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Allelopathic effects of emergent macrophyte, *Acorus calamus* L. on *Microcystis aeruginosa* Küetzing and *Chlorella pyrenoidosa* Chick

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ABSTRACT

In controlled laboratory conditions, the allelopathic effects of emergent macrophyte *Acorus calamus* L. were studied on the growth of two phytoplankton species: *Microcystis aeruginosa* Küetzing and *Chlorella pyrenoidosa* Chick. In co-culture experiment, *A. calamus* grew normally in the medium but the growth of *M. aeruginosa* and *C. pyrenoidosa* was significantly inhibited at initial cell densities by live plants and the inhibitory effect was less on *C. pyrenoidosa*. Within 10 days, the highest IR value of live *A. calamus* on *M. aeruginosa* and *C. pyrenoidosa* were 97.9% and 82.2%, respectively. The aqueous extracts of *A. calamus* dry biomass at high concentrations inhibited the growth of both phytoplankton spp. but the growth of *M. aeruginosa* was revived on 4th day with aqueous extracts concentration of 30 mL·L^{-1}, and growth of *C. pyrenoidosa* was promoted by the concentrations < 30 mL·L^{-1}. The same amount of aqueous extracts added to *M. aeruginosa* in single application or in multiple application, caused more inhibition in later. The result suggested that the allelochemicals are degradable and a long-term inhibition might need the continuous addition of compounds from *A. calamus*. This study showed that *A. calamus* was strongly inhibitory to phytoplankton and may have great potential in HABs control and prevention.

Key words: Allelopathy, *Acorus calamus*, *Chlorella pyrenoidosa*, Emergent macrophyte, Growth inhibition, *Microcystis aeruginosa*

INTRODUCTION

World over the Harmful Algal Blooms (HABs) are becoming an increasing problem to human health and environment due to both phytoplankton and non-planktonic algae (dinoflagellates, cyanobacterias and tychoplanktonic). The dense populations of HABs may lead to high consumption of oxygen and liberation of toxic substances (e.g., hydrogen sulphide) that can kill fish, or interfere with the digestion of flagellates by shellfish, causing deleterious effects on the biodiversity and equilibrium of aquatic ecosystems and economic losses. Furthermore, various toxins produced by HABs planktonic and non-planktonic microalgae can be great threats to human health. For instance in Mexico, a number of human fatalities have occurred in the last 30 years owing to poisonings like Paralytic Shellfish Poisoning (PSP) and Neurotoxic Shellfish Poisoning

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(NSP) (3). Therefore, the control of HABs is an important issue in aquatic environment (11). Many studies [using clay (29), copper sulfate (28), bacteria (15) and viruses (5)] have been done to control the harmful phytoplankton growth against HABs, but in the long term they may have potential risk on ecology (12). Recently, allelopathy had been used effectively as ecological-chemistry method for HABs control.

Since Hasler and Jones (9) first reported the inhibitory effect of hydrophytes on microalgae, numerous studies have shown that certain macrophytes [*Ceratophyllum demersum* (16), *Chara* (22), *Najas marina* (8), *Stratiotes aloides* (21) and *Elodea* (4)] can inhibit the growth of their neighboring phytoplankton by secreting the allelopathic substances against cyanobacteria and some common green algae, and some bioactive substances have been extracted and purified. Thus far, most researches of allelopathic inhibition on harmful phytoplankton growth were confined to using submerged macrophytes and there are very limited investigations to directly control the phytoplankton in freshwaters by emergent macrophytes. Emerged macrophytes plays important role not only in material cycling, but also in both abiotic and biotic process in shallow fresh waters, many emerged macrophytes are used to purify the domestic and agricultural wastewater (1). Some emergent macrophytes are also inhibitory to phytoplankton growth as submerged macrophytes e.g. *Phragmites australis* (18). Compared with submerged macrophytes, the emergent macrophytes have advantages that they are more favorable to the light, nutrient and space when competition with phytoplankton occurs.

