

Response of Microbial Community Structure to Clay Flocculation of Harmful Algal Blooms

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This dissertation is dedicated to my parents and my husband.

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ABSTRACT

Harmful algal blooms are cosmopolitan and can produce extremely dangerous toxins that can sicken or kill people and animals, and create dead zones in the water. The U.S. economic loss caused by algal blooms is \$82 million every year. Clay-based flocculation techniques have been developed to mitigate algal blooms; however, the potential impacts on the microbial community are poorly understood. This dissertation includes multi-scale experiments (Jar, field, microcosms) to study the response of microbial community structure and function to clay flocculation of algal blooms.

Jar tests of removal of *Microcystis aeruginosa* by chitosan and two types of commercially available clays was studied with Tennessee River water. Acridine Orange Direct Count results showed the bacterial biomass decreased, but not significantly, after clay flocculation. Water microbial community in high removal efficiency tests was significantly different from low removal efficiency tests using 16S sequencing measurements.

China field tests were conducted to verify laboratory tests by applying chitosan-modified-local-soil in 800 m² [meters squared] ponds. 16S sequencing revealed five bacterial groups that were increased in the water after flocculation. Total coliform tests showed clay flocculation significantly reduced the frequency of coliforms in the pond water.

In the China microcosm experiments, chemical measurements were combined with phospholipid fatty acid analysis and 16S sequencing to characterize the microbial community response to different flocculation techniques. This included clay flocculation only, clay flocculation with zeolite capping, and clay flocculation with oxygen loaded zeolite capping. Water bacterial biomass and sediment bacterial biomass measured by lipid concentration were not significantly altered by various flocculation techniques. However, sequencing results confirmed the presence of distinct water microbial communities between samples with and without zeolite-capping. The relative abundance of ammonia-oxidizing bacteria increased four-fold in zeolite capping sediments. Potential pathogens that are usually adapted to eutrophic water bodies were reduced

after clay flocculation. The increased monounsaturated-epoxide-ratio in post bacterial biomass indicated zeolite capping may help the sedimentary bacterial community recover from environmental stress. These studies demonstrated clay flocculation did not decrease overall bacterial biomass but may reduce pathogenic contaminants in water. Zeolite capping may help prevent nutrients from being released back into the water.

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CHAPTER 1 INTRODUCTION AND LITERATURE REVIEW

HARMFUL ALGAL BLOOMS

Harmful algal blooms (HABs) occur when certain microalgae species rapidly grow and accumulate in the water body, especially in the surface of seawater and freshwater. A small percentage of algal species produce toxins, such as hepatotoxin and neurotoxins, which can kill fish, mammals, and birds, and also cause human illness (1). Other nontoxic algal species cause environmental threats by clogging the gills of fish and invertebrates or by smothering submerged aquatic vegetation. Others discolor water, form smelly piles on beaches, or result in drinking water pollution (2). In early 2015, almost 500,000 residents in and around Toledo, Ohio, were without safe drinking water for one month while local water supplies were being polluted by harmful algal bloom on Lake Erie. HABs adversely impact economics of fishery industries, and tourism and create drains on resources from blooms monitoring and management.

The nature of the HAB problem has spread throughout the world. Great progresses have been achieved in HAB forecasting systems, which rely on satellite imagery, field observations, and buoy data to provide the large spatial scale and high frequency of observations required for assessing bloom location and movement (3). Another focus of past and ongoing processes is to obtain a fundamental understanding of the physical, chemical, biological and ecological processes to control HABs (4). A promising strategy for controlling HABs in the field is the application of suspended clay particles over the bloom to flocculate and settle the algal cells (5). Clay flocculation has been proven effective in many harmful algal bloom events in Japan and South Korea. Anderson (4) determined the removal efficiencies of selected clays on three HAB species found in U.S. waters (the Florida red tide dinoflagellate *Gymnodinium breve*, the New York brown tide chrysophyte *Aureococcus anophagefferens*, and to a lesser extent, fish-killing *Pfiesteria*-like dinoflagellate). In general, montmorillonite, kaolinite and phosphoric clay are preferred to be flocculants for controlling many algal species (6, 7). In 2011, a new method was proposed for HABs using local sand/soil modified with chitosan by Dr. Gang Pan (8). This technology was successfully applied in Meiliang Bay of Lake Taihu (China).

TRADITIONAL CLAY FLOCCULATION TECHNIQUES

The use of clays to control HABs has been explored in several countries (9). Applications of clay flocculation among countries are summarized in Table 1.1. In Japan, suspensions of pure montmorillonite and kaolinite were sprayed onto the surface of *Cochlodinium* sp. blooms at 200 g/m² near the fish enclosures (10). Within 12 hours, the number of algal cells decreased greatly at the surface, and the water transparency increased. Field trials in South Korea (11), China (12), United States (5, 9), Sweden (13) and Australia (14) demonstrated the effectiveness of the clay control. The target species, clay category and manipulation parameters are summarized in Table 1.1. The reported setting efficiency of HABs were 90%-99% with no mortality in the caged fish due to the clay treatment (7). Studies in large tanks and limncorrals were also performed during a red-tide event that occurred in Texas (Corpus Christi Bay, 1999) and Florida (Sarasota Bay, 2000) (9), respectively.

These studies showed that the turbidity declined immediately within the first hour and approached background levels after 4 h. However, the environmental implication was still unknown. To investigate the benthic impact of the clay dispersal, the USEPA Laboratory at Gulf Breeze (FL) employed standard EPA sediment toxicology procedures to measure the acute and chronic toxicities of clay flocks following the treatment of *Karenia brevis* to the infaunal amphipod, shrimp and clams (15). The results suggested that the use of this bloom control method may not result in toxicity to these types of organisms. However, when the flow conditions led to prolonged in situ suspension of clay, it was detrimental to clams, who rely on filter-feeding.

The main chemical compositions of the typical clays components are aluminum and iron salts, which give cationic hydrolysis products that are strongly adsorbed on negative particles, forming hydroxide precipitates. For example, bentonite clay has a very strong negative ionic charge, which attracts positively charged substances, such as heavy metals, toxins and pesticides. Clay can also connect with cationic metals, forming a bridge, which can absorb algae, bacteria and other particulates with a net negative surface charge. Rapid sedimentation of algal cells may be

Table 1.1 Countries where clay flocculation has been investigated (9)

Country	Target species	Clay	Loading rate
Japan	<i>Cochlodinium sp.</i>	montmorillonite	1.3-400 g/m ²
	<i>Chattonella spp.</i>	montmorillonite	-
	<i>Prorocentrum sigmoides</i>	montmorillonite	-
China	<i>Noctiluca scintillans</i>	montmorillonite	0.7 g/L
	<i>Prorocentrum minimum</i>	bentonite	0.7 g/L
South Korea	<i>Cochlodinium polykrikoides</i>	yellow loess	400 g/m ²
United States	<i>Karenia brevis</i>	bentonite	0.10-4 g/L
	<i>Aureococcus anophagefferens</i>	phosphatic clay	-
	<i>Pfiesteria piscicida</i>	kaolinite	-
	<i>Heterosigma akashiwo</i>	kaolinite	-
	<i>Alexandrium tamarense</i>	kaolinite	-
Sweden	<i>Prymnesium parvum</i>	phosphatic clay	0.10-4 g/L
Australia	<i>Microcystis aeruginosa</i>	"phoslock"	-

caused by the flocculation of clay particles with algal cells, and the entrainment of algal cells is due to the physico-chemical interactions with the clay particles. This process is known as flocculation. The principles and the action of hydrolyzing coagulants are well understood (16). In the study of equilibrium and kinetic properties of flocculation based on 26 clays, Pan et al. (17) proposed a kinetic equation for clay-cell flocculation, in which the parameters of t_{50} (time needed to remove 50% of the algae) and t_{80} (time needed to remove 80% of the algae) were calculated for all the clays. They found only sepiolite retained a high removal efficiency when the clay loading was reduced to 0.2 g/L due to its unique fibrous structure, suggesting the mechanism of netting and bridging regulated clay flocculation.

MODIFIED CLAY FLOCCULATION TECHNIQUES

Having demonstrated the effectiveness of clay flocculation in removing certain harmful algae, researchers focused on investigating whether the clay can be modified to enhance the removal efficiency. Several mechanisms such as bridging, netting, adsorption and charge neutralization (including electrostatic patch effects) have been generally accepted (18).

Modified by Cations

Algal cells tend to float in water due to the negatively charged cell surface, their low specific gravity and other unique structures (e.g. gas vesicles). Clays are also negatively charged in natural waters and hence electrostatic neutralization does not contribute significantly to the aggregation between clay particles and algal cells. Therefore, the surface charge modification in the clay by cations can facilitate the neutralization due to increased positive charge. Surface modification of montmorillonite by means of Mg^{2+} insertion reaction has been studied in coagulating *Heterosigma akashiwo* (19). The results showed that the modified clay enhanced the coagulation and decreased the amount of clay needed to 1/5–1/10 of the original. The removal rates of *H. akashiwo* were correlated positively with the positive charge on the clay in accordance with theoretical model. Pan et al. (20) compared the modification of surface charge by Fe^{3+} with the modification of polyaluminum chloride (PAC) and chitosan. Their research also showed similar results. After the sepiolite was modified with Fe^{3+} , the initial algal removal

rate increased significantly, but it was still lower than the removal efficiency of clays modified with PAC and chitosan.

Modified by Coagulants

Conventional coagulants, mainly inorganic metal salts (e.g. aluminum, ferric and chloride), were first induced into clay-coagulant combinations. The addition of these cations resulted in colloidal destabilization, as they especially interact with and neutralize the negatively charged colloids (21, 22). However, they were substituted by the pre-hydrolysed form of aluminum (PAC) and iron (polyferric sulfate or PFS), because the hydrolysis of the latter occurs in the preparation stage and is easy to control. Phosphatic clay with PAC could remove both intra- and extra-cellular brevetoxins (7). Some products have been reported, such as “biofloculants” including biopolymers (starches, chitosan, alginates) and biomaterials produced by microorganisms including bacteria, fungi and yeast (23). They are safe and biodegradable polymers without secondary pollutants (24). In particular, chitosan is a promising biofloculant for controlling harmful algal blooms, as reported in recent patents (25). It is a linear copolymer of D-glucosamine and N-acetyl-D-glucosamine hydrolyzed from exoskeletons of marine crustaceans, shrimp and crabs. Divakaran and Pillai (26) showed that chitosan reduced algal content effectively, due to its high cationic charge density, long polymer chains, and bridging of aggregates and precipitation. However, they noted that the flocculation was very sensitive to the pH value. Sun et al. proposed the combination of sophorolipid and loess for harmful algal bloom mitigation in laboratory experiments and demonstrated that it was a very effective method to control HABs due to the synergistic effect of sophorolipid and loess on both the removal efficiency and cost (27). However, the mechanism of the synergistic effect was not clear. Lee and his co-workers (28) also conducted field experiments on mitigation of harmful algal blooms using a sophorolipid and yellow clay mixture, during a *Cochlodinium* bloom near Miruk Island, South Korea in August 2002. A mixture of 5 mg/L sophorolipid and 1 g/L yellow clay was applied under similar conditions. The mixtures more efficiently mitigated the *Cochlodinium* bloom (95% removal efficiency after 30 min) than the yellow clay alone (79% after 30 min) (28).

Modification of Local Clay

A “modified local soil induced ecological restoration” (MLS-IER) technology was developed for the restoration of degraded shallow lakes (8). The technology is to flocculate the HABs using local sand modified with chitosan. The harmful algal cells precipitate with flocculants to the bottom of the lake. Another layer of sand and chitosan in which the macrophyte seeds were wrapped was applied to cover the algae flocs. This capping layer decreased the re-suspension of the algae cells and provided a clean layer for the macrophyte seeds to grow. The local soil used in the research is sandy soil, which mainly consists of clays, iron oxides, and sands. As an alternative strategy, the use of native ecological material such as local beach sands or soil that naturally enter the aquatic system through rivers or rainfall could minimize ecological risk to the aquatic environment. In addition, using local sands and soil is cost-effective by avoiding the transportation cost of those not immediately available clays (e.g. montmorillonite, yellow loess).

MOLECULAR TECHNIQUES FOR MICROBIAL COMMUNITY ANALYSES

Microbial community structure indicates the types of microorganisms and their abundance. As well as providing a better understanding of the basic pattern in microbial distributions, such as endemism, ubiquity, and interactions with environmental parameters (29). In addition, it can reveal genetic information of particular microorganisms associated with microbial processes and give us a systems engineering understanding of biological processes (30). Thus, understanding of microbial dynamics and discovering and characterizing associations of microorganisms and the environment become a major interest for environmental microbiology and microbial ecology. To answer the questions: (1) who are they, (2) what do they do, and (3) how do they work in the environment are impracticable with cultured methods only. The application of molecular methods has been developed to assess microbial community studies. The primary source of information that molecular methods rely on is biomolecules such as nucleic acids, proteins, fatty acids and other taxa-specific compounds (31). These molecules can be extracted directly from environmental samples without the need for culturing, and analysis of the molecular composition can be used to elucidate the composition of the microbial community (32, 33). Moreover, molecular methods are able to preserve *in situ* metabolic function and the microbial community composition since samples are usually immediately preserved at -80°C (34) or directly extracted

in the field (35). Over the last few years, tremendous techniques have been developed and applied in studies of microbial phylogenetic diversity and functionality in the environment. Two major categories are divided based on their capability of revealing the microbial community structure and function (Fig.1.1) (36).

High-throughput sequencing in environmental samples

High throughput sequencing methods have rapidly developed since 2000. These techniques are recognized as fast, cheap ways to sequence and analyze large genomes (37). They generally involve the amplification of DNA templates by the polymerase chain reaction, and then the DNA sample is sequenced. The bases of a small fragment of DNA are sequentially identified from signals emitted as each fragment is re-synthesized from a DNA template strand (38). Currently, ultra-high-throughput microbial community analyses are available on the Illumina HiSeq and MiSeq platforms (39). Freshwater bacteria are at the hub of biogeochemical cycles and control water quality in lakes. Little is known about the identity and ecology of functionally significant lake bacteria until the molecular methods were able to provide us with unprecedented access to the diversity and composition of freshwater lake bacterial communities (40). These technologies have for the first time enabled the field to identify the numerically dominant organisms in these ecosystems (41). In 2000, Pomati et al. (42) revealed that the dominant species of the HAB bloom was most closely related to the genus *Planktothrix* using 16S rRNA gene sequencing. Gernert (43) constructed the 16S rRNA gene libraries from experimental sponge tissues and from lake water to study the phylogenetic bacterial diversity of the freshwater sponge *Spongilla lacustris*. The phylogenetic composition of a bacterial community from a hypertrophic freshwater lake in China was investigated by sequencing cloned 16S rRNA genes (44). They successfully classified 336

bacterial clones into 142 operational taxonomic units (OTU). The results showed that most of them were affiliated with bacterial divisions commonly found in freshwater ecosystems, e.g. *Alpha*-, *Beta*-, *Gamma*- and *Deltaproteobacteria*, *Bacterioidetes* and *Actinobacteria*. Following the advances in molecular methods, photosynthesizing microscopic organisms that live in almost all fresh water like phytoplankton are also studied using high throughput sequencing (45). The

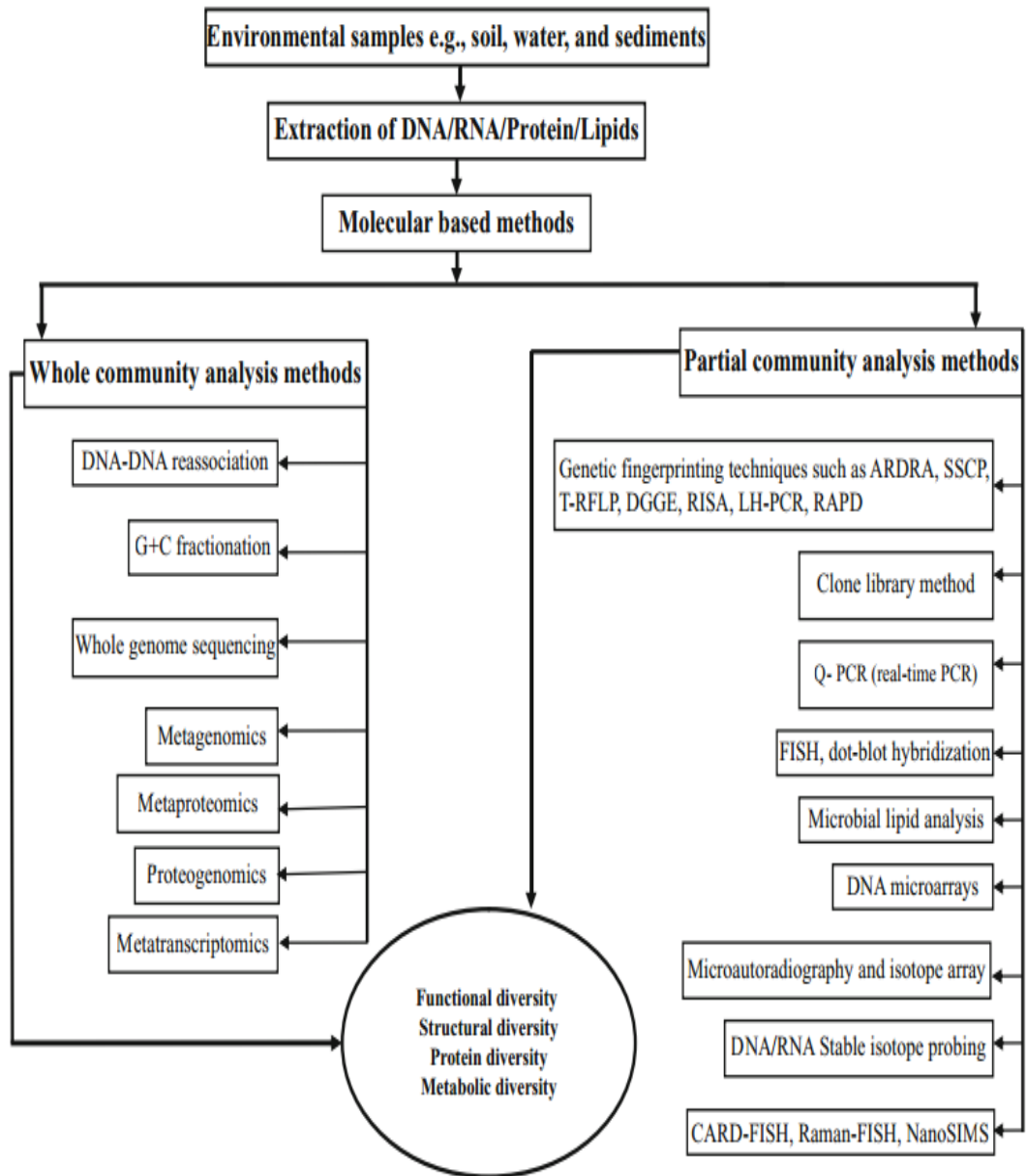


Figure 1.1 Culture-independent molecular toolbox to characterize the structural and functional diversity of microorganisms in the environment (38)

microbial community of sediment and soil is more complex than that of freshwater (46). Phospholipid fatty acids (PLFAs) are a potentially useful biomarker molecule that is being used to elucidate the structure of microbial community in soil because of their presence in all living cells and rapid degradation upon cell death (47). However, this technique provides more information on biomass and little information is available at deeper level of community compositions (48). Recently, Illumina sequencing platforms are the overwhelming method of choice for determining breadth and function of microbial diversity research (31, 49).

