth of A.S. were shown after 14 days of exposure at all tested dosages. Growth inhibition was significantly increased with the increase of MgAC concentration. With increasing concentrations of AC (0.01, 0.02, 0.05, 0.1, 0.5, and 1.0 g/L), reduced A.S. cell growth, for all treatments, was observed. High applied concentrations (0.05–1.0 g/L) significantly affected A.S. growth relative to the other concentrations ($p < 0.05$).

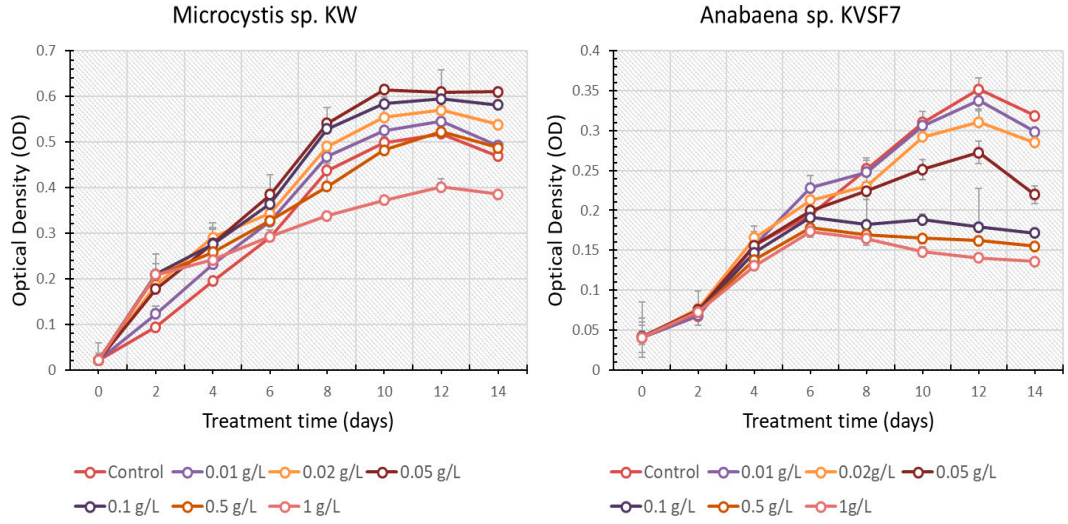

Figure 1. OD values at 680 nm of M.S. culture (**A**) and A.S. culture (**B**) where MgAC concentrations (0, 0.01, 0.02, 0.05, 0.1, 0.5, and 1.0 g/L) are treated in culturing for 0, 2, 4, 6, 8, 10, 12, and 14 days. The standard deviations of the mean ($n = 3$) are represented by the error bars. A $p < 0.05$ value is considered to represent significant difference.

As shown in Figure 1, the OD values of M.S. and A.S. cells, as compared with the control unit of each mono-culture, increased gradually from the beginning of the experiment ($A_{680;\ day\ 0}$: 0.0206) and reached the maximum value at the end of the experiment ($A_{680;\ day\ 14}$: 0.4690). The exposure of both cyanobacterial species to MgAC resulted in apparent differences in cell number between the controls and treated experiments ($p < 0.05$). The growth inhibition of the A.S. culture, according to the AC concentrations, was higher than that of the M.S. culture. The AC utilization at dosages of 1.0 g/L resulted in M.S.- and A.S.-inhibition efficiencies of 22.59 and 57.38% on day 14, respectively.

3.2. Cell Growth of Co-Culture of Two Cyanobacterial Strains in the Presence of Aminoclay (AC)

In nearly all of the mixed culture experiments, the mixture of *Microcystis* and AC strongly inhibited the growth of *Anabaena*, whereas the effects of the mixture of *Anabaena* and AC on *Microcystis* were trivial. In the case of the absence of AC, M.S. still individually showed an inhibition effect on A.S. growth. The biovolume inhibition of M.S. biomass after exposure to the mixtures of A.S. and AC (MgAC, CaAC, and AlAC) ranged from −23.03 to 27.42 for MgAC, −17.42 to 21.25 for CaAC, and 10.12 to 31.21% for AlAC (Figure 2A–C). There was greater and significantly enhanced inhibition of almost all of the A.S. cultures by the *Microcystis*/AC (MgAC, CaAC, and AlAC): 35.54–88.67%, 26.50–78.45%, 40.28–95.53%, respectively ($p < 0.05$; Figure 2D–F). Relative to the monoculture controls, the more sensitive strain to the presence of the "system" of microalgae-AC in co-culture model was A.S., which suffered $\gtrsim$80% inhibition of its biovolume by the end of the experiment (day).