The emergent macrophyte *Acorus calamus* (called sweetflag), is a common summer species in Chinese shallow freshwaters. It starts growing in early spring and reaches a biomass maximum in July-August in temperate regions. Its rhizomes contains active ingredients possessing insecticidal (26), antifungal (17), antibacterial (20) and allelopathic properties (25) and is widely used as medicinal material. However, its use to inhibit the phytoplankton (which might be promising) has not been reported yet.

In this research, the allelopathic inhibition of common emergent macrophyte *A. calamus* was studied on the most notorious HAB species in fresh shallow waters: *Microcystis aeruginosa* and on green algae species-*Chlorella pyrenoidosa*. Research was done using two approaches: (i). Co-culture experiment (effects of *A. calamus* on the growth of two phytoplankton spp. at two different initial cell densities) and (ii). the response of two phytoplankton species to aqueous extracts of *A. calamus* dry powder were also investigated.

MATERIALS AND METHODS

Healthy *Acorus calamus* was collected from a ditch in suburban Wuhan city and washed with tap water to remove the adhering soil. It was tested for its inhibitory effect on two phytoplankton species: *M. aeruginosa* and *C. pyrenoidosa*. These were supplied from Freshwater Algal Culture Collection, Institute of Hydrobiology.

Prior to the experiment, the phytoplankton was grown in 500 mL Erlenmeyer flasks containing 200 mL BG-11 medium for 7 days (i.e. exponential growth stage). All these phytoplankton were cultured aseptically at 25°C under 70 μmol m^{-2} s^{-1} with a 12:12-h Light/Day cycle in illuminating Incubator. All flasks containing phytoplankton cells were shaken twice daily to prevent wall growth. A high biomass with 5.5×10^{6}

cells·mL^{-1} concentration was obtained at the end of incubation and used as the inocula for the following experiments. The culture conditions in the following experiments were the same as for the phytoplankton culture unless stated.

Co-culture experimental device

To confirm the secretion of allelopathic substances from *A. calamus*, the isolation co-culture systems were built to prevent the direct contact between the phytoplankton and macrophyte and allowing the activity of allelochemicals.

An incubator of Plexiglas was prepared for co-culture assays (Fig.1), being transparent it ensured sufficient light for photosynthesis. Each incubator had 5 pools, the large pool in the center was used to grow *A. calamus* and the remaining smaller pools were used for phytoplankton growth. The holes between the pools were plugged with 0.45μm microporous filter membrane and gauze, to prevent the transfer of the rhizosphere bacteria and protozoa from the plant's roots. However, the nutrients and inhibitory chemicals exchanged freely within the five pools.

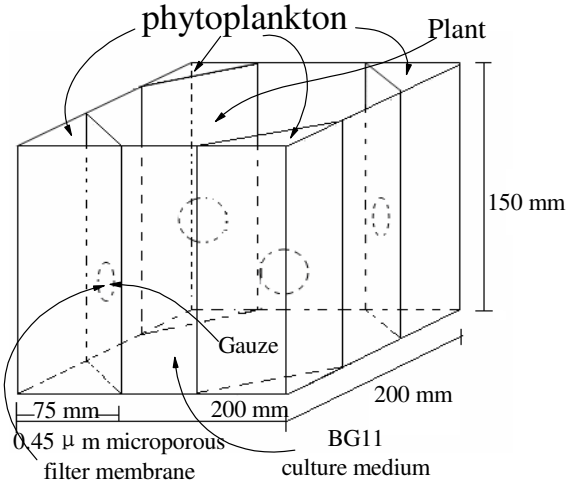

Figure 1. Diagram of co-culture experimental Appratus

Co-culture assays with live macrophyte

The experimental treatments consisted 3 factors: (i) Phytoplankton spp. 2 (*M. aeruginosa* and *C. pyrenoidosa*), (ii). Co-cultures combinations: 4(*A. calamus* + *M. aeruginosa, A. calamus* + *C. pyrenoidosa,* pure *M. aeruginosae,* pure *C. Pyrenoidosa*) and (iii). Initial inoculation doses 2 (2×10^6 Cells·mL^{-1} and 4×10^6 Cells·mL^{-1}). In control, fresh *A. calamus* plants were replaced by plastic plants to make uniform shading in all incubators The treatments and controls were replicated thrice in Completely Randomized Design. The study has been shown in flow diagram (Fig. 2).