Microbial community used as indicator of ecological conditions

Species abundance and diversity measurements are sensitive indicators of environmental conditions since microorganisms play crucial roles in environmental activities (50, 51). Microbial responses to geographic factors and contaminants have been summarized for surface waters and upland soils. Researchers in China studied the response of bacterial communities to environmental changes in a mesocosm scale subtropical watershed and found that benthic bacterial communities were clearly more diverse and uniform than surface bacterioplankton communities (52). Previous studies presented detailed analysis linking geochemistry and geology to microbiology (53, 54). A widely observed result provided supporting evidence that the structural diversity of a bacterial community is often sensitive to environmental changes and exhibits a shift in its composition (55, 56). The response of bacteria to contaminants indicates capabilities of particular microorganisms in biodegradation. Bioremediation potential of microorganisms from marine systems and intertidal sediments of a sandy beach affected by a major oil spill have been fully demonstrated (57, 58). Bacterial community composition associated with freshwater algae has also been studied. The results emphasize the ecological importance of species-specific bacteria–algae associations with important repercussions for other processes, such as the demineralization of nutrients, and organic matter dynamics (59, 60). Beazley et al. (61) strongly supported that bacterial communities are sensitive indicators of contaminant stress. In water resource monitoring and assessment programs, the United States Environmental Protection Agency has already adopted algal and microbial communities as indicators of wetland integrity (62).

RESEARCH AIMS AND HYPOTHESIS

Harmful algae blooms (HABs) present an important global danger. Although rigorous studies are currently underway, the majority focus either on establishing monitoring systems or establishing a highly efficient control technique for removing HABs. The impact of clay flocculation on water and sedimentary microbial community structure remains a rare topic in Environmental Microbiology/Ecology conferences, and periodicals. In most research, they only stated that clay addition is thought to have a low probability of causing environmental damage since they are naturally occurring particles that are commonly found in marine systems and lakes. Little has been studied in terms of looking at the biomass change, the microbial community structure change, and the response of potentially pathogenic bacteria.

The focus of this investigation is to develop fundamental new information on the effects of clay based HAB flocculation technology on sediment microbial community composition and residual microbial quality characteristics. The specific aims of this research are (1) to apply molecular microbial community assessment techniques to understand compositional changes in the microbial community following flocculation that may influence biogeochemical cycling and (2) To determine whether flocculation itself assists in improving water quality by removing microbial pathogens and indicator organisms during the flocculation procedure.

In conducting this research two specific hypotheses will be addressed and these are described as follows:

- (1) HAB flocculation deposits microbial biomass and nutrients on the surficial sediment resulting in a structural change in the composition of the pelagic microbial community. These changes may ultimately influence biogeochemical cycling affecting both water quality and possible recycling of nutrients after deposition by HAB flocculation.
- (2) The mechanism of clay mediated flocculation will indiscriminately result in flocculation of other microorganisms along with HAB biomass and this will reduce potential pathogens and/or water quality indicator organisms in the pelagic microbial community.

The resulting co-flocculation of organisms may be a secondary beneficial result of clay-based flocculation technology.

IMPLICATIONS OF RESEARCH

This research was an excellent opportunity to study response of microbial community structure to environmental engineering technologies. The main strategy incorporates usage of manipulated control techniques to look at structural changes in the microbial community, investigate the behaviors of pathogenic bacteria and indicator bacteria in the water body, and study the changes of geochemical parameters. These studies are important in terms of the environment, agriculture, and human health.

EXPERIMENTAL DESIGN

To study the response of microbial community structure to clay flocculation techniques, multiple scale experimental platforms were designed. The laboratory Jar test (Figure 1.2) can be repeated and conducted under controlled conditions to examine the validity of a hypothesis, however, the experimenter cannot account for every possible factor that would normally occur in the original environment. Thus we conducted pond tests which can maximally mimic natural environments (Figure 1.3). However, controlled studies in natural environments is never without challenges. During our experimental period severe storms happened unexpectedly, which adversely impacted the experimental results. The microcosm was used as an experimental tool that brings a small part of the natural environment under more controlled conditions (Figure 1.4). In this way microcosms provide a link between observational field studies that take place in natural environments (without replication), and controlled laboratory experiments that may take place under somewhat unnatural conditions. Results from multiple platform scales can better elicit what happens in natural system and provide multiple lines of evidence to make conclusions more robust.

Laboratory Jar tests were conducted to mimic the removal of algal cells with chitosan and clays (chapter 2). Water and bottom flocs were sampled after two week's incubation. Algal cells and bacterial cells were counted, and microbial compositions of all samples were determined by 16S rRNA sequencing. Results from this chapter provided systems model approaches to study impact of clay flocculation on water community structure. This experimental model was verified

in the field test. The field test studied the response of indigenous pond microbial communities to the clay flocculation of algal bloom water (chapter 3). The site is located beside Cetian reservoir in Datong, Shanxi province in China. It includes eight 800m² ponds for field tests as well as water supply and irrigation channels. The water utilized for experiments was pumped from Cetian reservoir which is already in a eutrophic state due to serious pollution from upstream. Flocculation experiments were conducted in an experimental pond according to the method described by Pan et al (63). Microbial community structure and function of control and experimental ponds over time were investigated. In addition, we conducted total coliform tests in the pond water to investigate the effectiveness of clay flocculation on indicator bacteria mitigation (regulatory guideline methods). When water was tested for total coliform, the results were given as the number of colony forming units per milliliter (CFU/mL) of water sample. The column experiments studied the influence of different flocculation techniques namely, clay flocculation only, clay flocculation plus zeolite capping layer, and clay flocculation plus O₂ loaded zeolite capping. PLFA analysis and 16S rRNA gene sequencing were the two methods used to provide view of uncultured microbial community. Multivariate analyses were used to elucidate how geochemical conditions correlated with microbial community structure.



Figure 1.2 Jar test platform



Figure 1.3. China Pond Test



Figure 1.4 Column structure and three treatments

CHAPTER 2 SYSTEMS MODEL APPROACHES FOR MICROBIAL COMMUNITY STUDIES OF HARMFUL ALGAL BLOOMS REMOVAL

**This chapter is a potential publication to Journal of Microbiological Methods.*

INTRODUCTION

Harmful algal blooms (HABs), known colloquially as red tides, are characterized by rapid growth and accumulation of toxic or otherwise harmful phytoplankton in aquatic systems. Such blooms are often marked by the discoloration of the water resulting from the high density of pigmented cells (64). Thus, HABs can have serious impacts on species' interactions, aquatic animal health and population growth, ecology, human health, ecosystem integrity, and even on major industries and economies (65). Hallegraeff (1) presented an apparent global increase of harmful algal blooms in a review and highlighted the need to develop effective methods to control the HABs directly, not only to minimize or prevent their effects. Control strategies proposed in the past involved chemical control such as cooper, aponin and ozone to inhibit HABs, and biological control to destroy harmful algal cells through the introduction of a predator or other pathogenic agent (66). These control techniques have not been applied in the field due to their negative effects on fish, clams and other plankton. The application of natural clays over the surface of a bloom has proved to be a promising strategy for controlling HABs. After successes in Japan (10) and South Korea (67) to control outbreaks of fish-killing marine algae, the research on the control of bloom-forming species through clay flocculation has been widely conducted (17).

However, clay flocculation brings high clay loading (> 200 mg/L) to seawater system (salinity ranges 30 -50 ppt) in order to achieve high removal efficiency (9). Natural clay flocculation was less effective in low salinity freshwaters. Kawd et al. (68) pointed out that the size, shape, structure, density, surface charge and chemical composition of clay particles can affect their inter-particle forces. Thus, modified clay flocculant was developed to decrease clay loading and increase removal efficiency in freshwater. Chitosan is a long chian biopolymer, produced by deacetylation of chitin using sodium hydroxide in excess as a reagent and water as a solvent (69).

It is an outstanding netting and bridging reagent. After modification with chitosan, the flocculating ability of most kinds of clays, minerals, local soils, and sediments is remarkably improved. The average removal efficiency of *Microcystis aeruginosa* is above 90% (70). The fact that high flocculation efficiency modified flocculants, implied that the netting and bridging ability is more important than the surface charge of the flocculant, even though electrostatic neutralization contributes to the aggregation between clay particles and algal cells. However, it is hard to reach a high removal efficiency (above 90%) because the chitosan-algae flocs are not heavy enough to settle down by themselves. Thus clays, soils, and sediments can add weight to the netting and bridging formed by chitosan and algal cells. A series of experiments were conducted to test the influence of ionic strength, pH, organic matter, cell concentration, and algal growth stage on chitosan-modified clays. The results indicated chitosan modified clays performed much better in low ionic strength water due to its unique chemical structure. The amino groups attached on its long chain structure are positively charged in solution, which can be attracted by negative ions in high ionic strength water (71). Chitosan modified clay was proven to be effective in a pH range of 6.0 to 9.0 and tolerant of organic matter (up to 40 mg/L humic acid) (72). It is suggested to perform clay flocculation during early senescence growth phase when pH is 6.0-9.0. A pre-test for optimal dosage is recommended prior to field test.

There are still several concerns with the clay flocculation. Bacteria associated with algae are highly precipitated after clay flocculation. This may decrease bacteria biomass in the water. Cations may be attractive to negatively charged bacteria and cause their sedimentation. Some bacteria may be trapped by the chitosan-algae network and precipitated to the bottom also. Therefore, the clay flocculation of *M. aeruginosa* by chitosan and two types of commercially available clays was studied in Jar tests. The removal efficiency of algal cell was tested using chitosan modified kaolinite and bentonite. Bacteria and algal biomass were estimated in the water and bottom flocs. 16S rRNA gene sequencing was performed to reveal the microbial community structure before and after clay flocculation.

MATERIALS AND METHODS

Algal species and culture

Microcystis aeruginosa was used as a target species, which is a unicellular colonial cyanobacterium. *M. aeruginosa* is considered a harmful algal bloom species because it can produce both neurotoxins and hepatotoxin. *M. aeruginosa* cells were supplied by UTEX-The Culture Collection of Algae at The University of Texas at Austin. The cells were grown in BG-11 medium which is media for freshwater, terrestrial, hot spring and salt water algae. *Cyanobacteria* BG-11 Freshwater Solution (Sigma-Aldrich Co. LLC.) was a 50x stock solution used to prepare *Cyanobacteria* BG-11 medium. The BG-11 50x solution was used at 20 ml/L with deionized water to prepare BG-11 medium. The final BG-11 medium was adjusted to pH 8.4 by adding 1 mol/L NaOH solutions and stored at 4°C. Algal batch cultures were maintained at 25°C on a 12 h light and 12 h darkness rotation in an environmental growth chamber. Each batch was shaken three times every day by hand, and it was transferred at a ratio of 1:5 every 7 to 10 days.

Clays and modifiers

Chitosan (Sigma-Aldrich Co. LLC.) had a medium molecular weight with the viscosity of 1 wt. % in 1% acetic acid. The powder (2 g of chitosan) was dissolved in 100mL diluted acetic acid solution (0.2% wt. /wt.). The chitosan solution was dissolved by adding 100 mg chitosan to 10 ml 1% acetic acid, then solution was diluted with distilled water to obtain a final concentration of 1 mg/mL before usage. The chitosan solution needs to be fresh before each experiment.

Water Sampling

Water samples were collected from the Tennessee River using a 50 liter sterilized water container. A minimum volume of 20.5 liters per depth per replicate was required for each analysis listed below.

- 1) 14 L for algae flocculation
- 2) 18 – 36 mL for acridine orange direct counts
- 3) 80 mL water for culture

4) 4 L for nucleic acid analysis

Table 2.1 Commercially available clays used in Jar test

Clays	Linear Formula	Grain Size	Supplier
Kaolinite	$\text{Al}_2\text{O}_3 \cdot 2\text{SiO}_2 \cdot 2\text{H}_2\text{O}$	<90 μm	Sigma-Aldrich
Bentonite	$\text{Al}_2\text{O}_3 \cdot 4\text{SiO}_2 \cdot 2\text{H}_2\text{O}$	<90 μm	Sigma-Aldrich

Flocculation Experiments

Flocculation experiments were conducted in a jar test apparatus (Nordic Scientific, Sweden). Algae culture was centrifuged by 10 min at 11000 rpm and the pellet was mixed with Tennessee River water. The initial algal cell density was adjusted to be around 5.82×10^9 cells/L. Then the 300 ml experimental culture was transferred into a 500 ml beaker and stirred at 300 rpm for 1 min and then 125 rpm for another 4 min followed by 40 rpm for 10 min (73). Chitosan alone and chitosan plus clays were tested under the following concentrations respectively:

- A. chitosan: 0, 20, 40, 60, 80, and 100 mg/L;
- B. Bentonite alone: 0, 200, 400, 600, 800, and 1000 mg/L;
- C. Kaolinite alone: 0, 200, 400, 600, 800, and 1000 mg/L;
- D. 20 mg/L chitosan + Bentonite: 0, 200, 400, 600, 800, and 1000 mg/L;
- E. 20 mg/L chitosan + Kaolinite: 0, 200, 400, 600, 800, and 1000 mg/L;

The control cultures were running without adding any clays or modifiers.

Acridine orange direct counts (AODC)

AODC provides a direct estimate of the total number of bacteria in the environment, regardless of ability to grow on any media that might be used. Biomass from unfiltered groundwater samples fixed with 4% formalin in the field were filtered through a 0.2 μm pore size black polycarbonate membrane (Whatman International Ltd., Piscataway, NJ) supported by a vacuum

filtration sampling manifold (Millipore Corp., Billerica, MA). Filtered cells were stained with 25 mg/ml of acridine orange for 2 minutes in the dark. Unbound Acridine orange was filtered through the membrane with 10 ml filter sterilized 1X Phosphate-buffered saline (PBS buffer) (Sigma Aldrich Corp., St. Louis, MO) and the rinsed membrane mounted on a slide for microscopy. Cells were imaged with a FITC filter on a Zeiss Axioskop (Carl Zeiss, Inc., Germany) epifluorescent microscope (74, 75).

16S rRNA gene sequencing

Genomic DNA of sediments was extracted in triplicate from 0.5 g sediment using the PowerSoil DNA Isolation Kit (MoBio Laboratories) following to manufacture's protocol. Genomic DNA of the water sample was extracted in triplicate using the PowerWater DNA Isolation Kit (MoBio Laboratories) following the manufacture's protocol. 16S rRNA genes were amplified using primer pair 515f and barcoded 806r and the DNA library was prepared according to procedures modified from Caporaso et al. (39). 16S rRNA gene sequencing was conducted on the Illumina MiSeq platform (San Diego, CA) at the University of Tennessee. Sequences were trimmed at a length of 250 bp and 3 million sequences were retrieved. Open reference operational taxonomic units (OTUs) were picked via the UPARSE pipeline and were filtered at 0.005% threshold. Taxonomic assignment, phylogenetic reconstruction were performed using QIIME v1.7.0 (76).

Analytical methods

Samples from 2 cm below the surface of the experimental beaker were collected after sedimentation at different times. Each sample was repeated three times. The cells were enumerated in epifluorescence microscope. The removal efficiency of cells was calculated using formula (1).

$$\varphi = \frac{C - c}{C} \times 100\% \quad (1)$$

Where φ = Removal Efficiency;

C= Initial cell concentration;

c= Sample cell concentration.

RESULTS

Removal efficiency

The removal efficiency of chitosan and different chitosan modified clays are shown in Figure 2.1. The removal efficiency of commercially available bentonite and kaolinite were below 40% when they were used alone (data not shown). After clay flocculation, the suspension still maintained high turbidity after 1 h. Chitosan alone showed best removal efficiency at a dosage of 20 mg/L. The removal efficiency increased with the concentration until it reached the optimal dosage, and then decreased dramatically. This decrease is expected because excess chitosan led to restabilization of the chitosan-algae flocs. Modified kaolinite and bentonite indicated high overall removal efficiency as compared to clay alone and chitosan alone. The efficiency of modified kaolinite kept increasing as the clay concentration increased. It reached 95% after 12 h of sedimentation. Modified bentonite achieved maximum removal efficiency at a dosage of 400 mg/L. The overall removal efficiency was above 95% after 5 min, and the water remained clear for 12 h.

Cell counts in water and bottom flocs

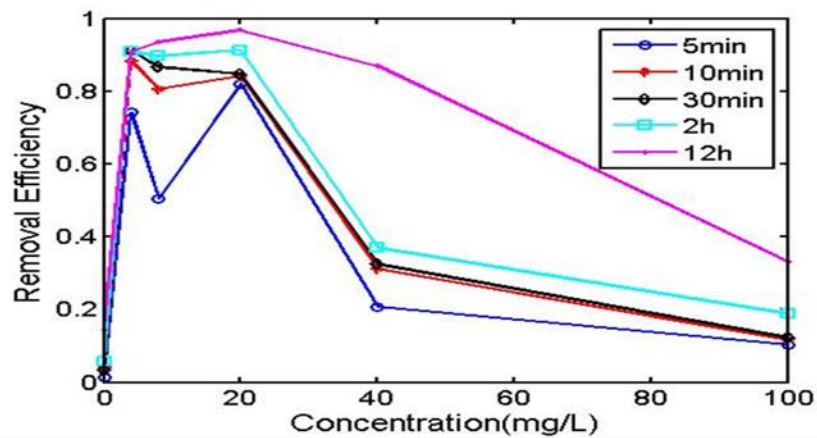
After flocculation most algae cells were brought to the bottom. The algae cells were counted right before flocculation and after two weeks' incubation by acridine orange direct cell count. Algal cells were not resuspended or grown in the water column after incubation. It showed a small drop in bacteria cell numbers in all modified kaolinite supernatant (Figure 2.2), but there was no significant change of bacteria cell number after flocculation as compared to before flocculation ($P=0.075$). The algal cell concentration decreased significantly ($P=0.0039$) after 2 weeks incubation (Figure 2.3). This trend was observed in all modified clay concentrations. The cell density decreased from 10^6 cells/ml to 10^4 cells/ml. Bacteria cell density increased significantly after two weeks incubation ($P=0.0051$).