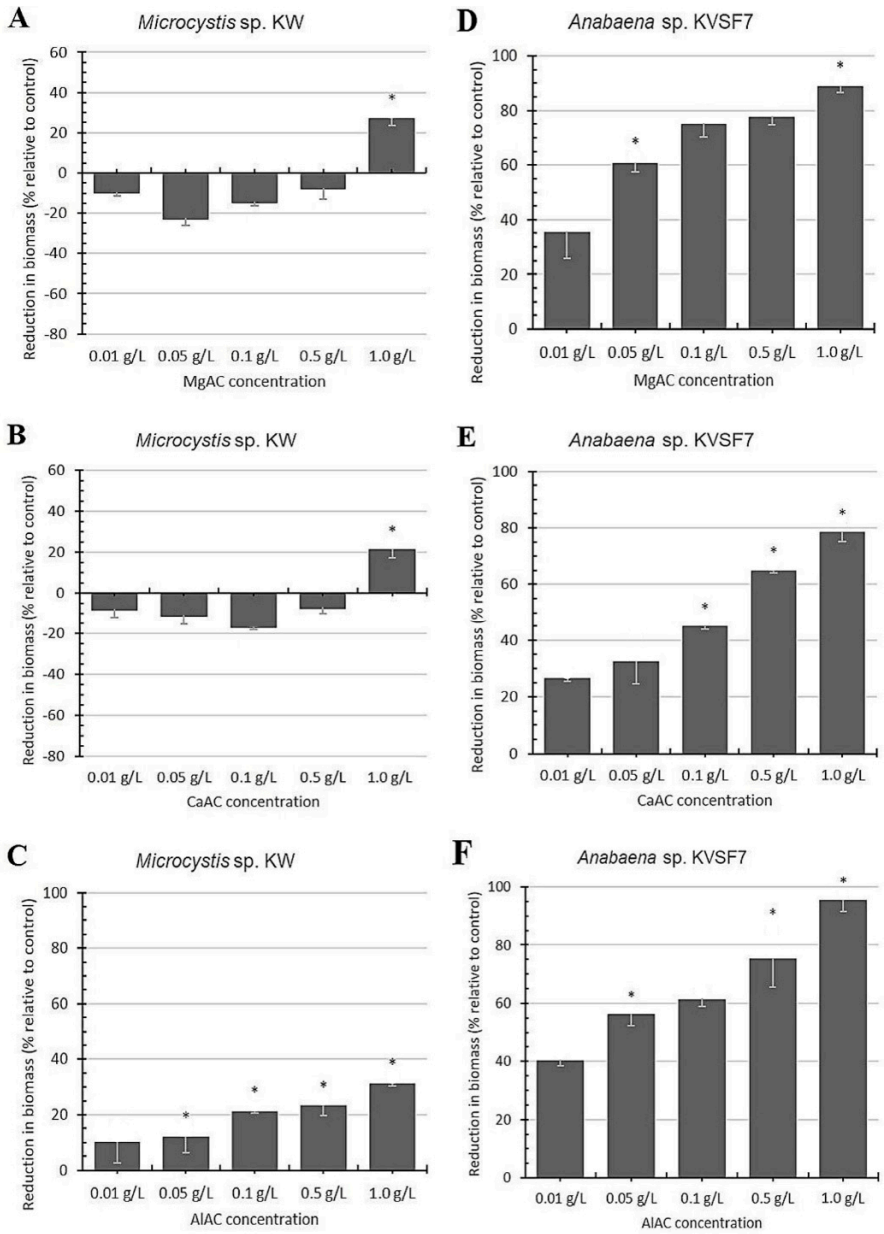

Figure 2. Reduction in biomass of M.S. culture (**A**–**C**) and A.S. culture (**D**–**F**) in co-cultures according to type and concentration of ACs. The standard deviations of the mean ($n = 3$) are represented by the error bars. A $p < 0.05$ value is considered to represent significant difference. The "*" sign is applied for this significant difference.

