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Effects of wattle extract on *Microcystic aeruginosa* growth and the simulated mini fresh water ecosystem

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Abstract: *In recent years, there has been an apparent increase in the occurrence of harmful algal blooms in fresh waters. The value of applying the novel wattle extract (Acacia mimosa) to inhibit algal growth was assessed. Our results showed that the growth of Microcystis aeruginosa was successfully repressed by the plant extract and resulted in decreased extracellular microcystin-LR production. In the experiments, it showed a very effective inhibition in the stage of exponential growth (the largest decrease in level is 47.3% of the control) especially in nonsterile conditions, and the extract can reduce 14.5-24.7% cell density of the control in the stationary stage. In outdoor experiments, the extract reduced dissolved oxygen and pH, and selectively cut down cyanobacterial cell density to one-third of the control after 36 d of treatment. Accordingly, due to competitive inhibition in interspecies, other nanoalgae and small-sized aquatic animals declined, while macrozooplankton increased. Finally, more large algae were eliminated and thereby the water treated was clarified and the recovery of the freshwater ecosystem was promoted. Hence, the present study suggested a new and more effective and very low ecological risk approach to reduce nuisance blooms cyanobacteria in eutrophic water.*

Key words: *Acacia mimosa, Algal bloom control, Fresh water ecosystem, Microcystis aeruginosa, Microcystin, Wattle extract*
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Introduction

The occurrence of harmful algal blooms is a problematic issue in many aquatic environments (Hallegraef, 1993; Newman and Barrett, 1993). Summer blooms in eutrophic lakes are usually composed of cyanobacteria, especially the genera *Microcystis*. *Microcystis* is dark green and often becomes groups gathered together with spherical cells. It is very widespread and its competitive dominance is fostered by massive accumulation of nutrients (Allen, 1984), favorable temperature and sufficient sunlight (Chaumont and Thtpenier, 1995). It will float up into the top of the water when it thrives (personal observation). The over growth of *Microcystis* deprives fish and invertebrates from oxygen (Ball *et al*., 2001) and leads to bad taste/odor (Izaguirre *et al*., 1982), increased water treatment costs. Furthermore, a potent toxins called microcystins (MCs) are produced mainly by freshwater *Microcystis* blooms and are responsible for liver failure in wild animals, livestock and aquatic life (Carmichael, 1992, 2001; Codd *et al*., 1988; Sivonen and Jones, 1999). MCs have also been identified as the cause of human illnesses and even death due to the exposure to hepatotoxin-contaminated water (Azevedo *et al*., 2002).

A variety of methods have already been developed and applied to cope with the problems related to cyanobacterial blooms, including UV-radiation (Alam *et al*., 2001), nutrient diversion, artificial destratification, hypolimnetic aeration/withdrawal, sediment oxidation/ removal, ultrasonication and biomanipulation (Wetzel, 2001). Even though such methods are often effective, many of them are very expensive and sometimes give rise to secondary pollution, or act for a short function time. Consequently, more long-term effective and pollution free methods are still required, particularly in developing countries (Ahn *et al*., 2003).

Plant extract has not been recognized as a control method for cyanobacterial blooms until quite recently. In present, although the application of plant extract such as barley straw extract (Ball *et al*., 2001) has certain inhibiton on algal bloom, it has not produced satisfactory results yet. Currently, there is still little plant extract to be used in bloom control. Furthermore, the current studies of plant extract inhibition have not been given sufficient attention on the effects of plant extract on cyanobacteria, for example, the influence on aquatic biological environment, efficient timing of treatment based on both laboratory and field studies.

Hence, in this paper, in order to find a more environment-friendly and more efficient method for cyanobacteria controlling, we assessed the value of application to control algal blooms and the effects of the novel plant extract of wattle (*Acacia mimosa*) on *Microcystis aeruginosa* inhibition, extra microcystin production and the simulated mini aquatic ecosystem. The extract used in this experiment is a natural substance extracted from *Acacia mimosa*. The report about acute toxicity tests of wattle extract made by Japanese Food Research Laboratory is, LC_{50} on *Oryzias latipes* for 96h is 67 mg l^{-1} and on

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mice is 100 mg kg^{-1} (personal data). Therefore, the general applied concentration of 2-6 mg l^{-1} is safe for continuous life. It has been applied in more than 20 eutrophic water bodies in Japan and China etc. and has produced good long-term effects on the improvement of the aquatic ecological environment (Ding *et al*., 2007).

Materials and Methods

Measurement of *M. aeruginosa* growth after treatment by the wattle extract: The toxic *M. aeruginosa* purchased from the Culture Collection of Algae at the Institute of Hydrobiology in Wuhan, was pre-cultivated in a BG11 medium (Stanier *et al*., 1971) to maximum density standing under the conditions of 25°C, 140 μmol m^{-2} s^{-1} and light/dark (L/D) 12 / 12 hr cycle in a light incubator. The strain was inoculated in 250 ml Erlenmeyer flasks with 100 ml growth medium. The wattle extract used in this study is readymade and provided by Applied Microbiology Research Co. Ltd. in Japan, Shanli.