Microbial community structure change

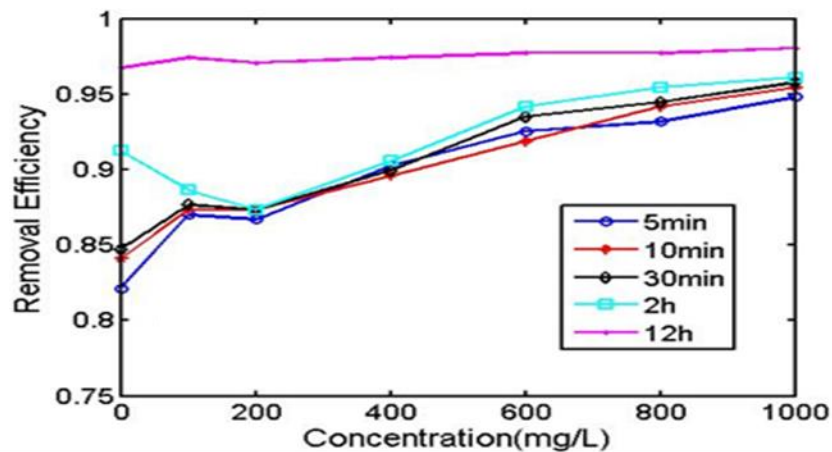
Changes in microbial community structure at the phylum level were shown in Figure 2.5. Microbial community change was observed in Tennessee River water, bloom water, low removal efficiency treatments (bentonite and kaolinite alone), and high removal efficiency treatments

(chitosan alone, chitosan modified bentonite, and chitosan modified kaolinite). Untreated Tennessee River water was dominated by 80% *Proteobacteria*, and 8% *Bacteroidetes*. The bloom water was dominated by *Cyanobacteria*, as high as 90% of the total microbial community. All the low removal efficiency treatments had similar community structure. The dominant phyla were *Cyanobacteria*, *Proteobacteria*, *Verrucomicrobia*, *Bacteroidetes*, and *Actinobacteria*. *Proteobacteria*, *Verrucomicrobia*, *Bacteroidetes*, and *Planctomycetes* dominated the high removal efficiency samples. The relative percentage of *Proteobacteria* was around 40%. Compared with bloom water, *Cyanobacteria* decreased dramatically after clay flocculation and there is only 20% to 30% left in the low removal efficiency samples. Almost 95% *Cyanobacteria* disappeared in high removal efficiency treatments. The percentage of *Actinobacteria* was only observed in low removal efficiency treatments, ranging from 5% to 10%. *Verrucomicrobia* was observed in all treatment water samples, the percentage was 15% in bentonite alone, 3% in kaolinite alone, 10% in chitosan alone, 23% in modified bentonite, and 10% in modified kaolinite. *Planctomycetes* was only observed in high removal efficiency treatment water samples. After two weeks' incubation, microbial communities of water and bottom floc in the treatments were similar at phylum level (Figure 2.7). The dominant phyla were *Proteobacteria*, *Verrucomicrobia*, *Bacteroidetes*, and *Planctomycetes*. *Proteobacteria* were slightly elevated but not significantly ($P=0.21$).

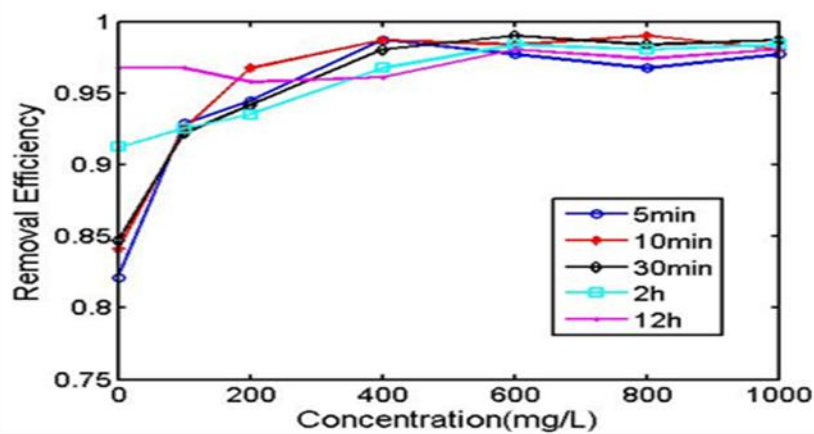
Beta diversity was analyzed based on a weighted unifrac distance matrix. Non metric multidimensional scaling (NMDS) compares pairwise distance among samples and classifies them into clusters. Each dot in the NMDS plot represents one community, and the dot clustered together indicates they have similar community structure. We can find all the samples from high removal efficiency treatments were clustered together within a 95% confidence interval circle (PerMANOVA test $P=0.001$) (Figure 2.6). The results also indicated that there was no significant effect of flocculant dosage on the microbial community structure.



A



B



C

Figure 2.1 Removal efficiency and concentration of flocculants: A represents chitosan only; B represents 20 mg/L chitosan + kaolinite; C represents 20 mg/L chitosan + bentonite.

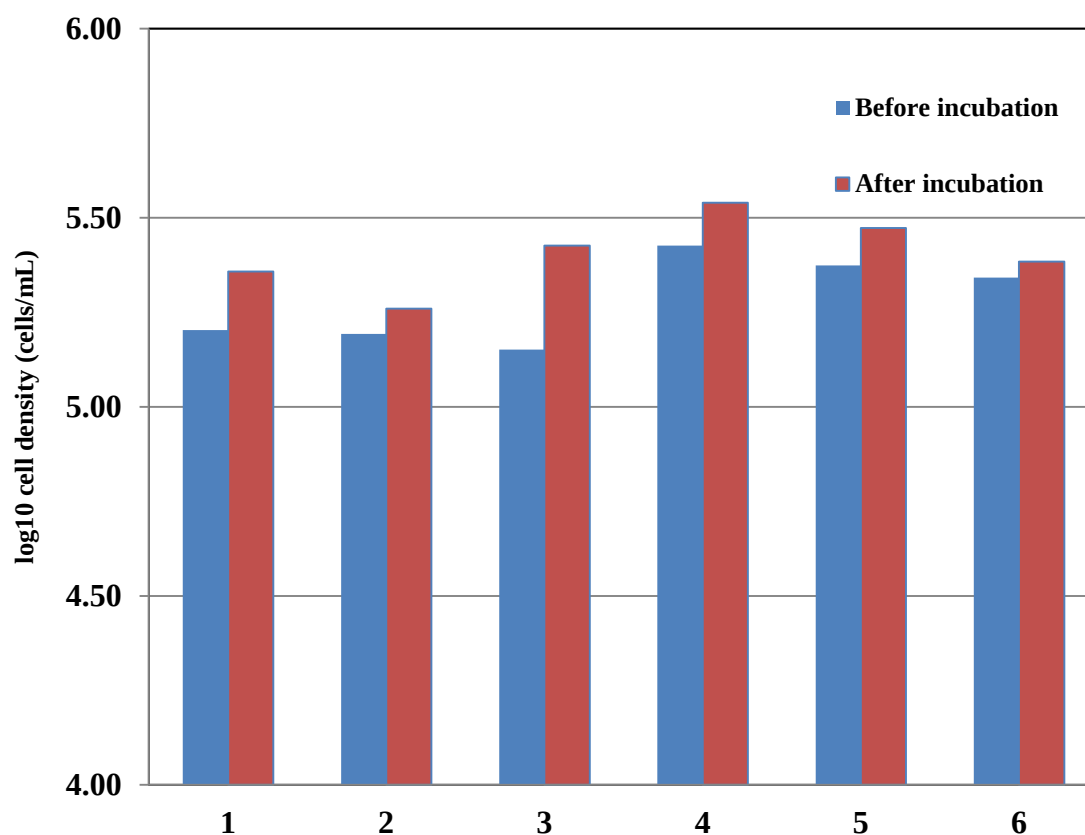


Figure 2.2 Bacteria cell counts in water. Sample group 1 represents 20 mg/L chitosan only; 2 represents 20 mg/L chitosan + 200 mg/L kaolinite; 3 represents 20 mg/L chitosan + 400 mg/L kaolinite; 4 represents 20 mg/L chitosan + 600 mg/L kaolinite; 5 represents 20 mg/L chitosan + 800 mg/L kaolinite; 6 represents 20 mg/L chitosan + 1000 mg/L kaolinite.

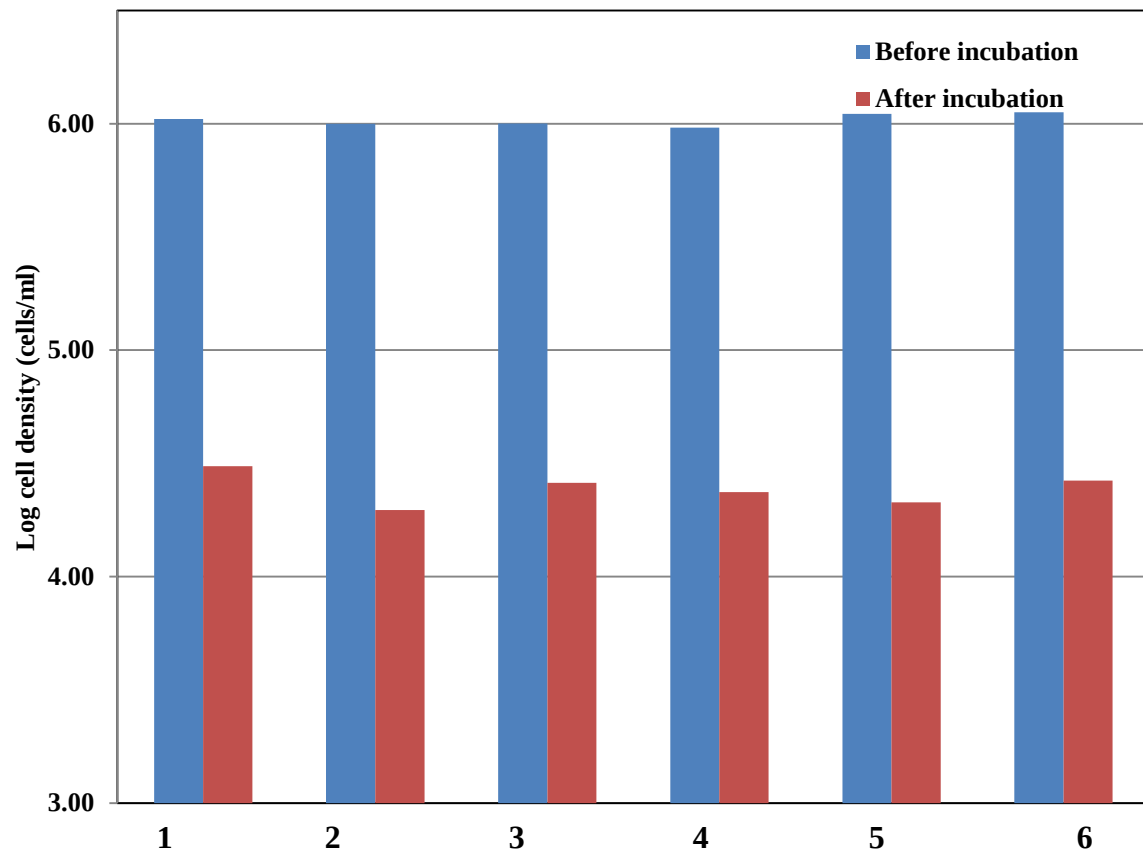


Figure 2.3 Algae cell counts in bottom flocs. Sample group 1 represents 20 mg/L chitosan only; 2 represents 20 mg/L chitosan + 200 mg/L kaolinite; 3 represents 20 mg/L chitosan + 400 mg/L kaolinite; 4 represents 20 mg/L chitosan + 600 mg/L kaolinite; 5 represents 20 mg/L chitosan + 800 mg/L kaolinite; 6 represents 20 mg/L chitosan + 1000 mg/L kaolinite.

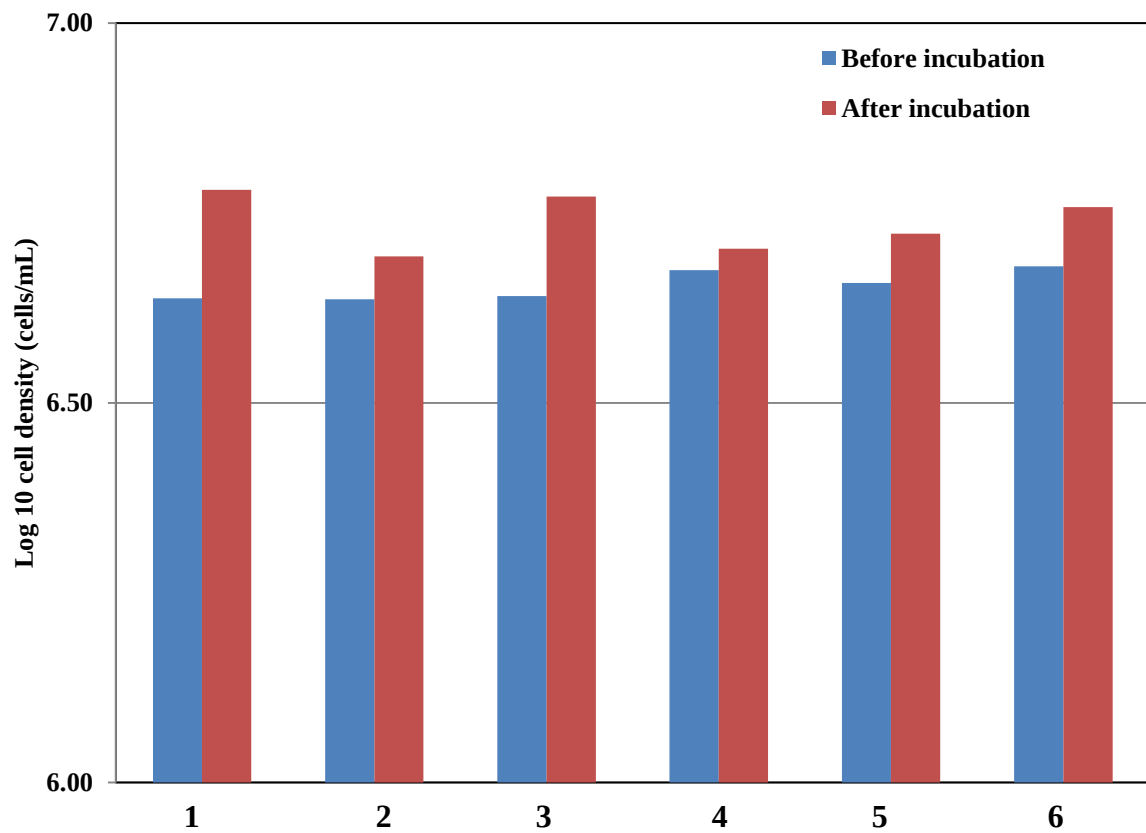


Figure 2.4 Bacteria cell counts in bottom flocs. Sample group 1 represents 20 mg/L chitosan only; 2 represents 20 mg/L chitosan + 200 mg/L kaolinite; 3 represents 20 mg/L chitosan + 400 mg/L kaolinite; 4 represents 20 mg/L chitosan + 600 mg/L kaolinite; 5 represents 20 mg/L chitosan + 800 mg/L kaolinite; 6 represents 20 mg/L chitosan + 1000 mg/L kaolinite.

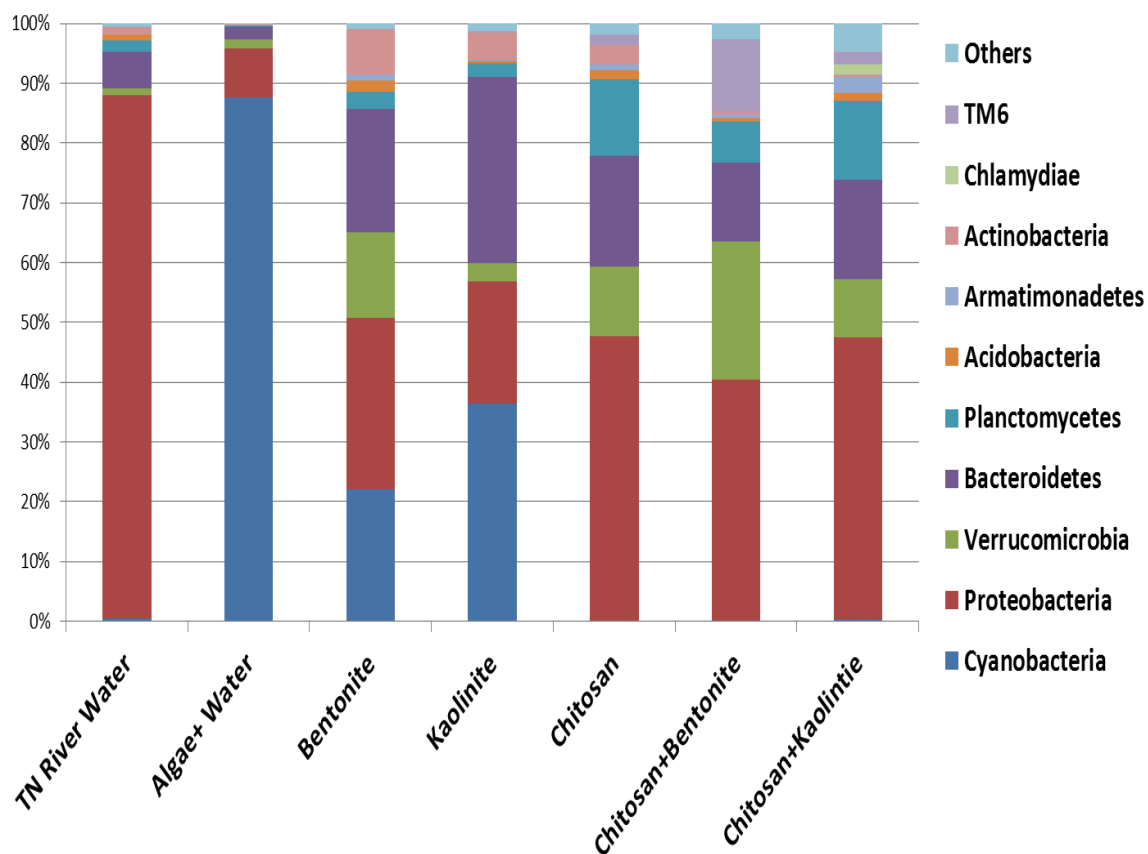


Figure 2.5 Microbial community structure comparison at phylum level of TN river water with water samples treated by clays (bentonite, kaolinite), chitosan, and chitosan modified clays (chitosan modified bentonite and chitosan modified kaolinite).