3.3. Reactive Oxygen Species (ROS) Formation in Cyanobacterial Co-Culture in Presence of Aminoclay (AC)

To determine the levels of ROS production in the tested algal cultures, the contents of ROS under MgAC, CaAC, AlAC, and cell-free medium/CaAC exposures were analyzed (Figure 3). As noted in Section 2.5, CaAC was the lowest-affected type among the 3 tested ACs, but still inhibited almost all of the A.S. cultures when in combination with the cell-free medium. Therefore, there was no need to test the two remaining, stronger ACs. When the AC concentration was 1.0 g/L, the ROS content differed between the other treatment groups and increased, compared with the control ($p < 0.05$). The ROS content demonstrated changes in the order of AlAC > cell-free medium/CaAC > MgAC > CaAC. The controls showed an increase of 7.64–8.18% in the level of intracellular ROS (Figure 3). After exposure for 6 days at 1.0 g/L, a significant increase in ROS, compared with the controls was found for the population exposed to MgAC, CaAC, and AlAC, and the "mixture" of cell-free medium/CaAC, respectively (Figure 3), relatively resulting in 20.80–22.27%, 18.87–20.21%, 18.95–20.29%, and 18.15–19.44% of AlAC, cell-free medium/CaAC, MgAC, and CaAC. AlAC showed the greatest increase of ROS relative to the controls, as recorded after exposure for 6 days at 1.0 g/L. It means that ROS has been produced and accumulated in the microalgal cells.

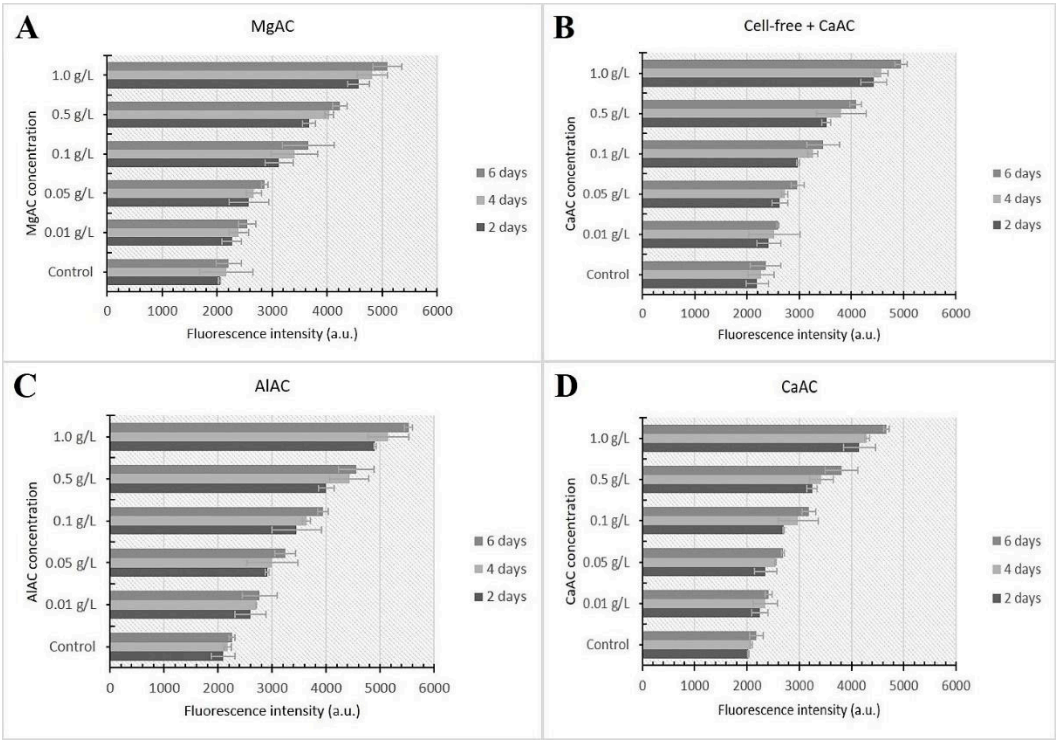

Figure 3. ROS formation in cyanobacterial co-culture according to different concentrations (0.01, 0.05, 0.1, 0.5, and 1.0 g/L) of (**A**) MgAC, (**B**) CaAC in cell-free medium/CaAC, (**C**) AlAC, and (**D**) CaAC. The standard deviations of the mean ($n = 3$) are represented by the error bars. A $p < 0.05$ value is considered to represent significant difference.