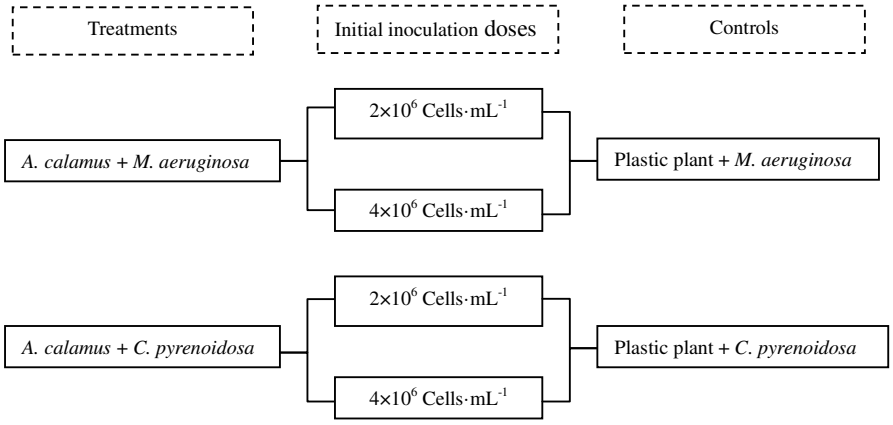

Figure 2. The flow diagram of co-culture experiment

The assays were performed with co-cultures of fresh *A. calamus* (a phytoplankton specie). First, healthy macrophytes were selected, then washed with tap water to remove the soil. To ensure normal photosynthesis, the old leaves were pruned to reduce its weight to 300 g, (this weight significantly inhibits the phytoplankton growth and made the weight uniform in all treatments). Finally, macrophytes were cultivated in 25% diluted BG-11 culture medium for 3-4 days to make them adapted to the co-culture environment, after these macrophytes were soaked in 0.5% $KMnO_4$ solution for 30-min to disinfect from all types of bacteria on plants, then rinsed by distilled water to remove the $KMnO_4$ residues. Lastly *A. calamus* was cultivated in the central large pool of incubator with 2600 mL water.

The *M. aeruginosa* and *C. pyrenoidosa* were inoculated into the remaining 4 smaller pools with 250 mL water respectively in each. Then incubators were closed with adhesive porous tape to prevent dust and provide optimal CO_2 concentration. The $Ca(NO_3)_2 \cdot 4H_2O$ (756 mg), KNO_3 (485.6 mg) and KH_2PO_4 (108.8 mg) were added to co-culture every 3 days to ensure adequate nitrogen or phosphorus supply (15). The initial and final pH of co-culture were measured.

These assays were conducted for 10 days. To find the cell density phytoplankton cells were counted daily with haemacytometer under Olympus optical microscope.

Algicidal assays of aqueous extracts from macrophyte on phytoplankton

The *A. calamus* aqueous extracts was prepared as under: One week before the experiment, fresh samples of *A. calamus* were rinsed with cell-free water and dried (60°C, 48 h), then ground into fine powder and 50 g dry powder was soaked in 1 L cell-free water (25°C, 24 h, in dark). The mixed extracts were centrifuged (4000 rpm, 30 min) and the supernatants were filtered through glass-fibre filter (0.22μm) to remove bacteria and protozoa. The resultant aqueous extracts of *A. calamus* were kept in a sealed aseptic beaker.