In order to studied the sensitivities of the plant extract on different *M. aeruginosa* growth stages, we selected three *M. aeruginosa* density for testing in the lab: one is low density for testing the preventive effect of the plant extract; one is higher density for testing its effects on stage of exponential growth; and another is the maximum density for testing its influences on the stage of the stationary phrase. Four different wattle extract concentrations (0, 2, 6, 12 mg l^{-1}) were chosen for algal experiments. Three groups were set up with different starting cell density or different conditions described in Table 1. Three groups of cells were treated by different extract doses on 0 hr and growth was then monitored on 8, 12, 24, 36, 48, 72 hr, respectively.

The growth test includes cell number and chlorophyll a. The experiment was repeated three times and each treatment had at least two replicates. Approximately 3 ml of each culture was removed for analysis using these parameters. The cell number was counted using a hemacytometer under an Olympus microscope (×400). Chlorophyll *a* (Chl-*a*) was measured by spectrophotometer every day according to Jeffrey and Humphrey (1975). After extraction with 90% acetone at 4°C in darkness for 24 hr, optical density was measured at 630 and 663, 750 and 645 nm and chlorophyll a was calculated according to the following equations:

$$Ca = 11.64\ (A_{663}-A_{750})\ y2.16\ (A_{645}-A_{750}) + 0.1\ (A_{630}-A_{750}).$$

*C*a represents chlorophyll a.

Growth curves based on changes in the cell number were constructed. The percent inhibition of cell growth at each extract dose was calculated by comparison of growth curves for each concentration. The instantaneous growth rate was determined by the following equation (OECD, 1984):

$$\mu = \frac{\ln N_t - \ln N_0}{t}$$

where t is the instantaneous growth rate (d^{-1}), N_t is the cell density at time t, N_0 is the cell density at time 0, and t is the time interval.

Toxin analysis: 50 ml samples of the growth medium were collected at both the beginning and end of 3 days' incubation for extracellular microcystin-LR detecting. For dissolved toxin analysis, the growth mediums of all groups were filtered through a 0.45 μm membrane. After which, they were concentrated and purified via C18 carbograph SPE columns (America, SC-103) by washing the column with 15 ml H_2O and 10 ml methanol followed by the samples. The cartridge containing the microcystin-LR was rinsed with 30 ml 20% methanol and the microcystin (MC) were finally eluted twice from the C18 cartridge with 10 ml of methanol. The eluate was evaporated under nitrogen and then the residue was dissolved in 0.5 ml methanol.

Microcystin-LR dissolved in growth mediums were analyzed by a reverse-phase high performance liquid chromatography (HPLC) (Shimadzu, LC-10AD) equipped with an ODS column (Cosmosil 5C18-AR, 4.6×150mm, Nacalai, Japan) and a SPD-10A UV–VIS spectrophotometer set at 238 nm. The samples were separated by using the mobile phases of 60% aqueous methanol containing 0.1% trifluoroacetyl (TFA) in 30 min at a flow rate of 1 ml min^{-1}. Column temperature was maintained at 25°C and the injection volume was 20 μl (Isabel *et al*., 2004). The microcystin-LR was identified by their UV spectra and retention times, and by using commercial microcystin-LR (Wako Pure Chemical Industries, Japan) as standards. The MC peaks were isolated and identified according to their mass spectra. The detection limits for intracellular MC and extracellular MC were 0.1 mg l^{-1}. Each analysis was performed in duplicate.

Outdoor exposed test: In order to further study the effects of the natural agent on the fresh water ecosystem, the simulated mini model aquatic ecosystem was made by two aquariums, which were constructed in 0.6 cm length, 0.3 cm depth and width and contained approximately 59.4 lit. freshwater from one eutrophic pond water in Kofu, Shanli, Japan. During 36 days test period (from August 28 to October 4), 2 mg l^{-1} extract was added in one aquarium weekly and the other one was used as the control. Samples of 100ml of the surface water were collected from the aquariums at fixed period and used to determine the algae cell count and pH, DO, water temperature, zooplanktons and phytoplanktons contents.

The water temperature and pH were measured in situ using a YSI meter (63/100 FT, YSI Inc., Yellow Springs, OH). The dissolved oxygen (DO) was measured with a DO meter (95/100 FT, YSI Inc., Yellow Springs, OH). The phytoplankton and zooplankton contents were counted using a Fuchs-Rosenthal counting chamber (Paul Marienfeld GmbH and Co., Lauda-Konigshofen, Germany) under an optical microscope (Microphot-FXA, Nikon Corp., Tokyo, Japan).

Results and Discussion

The growth inhibition of *M. aeruginosa* by the wattle extract: In our experiment, biomass or growth rate is used as indicator of algal growth inhibition. Compared with the control, the Chl-*a* concentration and cell density of *M. aeruginosa* treated with different wattle extract concentrations was obviously decreased in different amounts (Fig. 1, 2). In general, the effects were gradually enhanced with the increasing of the plant extract concentration. Our results