3.4. Zeta Potentials of Aminoclays for Two Microalgal Species

The zeta potentials of the three types of AC (MgAC, CaAC, and AlAC) and two microalgal species (M.S. and A.S.) were evaluated (Figure 4). The averaged zeta potentials of the AlAC, MgAC, and CaAC were ~+36, ~+27, and ~+4 mV, respectively, while the zeta potentials of the feedstock blue-green microalgae containing *Microcystis* and *Anabaena* were ~−14.3 and −32.8 mV. A possible reason for this difference in ACs is that the size distribution (degree of delamination) and valence charge, compared to those of the MgAC

and CaAC dispersions. Contrastingly, the surface charge potentials of the microalgal strains were indicated to be in a negatively charged state in which A.S. shows more negatively charged surface than that of M.S. (Figure 4). Due to electrostatic interaction between microalgal cells and ACs, the degree of aggregation or flocs of microalgal cells were formed, leading to ultimately the inhibition of growth or cell death.

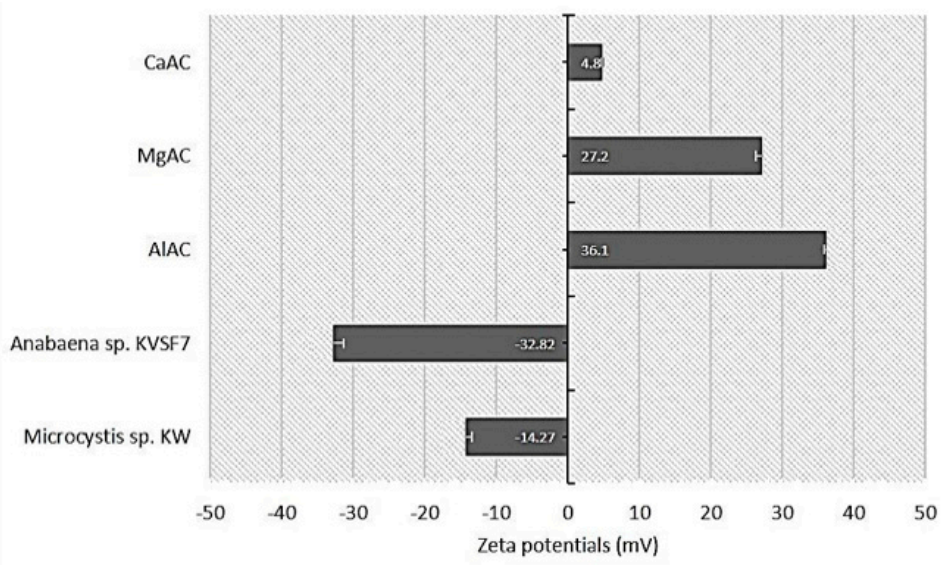

Figure 4. Zeta potentials of two cyanobacterial species (M.S. and A.S.) and three types of AC (MgAC, CaAC, and AlAC). The standard deviations of the mean (n = 3) are represented by the error bars. A $p < 0.05$ value is considered to represent significant difference.

3.5. Effects of Microcystis sp. KW Cell-Free Medium and CaAC on Anabaena sp. KVSF7

We affirmed the allelopathic interaction of M.S. cell-free medium containing AC with A.S. Cell-free medium of M.S. was preferred, because M.S.-free medium has been reported to more efficiently inhibit the growth of A.S. due to its allelochemicals [32,34]. The M.S.-free BG-11 medium was collected from the normal culture medium of M.S. at the exponential stage. It was then centrifuged at 3134 G-force in 10 min. This method was confirmed to have no effect on the cells of that strain.

M.S. cell-free media including different CaAC (the lowest-affected AC among the 3 tested types) concentrations were utilized in the A.S. culture. With the increase in the CaAC concentration, the cell-free medium of M.S. and CaAC had a significant adverse effect on the cell density of the A.S. culture, inhibiting biomass levels by 26.54–93.67% after 6 days ($p < 0.05$; Figure 5). The overall biomass of the A.S. culture was significantly inhibited by the CaAC-containing M.S. cell-free medium (Figure 5), whose effect was greater than that of the mixture of *Microcystis* and CaAC (Section 3.2; Figure 2E).