To determine the inhibitory algicidal effects of *A. calamus* aqueous extracts on *M. aeruginosa* and *C. pyrenoidosa*, two following experiments were done using the same Algal cell density (6.5×10^5 Cells·mL^{-1}), same method and same duration of 7 days:

Experiment 1: Test algal spp. *M. aeruginosa, A. calamus* extracts concentrations 6 (0,10,20,30,40,50 mL·L^{-1})

Experiment 2: Test algal spp. *C. pyrenoidosa, A. calamus* extracts concentrations 8 (0,10,20,30,40,50,75,100 mL·L^{-1}). These experiment were done with 200 ml culture volume in 500 ml Erlenmeyer Flask. Phytoplankton cultures without aqueous extracts served as controls. All flasks were shaken twice daily to prevent wall growth and these assays lasted 7 days Phytoplankton cells number were counted as the same method of co-culture assays.

Algicidal assays with different additions

From the results of algicidal assays of aqueous extracts on *M. aeruginosa*, we presumed that the allelochemicals released from *A. calamus* may degrade quickly. To confirm the instability of these allelochemicals, assays were done with different volumes of aqueous extracts. In one assay, the aqueous extracts were added in two ways: Single addition and multiple addition. **(i). Single addition:** The 5 mL aqueous extracts was added into 500 mL Erlenmeyer flasks in the beginning and the total culture volume was kept as 200 mL. **(ii)**. **Multiple addition**: The 2 mL aqueous extract was added into flasks on first day. Then every 2 days, 1 mL culture medium was removed from each flask (200 mL) and 1 mL fresh aqueous extracts was added to keep the culture volume constant in flask. In control cell-free water was used.

Allelopathic inhibition was evaluated by comparing the inhibition ratio (IR) curves and was calculated as under:

$$IR\ (\%) = [1 - (N/N_0)] \times 100\%$$

Where, N_0 and N are the number of phytoplankton cells in the control and treated cultures, respectively.

Data analysis and statistics

All experiments were carried out three times. Mean values and standard deviations were calculated from the different replicates (n=3). Statistical analyses of the data were performed using SPSS 13.0. ANOVA was used to test the significance levels of difference between the treatment and control set, using $p<0.05$ as significant.

RESULTS

Effects of live macrophyte on two phytoplankton species in co-culture assays

In co-culture experiment, *A. calamus* inhibited the growth of *M. aeruginosa* and *C. pyrenoidosa* at two initial cell densities (Fig. 3). After 10 days' cultivation, the

macrophyte had normal colour of leaves and normal weight of biomass. At the end of co-culture experiment, the pH was 8.3 i.e. similar to 8.0 at the beginning. At the initial inoculation dose (2.0×10^6 Cells·mL^{-1}), the live macrophyte drastically inhibited the growth of *M. aeruginosa*. The inhibition in cell growth started 3 days after co-culture, while after 8 days, *M. aeruginosa* population was sharply decreased than control. The culture medium became nearly colourless and transparent in the phytoplankton pool of experiment device, showing that the *M. aeruginosa* was completed inhibited.

