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Allelopathic control of cyanobacterial blooms by periphyton biofilms

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Summary

Periphyton biofilms are natural mixtures comprised of photoautotrophic and heterotrophic complex microorganisms. In this work, the inhibition effects of periphyton biofilms on cyanobacterial blooms were studied in pilot and field trials. Results show that the cyanobacterial species responsible for the blooms had an upper nutrient concentration threshold, below which it could not effectively compete with other organisms in the periphyton. The disappearance of the cyanobacterial blooms was due to the allelopathy between the cyanobacteria and periphyton biofilm. In particular, it was found that the periphyton biofilm could produce water-soluble allelochemicals such as indole and 3-oxo-α-ionone to significantly inhibit the growth of the cyanobacteria. These allelochemicals are able to damage the thylakoid membranes of the cyanobacteria, interrupt the electron transport in photosystem II, decrease effective quantum yields, and eventually lead to the failure of photosynthesis. A comprehensive discussion on the ecological consequences of these findings is also presented. This work demonstrates the potential of periphyton biofilm to be used as an environmentally friendly ecological engineering solution for (i) the control of cyanobacterial blooms and (ii) a transitional means for the construction of beneficial conditions for ecosystem restoration. In addition, this work provides significant insights into the competitive relationships between algae and biofilms.

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Introduction

Algal blooms have become a global problem as a result of continuous growth of population and increasing water demand (Chorus and Bartram, 1999). In particular, cyanobacterial blooms can impose strong physiological, chemical and biological impacts on the biogeochemical properties and function of water systems (Paerl *et al.*, 2001).

Direct physical and chemical treatment of algal blooms, such as ultrasonic treatment, ozonation, bluestone and potassium permanganate have been proposed to control cyanobacterial blooms (Chorus and Bartram, 1999). However, the performance of these direct treatments is not long-lasting and complete removal of the cyanobacteria is not normally achievable in large systems. Most importantly, these methods are not economically viable for large water bodies, such as lakes and reservoirs. Biomanipulation technologies, such as the use of filter-feeding fish (Xie and Liu, 2001) and biopond-wetland systems (Wu *et al.*, 2010), are the most feasible technologies to address the issues of the cyanobacterial blooms. They are usually environmental friendly, cost-effective and do not produce secondary pollutants. However, most of these bio-measures have not been validated and have yet to be reliably applied in field trials (Sengco and Anderson, 2004).

Biofilms are defined as the thin layer of microorganisms that cover rocks and plants in aquatic environments and can facilitate microbial growth (Karsten and Kühl, 1996). The succession (including structure and components) of periphyton biofilms changes with their living conditions (Lambert *et al.*, 2008). For instance, eutrophication at moderate levels may increase and speed up the development of biofilm communities in originally oligotrophic areas. At higher levels, however, eutrophication causes a decrease in biomass and diversity, and favours opportunistic species (Moran and Grant, 1989). In addition, the excessive growth of phytoplankton in eutrophic conditions may reduce the biofilm biomass due to shading (Strom,

2008). Some authors have suggested that the changes in the development of the periphyton biofilms communities in eutrophic areas are caused by the competition between tolerant and non-tolerant species instead of the toxic conditions (Moran and Grant, 1989).

Studies have been conducted to evaluate the allelopathic effects of single-species photoautotrophic microorganisms or their biofilms on other single-species bacterioplankton, such as *Diatoma* (Jüttner, 2001), *Desmodesmus quadrispina*, *Uronema confervicolum* (Leflaive *et al*., 2008; Leflaive and Ten-Hage, 2009), *Alexandrium tamarense*, *Karenia mikimotoi* and *Chrysochromulina polylepis* (Flstarol *et al*., 2004). Additionally, many studies have examined the allelopathic effects of heterotrophic microorganisms or the biofilms that consist of heterotrophic microorganisms in aquatic ecosystems, such as algal-lysing fungi (Redhead and Wright, 1978) and bacteria (*Alphaproteobacteria*, *Betaproteobacteria*, *Gammaproteobacteria*, *Bacteroidetes*, *Firmicutes* and *Actinobacteria*) (Jutta *et al*., 2009).

Some of the aforementioned single-species biofilms showed strong allelopathic effects on bacterioplankton (Jüttner and Wu, 2000). However, such simple-biofilm compositions may be susceptible to the variable conditions of surface waters and will find it difficult to form a stable and self-sustaining micro-ecosystem. Periphyton biofilms often consist of complex communities of photoautrophic and heterotrophic microorganisms (microalgae, bacteria, fungi, protozoa, metazoa and epiphytes) (Zippel *et al*., 2007). The organisms in biofilms create their own microhabitats with pronounced gradients of biological and chemical parameters, enabling the efficient and effective use of substrates and energy (Meyer-Reil and Köster, 2000). Therefore, the integrated nature and ability to self-organize may make periphyton biofilms useful for the production of compounds that have allelopathic effects, including negative effects on harmful bacterioplankton and algae. To our knowledge, the ecological relevance of the allelopathic processes of periphyton biofilms has not been previously explored in the literature (Legrand *et al*., 2003).

In this work, considering the periphyton biofilm as an ensemble, we examined the allelopathic effect of periphytic biofilms on the inhibition of cyanobacterial blooms. The main objective of this study was to determine (i) the effects of the periphyton biofilms on the growth of the cyanobacteria, (ii) the effect of periphyton biofilm extract on the growth of the cyanobacteria and (iii) the compounds responsible for the inhibition of cyanobacterial growth. With these findings we hoped to understand the allelopathy mechanism between multi-community biofilms and single-species bacterioplankton, and then provide an environmentally benign bio-measure to control cyanobacterial blooms as a transitional means of aquatic ecosystem restoration in surface waters. The use of periphyton biofilms for inhibiting cyanobacterial blooms in eutrophic waters has the following advantages: (i) the acclimation and maturation phases of periphyton biofilm are short because of the use of native microorganisms (Bourne *et al*., 2006), (ii) the incubation, cultivation and enrichment of the periphyton biofilm are easy to manage and are potentially applicable in various waters, (iii) the method is acceptable by the public (compared with the use of chemicals), and (iv) it may also degrade some microcystins such as microcystin-LR and microcystin-YR (Babica *et al*., 2005).