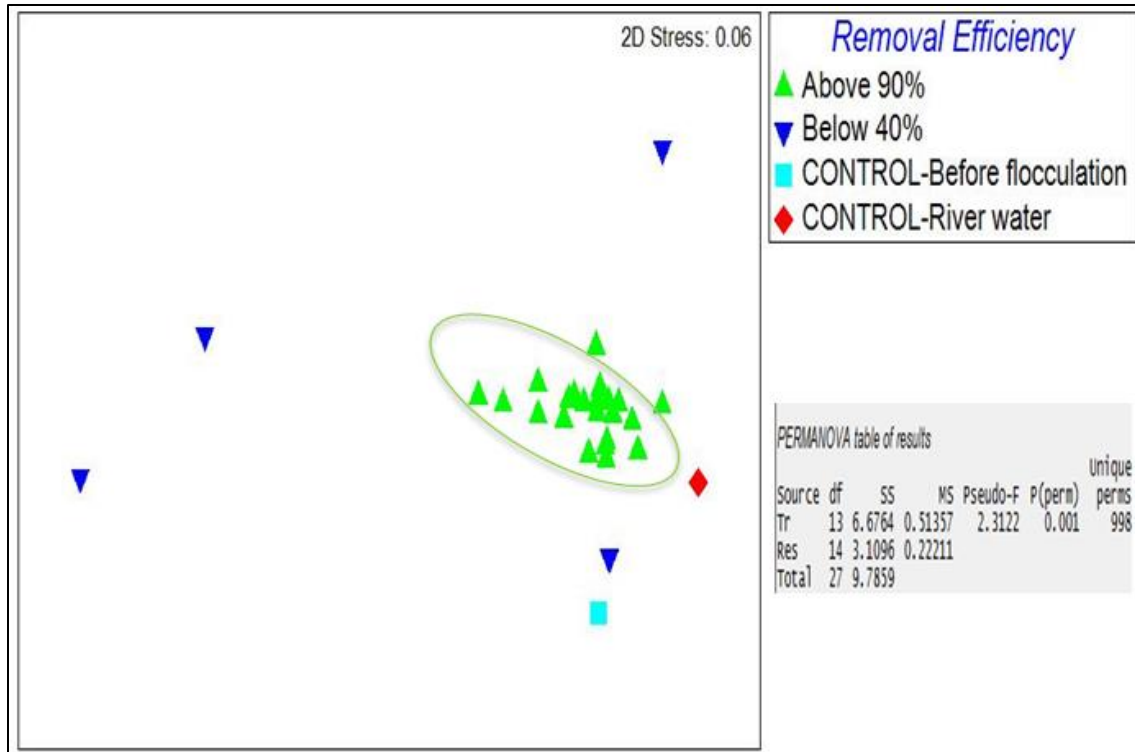


Figure 2.6 NMDS of microbial community with removal efficiency. Each sample represents 1067 unique OTUs (clustered at 97% similarity). The green circle is a 95% confidence interval. Stress value below 0.2 indicates the data was well fitted in the model.

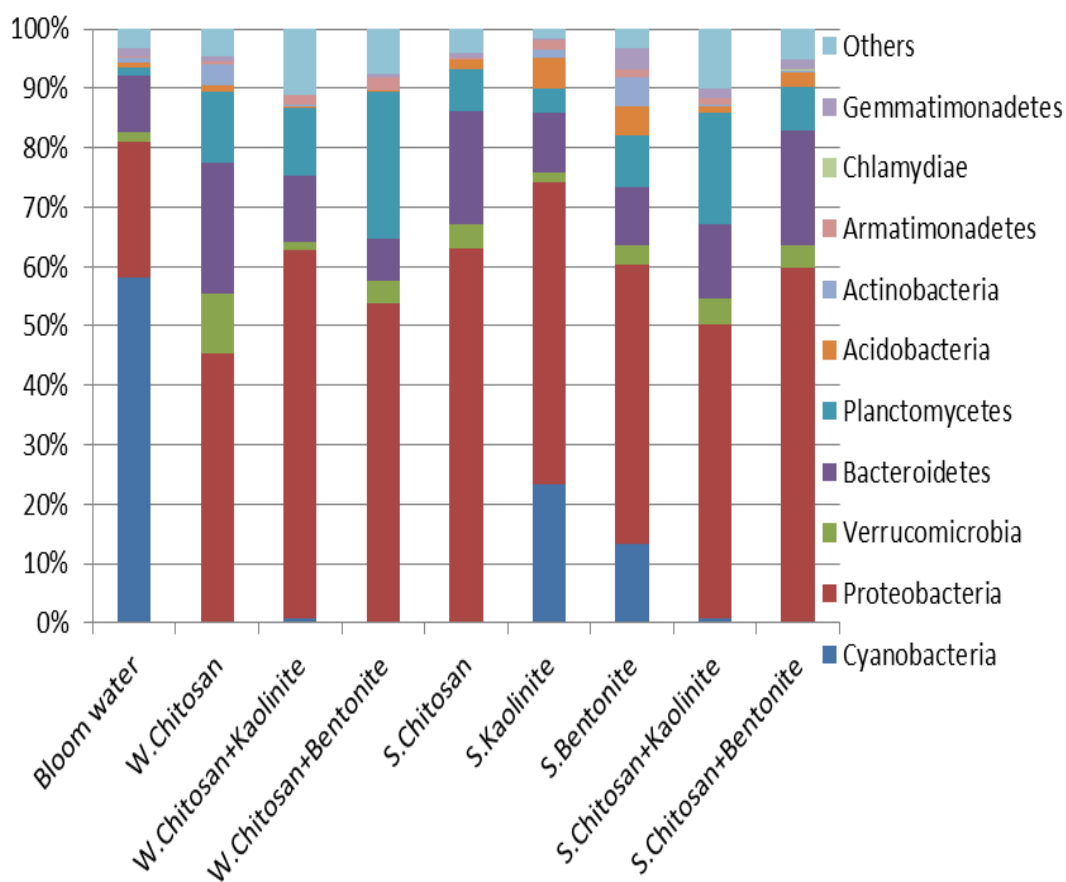


Figure 2.7 Microbial community structure comparison at phylum level of TN river water with water samples (treated by chitosan, chitosan modified kaolinite, and chitosan modified bentonite respectively) and bottom floc samples (treated by chitosan, kaolinite, bentonite, chitosan modified kaolinite, and chitosan modified bentonite, respectively).

DISCUSSION AND CONCLUSIONS

Clay flocculation mechanism

High removal efficiency of *M. aeruginosa* cells can be achieved with the clays modified with 20 mg/L chitosan over a short time, bentonite and kaolinite alone were not effective. Theoretically, bentonite is composed of 3 sheet-like layers which has a high swelling index. The ability to absorb and retain water between the layers causes an expansion in the crystal (77). This property may increase the contact frequency between the clay particles and the algae cells, since the larger particles can sweep through a larger surface than small particles. However, our results were not consistent with previous study (78). Bentonite and kaolinite are both negatively charged clays, so they are not attracted by negatively charged algal cells in river water. In addition, the commercially available clays used in this study had large particle size (larger than 90 μm), which is about 30 times larger than *M. aeruginosa* cell (3.4 μm). Han and Kim (79) reported the highest removal efficiency achieved when the clay particle size was similar to the size of algal cells in seawater, but the removal efficiency was low regardless of clay particle size in freshwater when clay was the only flocculant. Despite the colloidal properties, it failed to achieve high removal efficiency. The long chain algae-chitosan flocs observed under epifluorescent microscope (Figure 2.8) confirmed the results from Pan et al (20). The dominant mechanism in this clay flocculation experiment was netting and bridging formed by chitosan and algae flocs.

Effect on water microbial community

Bacterial biomass in the supernatant slightly decreased after clay flocculation but this change was not significant. It was evident that bacteria can be brought to the bottom with clay flocculation but clay flocculation may not significantly affect the whole community. The decrease of biomass could be caused by bacteria-algae interaction as well as bacteria-flocculant interaction. Several models suggest bacteria and algae interactions, including, symbioses (bacteria benefit from algae exudates), parasites (lysis of algae), commensalism (no negative effects), and competition (bacteria loosely associated with algae for limiting PO_4^{3-}) (80). Sallal (81) reported lysis of cyanobacteria by *Flexibacter spp.* Isolation indicated most of them are Gram-negative bacteria. They co-exist with algae and their abundance is positively

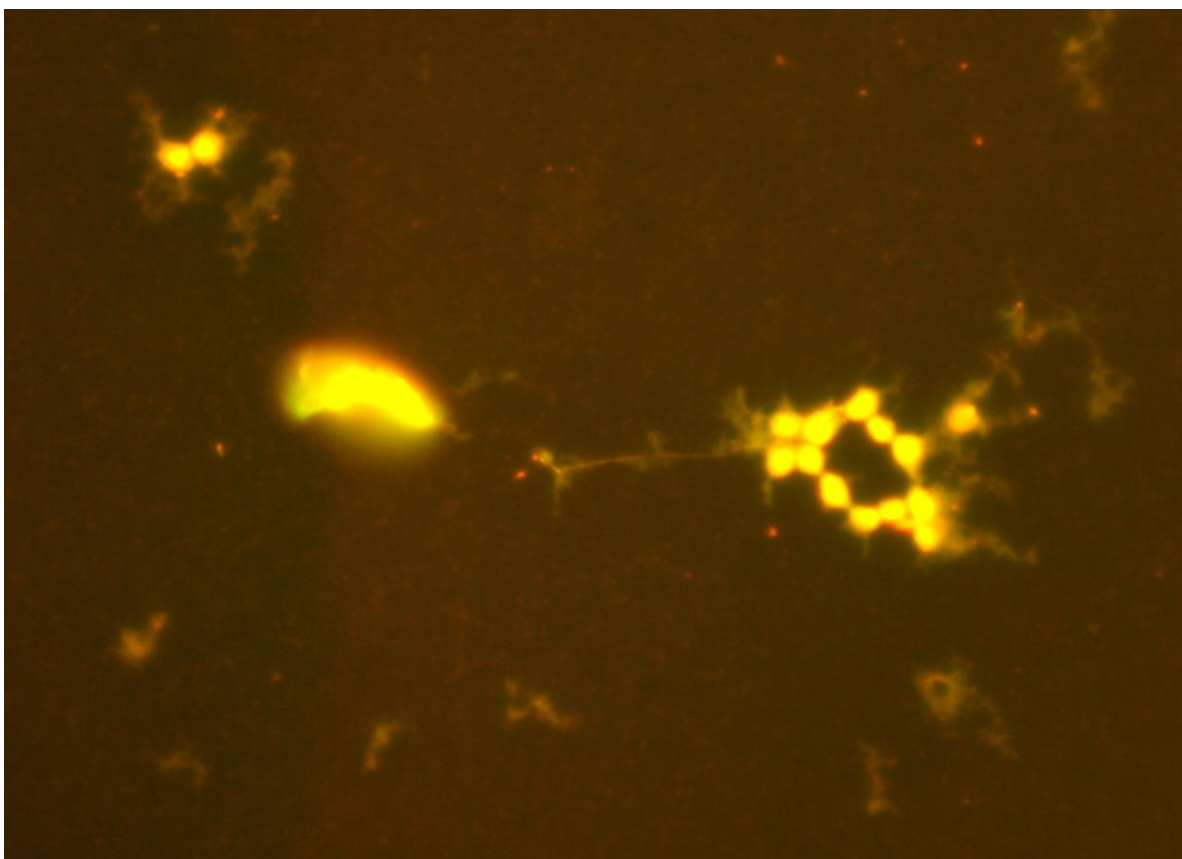


Figure 2.8 Image of algae-chitosan floc under epifluorescent microscope

Table 2.2 Pathogenic bacteria summary

Genus	Pathogenic bacteria	Disease
<i>Pseudomonas</i>	<i>P. aeruginosa</i>	Inflammation and sepsis
<i>Legionella</i>	<i>Legionella pneumophila</i>	Legionnaires disease
<i>Staphylococcus</i>	<i>S. aureus</i>	Abscesses and food poisoning
<i>Microcystis</i>	<i>Microcystis aeruginosa</i>	Neuromuscular poison

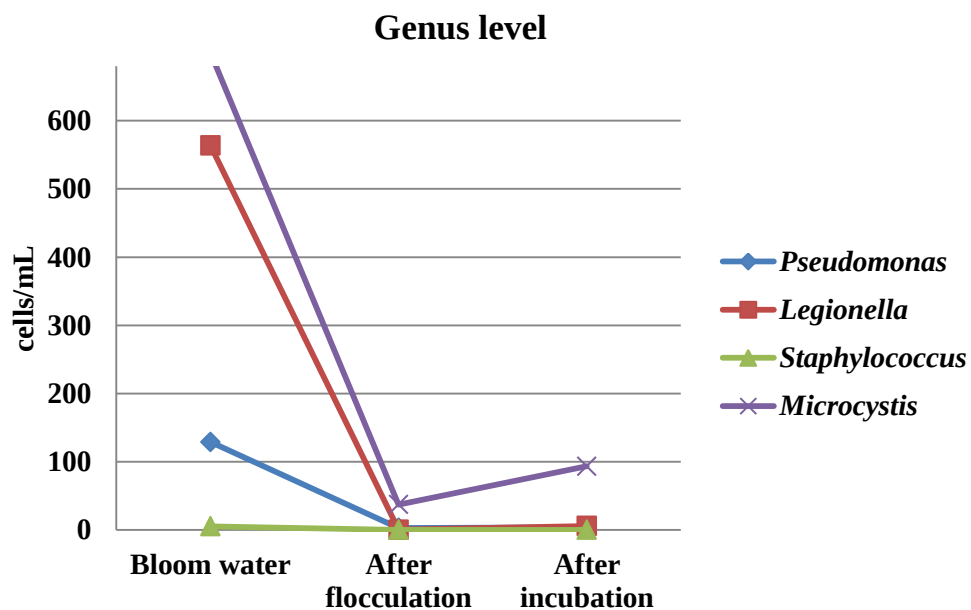


Figure 2.9 Abundance changes of pathogenic bacteria

correlated with the abundance of *Cyanobacteria*. In this study microbial community structure showed the percentage of *Proteobacteria* decreased after clay flocculation in the supernatant (Figure 2.5). This suggested *Proteobacteria* might be easier to precipitate with algae. The composition of freshwater bacterial community with the cyanobacteria blooms study distinguished that algal blooms were accompanied by the high abundance of heterotrophic bacteria (82). The results showed similar community structure between supernatant community structure and bottom-floc community structure. The microbial community structure of bottom flocs was influenced by supernatant, because those loose and light flocs were soaked in water, and it was hard to separate flocs from water adequately. In addition, bacteria can grow on flocs by degrading chitosan and/or algae exudates (83).

Bacteria can be also brought to bottom by clays due to electrostatic neutralization. The surface charge of bacteria depends on ionizable groups present on their cell wall. Gram negative bacteria usually process negative charges because of ionizable amino group (NH_2) and carboxyl (COOH) on the cell wall, as well as lipopolysaccharides on their membranes (84). Gram-positive bacteria are negatively charged primarily due to the presence of teichoic, teichuronic acids, and acidic polypeptides. The overall negative charges on bacteria may compete with negatively charged algal cells during clay flocculation. There is not much research on the comparison of surface charge of bacterial and algal cells. Different studies reported that the Gram positive bacteria cell wall charge density can be as high as $0.5\text{-}1.0\text{ C/m}^2$ (85), while that of algae have about 10 C/m^2 (86). Furthermore, cell size may also influence the electrostatic neutralization of chitosan and particles/cells. The results suggested that algal cells may be more attractive to positively charged chitosan.

In the bottom flocs, algal cells died after flocculation due to the netting formed by chitosan and clay that can immobilize cells and keep them from light. Viable cells can release dissolved organic compounds that are highly available substrates for heterotrophic bacteria. Dead algal cells released nutrients and provided organic carbon for heterotrophic bacteria in the bottom flocs. This can be a possible explanation of the bacteria cell number increasing significantly after flocculation.

Reduced pathogenic bacteria

Figure 2.9 shows the abundance decrease of four pathogenic bacteria and Table 2.2 lists the diseases caused by infection of those potential pathogens, including *Pseudomonas*, *Legionella*, *Staphylococcus*, and *Microcystis*. *Pseudomonas* is usually attached on *Cyanobacterium* and benefits growth of *M. aeruginosa* (87). These pathogenic bacteria usually are harmless, but they can cause infections under compromised immune conditions. Hui et al. (88) studied the effect of chitosan against *Escherichia coli* and *Staphylococcus* by evaluating bacterial viability at different incubation times. They indicated that NH_3^+ groups on chitosan can electrostatically neutralize phosphoryl groups of the phospholipid component of bacteria cell membrane. This led to leakage of intracellular constituents. Our results suggested that clay flocculation would bring colonial cyanobacteria as well as attached bacteria to the bottom flocs.

In this study, the laboratory Jar tests enabled an evaluation of the removal efficiency of modified commercially available clays. The dominant mechanism in this clay flocculation experiment was netting and bridging formed by chitosan and algae flocs. 16S rRNA gene sequencing was employed to elucidate the taxonomic information of bacteria and algae. Clay flocculation of harmful algal blooms may also cause precipitation of bacteria, but this effect was not significant in this experiment. Microbial community structure significantly changed after clay flocculation. The treatments with higher removal efficiency have similar microbial community structure. Microbial community structures of bottom flocs were biased by water column. They were similar to the microbial community in supernatant. The decrease of potential pathogenic bacteria indicates the risk of waterborne diseases can be reduced by chitosan modified clay flocculation. Clay flocculation study with Tennessee River water has provided an effective technique and experimental model to study impacts of clay flocculation on water community structure. Furthermore, field tests need to be conducted to verify the results of laboratory experiments.

CHAPTER 3 CHINA POND TEST: IMPACT OF CLAY FLOCCULATION OF ALGAL BLOOMS ON POND MICROBIAL COMMUNITY

**This chapter is potential publication for Journal of Microbial Ecology.*

INTRODUCTION

Harmful algal blooms (HABs) occur as consequences of eutrophication (89). Certain phytoplankton or microalgae species can rapidly grow and accumulate, especially in the surface of marine and freshwater environments (90-92). One of the primary environmental impacts of algal blooms from eutrophication includes excessive phytoplankton growth, depressed dissolved oxygen (DO) levels, and fish kills. These algal blooms have been increasing in number and severity during the past two decades, and have negatively affected coastal cities as well as lake and river systems worldwide. One of the most notable causes of these blooms is the environmental conditions of the water. Nutrients, mixing of water, sediment, and density of the algae population all affect the appearance of these blooms.

HAB events cause adverse impacts on human health due to the production of toxins. Accumulated biomass consumes oxygen, reduces light penetration, and alters food web dynamics (93). The first national survey reported 80% of 677 samples exceeded the World Health Organization's (WHO) advisory toxin limit in the United States and Canada between 1996 and 1998 (94). Human illness or death associated with CyanoHABs occurred worldwide, including Australia (illness of 141, 1979), Brazil (death of 88, illness of 2000, 1988), Canada (muscular pains, gastrointestinal of 13 people, 1959), China (liver cancer, 1993), England, Sweden (121 out of 304 village drinking water supplies affected, 1994), and USA (500,000 people out of drinking water, Ohio, 2014) (95). Common poisoning syndromes are paralytic, diarrhetic, neurotoxic, amnesic, and azaspiracid shellfish poisoning (1). Fish, seabirds, whales, dolphins, and other animals are threatened by these toxins (96). The estimated annual economic impact resulting from HABs in the U.S. is \$75 million from 1987 to 2000 (97).