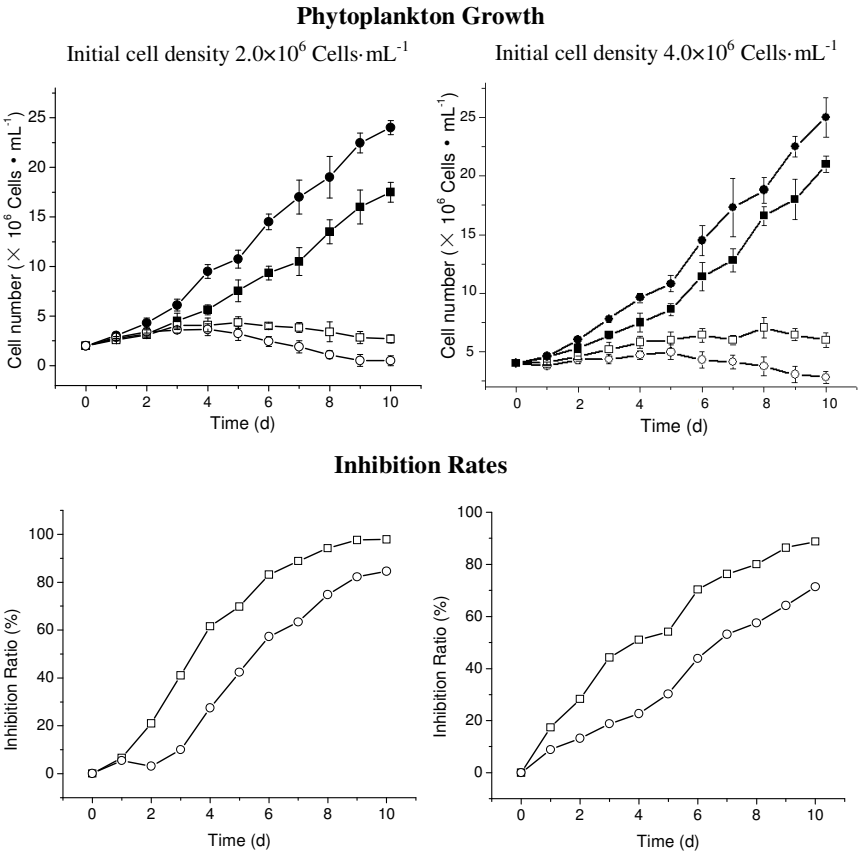

Figure 3. Effects of two densities of *A. calamus* in Co-culture on the growth of two phytoplankton spp. *M. aeruginosa* and *C. pyrenoidosa* (-●-*M. aeruginosa* control; -■-*C. pyrenoidosa* control; -○- *M. aeruginosa* treatment; -□- *C. pyrenoidosa* treatment). Data represent means (n=3) with associated error bars (S.D.)

The *A. calamus* extracts were more inhibitory to *M. aeruginosa* than to *C. pyrenoidosa*. The cell density of *C. pyrenoidosa* in control increased slowly than with *M. aeruginosa* (Fig. 3). In co-culture assay, the live macrophyte significantly inhibited the growth of *C. pyrenoidosa* (IR_{10} value: 82.2%), but it was more resistant than *M. aeruginosa* (IR_{10} value: 97.9%).

In trial with higher initial inoculation dose (4.0×10^6), *A. calamus* was most inhibitory to growth of *M. aeruginosa* ($p<0.01$). During the first 4 days of experiment, the cell density of *M. aeruginosa* was near the initial inoculation dose. After 6 days, the cell density was decreased, the culture turned brown, micro-sedimentation was formed and ultimately the IR_{10} value reached 88.8%. However the inhibitory effects of higher inoculation dose, on *C. pyrenoidosa* were little different than lower dose and the growth of algae was considerably inhibited ($p<0.05$). The IR_{10} value of *C. pyrenoidosa* was 71.4%, i.e. lower than 82.2% of precious assays.

Thus in co-culture experiment, the live *A. calamus* significantly inhibited the growth of both phytoplankton species at two initial inoculation doses (2×10^6 Cells·mL^{-1}; 4×10^6 Cells·mL^{-1}). *M. aeruginosa* was more sensitive to the allelochemicals from *A. calamus* than *C. pyrenoidosa*.