Results and discussion

Characteristics of the periphyton biofilms

To obtain native microorganisms and facilitate industrial application on a large scale, the periphyton was cultured and incubated using sediments and water collected directly from the control-enclosures (the controls of field experiments), Moon Lake, China. The morphologies of the periphyton biofilms were observed under SEM and optical microscope. The results show that the biofilms in both the pilot and field trials were very similar and were dominated by diatoms and bacteria (Fig. S1). The diatom mainly consisted of *Synedra ulna* Kütz., *Gomphonema parvulum* Kütz., *Fragilaria vaucheriae* Kütz. *Melosira varians* Ag., *Nitzschia amphibia* Grun, while the bacteria mainly consisted of bacilli and cocci.

Inhibition effect of the periphyton biofilms

Microscopic analyses revealed that the algae in both the biofilm-containing and control ponds were cyanobacteria (*Microsystis aeruginosa*). For control samples, the water was completely covered by a thick layer of the cyanobacteria for 36 days. In strong contrast to the control ponds, the water in the biofilm-containing ponds remained clear enough so see the sediment at the bottom through the overlying water after the same period of time. The quantitative change in algae in terms of chlorophyll *a* concentration over the 36 days in the overlying water in the simulated biofilm-containing and control ponds is displayed in Fig. 1A. In the control samples, the chlorophyll *a* concentration increased with time. In particular, after 17 days, the chlorophyll *a* concentration had increased exponentially in the control ponds and the algal bloom occurred. In contrast, the chlorophyll *a* content in the biofilm-containing ponds was undetectable after the 17th day.

Roles of the nutrients and zooplankton

The aforementioned inhibition effect of the biofilms may possibly be due to the following reasons: (i) the depletion

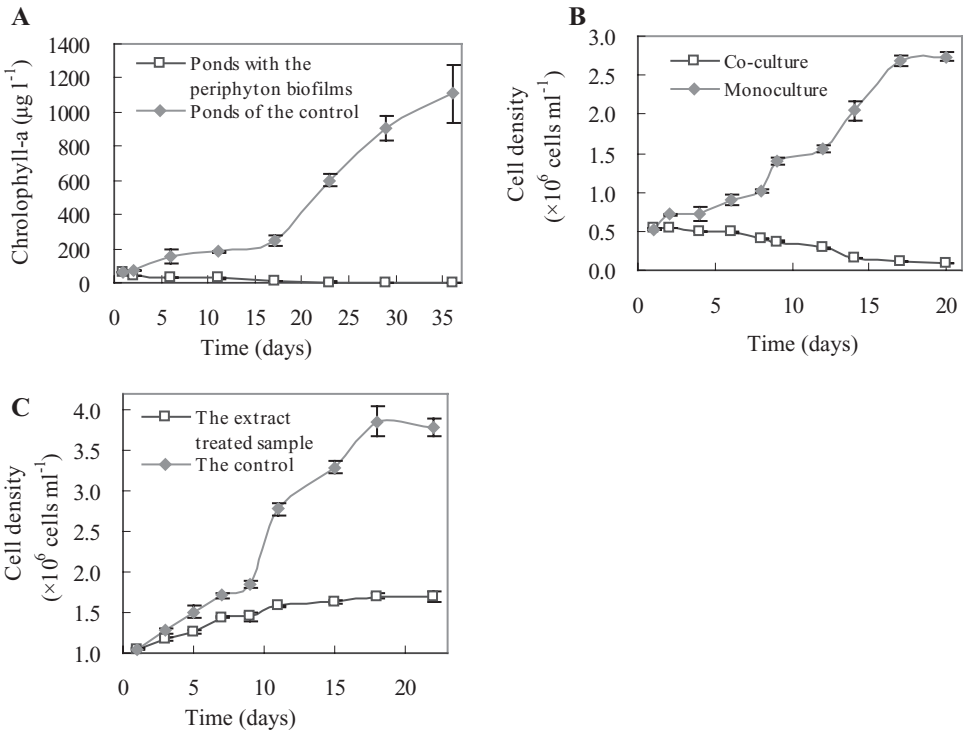

Fig. 1. A. The changes in chlorophyll *a* concentration in overlying water in the simulated ponds with periphyton biofilms and the controls.
B. Inhibition effects of the periphyton biofilms on the cyanobacterial growth in the monoculture and co-cultured experiments.
C. Inhibition effects on the cyanobacterial growth with extract and the controls.
The vertical bar represents the standard deviation of triplicate samples.

of the nutrients due to the introduction of the biofilms, (ii) the consumption of the algae by the zooplankton and (iii) the allelochemicals released by the biofilms. First, the roles of the nutrients and zooplankton were investigated.

The average concentrations of ammonium, nitrate and total dissolved phosphorus in the overlying water in the biofilm-containing ponds were 0.54, 0.67 and 0.12 mg l^{-1}, respectively, while the concentrations in the control samples were 1.40, 2.46 and 0.41 mg l^{-1} respectively. The fact that the nutrient concentrations of the treatment samples were much lower than the controls suggests that the biofilms consume the nutrients and can compete for nutrients with the algae. Although previous studies showed that the growth rate of the cyanobacteria decreased as phosphorus concentration declined from 4.6 to 0 mg l^{-1} and ammonium concentrations from 1.83 to 0 mg l^{-1} (Zhang *et al.*, 2002; 2006), the nutrient concentrations in the biofilm-containing ponds were still sufficient to support the rapid growth of the cyanobacteria (Yusoff and McNabb, 2008). Therefore, it is possible that the cyanobacterium had an upper nutrient concentration threshold, below which it could not effectively complete with other organisms in the periphyton.