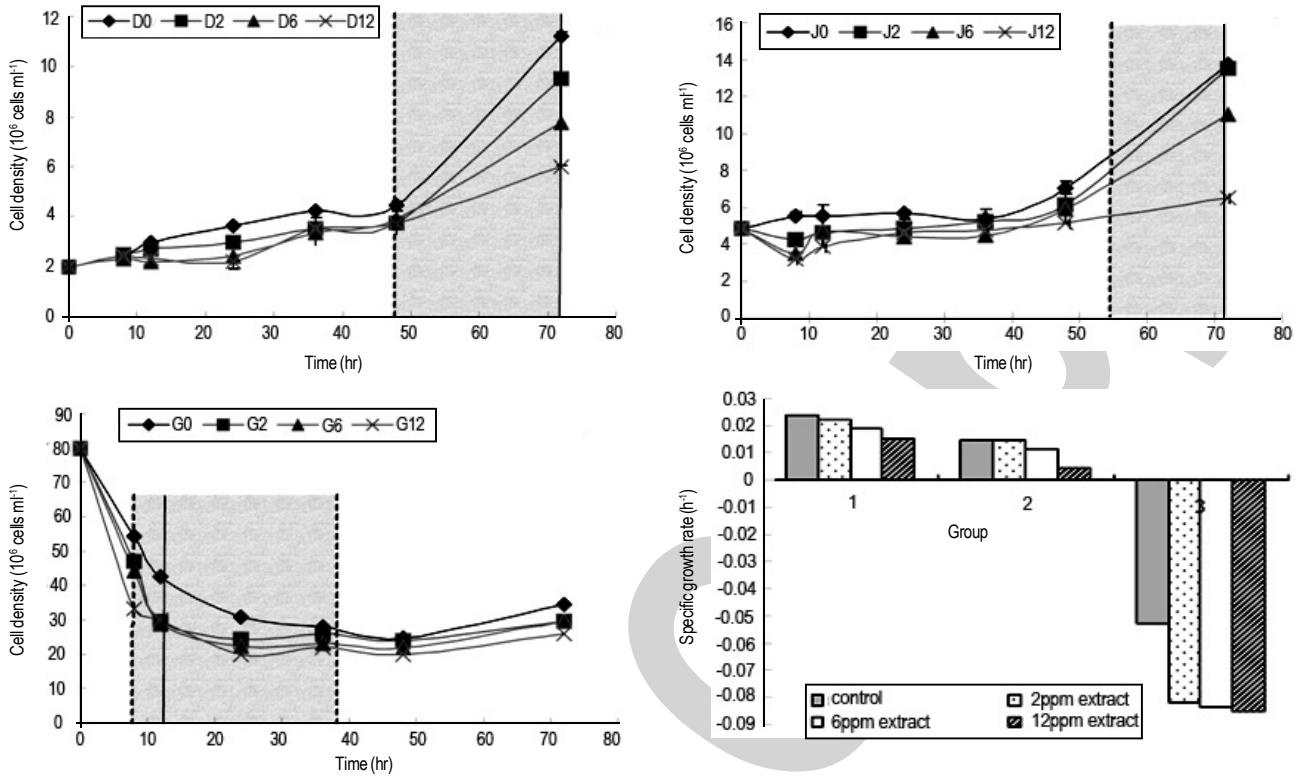

Fig. 1: Effect of different concentrations of the wattle extract on the growth of *M. aeruginosa* in 3 daysand specific growth rates (D) of samples in group 1, 2, 3 (A is the rates of samples in group 1; B is the rates of samples in group 2; C is the rates of samples in group 3) at the time of the best efficacy of the wattle extract (indicated by black vertical lines). The gray areas indicate the effective acting times of the extract on different groups with different cell density and growth conditions. The vertical bars indicate the SD