Effects of aqueous extracts of macrophyte on two phytoplankton species

In this assay, the lowest concentration of aqueous extracts (10 mL·L^{-1}) did not affect the growth of *M. aeruginosa* (Fig. 4). In first 4 days at 30 mL·L^{-1} aqueous extracts concentration, the cell density was maintained at 7.0×10^5 and IR value was decreased from 82.8% to 48.2%, these may result in degradation of allelochemicals. Afterwards, the growth of *M. aeruginosa* was revived during the remaining 3 days. On the contrary at high aqueous extracts concentrations (40 and 50 mL·L^{-1}), *M. aeruginosa* was almost eliminated in the cultures and there was no sign of growth recovery in cells, finally the IR_7 value reached to 97.8% and 98.7% respectively. The $EC_{50\ 7d}$ value of aqueous extracts of *M. aeruginosa* was 21 mL·L^{-1}.

The inhibitory effects of aqueous extracts on the growth of *C. pyrenoidosa* showed a great discrepancy from *M. aeruginosa*. The aqueous extracts of 10 and 20 mL·L^{-1} concentrations, stimulated the growth of algae on 2nd day and thus the culture appearance was much dense than control. While at 20 mL·L^{-1} concentration, the lowest IR_7 value decreased to -49.5%. The aqueous extracts < 40 mL·L^{-1} concentration did not influence the algae growth. As the extracts concentration increased, the inhibitory effects became more stronger. At 50 mL·L^{-1}, the algae growth was slightly inhibited, but was drastically inhibited at 75 and 100 mL·L^{-1} conc. The highest IR_7 value of aqueous extracts against *C. pyrenoidosa* reached 92.4%. The $EC_{50\ 7d}$ value was 62 mL·L^{-1} in this assay.

Effects of aqueous extracts with different additions on *M. aeruginosa*

Based on the results of aqueous extracts (30 mL·L^{-1}) on growth of *M. aeruginosa*, we assumed that the allelochemicals in the aqueous extracts may be decomposed in short time. The result of this assay, confirmed the assumption. In first 4 days, the growth of *M. aeruginosa* in single addition cultures, was inhibited in aqueous extracts of 25 mL·L^{-1} concentration (Fig. 5), but the growth of cyanobacteria was revived on 3rd day and finally

