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Mechanism of microcystin removal from eutrophicated source water by aquatic vegetable bed

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Abstract: For purifying raw water for tap water treatment, the aquatic vegetable bed (AVB) experiment has been carried out in a hypertrophic waterfront of Taihu Lake, China. The average removal rates of total microcystin-RR and microcystin-LR are 63.0% and 66.7%, respectively. Experiments indicate that *Ipomoea aquatica* can absorb microcystin by using enzyme-linked immunosorbent assay (ELISA), and the roots absorb more toxins than leaves and stems. Fluorescence *in situ* hybridization (FISH) is used to analyze the density of microcystin degrading bacteria in the AVB sediment. Two species of microcystin degrading bacteria are detected, which indicate that microcystin bio-degradation process happened in the AVB. Protozoa and metazoa are abundant in root spheres. *Aspidisca* sp., *Vorticella* sp., *Philodina* sp., and *Lecane* sp. are dominant species. The predation functions of protozoa and metazoa have a positive effect on the removal of cyanobacteria and microcystin.

Key words: microcystin; aquatic vegetable bed; biodegradation; protozoa; metazoa; accumulation of plant

Increasing eutrophication of freshwater ecosystems has favored the occurrence of cyanobacterial blooms. The toxic cyanobacterial blooms have caused the death of livestock and wildlife[1].

Safety of drinking water is one of the most critical factors to guarantee the health of humanity over time. Since many conventional water treatment methods are ineffectual in removing microcystin, it is important to investigate remedial actions that can be taken to reduce the risk to consumers[2]. Many attempts such as advanced oxidation[3-4], and adsorption by activated carbon[5] etc. have been carried out to remove microcystins in drinking water. However, the higher investment and operating costs restrain their application in production. Therefore, researchers have paid more attention to ecological techniques for the control of eutrophication[6].

The potential advantages of hydroponic treatment for BOD, suspended solids, nutrient removal from municipal wastewater have been widely studied[7]. But applying hydroponics to remove microcystins has rarely been reported. The aquatic vegetable bed (AVB) is an ecosystem, a simplification of nutrient film technique (NFT). Water is being treated by running through the plants' roots in the cultivatable channel. Specific processes are as follows. Plant roots spreading like fiber mats in water perform as filter elements, and catch floating objects. In the plant root sphere, zooplanktons and small creatures feed on the substances filtered by the plants' roots multiply, and food chains from smaller creatures to higher ones are formed. Aquatic vegetables uptake nutrients dissolved in the water or nutrients derived from the droppings and dead bodies of water creatures. Grown-up vegetables are then harvested. The use of eutrophicated surface water as a nutrition solution may, therefore, offer a solution.

Microcystins are the most studied hepatotoxins produced by cyanobacteria. Microcystin-RR (MC-RR) and microcystin-LR (MC-LR) are the main components of the cyanotoxins contained in *Microcystis* blooms[8]. This paper surveys the removal efficiency of the two microcystin variants by the AVB. The uptake of microcystin by plant is investigated. The microcystin degrading bacteria in sediments are measured. The protozoa and metazoa in the root sphere are also observed.

1 Methods

1.1 Location and design of the AVB

The study site is located on a waterfront of an influent river (Chendonggang River) of Taihu Lake, approximately 40 km away from Wuxi city, China (31°18.87′N, 119°56.73′E). The AVB was constructed for demonstration and research purposes.

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Every ditch of the AVB system consists of concrete and brick gullies 1.5 m wide, 0.25 m deep and 15.0 m long (see Fig. 1). Water was elevated from the river by two electrical centrifugal pumps and delivered to the AVB gullies in a parallel manner via twenty-one 60 mm diameter PVC pipes. Water level in the bed was maintained at a 10 cm depth throughout the experiment. The bottom of the bed was sloped approximately 1‰ from inlet to outlet.