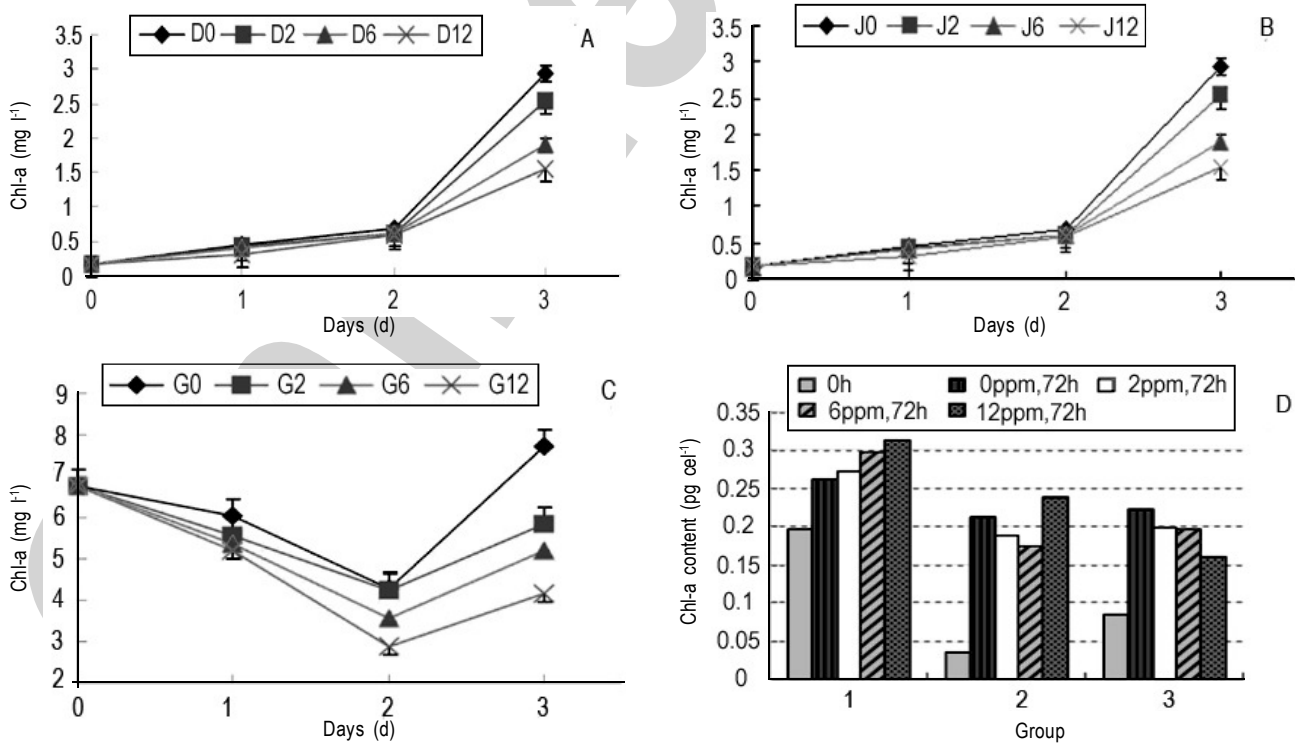

Fig. 2: Growth inhibition of *M. aeruginosa* by adding different wattle extract concentrations into different groups (A is the rates of samples in group 1; B is the rates of samples in group 2; C is the rates of samples in group 3) expressed by Chl-a concentration in 3days. The vertical bars indicate the SD

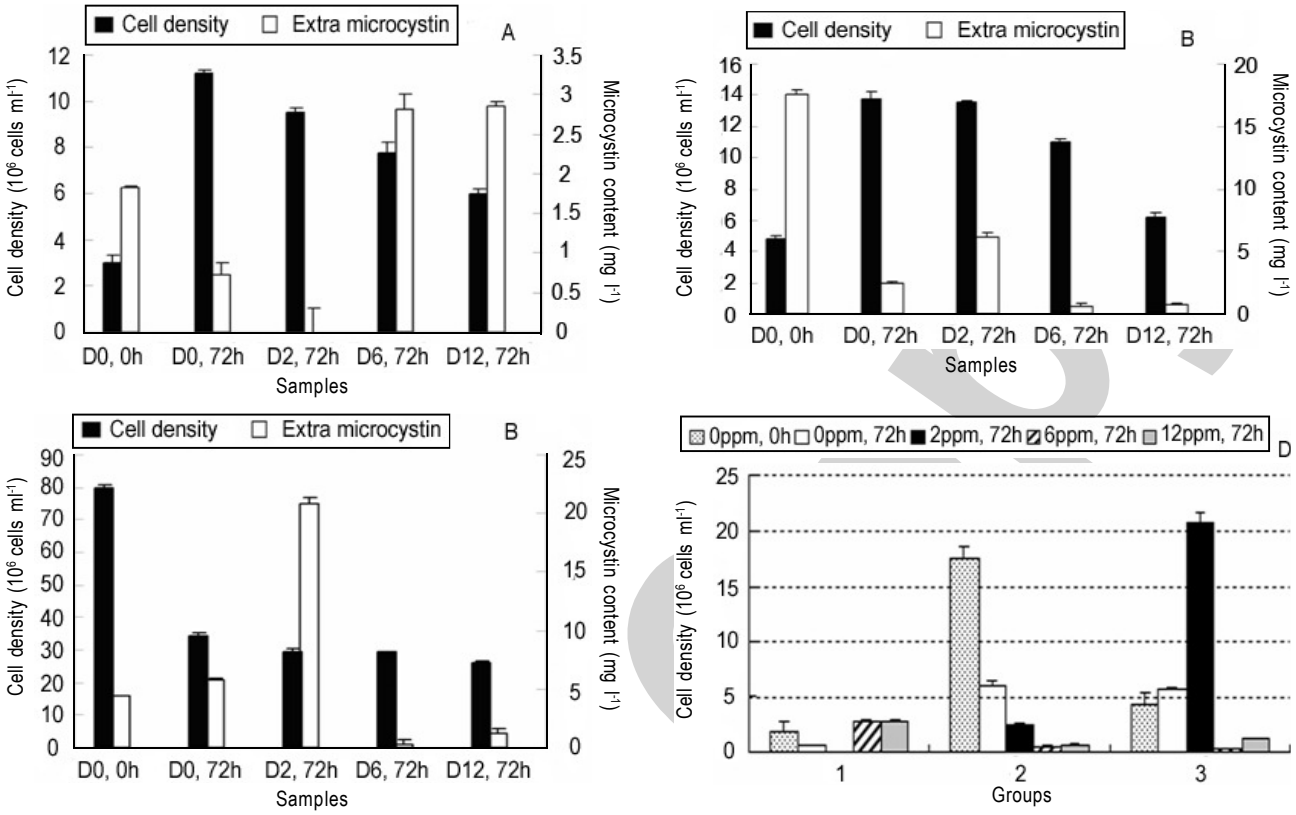

Fig. 3: Extracellular microcystin-LR contents of *M. aeruginosa* after 72 hr incubation with different concentrations of wattle extract in 3 groups. Vertical bars show standard deviation. The codes of samples are followed Table 1

showed that, 12 mg l^{-1} wattle extract in the medium of group 2 under nonsterile condition led to the largest decline of cell density and specific growth rate level, which were 47.3 and 70.2% lower than the control group during the 3 day period, while 2 mg l^{-1} wattle extract treatment in group 3 played a smaller role on the *M. aeruginosa* growth descending level. It is possible that the bacteria in the water can promote the inhibition of wattle extract on the algae by nutrition contest, and thereby lead to starving the algae, resulting in its death. A bacterial supremacy was found while in competition for both inorganic and organic P with algae (Trond *et al*., 2007).