The role of the zooplankton was investigated in absence of the zooplankton. To obtain periphyton biofilm without zooplankton, the biofilm was cultured with sterilized water (though 0.22 μm pore). The experiments were conducted in the same media (BG 11 media) in the absence of the zooplankton. Two different samples were compared: (i) a monoculture containing cyanobacteria only and (ii) a co-culture containing cyanobacteria and periphyton biofilms. The average cell density of the cyanobacteria gradually decreased from 0.54×10^6 to 0.01×10^6 cells ml^{-1} in the co-culture experiment over 20 days. In contrast, the average cyanobacteria cell density in the monoculture experiments increased rapidly from 0.54×10^6 to 2.8×10^6 cells ml^{-1} at the end of the experiment (Fig. 1B). This observation in Fig. 1B is in line with that demonstrated in Fig. 1A and shows that the inhibitory growth of the cyanobacteria was not directly associated with the presence of zooplankton in the simulated ponds.

During the non-zooplankton experiments, the nutrient concentrations decreased from 33.78 to 28.95 mg l^{-1} for ammonium, from 20.30 to 14.48 mg l^{-1} for nitrate and from 0.48 to 0.27 mg l^{-1} for total dissolved phosphorus. These decreases, however, did not significantly affect the growth rate of the cyanobacteria (Schindler, 1977; Zhang *et al.*, 2002; Yusoff and McNabb, 2008). It is possible that the nutrient decrease was beneficial and enhanced the competition between periphyton and the cyanobacteria.

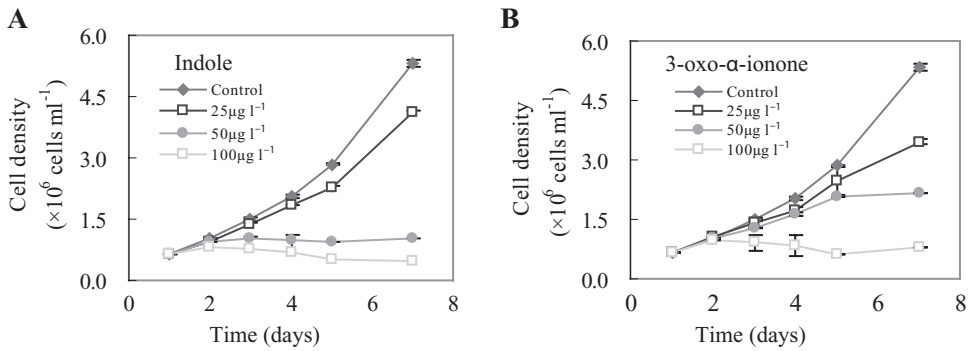

Fig. 2. Inhibition effects of indole (A) and 3-oxo-α-ionone (B) on the cyanobacterial growth. The vertical bar represents the standard deviation of triplicate samples.

Overall, these findings suggest that the periphyton biofilms are mainly responsible for the inhibition effects on the cyanobacteria.

Inhibition effects of the periphyton biofilms extract

As nutrient competition is not the main factor inhibiting growth of the cyanobacteria, we propose that the biofilms may release certain compounds affecting cyanobacterial growth. Extracts were collected from the biofilms and subsequently investigated to determine their inhibitory effects.

The cell density of cyanobacteria increased from 1.1×10^6 to 3.8×10^6 cells ml^{-1} in the control sample (*M. aeruginosa* monoculture in BG11 solution), representing a significant biomass increase (Fig. 1C). Under the same experimental conditions, only a small increase in cell density was observed for the biofilm extract treated sample (*M. aeruginosa* monoculture in BG11 solution in the presence of the extract), i.e. 1.1×10^6 to 1.6×10^6 cells ml^{-1} in 20 days. These results suggest that the biofilm extract inhibited the cyanobacterial growth.

Organic compounds in the periphyton biofilms

In order to identify the composition of the allelochemicals in the biofilms, 32 compounds were separated from the biofilm extract, of which 30 compounds were identified (Fig. S2 and Table S1).

It is well known that the allelopathy among phytoplankton algae might be the simple effect of a specific allelochemical or synergistic reaction of multiple allelochemicals. At present, little information is available about the latter due to technological limitations (Lewis, 1986; Jonsson *et al*., 2009). Considering the accessibility of experimental operation, some specific (or typical) allelochemicals were chosen to elucidate the roles of the identified compounds in inhibiting cyanobacterial growth in the following experiments. Among the identified compounds, indole and 3-oxo-α-ionone (Fig. S3) have the most potential for the inhibition effect. Indole is an aromatic heterocyclic organic compound. It can be produced by bacteria as a degradation product of the amino acid tryptophan (de Sá Alves *et al*., 2009). α-Ionone is an oxidation product of the carotenoids, flavoxanthin and lutein (from *Tagetes erecta*). It is also a precursor of other norisoprenoids with aroma and industrial interest like 3-oxo-α-ionone (Wahlberg and Eklund, 1998; Del Toro-Sánchez *et al*., 2006). Indole and 3-oxo-α-ionone (see Fig. S2) are two micro-water-soluble organic compounds, and their solubility in water were 1.9 g l^{-1} (20°C) and 1.69 g l^{-1} (20°C) for indole and 3-oxo-α-ionone respectively (Ni, 2007). It has been established that the indole and ionone can be toxic to a range of aquatic microorganisms. For example, indole can kill the larvae of aquatic insects (*Chironomus* sp.) (Becher and Juttner, 2005), and 6-hydroxy-3-oxo-α-ionone has strong adverse effects on algal growth (Xian *et al*., 2006). Indole and 3-oxo-α-ionone were not detected in the control (Fig. S4 and Table S2).

Using various concentrations of indole and 3-oxo-α-ionone, bioassays of the inhibition effect on the cyanobacteria (*M. aeruginosa*) were conducted quantitatively. The results show that both indole and 3-oxo-α-ionone significantly reduced cyanobacterial growth, and the degree of inhibition increased as concentration increased. The cyanobacterial growth was markedly inhibited when the concentrations were more than 25 μg l^{-1} for indole and 3-oxo-α-ionone, and was completely inhibited when the concentrations were greater than 100 μg l^{-1} (Fig. 2). Figure 2 also indicates that the inhibition effects of indole and 3-oxo-α-ionone on the cyanobacterial growth were time- and dose-dependent.