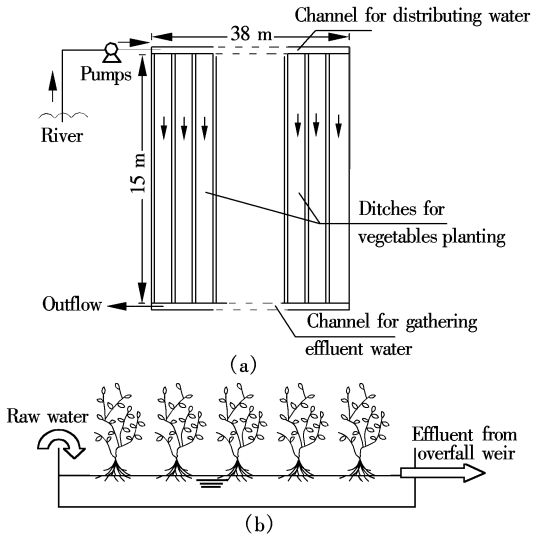

Fig. 1 Schematic diagram of the AVB system. (a) Ichnography; (b) Profile

Ipomoea aquatica is transplanted in the AVB during the experiment. *Ipomoea aquatica* is an annual plant, of a herbaceous species, belonging to the *convolvulus* family. They are distributed in semi-tropical regions of the world. This plant is a familiar vegetable in China.

1.2 Analysis of microcystins

Water samples are analyzed for microcystins using high performance liquid chromatographic (HPLC) methods.

1.2.1 Pretreatment of water samples

For total microcystin (sum of intracellular and extracellular microcystin) quantification, 500 mL water samples were fixed with glacial acetic acid at 5% (volume fraction) final concentration for 24 h and then filtered through Whatman GF/C filter paper. For extracellular microcystin quantification, 1 000 mL water samples were directly filtered through Whatman GF/C filter paper. All the filtrate was stored at −20 ℃ until used.

1.2.2 Concentration of raw water samples by solid phase extraction (SPE)

The filtrate was then concentrated over a preprimed (10 mL methanol followed by 10 mL distilled/deionized water) ODS C_{18} cartridge (waters sep-pak). The cartridges were then washed with 40 mL of distilled/deionized water and 20 mL of 10% methanol followed by 20 mL of 20% methanol to eliminate many of the other dissolved organics.

The microcystins were eluted from the C_{18} cartridge by 5 mL methanol with 0.1% (volume fraction) trifluoroacetic acid (TFA) and after filtration the supernatant was evaporated to dryness at 45 ℃ under vacuum. The dry residue was dissolved in 400 μL methanol with 0.1% (volume fraction) TFA and then stored at −20 ℃ before analysis by HPLC.

1.2.3 Detection by HPLC

MC concentrations were determined by comparing the peak areas of the test samples with the commercial standards available (MC-LR and MC-RR, Wako Pure Chemical Industries, Japan). Sample injection volumes were 20 μL. The conditions of HPLC (Agilent 1100) for analysis of microcystin were as follows: absorbance at 238 nm; capillary temperature 40 ℃; gradient elution starting at 45% (volume fraction) aqueous methanol with 0.05% (volume fraction) TFA was increased to 70% (volume fraction) in 25 min at a flow rate of 1 mL/min.

Intracellular microcystin was estimated as the difference between total microcystin and extracellular microcystin.

1.3 Algae cell density

Algae cells were counted on a compound microscope in a counting chamber after preservation in Lugol's iodine. Cell counts were carried out to a minimum precision of 20%.

1.4 FISH

Sediment samples were fixed in paraformaldehyde (final volume fraction 4%) overnight at 4 ℃. Fixed samples were washed two times with PBS (phosphate buffered saline), with centrifugation at 10^4 r/min for 10 min between washings, and stored in PBS/ethanol (1∶1) at −20 ℃ until further processing.