Clay based flocculation techniques have been developed to mitigate harmful algal blooms by algal sedimentation and have been implanted in numerous cases worldwide (9, 98-100). A novel

HAB control technology using modified local soil/sand flocculation (MLS-C) controlled a serious HAB event in Tai Lake, China (17, 20, 70). Practical applications of clay based flocculation strategies demonstrated high cell removal capacity (average > 80% cell removal efficiency) (101). Rapid sedimentation of algal cells is attributable to positively charged flocculant particles binding algal cells, the entrainment of algal cells into settling clay flocs, and/or the loss of algal cell motility (102), and long polymer chains that may link small flocs into bigger ones. Clay flocculation helps remove massive algal cells in water bodies. Natural clay/soil was modified by infusing in chitosan, which is a derivative of the polysaccharide chitin. The modified clay particles were then quickly sprayed over the entire surface of pond. Since chitosan contains positively charged molecules, the negatively charged algal cells are attracted to them and bind together. These clumps of clay and algae cells, called flocs, become dense and rapidly sink to the bottom of the water into the sedimentary layers. This technique has been applied in lake restoration in China. Some studies had shown that a low dosage of flocculation (0.25 g/L) can remove up to 90% of algal cells in less than 4 h. TN, TP, NH_4^+ , and PO_4^{3-} concentration in water were found to decrease dramatically (100, 103). The high cell removal over a short period made clay flocculation a very successful and widely used method for the past ten years (63, 104).

Although clay flocculation has been demonstrated extremely effective, its impact on both water and sediment microbial community has not been thoroughly studied. The nutrients and algal cells deposited onto surficial sediments can be nitrogen source and carbon source for sedimentary microbial community. In addition, algal sedimentation could potentially bring unknown environmental stress to sedimentary microbial community. Thus, we hypothesize that microbial community of the water and sediment will respond to these environmental stresses in terms of biomass and species diversity changes as well as community structure shifts. In this study, 16S rRNA gene sequencing was conducted to measure changes in water and sediment microbial community structure before and after clay flocculation. Total coliform tests were used to examine if water quality improved after clay flocculation.

MATERIALS AND METHODS

Experimental design and sample description

The site was located beside Cetian reservoir in Datong, Shanxi province, China. The water in the Cetian reservoir was already in eutrophic state due to serious pollution from upstream. The algal contaminated water was pumped from the Cetian reservoir to two 800 m² ponds, one of which was the control pond and the other one was the treatment pond (Figure 3.1). The dominate algae were *Scenedesmus quadricauda* and *Cyanobacteria*. On day 0 the geochemical parameters (temperature, pH, dissolved oxygen, oxidation-reduction potential) were measured and then both control and treatment pond were sampled for water and sediment. On day 1, treatment pond was flocculated by chitosan modified local soil. After the treatment was added, geochemical parameters were measured in control and treatment pond. Water and sediment samples were collected from each pond on day 2, 7, 11, and 14. Each time point was triplicated. Water samples were further analyzed for TN, TP, NH₄⁺, and Phosphate. General geo-properties of overlay water were shown in Table 3.1. Sediment samples were lyophilized and stored at -80°C for PLFA and 16S RNA sequencing. 400 ml water for each sample was filtered through 47 mm diameter, 0.22 µm pore size polyethylsulfone membranes filter (MO BIO Laboratories, Inc., Carlsbad, CA). The filters were immediately stored at -80°C for DNA extraction.

Geochemistry measurements

Temperature (Temp), pH, dissolved oxygen (DO), and oxidation-reduction potential (ORP) were measured by a YIS 556 Handheld Multiparameter Instrument (Xylem Inc., OH, USA) before sampling. Total nitrogen (TN), ammonium (NH₄-N), nitrate (NO₃-N), total phosphorus (TP) and soluble reactive phosphate (PO₄-P) were measured by Research Center for Eco-Environmental Research Sciences, Chinese Academy of Sciences, Beijing, China according to the procedures in Pan et al (73)

PCR amplification and 16S rRNA gene sequencing

Genomic DNA of sediments were extracted in triplicate from 0.5 g sediment using the PowerSoil DNA Isolation Kit (MoBio Laboratories) following to manufacture's protocol. Genomic DNA of the water sample was extracted in triplicate using the PowerWater DNA Isolation Kit (MoBio

Laboratories) following the manufacture's protocol. 16S rRNA genes were amplified using primer pair 515f and barcoded 806r. The DNA library was prepared according to published procedures (105). 16S rRNA gene sequencing was conducted on the Illumina MiSeq platform (San Diego, CA) at the University of Tennessee. Taxonomic assignment, and phylogenetic reconstruction, diversity analyses and visualizations were performed using Qiime and R packages.

Statistical analysis

ANOVA analysis, Tukey's HSD Test, and multivariate analysis were performed in JMP pro 10.0 (SAS Institute Inc., NC). All tests were considered significant at a level of $\alpha=0.05$. The Weighted unifracs profile was analyzed with various methods in Prime 6 and PERMANOVA+ (Primer Inc. UK). Non-metric multidimensional scaling (NMDS) based on the weighted unifracs distance matrix and environmental variables were fitted using canonical analysis of principal components.

RESULTS

Geochemistry properties

Temperature (Temp.), DO, pH, ORP, pH and Secchi Disk depth (SD) in the control and treatment ponds were shown in Table 3.1. The temperature was 22°C to 27 °C at the surface water and 20°C to 25°C at the bottom. DO and ORP decreased dramatically in both control and treatment ponds on day 2. DO was maintained around 6 mg/L at the surface water in the treatment ponds, and around 4 mg/L in the control ponds. The bottom of the control ponds was anaerobic after day 2. The pH decreased to a neutral pH range after clay flocculation but it was kept at high pH in the control ponds. The geochemical profiles of nutrients are shown in Figure 3.2 -3.6. TN and TP concentrations decreased in the treatment ponds right after clay flocculation and increased gradually after day 2. NO_3^- , PO_4^{3-} and NH_4^+ concentrations were fluctuant gradually in both control and treatment ponds, but there was no significant difference between ponds (T-test, $P=0.73$, $P=0.103$ $P=0.08$, respectively). The mean of NO_3^- , PO_4^{3-} and NH_4^+ concentrations was compared between 1-7 days and 7-14 after clay flocculation. None of the three concentrations changed significantly in the treatment ponds ($P=0.53$, $P=0.200$, $P=0.124$).



Figure 3.1 Photograph of control pond and modified local soil (MLS) treatment pond.

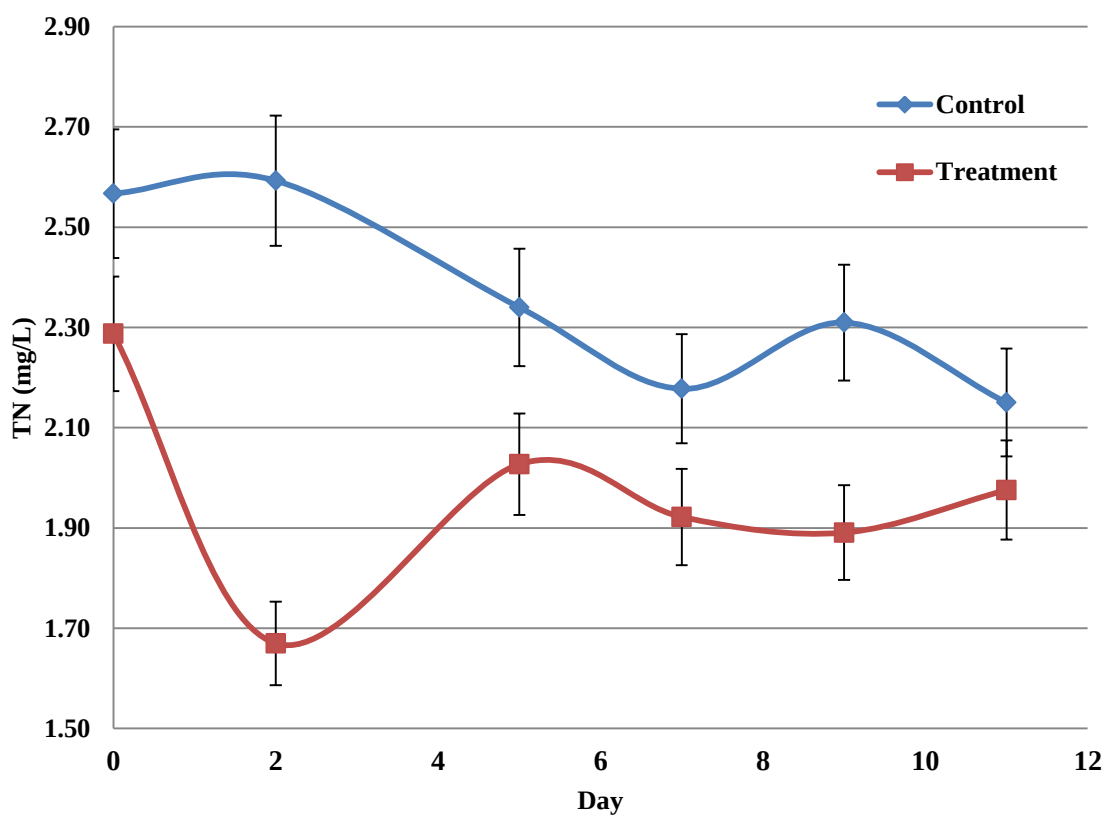


Figure 3.2 Total nitrogen concentration (mg/L) change with time in control and treatment ponds. The dots represent the mean value and the error bars indicate + /- standard deviation.

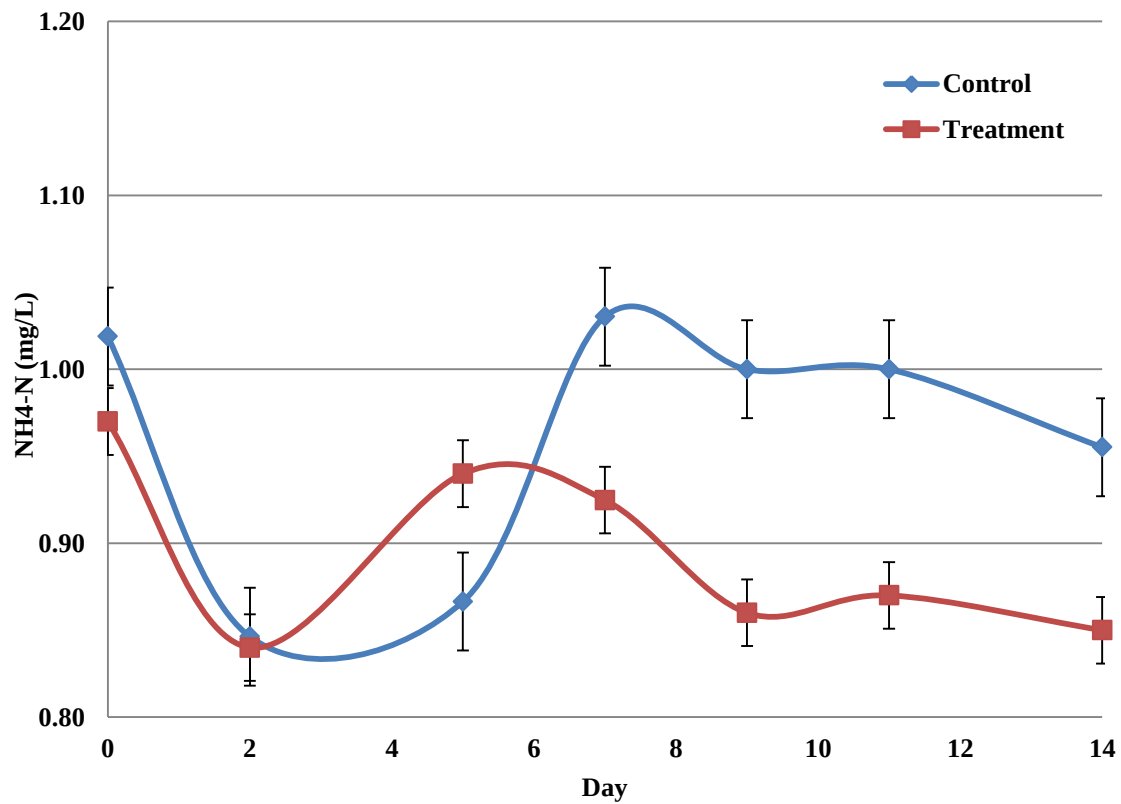


Figure 3.3 Ammonia concentration (mg/L) change with time in control and treatment ponds. The dots represent the mean value and the error bars indicate + /- standard deviation.

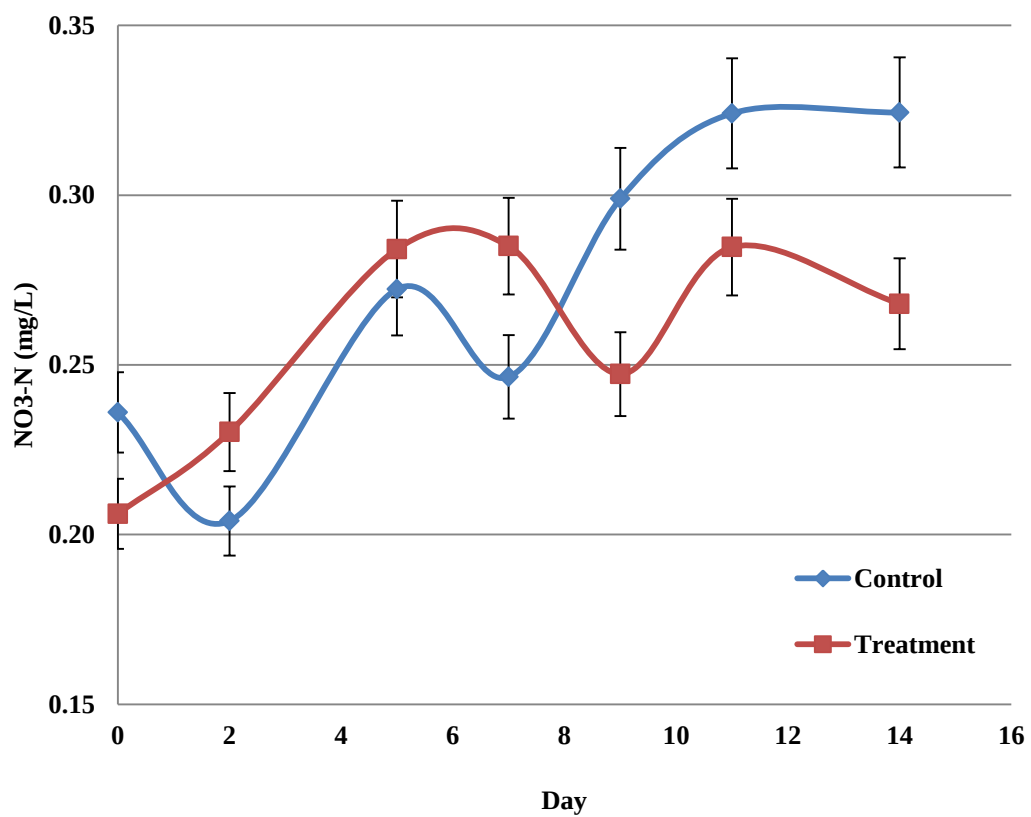


Figure 3.4 Nitrate concentration (mg/L) change with time in control and treatment ponds. The dots represent the mean value and the error bars indicate + /- standard deviation.

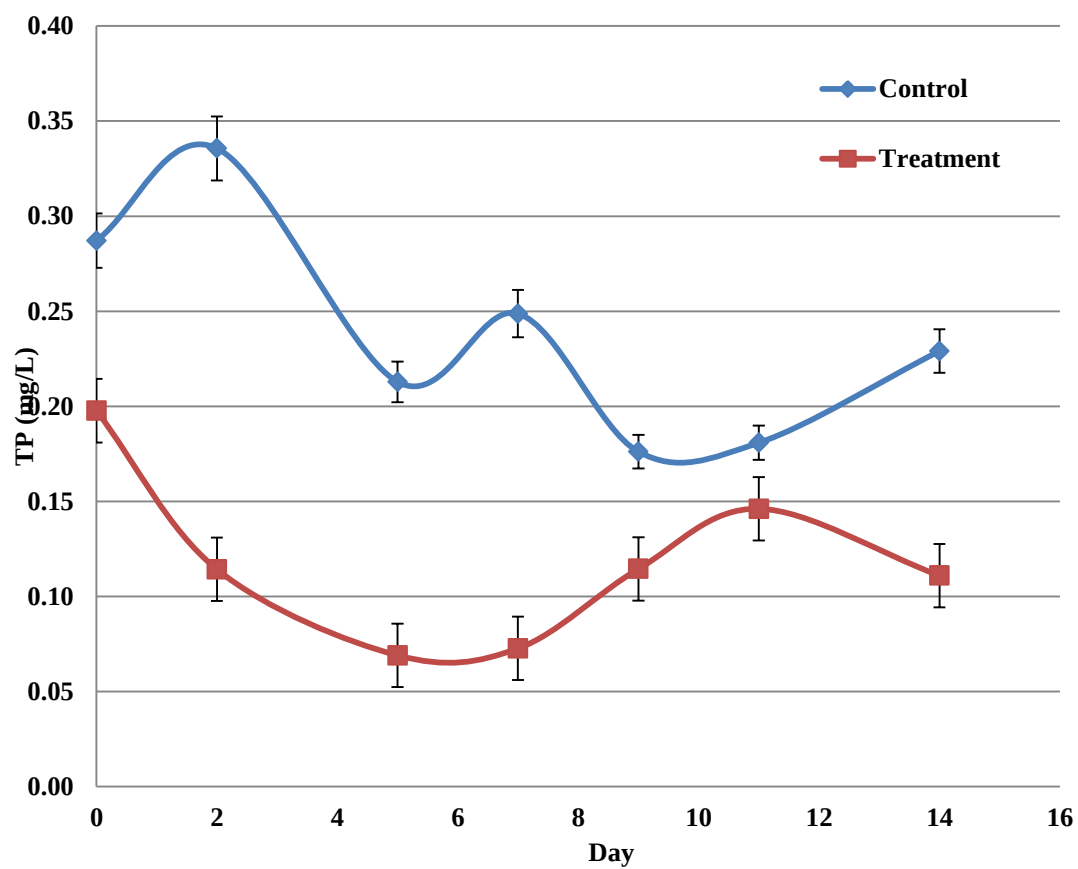


Figure 3.5 Total phosphorus concentration (mg/L) change with time in control and treatment pond. The dots represent the mean value and the error bars indicate + /- standard deviation.