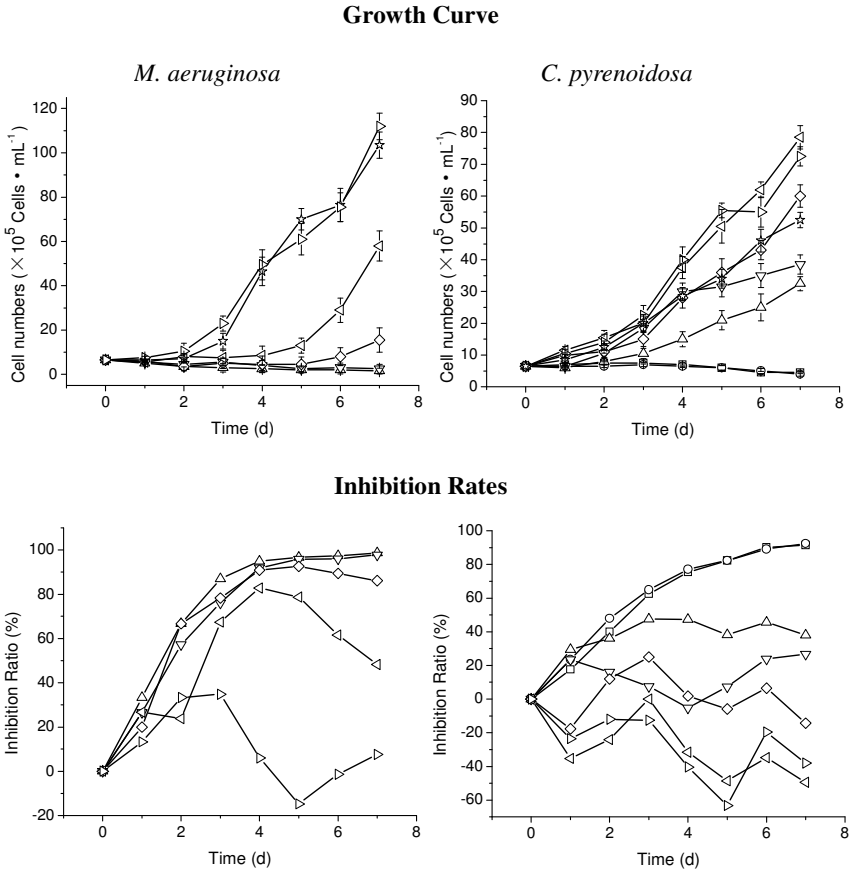

Figure 4. Effects of different concentrations of aqueous extracts of *A. calamus* on the growth curve and inhibition rates of *M. aeruginosa* and *C. pyrenoidosa* during 7 days' cultivation. (-☆- Control; -▷-10 mL·L^{-1}; -◁-20 mL·L^{-1}; -▽-30 mL·L^{-1}; -◇-40 mL·L^{-1}; -▽-50 mL·L^{-1}; -○-75 mL·L^{-1}; -□-100 mL·L^{-1}) of . Data represent means (n=3) with associated error bars (S.D.)

the cell density reached 3.5×10^6 Cells·mL^{-1}. On the contrary, the growth curve of cells in 'multiple addition' cultures showed different trend. In first 3 days, the growth of *M. aeruginosa* was slightly promoted (as the allelochemicals accumulated in every application) but the growth of algae was inhibited on 5th day, then the cell density kept decreasing during the last 2 days of assay. Ultimately the cell density was controlled at 2.7×10^6 Cells·mL^{-1} and the IR_7 value of extracts was 64% (Fig. 5), i.e. higher than 55.3% of extracts in 'single addition' groups.

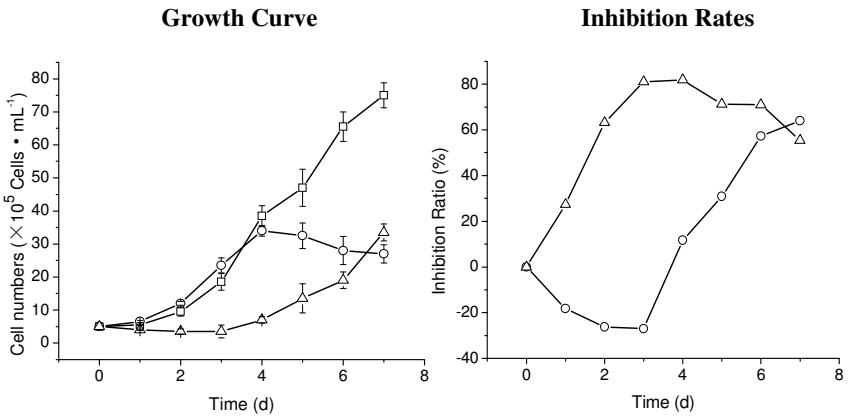

Figure 5. Effects of single or multiple addition of aqueous extracts of *A. calamus* on the growth curve and inhibition rates of *M. aeruginosa*. (-□-Control; -○- Multiple addition; -□- Single addition). Data represent means (n=3) with associated error bars (S.D.)

DISCUSSION

Allelopathy as a natural phenomenon and has been extensively studied in terrestrial plants (2). However allelopathy also occurs in aquatic habitats, because all organisms are capable to produce and release the allelochemicals (6,7,16). Although most co-existence laboratory studies have shown the allelopathic effects of many macrophytes on phytoplanktons (16,19,22,23,30,32), but it is difficult to study the allelopathic effects between macrophytes and phytoplanktons under natural conditions, due to many factors (Nutrients and light, temperature, pH change) which mask the allelopathic effect (14).