Aliquots of 10 μL fixed sample were spotted onto glass slides and dried at room temperature. The slides were then dehydrated in ethanol (50% for 5 min, 80% for 1 min and 96% for 1 min), and then dried at room temperature. Samples were hybridized for 3 h at 46 ℃ in isotonically equilibrated humid chambers. After hybridization, specific binding and excess probes were washed away with solution (0.2 mol/L NaCl, 20 mmol/L Tris-HCl (pH 7.2), 0.1% SDS, 5 mmol/L EDTA) for 20 min at 48 ℃, and subsequently rinsed

with distilled/deionized water and air-dried.

Epifluorescence microscopy was performed with a Nikon 26188 microscope (Nikon, USA). The oligonucleotide probes used in this paper are shown in Tab. 1.

Tab. 1 Probe sequences and target sites

Probe name	Target organism	Sequence	Target site, rRNA position
ALF1b[9]	α-Proteobacteria	5′-CGTTCG(C/T)TCTGAGCCAG-3′	16S, 19 − 35
CF319a[10]	*Cytophaga/Flavobacterium* group	5′-TGGTCCGTGTCTCAGTAC-3′	16S, 319 − 336
MCD[11]	MCD-bacteria	5′-CGCCACCAAAGCCTAAAAGG-3′	16S, 839 − 858

1.5 Extract and determination of MC in *Ipomoea aquatica* tissues

The plant samples were rinsed thoroughly with distilled/deionized water and methanol to remove matrix contaminants, and dried on blotting paper, then dissected into root, stem and leaf. After weighing, the tissues of plant were then frozen in liquid nitrogen and ground to fine powder, then extracted in acetic acid followed by methanol. The extracts were combined, rotary evaporated to dryness, and resuspended in 1 mL of methanol. The presence of MC in plant tissues was then determined using an enzyme microplate reader (S/N 5810 Vmax kinetic microplate reader, Molecular Devices Inc., USA) by enzyme-linked immunosorbent assay (ELISA).

2 Results

2.1 Removal of algae

Tab. 2 shows the removal effect on algae by the AVB. The dominant category algae density decreases sharply when raw water is treated by the AVB, especially the typical Microcystis sp. which producing microcystin reduces most algae with the average removal efficiency of 78.0%. *Microcystis* sp. is surrounded by mucilage, which mainly consists of polysaccharide[12]. And the mucilage is of high viscosity. Thus *Microcystis* sp. is more easily trapped or adsorbed by plant roots and then removed from raw water.

Tab. 2 The dominant category algae density of raw water and treated water cells/mL

Date	*Phormidium* sp.		*Melosira* sp.		*Microcystis* sp.		*Oscillatoria* sp.	
	Raw water	Treated water	Raw water	Treated water	Raw water	Treated water	Raw water	Treated water
2005-07-27	6 100	900	700	160	6 900	700	600	200
2005-07-30	400	80	400	100	800	200	200	100
2005-08-05	700	100	1 700	640	2 500	600	200	80
2005-08-16	1 300	500	900	460	2 300	460	200	100
2005-08-23	100	40	800	320	1 100	300	900	300
2005-09-01	400	100	1 000	280	1 700	320	700	200
2005-09-07	800	200	700	200	1 800	500	500	100
2005-09-25	1 500	600	700	300	2 600	600	300	120
2005-10-07	800	380	500	180	1 800	400	300	80

2.2 Removal of MC-RR and MC-LR

The experiment was conducted for 6 months (May to October). The hydraulic loading rate was 1.0 $m^3/(m^2 \cdot d)$, the correspondingly theoretical hydraulic retention time (HRT) was 110 min.

For the influent intracellular MC-RR concentration of 0.193 to 0.715 μg/L, its removal efficiency reaches 59.2% to 86.6% and averages 76.1%. For the extracellular MC-RR concentration of 0.174 to 0.916 μg/L, the removal efficiency reaches 34.4% to 67.8% and averages 50.2%. The removal efficiency of total MC-RR reaches 51.2% to 82.9% and averages 63.0%.