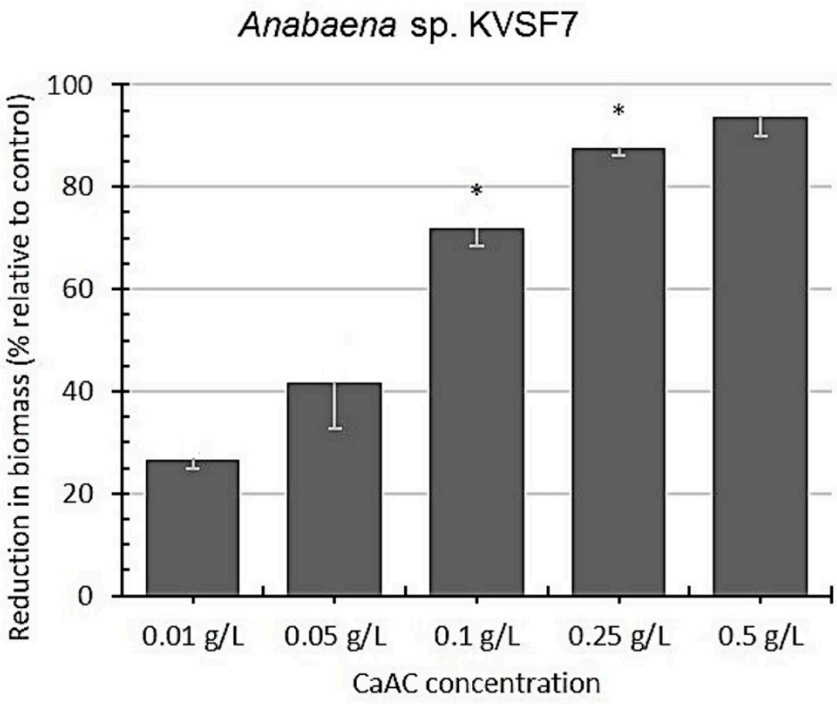

Figure 5. Reduction in biomass of A.S. culture in *Microcystis* cell-free medium containing different dosages of CaAC (0.01, 0.05, 0.1, 0.25, and 0.5 g/L). The standard deviations of the mean (n = 3) are represented by the error bars. A $p < 0.05$ value is considered to represent significant difference. The "*" sign is applied for this significant difference.

3.6. Microalgal Cell Disruptions in Aminoclay (AC)-Treated Cultures

The state changes of algal cells in co-cultures in the presence of AC were observed under optical microscopy. Microalgae images under bright optical microscopy also showed that the AC was surrounded by microalgal cells in sets, due to the cell-wall instability affected by the cationic charged groups of AC [19]. In positively charged MgAC, expressed by zetapotentials (Figure 4), the electrostatic interaction and the coagulation mechanism of MgAC with microalgal cells was an initial driving force in microalgal cell disruption (Figure 4). Specifically, MgAC acted as an adhesive to the algal cells, causing them to stick together. According to the test time, microalgal cells in co-culture were severely damaged after exposure to AC. Specifically, they were greatly aggregated and unstable. It was clear that A.S. was more sensitive than M.S., showing some marked cell distortion and death (Figure 6). Along with that, Figure S2 presents that ACs are adhered to cell surfaces during M.S. division with slight inhibition of cell division at 0.1 g/L of MgAC. In addition, fluorescent red images, according to days, A.S. cell were aggregated and some cells were lysed and ultimately led to death at 10 days (Figure S3).