In this study, we did laboratory co-culture experiments under controlled conditions (excluding the nutrient and light competition, effects of bacteria etc.), to investigate the allelopathic effects of *Acorus calamus* on two typical phytoplankton species (*Microcystis aeruginosa* and *Chlorella pyrenoidosa*). The co-culture assays results showed that (i). live plants of *A. calamus* drastically inhibited the growth of *M. aeruginosa* and *C. pyrenoidosa* and (ii). nitrogen and phosphorus were adequate for the growth of phytoplankton (i.e. nutrients were not limiting). During the experiment, we kept *A. calamus* and plastic plants far away from the light source, to avoid shading effects of macrophyte on two phytoplankton species. Schmidt and Hansen (27) showed that macrophyte may change the pH of culture medium in which it was grown, making it unsuitable for the growth of microalgae. We measured the pH of culture medium in the beginning and at the end of experiment, there was little change in pH, hence it did not play important role in growth inhibition of two phytoplankton species. Therefore, the growth reductions in *M. aeruginosa* and *C. pyrenoidosa* were not due to pH or light or nutrients competition with *A. calamus*.

In co-culture assays, the inhibitory effect on the growth was stronger on cyanobacteria (*M. aeruginosa*) than chlorophyte (*C. pyrenoidosa*). The diatoms and cyanobacteria are often significantly inhibited by the allelochemicals of submerged macrophytes, whereas, chlorophytes are less sensitive (10). A stronger allelopathic effect on diatoms and cyanobacteria seems ecologically more reasonable, because these groups are more abundant in shallow lakes than chlorophytes (13). The competition for growth resources (nutrients and light) between cyanobacteria and macrophyte was stronger that may induce the macrophyte to release more active chemicals against cyanobacteria. It may be the result of long-term natural evolution in shallow freshwaters, that may account for the different responses of these two phytoplankton species to the allelochemicals.

The aqueous extracts of *A. calamus* were inhibitory to both phytoplankton species at higher concentrations. Similar to co-culture assays, *C. pyrenoidosa* proved less sensitive to active chemicals in aqueous extracts and the $EC_{50\ 8d}$ value of aqueous extracts on *C. pyrenoidosa* was much higher (62 $mL \cdot L^{-1}$) than on *M. aeruginosa* (21 $mL \cdot L^{-1}$). The aqueous extracts of < 30 $mL \cdot L^{-1}$ concentrations stimulated the growth of *C. pyrenoidosa* and some allelochemicals are stimulatory to target organism at lower concentrations and exert negative effects at higher concentrations (31).

The initial aqueous extracts concentration of 25 $mL \cdot L^{-1}$, showed inhibitory effect on *M. aeruginosa* in first 4 days, but its growth was re-established afterwards. On the contrary, the same amount of aqueous extracts added in multiple applications showed great inhibitory effects. The growth of phytoplankton was slightly promoted by low concentrations but the multiple applications caused growth-inhibition. The growth recovery might be attributed to the "allelochemical supply" patterns (24). In co-cultures, *A. calamus* may have continuously released the growth-inhibiting allelochemicals throughout the cultivation period, whereas in the single addition of aqueous extracts, the allelochemicals may inhibit the growth of phytoplankton at the start of cultivation, because they are rapidly degradable. The 'single addition' of allelochemicals solution might underestimate the allelopathic inhibitory effects than in coexistence assays (23, 32).

Our results have demonstrated that the common freshwater macrophyte *A. calamus* had detrimental allelopathic effects on HAB species: *M. aeruginosa* and common green algae *C. pyrenoidosa*. The aqueous extracts of *A. calamus* have allelopathic properties. Emergent macrophyte such as (i). *A. calamus*, are widely distributed and are indigenous in the fresh water environment. Hence the collection and cultivation of abundant emergent macrophyte species is an easy and economical method to control potential HAB in selected areas, (ii). it is ecofriendly and (iii). emergent macrophytes are more favourable to the light, nutrient and space than harmful phytoplankton. Our results not only showed the interactions between the emergent macrophyte and phytoplankton in fresh water areas but also lead us to isolate and characterize these allelopathic substances and study their allelopathic mechanism in future researches.

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