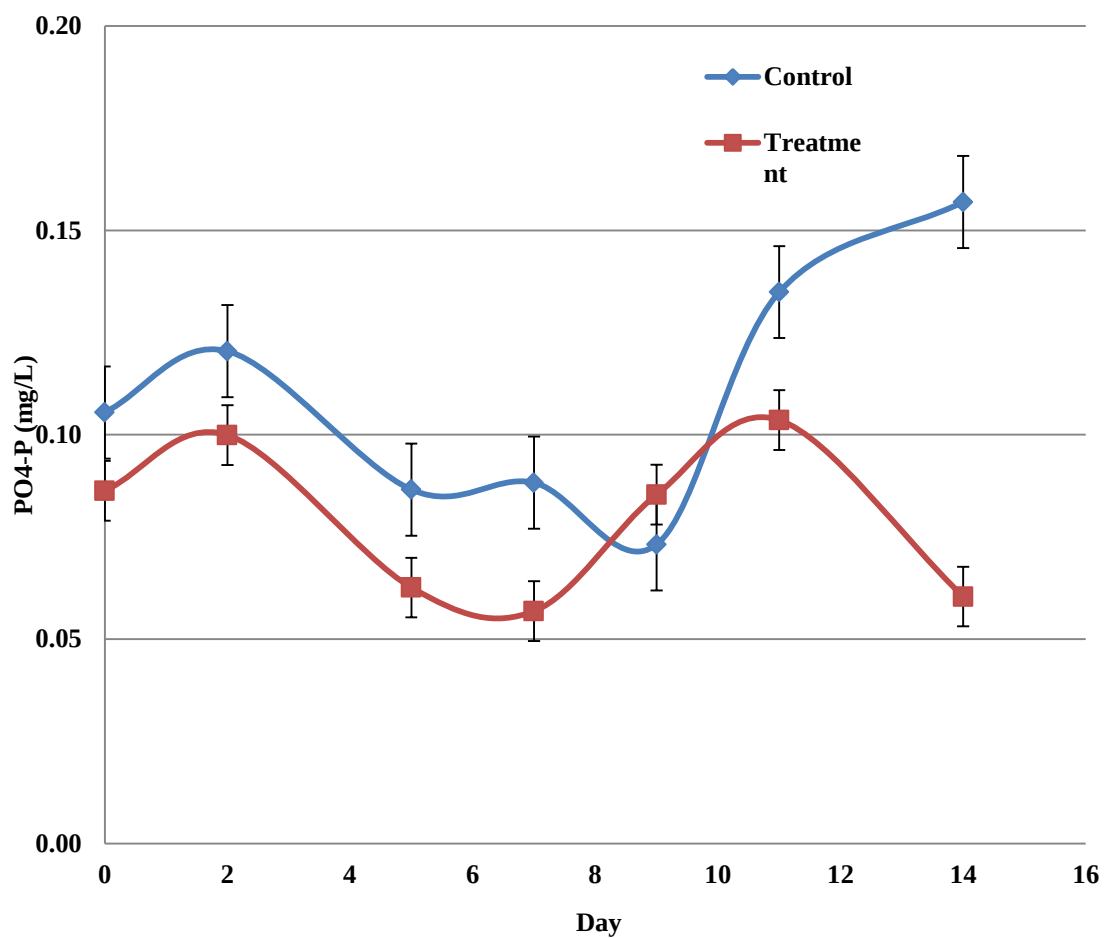


Figure 3.6 Phosphate concentration (mg/L) change with time in control and treatment pond. The dots represent the mean value and the error bars indicate + /- standard deviation.

Table 3.1 Geochemical properties of control and treatment ponds

Day	Pond	Location	Temp. (°C)	DO (mg/L)	ORP (mV)	pH	SSD (m)
0	Control	water_surface	27.2	10.5	152.9	8.52	60
		water_bottom	20.8	4.95			
	Treatment	water_surface	25.2	11.1	160	8.45	60
		Water_bottom	21.6	6.13			
2	Control	water_surface	23.1	1.43	-164.5	8.12	70
		water_bottom	22.5	0.27			
	Treatment	water_surface	22.3	6.6	-210.7	7.02	80
		water_bottom	23.1	2.76			
7	Control	water_surface	24.8	4	-173.5	8.34	85
		water_bottom	24.7	1.78			
	Treatment	water_surface	25	6.42	-174.1	7.15	120
		water_bottom	24.9	2.56			
11	Control	water_surface	24.8	4.09	-185.2	8.68	75
		water_bottom	24.7	1.72			
	Treatment	water_surface	25	6.41	-165.3	7.19	90
		water_bottom	24.9	3.01			
14	Control	water_surface	25.2	4.35	-183.8	8.49	120
		water_bottom	23.1	0.43			
	Treatment	water_surface	25	6.38	-158.2	7.05	120
		water_bottom	22.9	2.45			

Water microbial community structure

Changes in microbial community structure of control and treatment ponds at the phylum level were shown in Figure 3.7. *Cyanobacteria*, *Proteobacteria*, *Actinobacteria*, and *Verrucomicrobia* dominated control pond. *Cyanobacteria* indicated algal cells took up 22% of total microbial community. This percentage varied with experimental time points. The percentage of *Proteobacteria* started at 10% and increased to 15% on day 2. It decreased dramatically after five days and then rebounded to 50% in day 7 to 10. The percentage of *Actinobacteria* was also unstable, varying from 35% to 5%. *Verrucomicrobia* (30%) was relatively stable in the control pond. After clay flocculation, *Cyanobacteria* were significantly reduced in the treatment pond. The dominant phyla were *Proteobacteria*, *Actinobacteria*, and *Bacteroidetes*. The percentage of *Verrucomicrobia* decreased during the experimental period, and *Bacteroidetes* increased gradually after clay flocculation. *Actinobacteria* was extremely dominating during first 11 days. The microbial community composition suddenly changed day 14 with an overwhelming dominance of *Proteobacteria*.

Sediment microbial community structure

The microbial community structures were similar in the control and treatment ponds, dominated by *Proteobacteria*, *Chloroflexi*, *Acidobacteria*, *Actinobacteria*, *Bacteroidetes*, *Verrucomicrobia*, and *Firmicutes*. However, the percentage of dominated phyla was changing inversely between control pond and treatment pond. In both control and treatment, *Proteobacteria* took up 30% to 40%, and *Chloroflexi*, *Acidobacteria*, *Actinobacteria*, and *Bacteroidetes* were in similar abundances. The depth 16S rRNA gene sequencing information will be demonstrated in discussion section (Figure 3.8).

DISCUSSIONS AND CONCLUSIONS

Geochemistry parameter changes in water

The photosynthetic activity of dense algal blooms tends to deplete free CO₂ in natural waters, which leads to an increase in pH (106). The pH decreased to a neutral range after clay flocculation due to the removal of algal cells from pond water. The DO changed dramatically in the first two days due to the stormy weather. The nutrient profiles of the control and treatment

ponds showed that samples collected from treatment pond had lower TN and TP concentrations than those from control pond. Phosphorus and nitrogen combined with carbon dioxide are the main sources for algal growth. Algae are able to assimilate various nitrogen forms, including ammonia, nitrite, nitrate and many dissolved organic nitrogen (107). The decrease of TN and TP in water was contributed by the algae's uptake and sedimentation. Insignificant changes of NO_3^- , PO_4^{3-} and NH_4^+ concentrations in treatment pond suggested that clay flocculation was unable to remove dissolved nutrient directly. The rebounded trend of TP and phosphate may be caused by algal cells releasing nutrients. Force et al. (108) reported three phases of nutrient regeneration from algae when they were hidden from light. Algal cells may either release or absorb nitrogen and phosphorus during the first 24 h and they went to inactive phase, which would last for several days. They then actively release the nutrients into the environment and this phase can be as long as 3 to 4 months. Other previous studies showed phosphorus compounds were released faster than nitrogen compounds, 50% phosphorus was released by autolysis in the first few hours.

Distinct water microbial community structures

The in-depth microbial community profile showed different microbial community structures between the control and treatment ponds (Figure 3.9- 3.12). Members of *Verrucomicrobia* were observed in the surface and hypolimnetic water and there were a variety of metabolic bacteria within this group (109). *Mycobacteriaceae* under *Actinobacteria* phylum was dominating at the family level, the abundance was much higher in treatment ponds than that in control ponds. Many *Mycobacterium* species readily grow with ammonia or amino acids as nitrogen sources and glycerol as a carbon source in the presence of mineral salts. This might be a possible explanation of elevated *Mycobacteriaceae* in treatment pond water samples.

Alphaproteobacteria, *Betaproteobacteria*, *Gammaproteobacteria* and *Deltaproteobacteria* dominated the control pond water, but only *Alphaproteobacteria* and *Betaproteobacteria* dominated in the treatment ponds (Figure 3.10). The *Deltaproteobacteria* belongs to a physiologically diverse group, SAR324, which has ability to grow autotrophically and heterotrophically involved in carbon, sulfur and nitrogen cycling. *Sphingobacteria* and

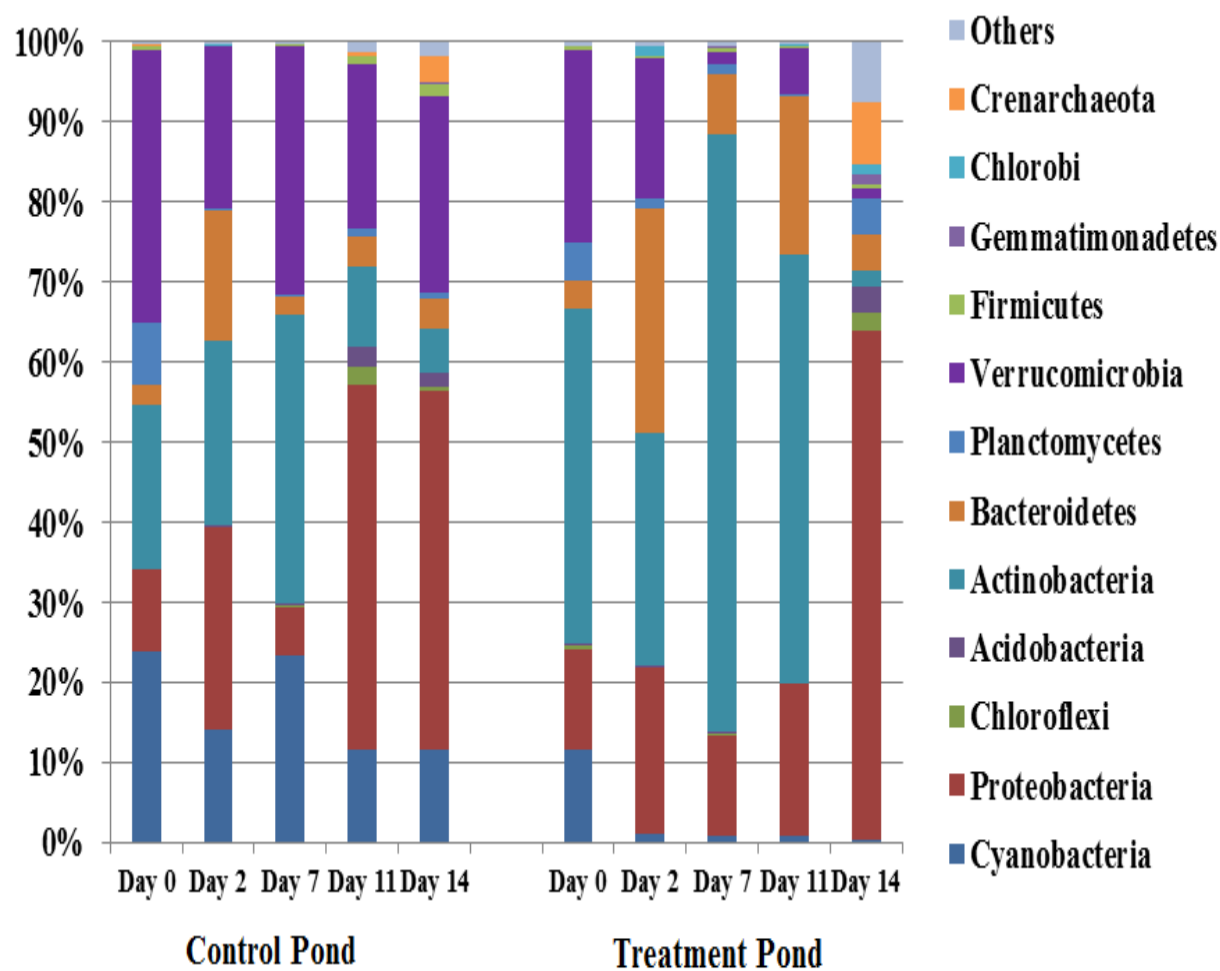


Figure 3.7 Changes in water microbial community structure of control and treatment pond at phylum level

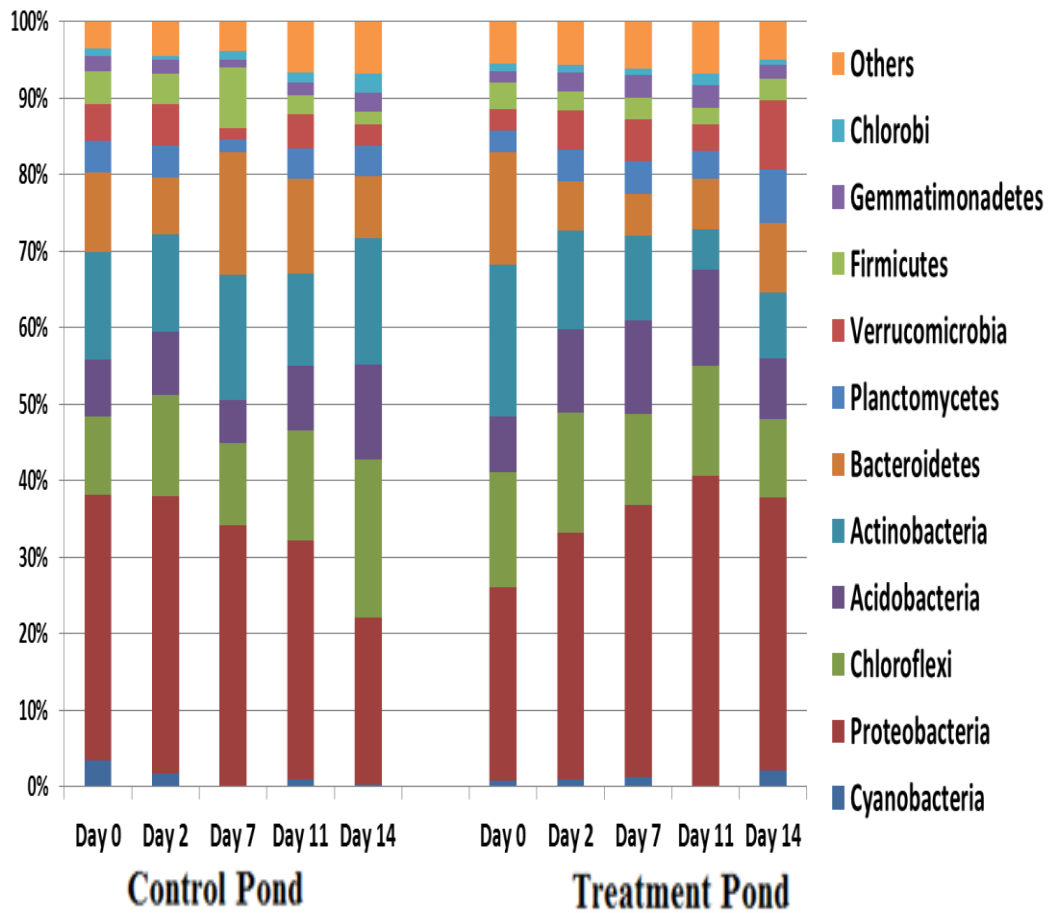


Figure 3.8 Changes in sediment microbial community structure of control and treatment pond at phylum level

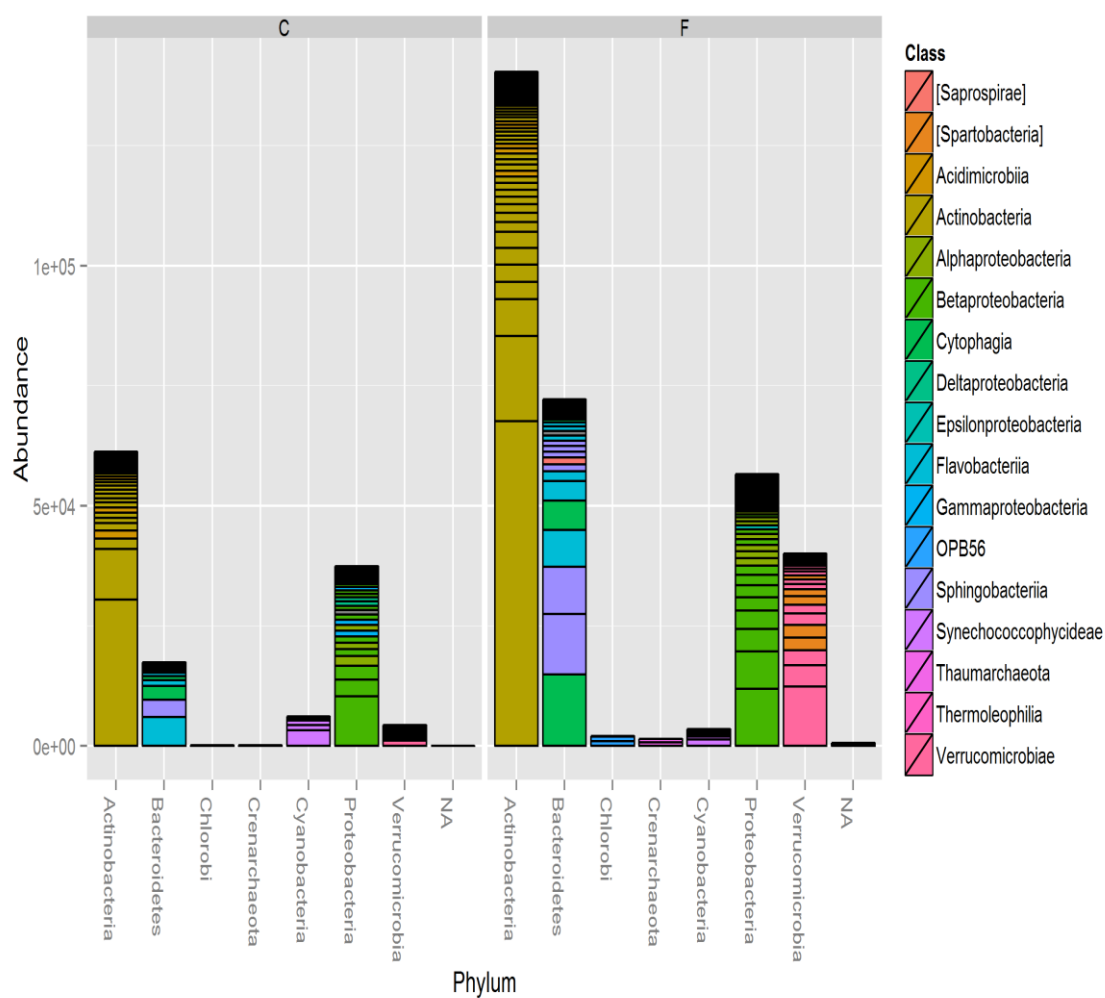


Figure 3.9 Most abundant Phyla change of top 100 OTUs in water samples. Filled colors represent the Classes to which each OTU belongs.