Additionally, different conditions of algae in polluted water treated with the wattle extract had different results. The growth patterns of the two lower initial cell density groups (group1 and 2) were similar, but differed from that of the maximum initial cell density group (group 3). When the initial cell density is low below 5×10^6 cells ml^{-1} (group 1 and 2), the plant extract exhibited a distinct inhibition of the algae growth after adding 48 hr and its effects reached the maximum after 72 hr treatment (Fig. 1 A,B). Whereas, when the cell density is at the maximum about 8×10^7 cells ml^{-1}, application of wattle extract promoted deeper decline of cell density to some degree, but lost its action soon after 36 hr incubation (Fig. 1C). Thus, these results also indicated that the application of wattle extract early in the initial stage before the exponential phase can cause a better effect on cyanobacterial bloom control.

In general, the changes of Chl-*a* concentration in three groups were similar to that of cell density after adding the wattle extract (Fig. 2). The results of Chl-a content also suggested a stronger inhibition of the wattle extract in group1, in which the cell size indicated by Chl-*a* content of every cell became larger with the increasing of the wattle extract concentration (Fig. 2D). As for group 2, cell size was only enlarged by 12 mg l^{-1} wattle extract. Kruger and Eloff (1981) suggest that cell size is a likely indicator of the physiological state of a cell with stressed cells being larger. Visual observation of ecdysteroid treated *C. vulgaris* cells shows that ecdysteroids stimulate enlargement of the algal cells (Bajguz and Dinan, 2004). However in group 3, Chl-*a* of every cell was decreased with the increasing of the wattle extract concentration (Fig. 2 D). This may be due to the maximum cell density decreased by the wattle extract inhibition, which weakened the interspecific competition in algae and subsequently became in favor for the algal growth.

From our results, it is confirmed that the wattle extract have good positive effect on *M. aeruginosa* growth control. The major adverse effect of the wattle extract on cyanobacteria may inhibit algal growth in two ways: on one hand, its main composition of tannins could possibly combine with the proteins in cytoplasm and thereby interferes with cellular enzymatic activities (Di and Shi, 1999); on the other hand, it could also bind extracellular substrates and thus, limit C and N mineralization (Fierer *et al*., 2001) cutting off the nourishing

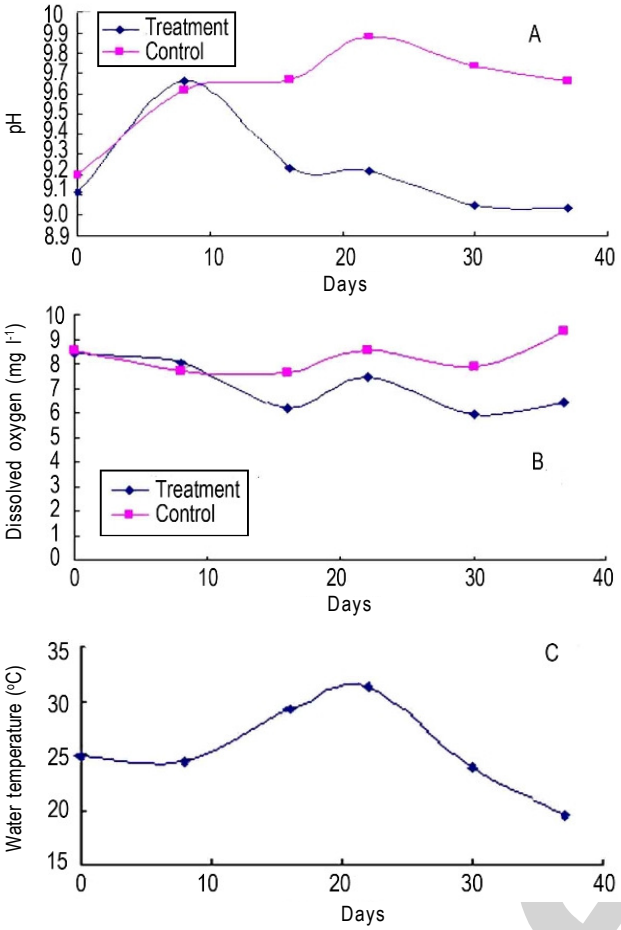

Fig. 4: Variations of pH (A), dissolved oxygen (B), and water temperature (C) resulting form wattle extract treated outdoor experiment. The condition of temperature in treatment and the control is the same

source of algae. However, this biological mechanism of the interaction among the wattle extract, algae and bacteria need to be studied further in the future work.

Effect of wattle extract on extracellular microcystin-LR content of *M. aeruginosa*: In the algal toxicity experiment, extracellular microcystin is in general used to safety evaluation the algae with the wattle extract treatment. Our study showed that different concentration of the wattle extract had a different extent of variation influence on extracellular microcystin production (only microcystin-LR was detected) of monoclonal *M. aeruginosa* with three groups of different conditions (Fig. 3).