Inhibition mechanism

To investigate the inhibition mechanism of indole and 3-oxo-α-ionone, the changes in cyanobacterial cell densi-

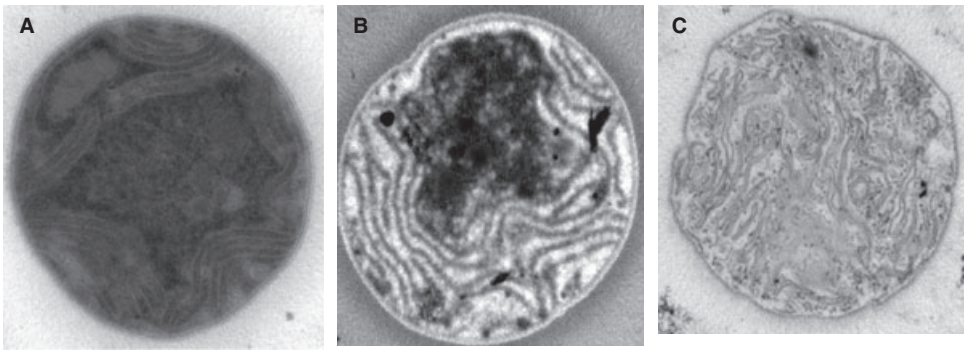

Fig. 3. A. The control (i.e. the cyanobacterial monoculture): the cell structure of cyanobacterium was intact.
B. Bioassay with 3-oxo-α-ionone at the concentration of 50 μg l^{-1}: the thylakoid membrane was detached from the cytoplasm in the algae cells.
C. Bioassay with indole at the concentration of 50 μg l^{-1}: the content within the thylakoid membrane deteriorated.

ties were monitored during the bioassay. The cell structure of the cyanobacterium was intact in the monoculture (control) sample (Fig. 3A). In contrast, in the presence of 50 μg l^{-1} 3-oxo-α-ionone on the fourth day, the thylakoid membrane of the cell was detached from the cyanobacterial cytoplasm (Fig. 3B). Similarly, in 25 μg l^{-1} indole on the fourth day, the shape of thylakoid membrane was contorted; and on the sixth day, the membrane was disconnected from the cyanobacterial cytoplasm. The inner materials within the thylakoid membrane started to deteriorate when the contact time between 50 μg l^{-1} 3-oxo-α-ionone and cyanobacteria reached the sixth day. Most of the content within the thylakoid membrane were lost on the fourth day in the presence of 50 μg l^{-1} indole (Fig. 3C). The above observations suggest that these two compounds are able to adversely affect the thylakoid membrane. As the thylakoid membrane is the site of the light-dependent reactions of photosynthesis, with the photosynthetic pigments embedded directly in the membrane, the loss of the membrane will lead to the dysfunction of photosynthesis and eventually result in the death of the cyanobacteria.

In the photosynthesis by the cyanobacteria, chlorophyll *a* in photosystem II (PS II) initially captures the photons with sufficient energy ($\lambda < 680$ nm) and produces electrons. The electrons are then transferred via cytochrome *bf* to photosystem I (PS I) and generate NADPH. The efficiency of photosynthesis (Smith, 1997) can be characterized by the electron transport rate and effective quantum yield. They are often used to represent the life competence of the plants and algae (Bolhar-Nordenkampf *et al*., 1989). Thus, the electron transport rate and effective quantum yield in PS II of the algae were investigated in the presence of 50 μg l^{-1} indole and 3-oxo-α-ionone separately. Both the electron transport rate and effective quantum yield for the control increased with the cultivation time due to the increased number of algae (Fig. 4). In contrast, these two parameters substantially decreased with the progress of time in both scenarios ($P < 0.05$). This suggests that indole and 3-oxo-α-ionone are able to interrupt the electron transport in PS II, and reduce the effective quantum yields.

Accordingly, a simplified model of the process of periphyton biofilm adversely affecting the cyanobacteria was

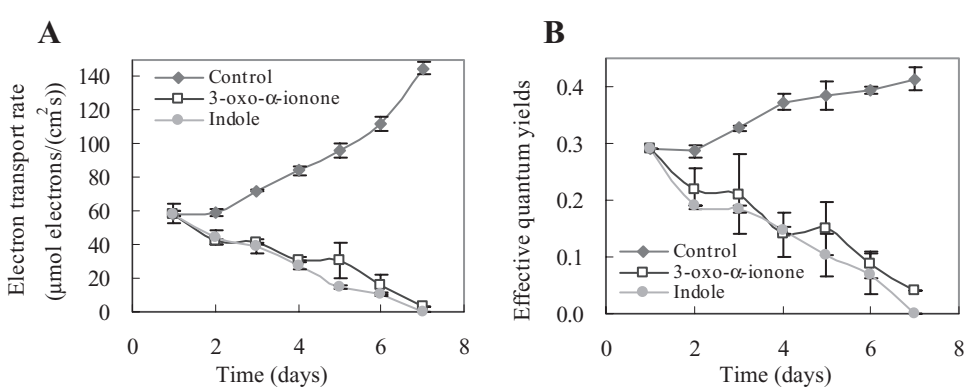

Fig. 4. The effects of the indole and 3-oxo-α-ionone on (A) the electron transport rate and (B) effective quantum yields of photosystem II reaction centre. The concentrations of both allelochemicals were 50 μg l^{-1}. The vertical bar represents the standard deviation of triplicate samples.