For the influent intracellular MC-LR concentration of 0.190 to 0.509 μg/L, the removal efficiency reaches 59.8% to 80.2% and averages 69.9%. For the influent extracellular MC-LR concentration of 0.133 to 0.668 μg/L, the removal efficiency reaches 43.6% to 74.1% and averages 60.6%. The removal efficiency of total MC-LR reaches 60.8% to 77.3% and averages 66.7%.

For both MC-RR and MC-LR, the average removal efficiency of intracellular microcystin is higher than that of extracellular microcystin with the surplus value of 25.9% and 9.3%, respectively.

The AVB achieves better removal efficiency under shorter HRT, and the removal mechanism of MC is discussed in the following.

2.3 Accumulation in plant

Experiments show that the roots, stems and leaves can uptake microcystin. Fig. 2 shows the MC content in different tissue of fresh *Ipomoea aquatica*. It can be seen that roots absorbed much more toxin than leaves and stems did and the MC sorbed in the stems and leaves are nearly equal.

Fig. 2 also shows that the toxin content in the plants is higher in the forward bed and the content decreases along with flow direction. Many research

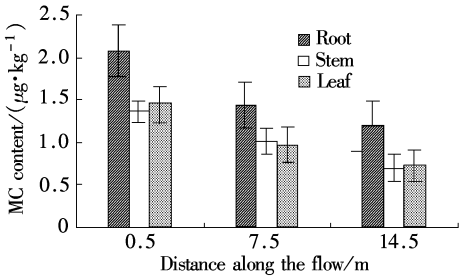

Fig. 2 Uptake of MC in *Ipomoea aquatica* tissues in different distances along the flow

achievements show that the process of toxin accumulation in plant tissues is dose-dependent[13]. Therefore, the reason for the toxin content in plants decreasing along with flow is the decline of the microcystin content in the water.

2.4 Effect of biodegradation

Abiotic removal of microcystin through adsorption to particulate and dissolved matters seems in general to play a minor role in the removal of dissolved microcystins[14]. Photosensitised degradation of microcystins has been shown to occur very slowly under natural conditions[15]. Therefore, we can assume that the observed disappearances of microcystin were caused by microbial degradation of microcystin instead of uptake or adsorption by plants.

There are a lot of MC degrading bacteria in a natural water body[16]. To our knowledge, members of the *Cytophaga/Flavobacterium* group can degrade not only into macromolecular compounds[17], but also into *Microcystis* cells[18]. It has also been confirmed that MCD-bacteria play a major role in microcystin degradation in a eutrophic natural water body.

We used FISH to analyze the population of bacterial consortia in AVB sediments which proved to be able to degrade microcystin. *Cytophaga/Flavobacterium* group and MCD-bacteria are all detected, but α-Proteobacteria are not detected. The density of *Cytophaga/Flavobacterium* group and MCD-bacteria increased with AVB run time (see Fig. 3), which indicates that the microbe has been acclimatizing while the AVB aperiodically received inflow contained microcystin. This clearly shows that the microcystin bio-degradation process does happen in the AVB.

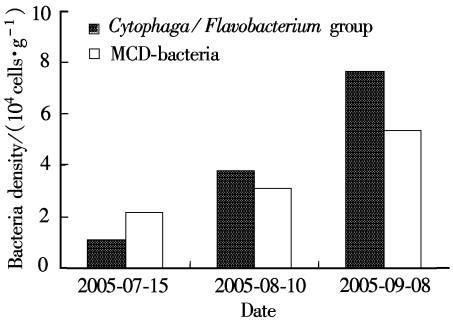

Fig. 3 Change in the density of *Cytophaga/Flavobacterium* group and MCD-bacteria with run time

2.5 Observation of micro-animals

Tabs. 3 and 4 show the amount of protozoa and metazoa accreted on fresh plant roots respectively in the AVB.