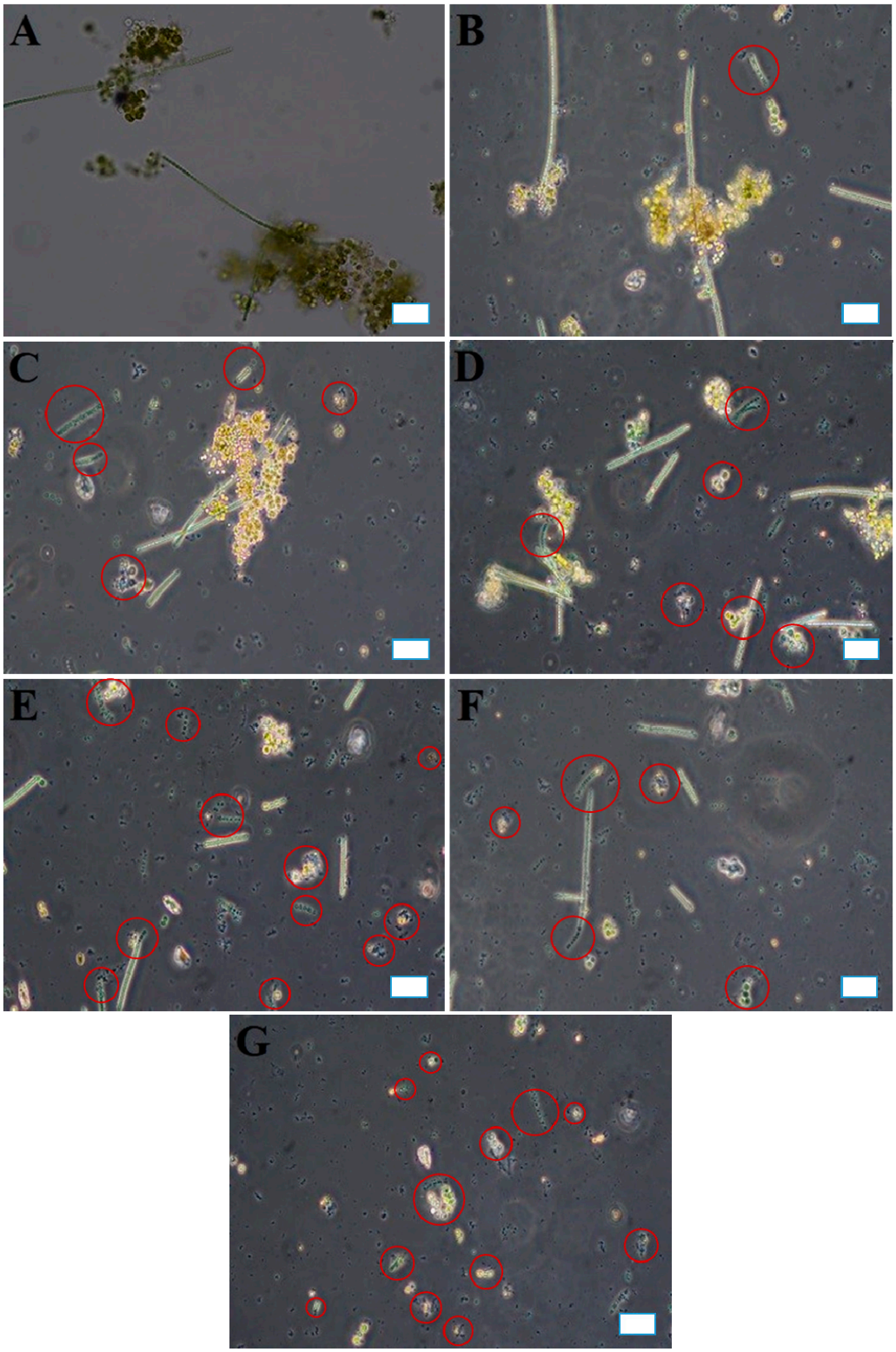

Figure 6. Images of algal cells in co-culture obtained under optical microscopy. (**A**) Control culture without MgAC; (**B**) (1.0 g/L) MgAC-treated mixture cells at day 1; (**C**) cell death occurrence at days 2; (**D**) more encapsulation, fragmentation, and cell death at day 3; (**E**) distortion and death of algal cells at day 4; (**F**) algal cell death at day 5; (**G**) algal cell death at day 6. The red circles indicate the cell fragmentation, lysis, and death. It is noted that the white bars are 10 μm scale.

4. Discussion

HAB-controlling methods are required to be environmentally friendly and safe. Ammonium compounds and their derivatives show some promising potential in specific concentrations, though there are issues with respect to toxicity. In the current study, the *Microcystis* and *Anabaena* strains were influenced by different growth inhibition or stimulation levels in terms of the effects of AC on the mono-culture. In the case of the co-culture, meanwhile, *Microcystis* strongly inhibited *Anabaena* growth, especially under AC treatment. Moreover, the change in AC dosage was associated with the interaction between the algal strains.