Flavobacteria were present in higher abundance in treatment water samples as compared to samples from control ponds (Figure 3.10). These members of *Bacteroidetes* are chemoorganotrophic and efficiently degrade a variety of high-molecular-weight compounds such as protein, cellulose, pectin, and chitin, typical components of the high molecular mass fraction of DOM (110). Chitosan used in clay flocculation is a long chain biopolymer and it may cause the elevated abundance of *Flavobacteria* members in the treatment. *Sphingomonadaceae* was observed in the treatment samples. Some species from this group are also known for their ability to degrade some aromatic compounds and it is actively involved in environmental remediation.

Beta diversity was analyzed based on the weighted unifrac distance matrix. Non metric multidimensional scaling (NMDS) compares pairwise distances among samples and classifies them into clusters. The NMDS plot of all water samples indicated significantly different community structures between the control pond and treatment pond (PerMANOVA test $P=0.017$) (Figure 3.13). Environmental factors were fitted with community structure using canonical analysis of principal coordinates (Figure 3.14). The microbial community structures were strongly affected by TN, $\text{NH}_4\text{-N}$, TP, $\text{NO}_3\text{-N}$ and DO. The total coliform tests are conducted in both ecosystem and control pond over three time points. The result showed the total coliform level was 10 times lower in treatment pond than that of the control pond (Figure 3.16).

Abundance of OTU level in sediment samples

Unlike the distinct microbial community structure in water samples, the sediment samples from the control and treatment ponds were not obviously different at phylum level. However, they showed differences in OTU level (Figure 3.15). This fact indicated that some species may take over a similar function from other species that are under the same phylum, or even genus. Members of *Actinobacteria*, *Acidobacteria*, *Bacteroidetes*, *Betaproteobacteria*, *Deltaproteobacteria* and *Chloroflexi* were presented higher abundance in treatment pond sediment. Elevated *Mycobacteriaceae* in the treatment sediment samples suggested an active nitrogen cycling process. *Flavobacteriaceae* were enriched in the treatment sediment, which indicated that members of this group might participate the degradation of chitosan used in clay flocculation. *Comamonadaceae* were observed in higher abundance in the treatment ponds as

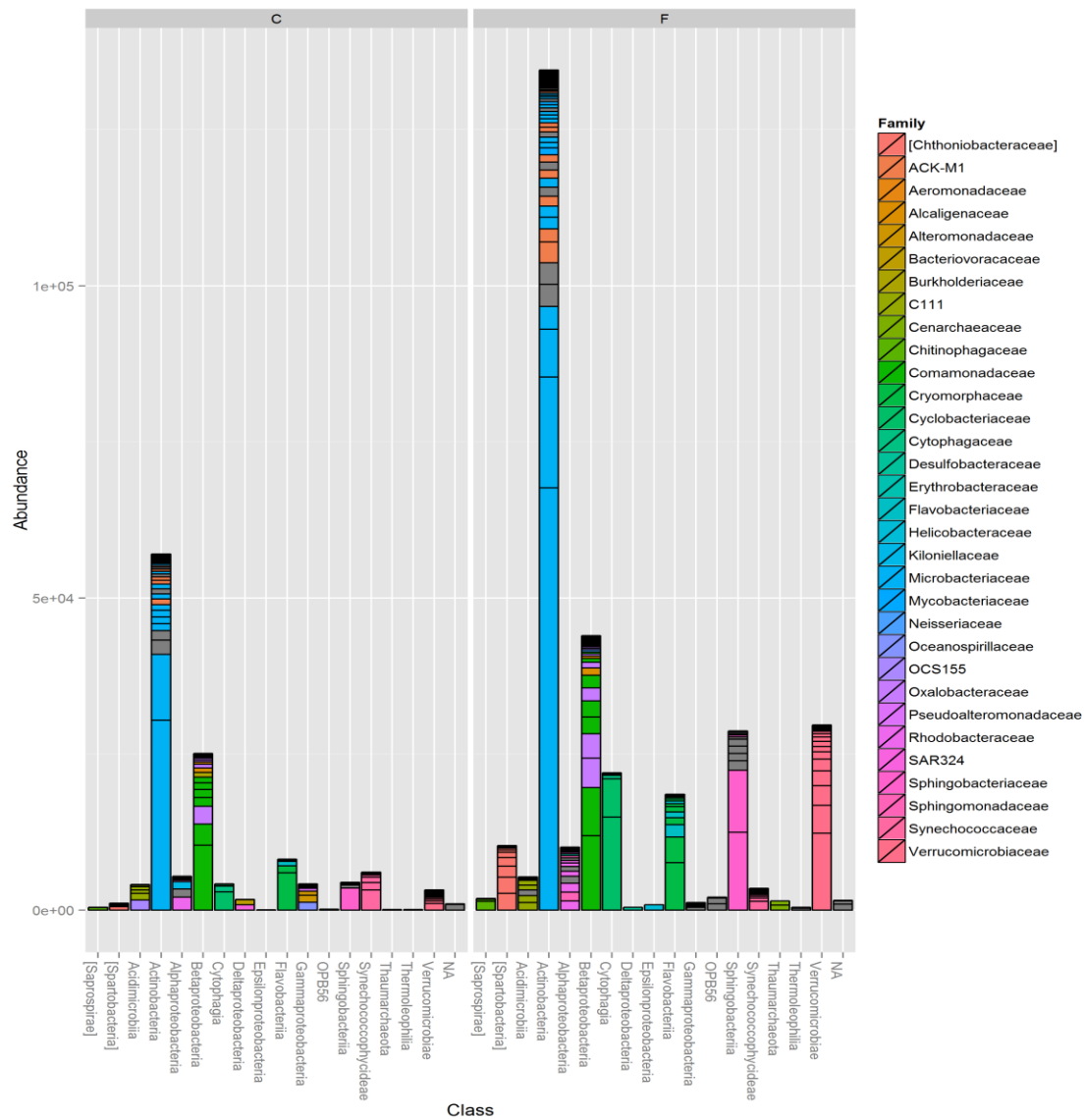


Figure 3.10 Most abundant Classes change of top 100 OTUs in water samples. Filled colors represent the Family to which each OTU belongs.

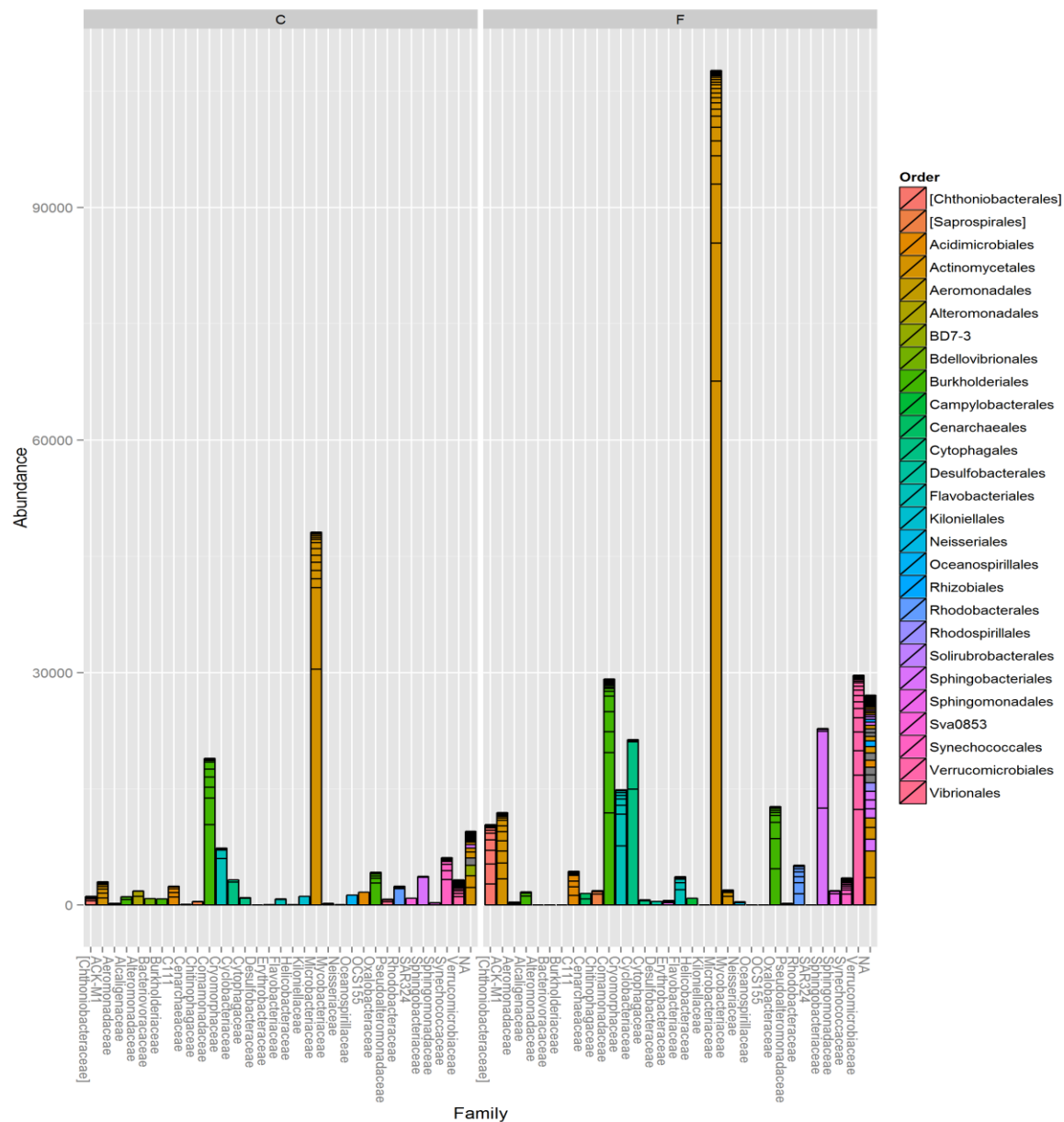


Figure 3.11 Most abundant Family change of top 100 OTUs in water samples. Filled colors represent the Order to which each OTU belongs.

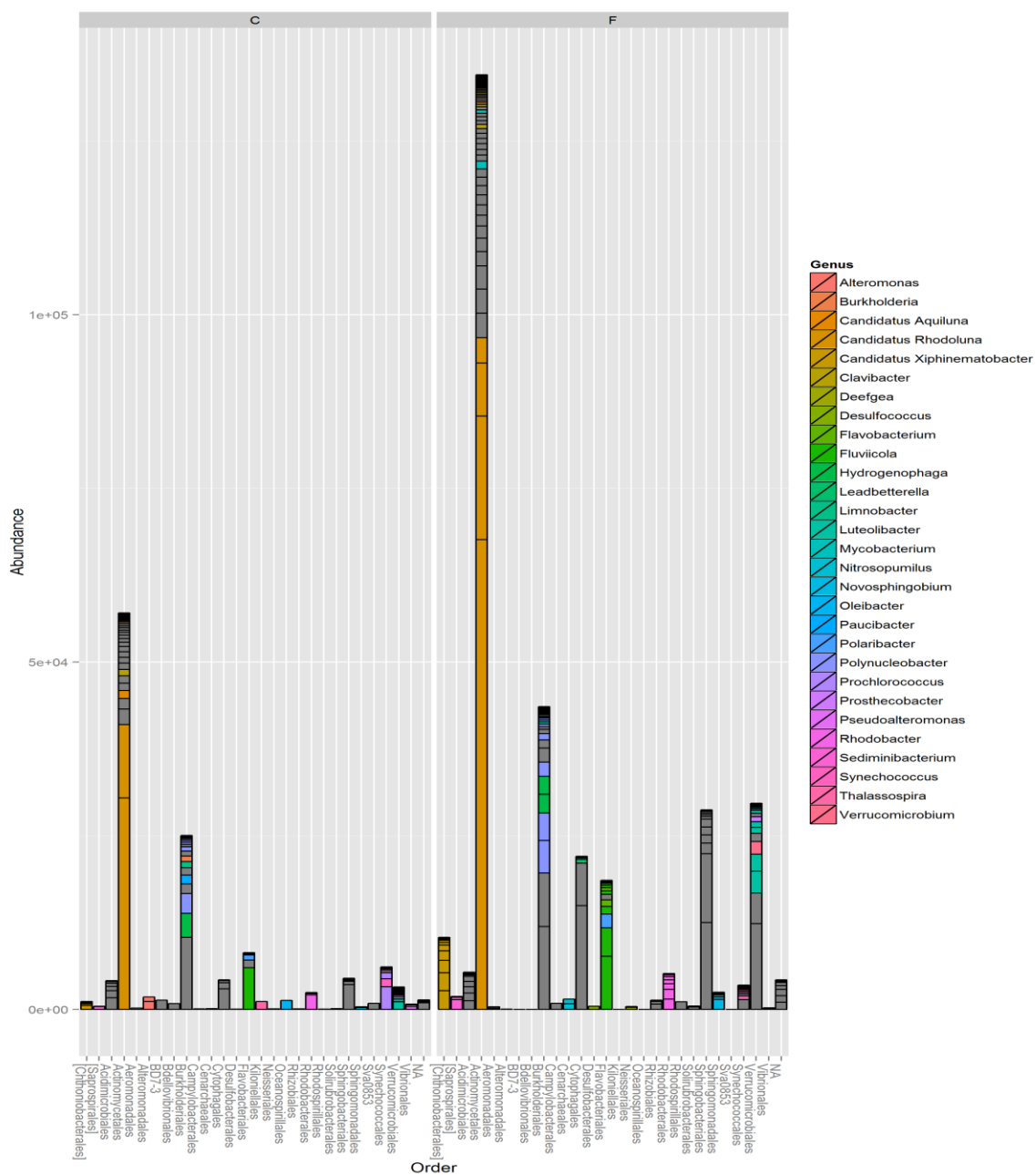


Figure 3.12 Most abundant Order change of top 100 OTUs in water samples. Filled colors represent the Genus to which each OTU belongs.

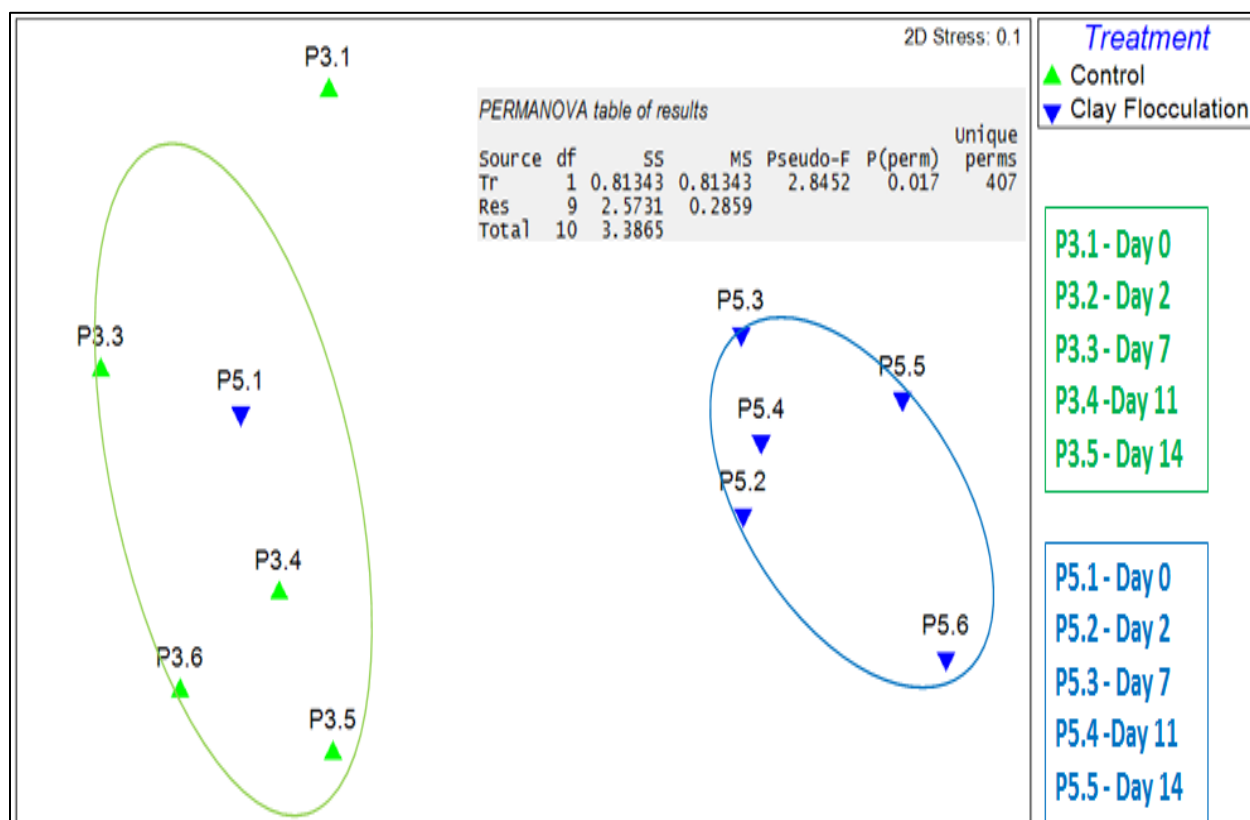


Figure 3.13 Non-metric Multidimensional scaling (NMDS) plot of weighted unifracs distance of water samples community. Circle indicates the 95% confidence interval.

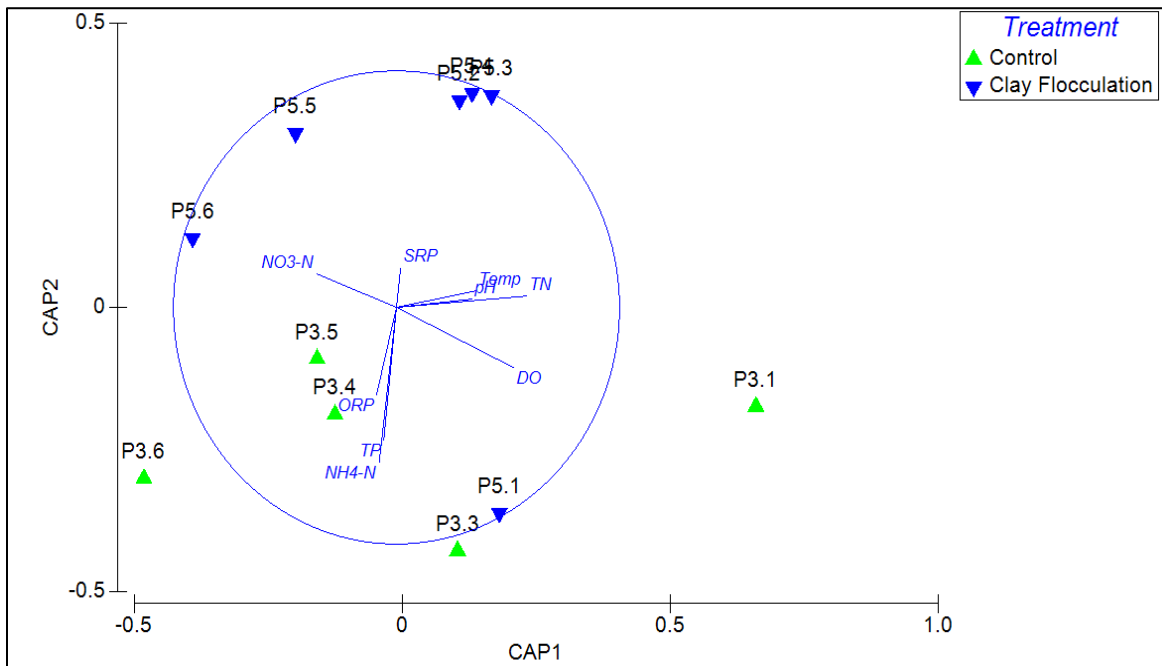


Figure 3.14 Environmental factors fitted with community structure using canonical analysis of principal coordinates.