Tab. 3 Amount of protozoa

Protozoa	Individuals/g
Arcella sp.	3 000
Centropyxis sp.	3 000
Euglypha sp.	2 500
Amoeba sp.	1 000
Aspidisca sp.	5 000
Colpidium sp.	2 000
Spirostomum sp.	1 000
Paramecium sp.	500
Trochilia sp.	2 000
Vorticella sp.	5 000
Vaginicola sp.	2 000
Cephalodella sp.	2 000

Tab. 4 Amount of metazoa

Metazoa	Individuals/g
Philodina sp.	10 000
Lecane sp.	3 000
Aeolosoma sp.	1 000
Nematoda	3 000

Of the protozoa, the planktonic ciliate is abundant, such as *Aspidisca* sp., *Spirostomum* sp., *Paramecium* sp., *Vorticella* sp., and *Vaginicola* sp.. Among them, *Aspidisca* sp. and *Vorticella* sp. are all dominant species. Flagellate (e. g. *Euglypha* sp.) is also a dominant species. Some species of ciliate[19] and flagellate[20] can consume algae.

Rotifer (*Philodina* sp., *Lecane* sp.) and oligochaete (*Aeolosoma* sp.) are abundant in the root sphere. The dominant species are *Philodina* sp. and *Lecane* sp. Rotifer and oligochaete can prey on algae including *Microcystis*[21].

The predation functions of protozoa and metazoa have a positive effect on the removal of cyanobacteria and microcystin.

3 Discussion

The removal process of microcystins produced in *Microcystis* that occurs in the AVB can be concluded as follows: The first process for the elimination of microcystins from a population is the physical filtration of intracellular microcystins by plant roots. The shade of leaves and stems of *Ipomoea aquatica* can prevent

healthy cyanobacteria from reproduction. The trapped *Microcystis* cell decays by autolysis, grazing by protozoa, zooplankton, or viral or bacterial activity, and the cellular products are then degraded by bacterial consortia or assimilation by plant tissue.

The contribution of bacterial degradation and plant uptake occurring in the AVB to remove dissolved microcystin should be paid more attention to. The accumulation and metabolism of microcystins in plant tissues should also be fully investigated.

Compared with other algae removal techniques (e. g. UV irradiation), the AVB is simple and has a low cost of construction. An additional advantage of the AVB compared to the application of algicides is that this technique does not threaten the safety of the aquatic ecosystem.

4 Conclusion

The removal efficiency of *Microcystis* sp. in the AVB averages 78% while the removal rates of total microcystin-RR and microcystin-LR average 63. 0% and 66. 7%, respectively.

The plant roots in the AVB were not only important for the filtration of the wastewater but also provided a microhabitat for microcystin degradation bacteria, which led to a marked improvement of the water quality for the eutrophicated water body serving as a source of drinking water.

Protozoa and metazoa are abundant in the root sphere. *Aspidisca* sp., *Vorticella* sp., *Philodina* sp., and *Lecane* sp. are dominant species. The predation functions of protozoa and metazoa have a positive effect on the removal of cyanobacteria and microcystin.

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水生植物床去除富营养化水源水中藻毒素的机制

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摘要:为净化富营养化水源水中的藻毒素,在太湖湖滨进行了水生植物床(AVB)技术试验. 该技术对 MC-RR 和 MC-LR 的平均去除率分别达 63.0% 和 66.7%. 用酶联免疫吸附法检测发现水生植物根部对藻毒素的富集能力大于茎叶部位. 用荧光原位杂交法在 AVB 的底泥中检测到 2 种已知的藻毒素降解细菌,证明微生物降解作用的存在. 植物根系原生动物相和后生动物相丰富,盾纤虫、钟虫、旋轮虫、腔轮虫是优势种,原生动物和后生动物的捕食作用在蓝藻和藻毒素的去除中发挥着积极作用.

关键词:藻毒素;水生植物床;生物降解;原生动物;后生动物;植物蓄积

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