Two strains of cyanobacteria were co-cultured in this study. Of these two types of algae, *Microcystis* often showed dominance in the culture population over the *Anabaena* strain when comparing the differences between the mono-culture and co-culture in the presence and absence of AC. These observations are consistent with previous studies on *Microcystis* sp.'s ability to inhibit or stimulate the growth of cyanobacteria or microalgae [32,35–37].

The significant reduction in photosynthetic efficiency also partially inhibited the growth of *Anabaena* strains, thereby affecting the biomass distribution and production. It has been reported that *Microcystis* cell-free media inhibit photosynthesis and interfere with the carbonic anhydrase function of dinoflagellate *Peridinium gatunense* [34]. Bártova et al. (2011) also reported that the glutathione-S-transferase (GST) activity of *Anabaena* sp. is promoted in the presence of *Microcystis aeruginosa*. These studies affirm, moreover, that allelochemicals can indirectly mediate interactions between microalgal strains. This phenomenon was not clearly shown in the present experimental results, but notably, it was in fact shown when comparing differences, more specifically, in the increase in the growth inhibition effect across cultures (from mono-culture to co-culture) at the same AC concentration. In terms of the impact of AC on cultures, the growth inhibition between the studied strains was likely due to allelopathic interactions. The demonstrated ability to significantly inhibit the growth of the *Anabaena* strain in a *Microcystis* cell-free medium further confirms this hypothesis. The release of secondary metabolites and biologically active substances into the growth medium by *Microcystis* likely inhibits the *Anabaena* strain, and it is consistent with observations of their impact on plankton [31,32,36–39]. Activities such as catalase, peroxidase, or superoxide dismutase are likely to be enhanced by the increase in the production of oxidative stress species due to allelochemicals from *Microcystis*, thereby inhibiting the growth of *Anabaena* [32]. Therefore, an increase in the production of ROS, due either to the *Anabaena* strain itself or the presence of *Microcystis*, is likely related to the disruption of nitrogenase activity as well as the suppression of N_2-fixation of *Anabaena* strains [32,40,41].

In this study, algicides were chosen as an approach using types of AC (MgAC, CaAC, and AlAC). These types of AC, having no apparent harmful effects on plankton, also provide the desired functions of ammonium. These ACs, including the organic parts of the ammonium sites, attaches to the cell surface. This causes mucus release that increases adhesion on the cell surface, and cell adhesion and coagulation in turn [42]. Consequently, the surface interactions of AC at the ammonium sites likely disrupt the internal environment of the cell, leading to cell lysis.

It can be assumed that MgAC, CaAC, and AlAC use the same HAB-control mechanism. All three ACs have relatively high algicidal activity, with a focus on the harmful toxin genera and somewhat lesser one on the non-N-fixing genera, in the order AlAC > MgAC > CaAC. *Anabaena* is more sensitive and highly disrupted. Indeed, under the same (0.1 g/L) loading of MgAC, there was 22.59% algicidal activity of M.S, compared with 57.38% of A.S, in the case of the mono-cultures. The same phenomenon was observed for co-cultures: 27.42 and 88.67% algicidal activity of M.S. and A.S., respectively. However, when the ACs loading was increased, the algicidal activity reached 100% in both strains and all three ACs. These results of growth inhibition by CaAC treatment were consistent with those for MgAC and AlAC. By contrast, CaAC, for comparison with the least positively charged

surface, showed an inferior level of algicidal selectivity and lower inhibition effects on HABs in the co-culture (Figure 2B,E).

ROS-producing capability is another factor affecting the impact levels of AC types on microalgal strains. Accordingly, AlAC exhibited an ability to create a highly oxidative stress environment, followed by MgAC and CaAC (Figure 3). This was noticeable, and confirmed, when comparing the growth-inhibiting effects of each AC on the mono-cultures or co-cultures (Sections 3.1, 3.2 and 3.5). The production of ROS contributes significantly to the level of cell disruption as well as the inhibition effects of the material employed [43].