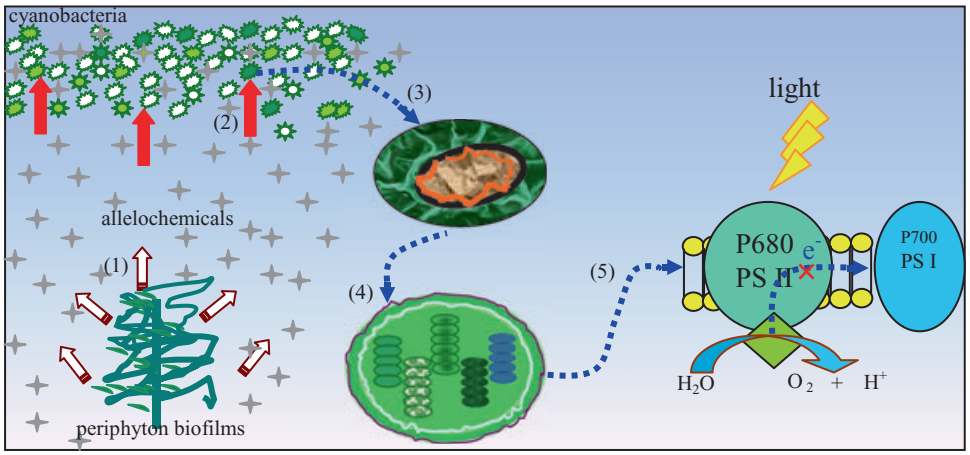

Fig. 5. The simplified model of the process of periphyton biofilms adversely affecting the growth of cyanobacteria. The process includes five steps in order as follows: (1) the periphyton biofilm releasing allelochemicals such as indole and 3-oxo-α-ionone, (2) the allelochemicals making contact with cyanobacteria, (3) the allelochemicals entering into the cells of cyanobacteria, (4) the thylakoid membrane suffers from allelochemical attack due to allelochemicals like indole and 3-oxo-α-ionone; for example, the membrane detaches from cyanobacterial cytoplasm; the inner materials in thylakoid membrane started to deteriorate, and (5) the electron transport in PS II reaction centre is interrupted and the effective quantum yields decreased leading to the failure of photosynthesis.

established. This process includes five main steps from the allelochemicals such as indole and 3-oxo-α-ionone being released by the periphyton biofilms to the failure of photosynthesis of cyanobacteria. A detailed description is shown in Fig. 5.

Inhibition effect of the periphyton biofilms in field tests

To determine whether the periphyton biofilms can become a practically useful biotechnology for controlling algal blooms, a series of field tests (fieldwork photo in Fig. S5) were conducted in Moon Lake, Wuhan City, China between June 2004 and March 2005. Before the beginning of the experiments, cyanobacterial blooms (*M. aeruginosa*) occurred frequently in the enclosures. Chlorophyll *a* levels in the treatment-enclosures in which periphyton biofilms were growing were always lower than those in the control-enclosure, although the trend varied during the periphyton biofilm deployment (Fig. 6). During the experiments, the cyanobacteria were dominated by *M. aeruginosa* in the control-enclosures, but in the treatment-enclosures the cyanobacterial blooms did not occur. The biofilms in the enclosure grew well in the practical conditions (Fig. S6). The biofilms consumed a certain amount of nutrients and grew from 1882 g m^{-3} to 2740 g m^{-3}. The concentrations of ammonium, nitrate and TDP in the overlying water in the enclosure were found to be 0.12–1.42, 0.06–0.22 and 0.07–0.54 mg l^{-1} respectively (Fig. 6). These nutrient concentrations are sufficient to support cyanobacterial growth (Yusoff and McNabb, 2008). To test whether the biofilm substrate [soft fibre carrier (SFC)] affects the cyanobacterial growth, a substrate-controlled experiment was conducted. The results showed that the effect of the biofilms substrate (SFC) on cyanobacterial

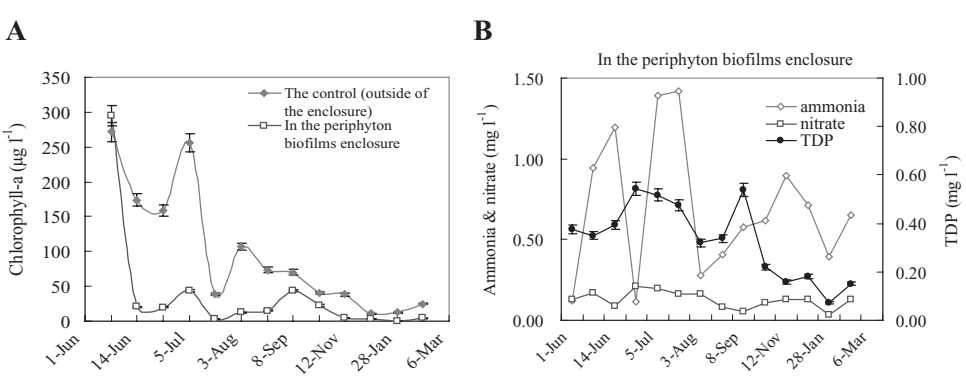

Fig. 6. (A) Concentration changes in chlorophyll *a* in the enclosures with periphyton biofilms and controls, and (B) nutrients (ammonia, nitrate and TDP) in the overlying water in the periphyton biofilms enclosure during the field deployment. The vertical bar represents the standard deviation of triplicate samples.

growth was negligible (Fig. S7). It was concluded that the disappearance of the cyanobacterial blooms was due to the periphyton biofilms.

To test whether the water in the control-enclosures and the aqueous extract of periphyton biofilm contained allelochemicals such as indole and 3-oxo-α-ionone, GC/MC analyses were conducted. The results showed that indole and 3-oxo-α-ionone were not detected in the water in the control-enclosures where the periphyton biofilm biomass on the inner enclosure walls was very low, i.e. from 50 to 200 g per cubic metre of water during the experimental period (Fig. S4 and Table S2), while the extract of periphyton biofilms contain these two compounds (Fig. S1 and Table S1). Consequently, these results suggest that the inhibition of cyanobacterial growth was mainly due to the allelochemicals such as indole and 3-oxo-α-ionone released from the dense periphyton biofilm.

The allelopathic interactions described in the literature are mainly between single microorganism (or microorganism biofilms of single species) and single phytoplanktons (Jonsson *et al*., 2009). In this study, the investigation of the allelopathy between the periphyton biofilms and bacterioplankton (cyanobacteria) provides insight into mechanisms which may inhibit the proliferation of cyanobacterial blooms. This is especially relevant in eutrophic and hypereutrophic systems where biofilms and phytoplankton frequently coexist. The results demonstrated that the integrated allelopathy of dense periphyton biofilm comprising autotrophic and heterotrophic microorganisms was responsible for significant reduction in harmful species.