The toxicity of cyanobacteria may be altered depending on varying culture conditions (Doers and Parker, 1988). The close relationship between cell density and extracellular microcystin found in previous work (Lyck, 2004; Wang *et al*., 2007) has also been confirmed by our data. Positive correlations between increasing concentrations of extra MCs (microcystins) and the dominancy of *Microcystis* spp. have been observed by Blahova *et al*. (2007). This result was partly supported by our experiment just after three days' incubation with no wattle extract treatment (Fig. 3 D). When the wattle extract did not exist, the samples of group 1 with the lowest cell density produced the lowest content of extra microcystin-LR (1.8297 mg l^{-1}), while samples of group 2 produced the highest content of microcystin (17.5787 mg l^{-1}) than the others at the initial time. It is possibly because the bacteria in group 2 stimulated *M. aeruginosa* to excrete extra Microcystin. Environmental stimuli (*e.g.*, a chemical signal or feeding related activity of fish) could cause levels of toxin expression in Microcystins to vary (Jang *et al*., 2003, 2004). Additionally, samples with maximum cell density (about 8×10^7 cells ml^{-1}) produced slightly higher microcystin content in the water 3 days later than the initial time (Fig. 3C). This result supports the assumption that microcystin excretion increases as the cell number in the culture reaches a maximum (Lyck, 2004). On the contrary, extra microcystin contents in two groups with lower density declined with the increase of cell density after 3 days of cultivation. This suggests that on the logarithmic phase of growth, the algae releases less microcystin under the favorable conditions than in the stationary phase.

Furthermore, our results showed a more complex relationship between the wattle extract concentration and extracellular microcystin production. In spite of a smaller inhibition of 2 mg l^{-1} wattle extract than that of 6 and 12 mg l^{-1} on algae growth, it still led to the similar variation of extra microcystin content in different groups. In group 1 with the lowest cell density than other groups, 2 mg l^{-1} wattle extract resulted in so much low extra microcystin content that it couldn't be detected 3 days later (Fig. 3A,D). It indicates that wattle extract can play an inhibiting role not only on algal growth but also on the process of microcystin excretion. The observation by Shi *et al*. (1995) of some localization of microcystin in the cell wall and sheath suggests that microcystin may act as transmembrane transporters of calcium, magnesium or other metals. Under stronger actions, the activity of membrane receptors is suppressed (Kuzin, 1995). Therefore, we speculate that wattle extract might bind with the enzymes related to the physiological activity in algal cell and accordingly influence the cell membrane receptors. Further experiments are needed to clarify the problem. However, 2 mg l^{-1} of the wattle extract produced higher microcystin content in group 2 than in group 1 (Fig. 3B,D). A possible reason may be, a big interspecific competition between algae and bacteria in group 2 under nonsterile condition was likely to stimulate the algae to release more microcystin as a defense response for this environmental stress. A similar phenomenon was found when the alga was exposed to some fish (Jang *et al*., 2004). Singh *et al*. (2001) found that one main function of microcystin is to inhibit the growth of other aquatic organism. Meanwhile, the highest extra microcystin content was found in group 3 with 2 mg l^{-1} wattle extract treatment (Fig. 3C). This may be due to more intracellular microcystin released to the water as the larger intraspecific competition of life source in stationary phase. Hence, the comparable weak ability of 2 mg l^{-1} wattle extract for inhibiting excretion of microcystin could not catch up with the microcystin released to the mediums in group 2 and 3 with high cell density. Hence, based on our results, we presume that more extra microcystin is excreted when there is larger inter- or intraspecific competition in the water.