As for the zeta potentials among the three ACs, they differed, showing the various capabilities of the respective approaches and their levels of electrostatic interaction. A.S. was most highly effected by MgAC, followed by M.S. According to ROS production and levels of interaction, every species behaved differently. These results are in accordance with our other data, which showed that M.S. behaved similarly and actively with MgAC, and less sensitively than A.S. It was noticed that when the level of interaction between ACs and microalgae attained a certain level, the effect was shifted, from the stimulation to the inhibition, for each microalgae strain. The speed of this shift is different for each strain. The amine functional groups of MgAC acted as a biocidal agent: via the electrostatic interaction between the negatively charged cell wall and the positively charged MgAC, they affected cell-wall instability [19].

The AC-treatment conditions strongly influenced the allelopathic interactions between the *Microcystis* and *Anabaena* strains. The *Anabaena* strain appeared to be negatively affected even at low doses of AC. Inhibition of biomass strongly occurred under exposure to the "mixture" of AC and *Microcystis*, while the reverse (by strain) occurred under high AC dosages. These observations are indications that the allelopathic effect of the *Microcystis* strain is very high under the conditions of "mixture" treatment. They also point to the effects of AC on the interactions between treated cells: improved cell-wall stability, inhibition/stimulation/metabolization of secondary metabolites, and greater susceptibility of cyanobacteria cells to allelochemicals (Section 3) [44].

Therefore, under high dosages of AC, the *Microcystis* strain appeared to be less inhibited than the *Anabaena* strain. This showed that the allelopathic effect was lower in the latter strain, or that the resistance was higher in the former strain, whose phenomenon is possibly related to the N-fixation capability of *Anabaena* under AC-treatment conditions. In other words, filamentous N-fixing cyanobacteria, such as *Anabaena* spp., are more strongly distinguished by *Microcystis*. Additionally, *Anabaena* was more sensitive to single-AC treatment, and more strongly affected under the "mixture"-treated conditions. This complicates the issue of the relationship between available substances in the environment such as nutrients, released metabolites, or algal culture motion/kinematic [38]. After MgAC is internalized, it can be seen that AC spills and takes over the entire cell as the consequence of the positively charged AC's effect on the plasma membrane. Specifically, the double-lipid layer breaks down, and cell damages subsequently follows, sometimes releasing intracellular compounds (Figure 6, Figures S2 and S3).

5. Conclusions

Aminoclays (ACs) offer an approach to the problem of HAB control. One-pot synthesis and mass production of these materials for control of HABs is feasible [25]. In addition, it has advantages such as optical transparency, superior dispersion, no need for surfactants, and maintained ammonium-like properties. It is suggested that AC is capable of providing effective HAB control in small amounts. In the previous works, especially using MgAC, it has been investigated with selective algicidal activity of redtides in seawater [19] and both in increase of cell size and lipid contents in green microalgal culturing systems. However, in this research, the combination of *Microcystis* cell-free medium and ACs could be considered in controlling cyanobacterial blooms.

However, further studies are needed in order to comprehensively assess the stimulating/inhibiting properties and potential biological risks of this material [45]. One of

the critical challenges is the selective elimination of HABs in environments against other non-harmful phytoplankton. However, there remain a few challenges. Current research demonstrates that the use of individual ACs, combinations of ACs, or further development of multi-functional hybrid clays can improve the efficiency and cost of management and testing for further control of HAB species. If such approaches are likely to harm other resources [46], these ACs can be immobilized on the substrate or mixed with other clays and compounds to reduce this risk. However, this may reduce algicidal activity. In any case, certainly, these approaches can be used to deal with "tides" of cyanobacterial blooms as well as membrane-fouling problems [47].

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/app11125607/s1, Figure S1: Ideal three-dimensional structure of MgAC, reproduced with the permission from reference (Yang et al., 2014)., Figure S2: Optical microscope images of cell lysis of Microcystis sp. KW due to AC (0.1 g/L) loading. It is noted that the white bars are 10 μm scale., Figure S3: Optical microscope and fluorescent images of cell lysis of Anabaena sp. KVSF7 due to aminoclay (0.1 g/L) loading after (A) 0 day; (B) 2 days; (C) 4 days; (D) 6 days; (E) 8 days; (F) 10 days. It is noted that the white bars are 10 μm scale., Table S1: Mono-culture and co-culture experimental designs of two cyanobacterial Microcystis and Anabaena species., Table S2: Experimental designs to test the effect of Microcystis sp. KW cell free medium (MSCFM*) and CaAC on Anabaena sp. KVSF7.

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