Many allelochemicals were identified (Dziga *et al*., 2007), of which many are produced by aquatic organisms (Gross *et al*., 1996). To avoid studying the complicated interactions among the individuals in periphyton biofilm, the periphyton biofilm was considered as an ensemble including both living organisms and dead (and lysis) of the biofilm organisms. This showed that the compounds from periphyton biofilms are the combination of those exuded by the biofilm and those released upon death (and lysis) of the biofilm organisms. This study is the first to report the presence of indole and 3-oxo-α-ionone in water extracts of periphyton biofilms. Previous studies indicated that the inhibitory mechanisms of aquatic organisms (e.g. pyrgallon) on cyanobacterial growth included interaction among proteins (Spencer *et al*., 1988), inhibition of alkaline phosphatase (Gross *et al*., 1996; Dziga *et al*., 2007), interruption of the electron transfer chain (Dziga *et al*., 2007) and oxidant damage from auto-oxidizing (Nakai *et al*., 2001). In this study, it was found that the allelochemicals, indole and 3-oxo-α-ionone could damage the thylakoid membrane and interrupt the electron transport in PS II reaction centre, leading to the dysfunction of key photosynthesis reactions.

At the ecosystem level, the formation of dense periphyton biofilm actually provides a habitat for aquatic organisms, such as *Rana utricularia* and *Daphnia laevis*, and also regulates food-web structure and nutrient movements (Leibold and Wilbur, 1992; Sainto and Reddy, 2003). These factors may have important consequences to the ways that primary productivity is regulated by biotic factors in eutrophic systems. Hence, it is proposed that allelopathy could serve as a means for helping control cyanobacterial blooms. In this study, the growing biofilms competed with cyanobacteria for nutrients, which decreased the growth rate of cyanobacteria. As a result, the cyanobacteria could not effectively compete with other organisms once a different trophic state was achieved. Moreover, existence in a biofilm provides many advantages over a planktonic (single-cell) existence, including increased resistance to predation and antimicrobial agents (Hickman *et al*., 2005).

From the view of competition, the competition ability of periphyton biofilm improved when nutrients decreased (even they decreased a little). This result implied that the reduction of nutrients is an effective approach to control cyanobacteria blooms in the long term. The introduction of periphyton biofilms could present an environmentally friendly measure to reduce nutrients in water, leading to a drop in the competitive ability of cyanobacteria.

In eutrophic waters, the growth of biofilm is limited by the availability of the biofilm substrates (i.e. macrophytes) and the shading effect of cyanobacterial blooms (Strom, 2008). In addition, the disappearance of macrophytes, especially the submerged macrophytes in these waters, is mainly owed to the shading effect of cyanobacterial blooms (Phillips *et al*., 1978). This disappearance easily leads the aquatic ecosystem from macrophyte-dominated state (clear water state) to phytoplankton-dominated state (turbid water state) (Mitchell, 1989; Hilt and Gross, 2008). In the long term, to improve the self-recycling capacity of the aquatic ecosystem and to keep the ecosystem in a clear water state, the reintroduction of macrophytes should be conducted once the cyanobacterial blooms are controlled. In this study, the 'replantation' of biofilm on substrates such as SFC facilitated the formation of dense periphyton biofilms in eutrophic waters. The introduction of substrates can increase biofilm mass and enhance the ratio of the periphyton biofilms biomass to water to 1.882:1000 (w/w) in 'real-world' scennarios such as lakes, which is beneficial for the control of cyanobacterial blooms by allelopathy. This biofilm approach also provides transitional benefit conditions for restoration of macrophytes.

For environmental safety, studies have shown that the periphyton biofilm biomass in a healthy aquatic ecosystem can reach 10 g dry matters cm^{-2} (equal to ~5800 g per cubic metre of water in this study) (Azim *et al*., 2003)

or 1200 mg chlorophyll *a* m^{-2} (Welch *et al*., 1992). These studies suggest that the introduction of periphyton biofilm to waters will not introduce a potential risk to the aquatic ecosystem. However, the ecologically effective concentrations of the allelochemicals exuded (or released) by biofilms need to be determined before the long-term effectiveness of biofilms in bloom control is considered in 'real-world' eutrophic waters. In addition, although no significant side-effects such as nutrient starvation, viral attack and general stress from being in containers and experimental ponds were observed in this study, these side-effects should be consider during a study over a long time span.

The use of periphyton biofilm to control harmful algal blooms would be a highly effective, safe and environmentally benign biomeasure. Therefore, the use of periphyton biofilm to control cyanobacterial blooms should be favourable to the general public in comparison with other alternatives such as chemicals. Considering that it is relatively easy to culture periphyton biofilms *in situ*, the application of periphyton biofilm has vast potential in practical restoration projects. In addition, there are more waves in waters to facilitate the regeneration of periphyton biofilm and oxygen to infiltrate through to the deep biofilm (Xu *et al*., 1998).

It is well known that sediments can play an important role in nutrient cycling and organic compound degradation in waters (Malcolm *et al*., 1986; Schultz and Urban, 2008). It is possible that sediments influence the results of periphyton biofilms inhibiting cyanobacterial growth by affecting nutrient flux and degradating allelochemicals. In this study, the nutrient and chlorophyll *a* concentrations in overlying water in the simulated ponds and the treatment-enclosures were investigated in the presence of sediments. The results showed that the presence of sediments did not significantly affect the inhibitory growth of cyanobacteria. In the non-sediment experiments, the BG 11 media was used for co-culturing periphyton biofilm and cyanobacteria (*M. aeruginosa*) and the biofilm aqueous extract was used for co-culturing biofilm and cyanobacteria. The results showed that the cyanobacterial growth was significantly inhibited. Accordingly, this suggests that the sediments have not significant effects on the inhibition of cyanobacterial growth.

In summary, although we are still in the infancy stage of understanding how allelopathic responses may be used in a lake or reservoir management strategy, our study represents a step forward. The experiments described here demonstrate that the formation of dense periphyton biofilms exhibit a strong ecological function, i.e. the reduction of nutrients levels and the decrease of competition ability of cyanobacteria. This leads to the control of the cyanobacterial blooms through the release of water-soluble allelochemicals such as indole and 3-oxo-α-ionone which interrupt photosynthesis. The introduction of periphyton biofilm is a promising approach to minimize harmful algal blooms and provide beneficially transitional conditions for the restoration of aquatic ecosystems. Recent studies have also demonstrated that the presence, abundance, composition and growth of periphyton biofilms are influenced by five broad classes of environmental variation: disturbances, stressors, resources, hydraulic conditions and biotic interactions (Dolédec and Statzner, 2010; Larned, 2010). Multifaceted investigational approaches that integrate functional modification of periphyton ecology in these five classes will be required for the development of systematically integrated technology based on allelopathy of periphyton biofilm for controlling harmful algal blooms and improving aquatic ecosystem health.