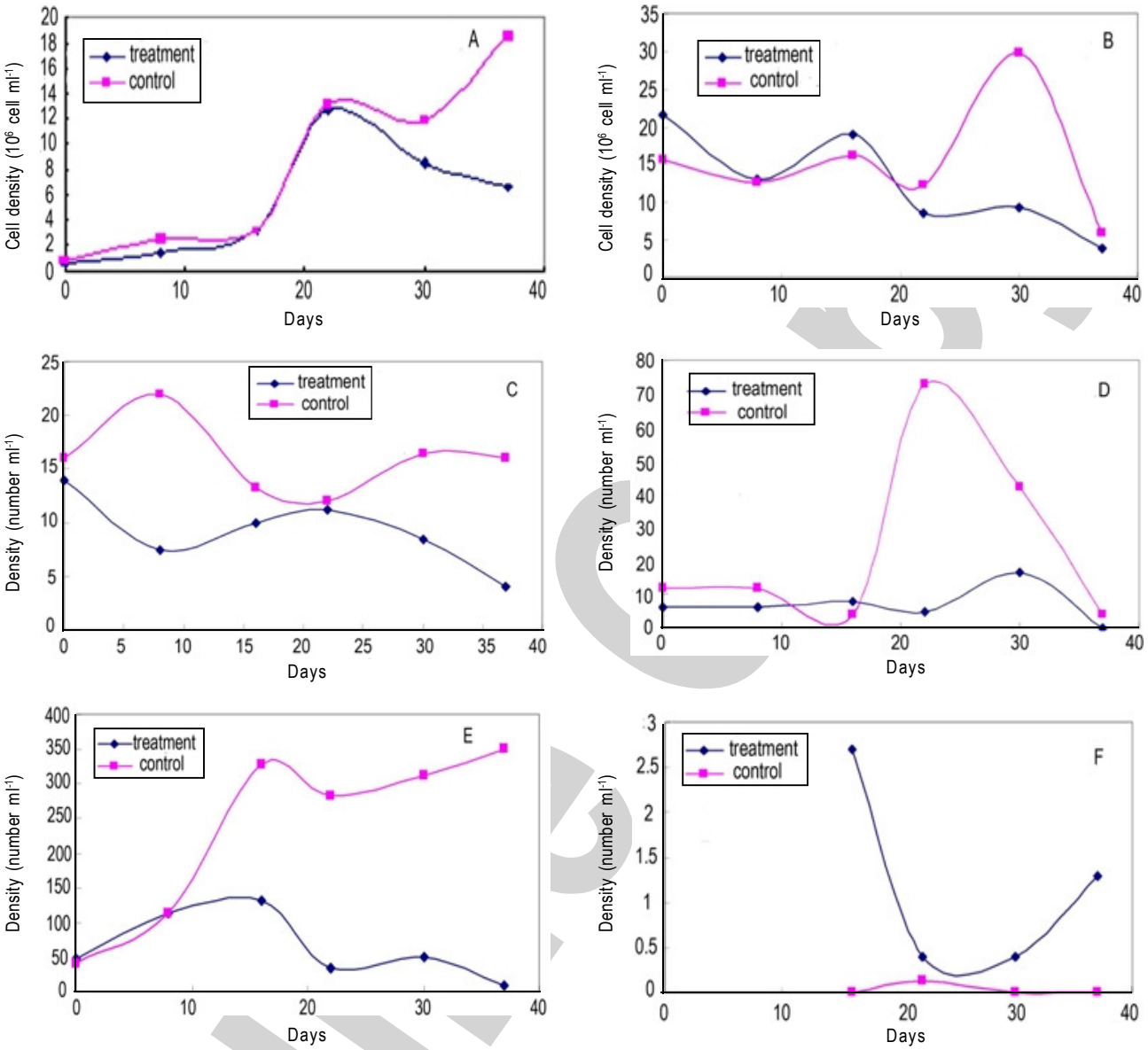

Fig. 5: The changes of algae and some aquatic animals number in the outdoor test water treated by the plant extract. A indicates the variations of the density of cyanobacteria; B is the variations of the density of diatoms; C presents the variations of the density of small-sized aquatic animals; D is the variations of the density of rotifer; E is the variations of the density of *Centropyxis* sp.; F is the variations of the density of macrozooplankton

Nevertheless, 6 and 12 mg l^{-1} wattle extract led to higher microcystin content in group 1 than in the other two groups with the same concentrate of the wattle extract treatment (Fig. 3A,D). In these cases, more microcystin was released from much more dead cells, which may have been a result of the stronger inhibition of the wattle extract. But, this situation is reversed in the samples of group 2 with nonsterile treatment and group 3 with maximum cell density. It has been confirmed that bacteria can decompose the microcystin (Imanishi *et al*., 2005) and this effect can be enhanced because of the development of their activities stimulated by higher concentration of the wattle extract and air flow. As for group 3, stronger inhibition on microcystin excretion than on the algal growth by high wattle extract concentration was contributed to the low extra microcystin content at the maximum cell density.

The significant Influence of wattle extract on the simulated mini aquatic ecosystem: During the experimental period which was 36 days long, the water temperature was at 20-30°C, which is suitable to cyanobacterial growth (Fig. 4C). On the day with the highest temperature, DO values increased slightly. In the water treated, the pH and DO values decreased when compared to the control (Fig. 4 A,B), which reflected lower photosynthesis and the suppression of algal growth by the wattle extract.

Table - 1: The experimental design of different growth conditions. All groups were incubated at 25°C, 140 μ mol m^{-2} s^{-1} and light $dark^{-1}$ (L/D) 12 hr 12 hr^{-1} cycle in a light incubator except for the different conditions described as follows

Groups	1	2	3
Cells number (cells ml^{-1})	2×10^6	4.8×10^6	8×10^7
Wattle extract concentration: 0 mg l^{-1}	DO	JO	GO
Wattle extract concentration: 2 mg l^{-1}	D2	J2	G2
Wattle extract concentration: 6 mg l^{-1}	D6	J6	G6
Wattle extract concentration: 12 mg l^{-1}	D12	J12	G12
Growth condition	Standing, sepsis	Ventilation, no asepsis	Standing, asepsis

D0=group 1 adding no extract; D2=group1 with 2 mg l^{-1} extract; D6=group1 with 6 mg l^{-1} extract; D12= group1 with 12 mg l^{-1} extract; J0= group2 with 0 mg l^{-1} extract; J2= group2 with 2 mg l^{-1} extract; J6= group2 with 6 mg l^{-1} extract; J12= group2 with 12 mg l^{-1} extract; G0= group3 with 0 mg l^{-1} extract; G2= group3 with 2mg l^{-1} extract; G6= group3 with 6 mg l^{-1} extract; G12=group3 with 12 mg l^{-1} extract.