Experimental procedures

Periphyton biofilm culture

Two simulated water ponds (Glass tanks, 1.2 m^3, 1.2 m × 1.0 m × 1.0 m) were filled with 15 cm of sediment and 85 cm of water. The water and sediment were collected from three enclosures in Moon Lake, Wuhan City, China where cyanobacterial blooms often occur in summer. The water and sediment were allowed to stabilize in the simulated ponds for 2 days. Thereafter, the periphyton biofilm substrate – SFC – was fixed at the surface water under 0.5 m with density of 0.3 m^3 SFC per cubic metre of water after the SFC was soaked in 0.1 M HCl solution for 24 h and rinsed with fresh double-distilled water three times. The SFC (diameter 12.0 cm, XinXing Water Treatment Plant Equipment, Xinghua, China) was made from polyethylene. The simulated ponds were placed outside and the light intensities entering the simulated ponds were kept between 0–200 Lux at night and 1250–6200 Lux during the day. The recorded water temperatures in the simulated ponds ranged from 13°C to 34°C during the experimental period. Tarpaulins were used to keep rain out of the experimental ponds on rainy days. After the brown biofilms were formed on the 63rd day, the periphyton biofilm was gently removed from the substrate (SFC) by hand in order to avoid breaking the microorganisms' cells.

Effect of periphyton biofilm on cyanobacterial blooms in simulated ponds

The experiments were conducted in the three similar simulated ponds mentioned above (Glass tanks, 1.2 m^3, 1.2 m × 1.0 m × 1.0 m). The sediment of 15 cm thickness and the overlying water of 85 cm depth were also collected from the three enclosures in Moon Lake, Wuhan City, China (same sites mentioned above). A total of 1600.0 g of periphyton biofilm (air-dried at 25–30°C, moisture 85 ± 2%) was placed in the pond 0.5 m under the surface of the water. The ratio of the periphyton biofilm biomass to the water was 1.882:1000 (w/w). The three control ponds did not contain any periphyton biofilms. The inner wall of the control ponds was cleaned once a day using clear gauze in order to avoid the growth of the periphyton biofilm on the inner walls of the ponds.

Inhibition effect of the periphyton biofilms

Cyanobacteria (*M. aeruginosa*) were grown in a sterile BG11 medium (Rippka *et al*., 1979) at 28 ± 1°C under an illumination of 3000 Lux during a 12 h light/dark cycle in illuminating incubators. The initial pH of the culture medium was 7.5 ± 0.02 and the salinity was 30%. All flasks containing the cyanobacteria were placed on a shaker (140 rpm) and shaken twice daily (each time was for 2 h) at the same time each day. The cyanobacteria were cultivated to the exponential growth phase for use in subsequent experiments. During the experiment, the cyanobacteria were inoculated into sterile 250 ml flasks containing 150 ml of fresh sterile BG11 medium in order to obtain cyanobacterial monocultures. These cultures were then used as the controls (*M. aeruginosa* monoculture) throughout the experiment.

In order to avoid the introduction of zooplanktons, the periphyton biofilms were cultivated using filtered (sterilized) nutrient-enriched water through a plankton net (20 μm mesh) and filter paper (0.22 μm pore), and then washed with double-distilled water until no zooplankton could be observed under an optical microscope. Then, 0.28 g of periphyton biofilm was added to sterilized 250 ml flasks that contained 150 ml of fresh cyanobacterial BG11 medium to produce co-cultures (the ratio of the biofilm biomass to the BG-11 medium was close to 1.882:1000). These co-cultures were then incubated under the same conditions as the *M. aeruginosa* monoculture to determine if the growth of the cyanobacteria were inhibited in the absence of zooplanktons. Dissolved oxygen (DO) in the water was maintained at 8.5–9.5 mg l^{-1} via aeration throughout the whole experiment.

In addition, 0.28 g of periphyton biofilm was also added to sterilized 250 ml flasks that contained 150 ml of sterile fresh BG11 medium without cyanobacteria to produce biofilm monocultures (the ratio of the biofilm biomass to the BG-11 medium was close to 1.882:1000). These biofilm monocultures were then incubated under the same conditions as the *M. aeruginosa* monoculture to determine whether the biofilms could grow normally in the BG11 medium.

These aforementioned processes were conducted under sterilized conditions.

Inhibition effect of the biofilm extract

Approximately 1.88 g of the biofilm with no zooplankton was immersed in double-distilled water (1.0 l) for 30 min (the ratio of the biofilm biomass to the water was close to 1.882:1000). It was then filtered using 0.22 μm filter papers. The sterilized filtrate was then used to prepare the BG11 medium and culture cyanobacteria under the same conditions as the *M. aeruginosa* monoculture. In the controls, samples were cultured in BG11 medium prepared using double-distilled water instead of the filtrate. These operation processes were conducted under sterilized conditions.

Isolation and identification of effective compounds from the biofilms

Approximately 1.88 g of the biofilm was immersed in 1.0 l of ultra-pure water (the ratio of the biofilm biomass to the water was close to 1.882:1000). After 24 h, it was filtered through filter papers (0.45 μm pore size). Next, the aqueous filtrate (450 ml) flowed through a Super-clean ENVI-18 cartridge [6 ml (1 g)] that had been activated with 5 ml of methanol and then 5 ml of water to enrich target compounds. This was followed by centrifugation at 4800 rpm^{-1} to remove the water in the ENVI-18 cartridge after the ENVI-18 cartridge was wrapped with filter paper. This procedure was repeated twice, then the ENVI-18 cartridge was eluted with 10 ml of ethyl acetate three times to enrich compounds of middle polarity. The three filtrates were then combined and evaporated to condense at 42°C *in vacuo* until about 3 ml of aqueous solution was left. The remaining aqueous solution was blown with nitrogen gas till almost dry. Finally, the residue was dissolved in 1.0 ml of ethyl acetate and the sample was subjected to GC/MS analysis.