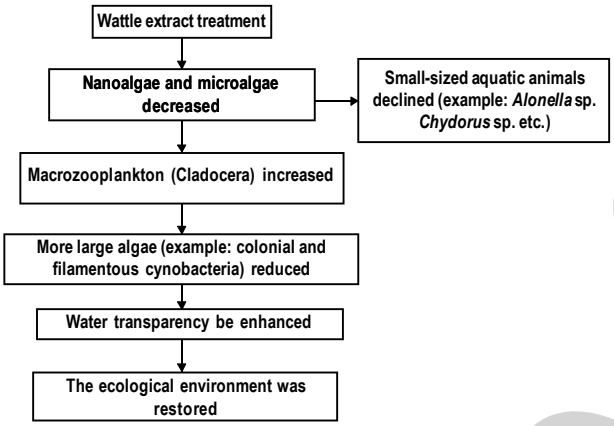

Fig. 6: The influence of the wattle extract on the aquatic ecosystem of eutrophic water attained from a fish pond in Japan

While much floating scum of algae was observed on the surface water in the control enclosure after the 36 days outdoor experiment, little was formed in the treated aquarium. After 16 days in the experiment, cyanobacteria in the two aquariums grew exponentially with the increased temperature from 28 to 31.2°C, but the cell density of cyanophytes in the treated one started to decrease at 6d later from then on (Fig. 5 A). At the end of the experiment, the cell density in the water with the wattle extract was nearly one-third of (6.3×10^3 cells ml^{-1}) of the control level (1.9×10^4 cells ml^{-1}). The dramatically greater decrease of cyanobacteria in the outdoor treatment, which differs in the lab, indicates a more effective inhibition of the wattle extract on algae in a natural water ecosystem than in monoclonal *M. aeruginosa* culture.

Moreover, the composition of the aquatic animal community was also influenced by the wattle extract. As the water temperature increased to the highest point (31.2°C), diatoms of the control grew exponentially and bloomed as the temperature dropped to 24.4°C, but diatoms of the treated sample remained nearly constant during this period (Fig. 5B). When the wattle extract was added to the water, nutrients limited by the wattle extract, especially Silicon, resulted in the decline of diatoms. Thus, there was not enough food provided for small-sized aquatic animals in the water, such as *Centropyxis* sp., whose main food is diotams. In the outdoor experiment, with the inhibition of cyanobacteria and diatoms, the water quality got better. Accordingly, the number of small-sized aquatic animals in the treated water, such as *Alonella* sp., *Chydorus* sp., *Trichocerca* sp. and *Centropyxis* sp. etc. (Fig. 5 C,D and E, respectively) decreased; whereas in the control, they multiplied during the exponential phase of cyanobacteria in the control water. Meanwhile, in the treated water, macrozooplankton such as *Simocephalus* sp., the good water quality indicator, was increased (Fig. 5F) and subsequently more large algae (example: colonial cyanobacteria) were reduced. Finally, water transparency was enhanced and the specific diversity was increased. Therefore, the outdoor experiments represented observable evidences for the algal bloom control and the improvement of the aquatic biological environment by the wattle extract (Fig. 6). However, it has not been fully investigated to conclude that inhibition is also applicable to eukaryotic algae yet. Further researches need to be done in this area.

Ding *et al.* (2007) had successfully applied the wattle extract in their BIOSS system to the Lake of Yanzhong Park in Shanghai and monitored the changes in water quality over a 3 year period. Major improvements were also attained in the transparency, suspended solids and chemical oxygen demand, TN, TP and biological diversity. However, many current chemical and physical treatments have somewhat harm effects on the aquatic ecosystem. For example, the ultrasonic radiation (Ahn *et al*., 2003) application is not only able to kill the cyanobacteria, but also able to kill other algae and may do harm to other zooplankton. Most chemical compounds application, such as $CuSO_4$ and aluminum sulphate (Chow *et al*., 1999), will introduce the new compounds, which have negative impacts on fish health, into the freshwater. The applications of algicidal bacteria to freshwater is also considered to be ecologically risky for bringing the alien organisms into the water (Mayali and Azam, 2004). While there is lack of the study on the freshwater aquatic ecosystem in the barley straw extract inhibition (Welch *et al*., 1990; Ridge and Pillinger, 1996; Ball *et al*., 2001). Our experiments proved that the plant extract can improve the ecological environment by promoting interspecific competition with algae in water. It can be decomposed in the fresh-water easily in a few days as described above. Therefore, our careful estimation on acute toxicity tests exhibited that the plant extract in the application concentration was safer than other chemical methods (personal data). However, the long-term ecological safety of the wattle extract still needs to be confirmed.

In conclusion, the current finding is very useful for effective and safe application in controlling cyanobacterial blooms. The effect of the wattle extract was fast and definitive in controlling cyanobacteria and cut down extracellular micryocystin content, especially with the help of bacteria decomposition. The variability of sensitivity to the wattle extract in different conditions also provides for more efficient and safer cyanobacterial growth inhibition. Finally, a field study based on an outdoor experiment confirmed the virtuous action of this plant extract to restore the fresh-water ecosystem. However, the mechanism of the plant extract inhibition on cyanobacteria needs to be further studied.

Acknowledgments

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