GC/MS analyses were performed using a Varian CP3800 gas chromatograph equipped with a Saturn 2200 mass spectrometer. The chromatographic parameters were as follows: column: CP5860, 30 m × 0.25 mm × 0.25 μm; carrier gas: highly pure helium; flow rate: 1.0 ml min^{-1}; injection chamber temperature: 260°C; loading volume: 1 μl; split ratio: 20:1; column temperature programme: initial temperature: 100°C, hold for 2 min; first ramp temperature: 100–150°C at 3°C min^{-1}; second-stage temperature: 150°C, hold for 3 min; second ramp temperature 150–250°C at 3°C min^{-1}; final-stage temperature: 300°C at 5°C min^{-1}. The mass spectroscopic conditions were set up as follows: ion trap temperature: 210°C; EI energy: 70 eV; mass number range: 45–650; solvent delay: 2 min; scan mode: entire scan mode. The compounds were identified by matching the mass spectrum with the one in the Nist libraries.

Due to the culture water of the periphyton biofilms being collected from three control-enclosures (without periphyton), the control water samples for isolation and identification of compounds from the periphyton biofilms extract were also collected from these control-enclosures. The analytical process was the same as that for the periphyton samples. All analyses were conducted in triplicate.

Inhibition effect of indole and 3-oxo-α-ionone

The inhibition experiments against the cyanobacteria were conducted as follows. Briefly, the cyanobacteria were cultured in 250 ml flasks that contained 150 ml of BG11 medium. Various amounts of indole and 3-oxo-α-ionone were then added directly to the cultures, which lead to the following concentrations: 0, 25.0, 50.0, 100.0 μg l^{-1} 3-oxo-α-ionone and indole respectively. All cultures were incubated at 25 ± 1°C under an illumination of 3000 Lux and a 12 h light/dark cycle in illuminating incubators.

Field tests

The field experiment was conducted in the enclosures that installed in Moon Lake, Wuhan City, China; a heavily polluted inner-city lake with hyper-eutrophication and frequent cyanobacterial blooms. The polyhexene enclosures (5.0 × 5.0 × 3.5 m) were sealed off from the sediments at the bottom at depth of *c.* 2.0 m. The sediments and water in the

different enclosures were fully mixed, while fish and meso- and macro-zooplankton were excluded by a nylon net (pore size 1.0 mm). These six enclosures were divided into two types, three for treatment (treatment-enclosures) and three for control (control-enclosures). In the treatment-enclosures, the periphyton biofilms with polyethylene substrate (SFC) were fixed on the surface of sediments after the overlying water in the enclosures had settled after 1 week. In the control-enclosures, no periphyton biofilm was added and the enclosure walls were cleaned once a day using clear gauze in order to avoid the growth of periphyton biofilm in the inner walls. Water samples were collected from these two test experiments and analysed at regular time intervals. The analyses were performed in triplicate.

Analysis and statistics

Chlorophyll *a* was measured using overnight extraction in acetone (90% v/v). The extracts were read at 663 and 750 nm in a spectrophotometer (Golterman and Clymo, 1969). The ammonia, nitrate and total dissolved phosphorus concentrations were determined using the Standard Methods (APHA, 1998). The cyanobacterial growth was determined by counting cells under a microscope. Effective quantum yield and electronic transport rate of PS II of the cyanobacterial cells were determined using a Phyto-PAM fluorescence analyser (Waltz, Germen). The cells of the cyanobacteria were characterized using transmission electron microscopy (TEM, TEM-H-70000FA, HITACHI).

The biomass of the biofilms was weighed after the biofilms were air-dried at 25–30°C and the moisture of the biofilms was kept at 85 $\pm$ 2%. The morphology of the periphyton biofilms was characterized by scanning electron microscope (SEM, Philips Xl30S-FEG). All extraction procedures and sample analyses were conducted in triplicate. Statistical analyses (paired-samples *t*-test) were performed using SPSS Version 12.0. $P < 0.05$ was considered to indicate statistical significance.

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Supporting information

Additional Supporting Information may be found in the online version of this article:

Fig. S1. Photo-image of periphyton biofilm characterized by scanning electron microscope (SEM).
Fig. S2. Chromatogram of compounds isolated and identified from the water extract of natural biofilm (corresponding numbers are listed in Table S1).
Fig. S3. Typical chromatograms (top) and mass spectra (bottom) of periphyton biofilm extract samples. The structures of indole (A) and 3-oxo-α-ionone (B) are inserted in the mass spectra respectively.
Fig. S4. The chromatogram of the control-enclosures (i.e. without periphyton biofilm). The corresponding numbered compounds are listed in Table S2.
Fig. S5. The enclosures in the field test experiment, Moon Lake, Wuhan City, China, between June 2004 and March 2005. The enclosures were isolated with rubber lining and bamboo wall to avoid the exchange of water and the damage of enclosures by waves.
Fig. S6. The periphyton biofilms growing well during the experiment. (A) After 3 months of the experiment, and (B) at the end of the experiment.
Fig. S7. Changes of chlorophyll *a* concentration in the substrate-controlled experiment. This experiment was designed to assess whether the SFC substrates of periphyton biofilms had affected the cyanobacterial growth. A 1000 ml measuring cylinder with 1000 ml of sterilized BG11 medium acted as the experimental microcosm. The sterile SFC was fixed at the 700 ml scale under the water surface. No SFC substrate was added in the control. All measuring cylinders were incubated at 28 ± 1°C with a light intensity of 2500 Lux under 12/12 h of a light/dark cycle. Results showed that the chlorophyll *a* concentration in the SFC substrate microcosm was not significantly different from that in the control sample. This indicates that the substrate itself did not affect the cyanobacterial growth.
Table S1. The identified compounds in the water extract of the biofilms.
Table S2. The identified compounds extracted from the control-enclosures (without the periphyton biofilms). Alkyl compounds are not listed in this table.

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