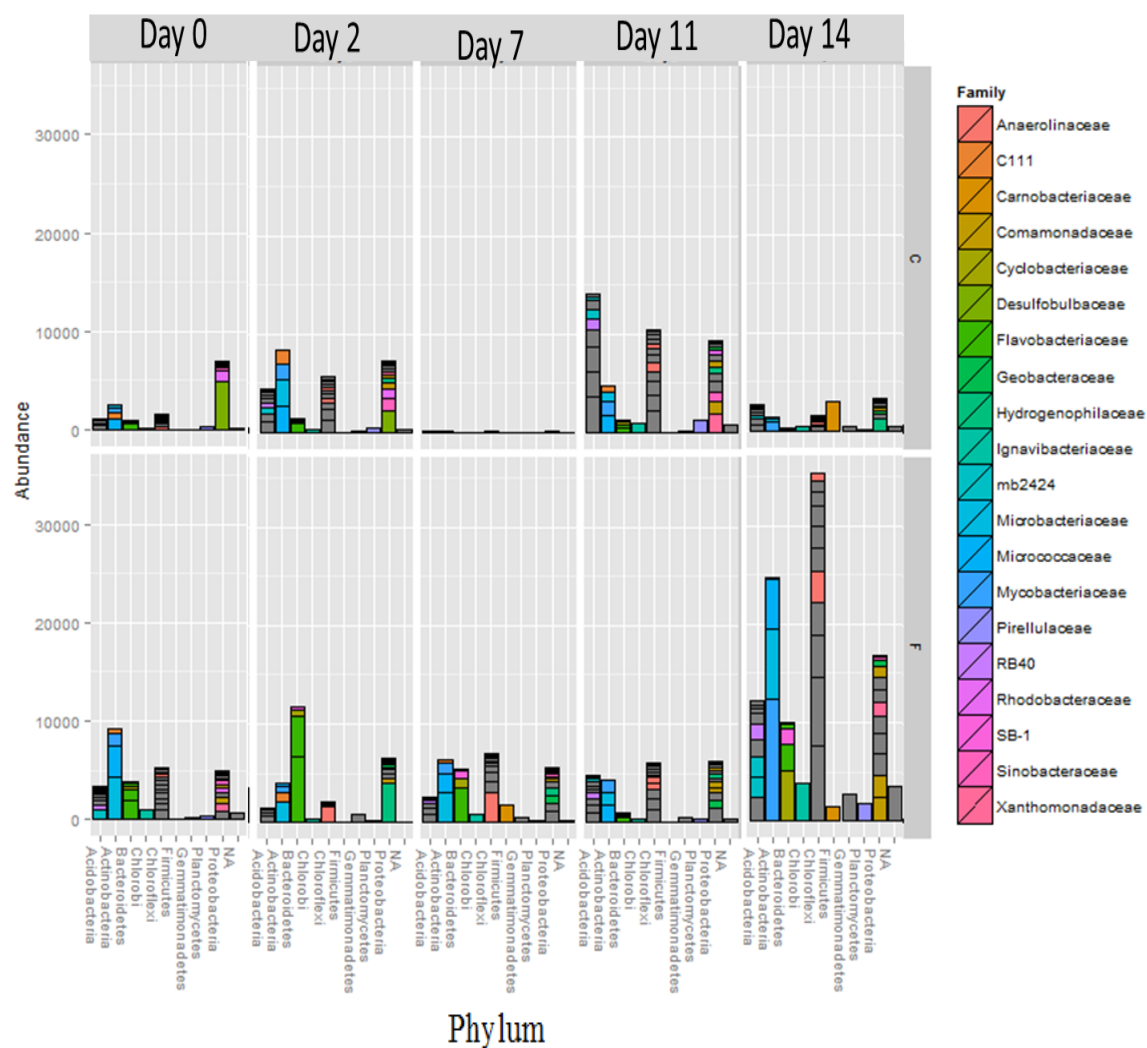


Figure 3.15 Most abundant phyla changes of top 100 OTUs in sediment samples. Filled colors represent the Family to which each OTU belongs. C represents the control pond; F represents the treatment pond.

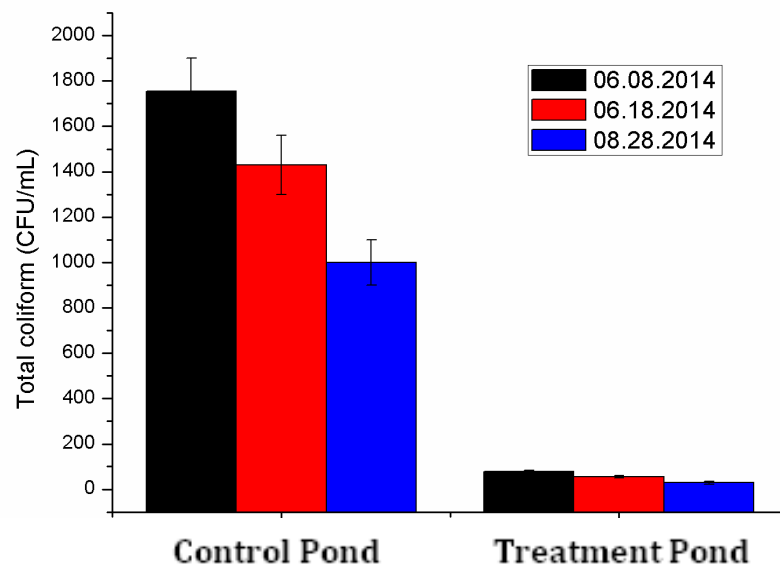


Figure 3.16 Total coliform test results

compared to the control ponds. *Comamonadaceae* belongs to *Betaproteobacteria*, and members of the family *Comamonadaceae* are primary PHBV-degrading denitrifiers in activated sludge. They were used to develop a new nitrogen removal system with solid biopolymer as an electron donor (111).

The pond tests investigated the response of the microbial community in both pond water and pond sediment to chitosan-modified-local-soil (MLS) clay flocculation. The results showed TP and TN were reduced in the water after treatment. 16S rRNA sequencing showed no major phylum division change in sediment samples, but OTU level abundance variations suggested some species may take place of others due to the algal biomass and nutrient deposits, as well as chitosan and local soil brought to the sedimentary layer. Total coliform tests showed MLS clay flocculation significantly reduced the risk of fecal contamination or standard indicators of recent human fecal contamination in pond water. Since more and more new flocculants are being developed for algal sedimentation, the response of microbial community structure to these techniques need be assessed. In addition, further studies should focus more on microbial community structure change in sediment.

CHAPTER 4 MICROCOSMS EXPERIMENT: THE RESPONSE OF MICROBIAL COMMUNITY IN WATER AND SEDIMENT TO VARIOUS CLAY FLOCCULATION TREATMENTS

**Portions of this chapter have been submitted to Ecotoxicology.*

INTRODUCTION

Initial research studies of clay flocculation mainly focused on the effect of autochthonous Phosphorus and Nitrogen load management and toxicity reduction in the impacted water body (112). Studies reported P can be trapped effectively by flocculant and buried in sediment as long as 15 years (113). One natural porous mineral, zeolite, which can absorb ammonia via ion exchange, can be used as an amendment with chitosan-modified clay flocculation (114). Addition of zeolite allows for the permanent removal of excess N by coupled nitrification and denitrification (115-117). Other studies have shown that paralytic shellfish toxins (PSTs) in water were mostly scavenged after clay flocculation, and were found to be 83% degraded in the flocs-algae-sediment mixture (118). In addition, several studies characterized the succession of the eukaryotic plankton community subject to clay flocculation based on 18S rRNA gene sequences. Increased diversity evenness and decreased phylogenetic species variability of algae and protozoa were observed after clay flocculation (119). TN, TP, NH_4^+ , and PO_4^{3-} were important factors structuring microplankton communities (120). However, few investigations have been published that examine the microbial community structure response to clay flocculation. Prokaryotes are fundamentally involved in the degradation of pollutants and they govern biogeochemical processes globally (29). In the current investigation, we used multiple lines of evidence to study how water and sediment microbial community respond to three clay flocculation approaches. The high throughput sequencing of the 16S rRNA gene was performed to compare bacteria biomass, species diversity and microbial community composition among various treatments; secondly, to elucidate latent association of microbial community with varying environmental parameters.

The deposited nutrient and algal cells can be utilized as nitrogen source and carbon source for sedimentary microbial community. In addition, algal sedimentation could potentially bring

unknown environmental stress to sedimentary microbial community. Thus, we hypothesize that the sedimentary microbial community will respond to these environmental stresses in terms of biomass and species diversity changes as well as community structure shifts. Bacterial cell membranes are made up mostly of different types of phospholipids, which are able to alter lipid configuration to respond to physiological and chemical changes. Furthermore, phospholipid will be degraded rapidly when the cells die, making PLFA a better indicator of viable biomass. It therefore allows for its use as valuable information on environmental stress and viable community structure. This study provided insight into how microbial communities and their activities will shift in a changing environmental engineering operation.

MATERIALS AND METHODS

Site description and experiment setup

The field study site was located near the Cetian reservoir in Datong, Shanxi province, China. The reservoir was eutrophic due to the nutrients from agricultural runoff. The site includes eight 800 m² ponds, which were filled with algal contaminated water from the reservoir. For this study, sediments were collected from one of the eight ponds, mixed, and placed in 72 columns to a height of 20 cm. Algal contaminated water from the pond was used to fill the columns to 1 cm below the top. The treatments were clay flocculation (F), clay flocculation with added zeolite capping (Z); clay flocculation with O₂-loaded zeolite capping (OZ), and control/no flocculation (C). These were triplicate columns for each treatment. The F columns were treated with chitosan modified local soil (2 mg/L chitosan and 75 mg/L soil). In addition to chitosan modified local soil, the Z columns received 1 cm layer of zeolite (sieved through 100 meshes), in order to act as a capping layer to prevent algae from getting back into the water. The OZ columns were treated with chitosan modified local soil and 1 cm O₂ loaded zeolite-capping layer. The O₂ loaded zeolite was prepared by putting sieved zeolite in a high-pressure cylinder and purging with pure O₂ overnight. Columns were destructively sampled on the day of treatment day 1 and on day 2, 4, 7, and 10. Initial samples were also collected before the treatment (day 0).

The overlaying water samples (50 mL) were collected from 10 cm above the sediment-water interface using a syringe and filtered through a 0.2 µm corning™ RC Syringe filter. The filters

were immediately stored at -20°C for nutrient analysis. Remaining water was filtered through 47 mm diameter, 0.22 µm pore size polyethylenesulfone membrane filters (MO BIO Laboratories, Inc., Carlsbad, CA). The filters were immediately stored at -80°C for DNA extraction. Sediment cores were collected from columns using auger and thin walled tube sampler. The cores were sectioned at 3 cm depth intervals. Sub-samples were frozen at -80°C for both DNA and lipid analyses.

Geochemistry measurements

Temperature (Temp), pH, dissolved oxygen (DO), and oxidation-reduction potential (ORP) were measured by a YSI 556 Handheld Multiparameter Instrument (Xylem Inc., OH, USA) before sampling. Total nitrogen (TN), ammonium (NH₄-N), nitrate (NO₃-N), total phosphorus (TP) and soluble reactive phosphate (PO₄-P) were measured by Research Center for Eco-Environmental Research Sciences, Chinese Academy of Sciences, Beijing, China according to the procedures in Pan et al (73).

Genomic DNA extraction and PCR amplification

Genomic DNA was extracted using the PowerWater DNA isolation kit for water samples and PowerSoil DNA Isolation kit for sediment samples (MO BIO Laboratories) following the manufacturer's protocol. The DNA extracts were purified using genomic DNA Clean and Concentrator kit (Zymo Research, Irvine, CA, USA). 16S rRNA genes were amplified using Phusion DNA polymerase (Thermo Scientific, Waltham, MA) with primer pair 515f and barcoded 806r. DNA quality was determined using the 2100 Bioanalyzer Instrument (Agilent Technologies, Santa Clara, CA, USA) and DNA concentration was determined by KAPA SYBR® FAST qPCR Kits. Samples were diluted and pooled to a final concentration of 4 nM for sequencing.

Barcoded amplicon 16S rRNA gene sequencing

16S DNA library was prepared according to the protocol published by Caporaso et al (39), and sequenced on the Illumina MiSeq platform at the University of Tennessee. Due to the poor quality of the reverse reads, only the forward reads were analyzed. Sequences were trimmed at a

length of 250 bp and analyzed using the QIIME v1.7.0 software package (39). Raw reads were assembled using join_paired_ends.py and then demultiplexed using split_libraries_fastq.py to remove low quality scores. Chimeric sequences were identified and a total of 11 million sequences were retrieved after quality filtering and chimera checking. Open reference operational taxonomic units (OTUs) were picked via the UPARSE pipeline (121) and taxonomy assignment was performed using rdp classifier trained against the Greengenes 16S rRNA gene database (May 2013 release, 97%). OTUs with less than 0.005% relative abundance were removed. Alpha diversity (within sample diversity) was calculated for each sample using a variety of alpha diversity metrics in QIIME. Beta diversity (pairwise sample dissimilarity) was also calculated in QIIME using metrics including unweighted and weighted Unifrac and Bray-Curtis.

Phospholipid fatty acid analysis

Approximately 10 g of lyophilized sediment from each water column was extracted for total lipid using a modified Bligh and Dyer method (122). Total lipid was fractionated on a glacial silicic acid column into non-polar, neutral, and polar lipids by chloroform, acetone and methanol elution, respectively. Intact polar lipid was then methylated to recover fatty acid methyl esters (FAMES). FAMES were identified and quantified using the Agilent 6890 gas chromatograph coupled to an Agilent 5973 mass spectrometer. Microbial biomass in sediment was measured by converting picomols of lipids per gram to viable cells per gram by multiplying bacterial size based conversion factor of 5.9×10^4 cells/pmol (123).

Real time PCR

Universal bacterial 16S rRNA genes were quantified using primer pairs of 341f (5'-CCTACGGGWWGGCWGCA-3') (124) and 519r (5'-TTACCGCGGCKGCTG-3') (125). The standard was obtained from purified PCR product of cloned *Alcanivorax* and the dilution series ranged from 20 to 2×10^{-3} pmol/μL. The standard curve was $y = -3.02x + 18.67$ with an estimated amplification efficiency of 114%. Real time PCR was performed with the thermal cycler Primus 96 (MWG-Biotech, Ebersburg, Germany) with YBR green PCR master mix (Applied Biosystems). The thermal cycle program was 95°C for 15 min, then 35 cycles of 95°C

for 15 s, 58°C for 30 s, and 72°C for 30 s (126). The quantitation cycle (Cq) mean was detected based on sample replicates and the Cq mean was substituted into standard curve for logarithms of copy number.

Statistical analyses

Water, pH, ORP, Temp, nutrients and biomass results were analyzed using ANOVA and Kruskal-Wallis tests in SPSS. The 16S rRNA gene sequencing data were further analyzed with various methods in Prime 6 and PERMANOVA+ (Primer Inc. UK) (127), including (1) tailed ANOVA test for microbial diversity alpha index, (2) hierarchical clustering for microbial community structure and composition, (3) non-metric multidimensional scaling (NMDS) based on weighted unfrac distance matrix, and (4) permutational multivariate analysis of variance using distance matrices to test whether the four treatments were different from each other. All tests were considered significant at $\alpha=0.05$. Graphs were generated in R using “vegan” and “ggplot” packages (128). The PLFA profile was analyzed using heatmap with clustering information to estimate the sedimentary microbial community succession after algal sedimentation with zeolite capping. Principal component analysis (PCA) was performed to investigate the latent factors structuring the community.

RESULTS

Geochemistry characteristics

Temperature (Temp.), DO, pH, and nutrients in the water column samples were measured in the field. The average geochemical characteristics and pairwise comparisons are shown in Table 4.1. In the four treatments, the average DO concentration in water column was 1.5 mg/L. The column was hypoxic because the freshly set anaerobic sediment consumed O_2 at the bottom. Geochemical profiles of the four treatments showed that TN and TP concentrations decreased dramatically in F, Z, and OZ. PO_4^{3-} and NH_4^+ concentrations remained constant in Z and OZ throughout the rest of the time points but increased gradually in C and F. Conversely, nitrate concentrations increased gradually in Z and OZ after treatment added and decreased in C and Z. Nitrite concentrations were not significantly different among four treatments.

Table 4.1 Geochemical parameters of water samples and ANOVA P value

ID	Temp. (°C)	DO (mg/L)	pH	TN (mg/L)	NH₄⁺ (mg/L)	TP (mg/L)	PO₄³⁻ (mg/L)
WC (n=16)	22.20	1.28	7.38	3.72 ^a	1.85 ^a	1.44 ^a	0.53 ^a
WF (n=14)	22	1.15	7.25	3.02 ^b	2.03 ^a	1.00 ^b	0.60 ^a
WZ (n=15)	21.9	1.49	7.27	2.50 ^{b,c}	1.36 ^{a,b}	0.56 ^{b,c}	0.38 ^{a,b}
WOZ (n=13)	22.1	1.22	7.25	2.02 ^c	0.81 ^b	0.78 ^c	0.09 ^b
ANOVA (P-value)	0.983	0.828	0.269	0.000*	0.000*	0.000*	0.006

* Indicates P value of ANOVA test ($\alpha=0.05$) is extremely significant but not equal to zero.

a, b, and c indicate levels not connected by the same letter are significantly different.

Biomass and diversity

Due to the small sample size for each treatment, we conducted non-parametric Kruskal-Wallis assay to test the differences of biomass among treatments. The result indicated there was no significant difference in the means of universal bacteria gene copy number (Table 4.2) among C, F, Z, and OZ (Kruskal Wallis, $P=0.359$). The average PLFA concentration in the sediments of the four treatments did not differ significantly (ANOVA $P=0.9078$). The cell density ranged from 9.75×10^7 cells/g to 1.28×10^8 cells/g (Figure 4.1).

The alpha diversity estimators are shown in Figure 4.2. The Chao 1 index is much higher in sediment samples, ranging from 350-1600. Based on Chao1 alpha diversity metrics, sediment samples possessed twice as much species richness as water samples. The evenness index, Shannon, indicates how close in numbers each species in an environment is. This index was similar for water and sediment samples, both ranging from 5 to 9. The median of the Chao1 index was 320 in water. Although a wider spread of estimated OTUs, richness was not significantly different among the four treatments (ANOVA, $P=0.39$). Species evenness measurements in water samples indicated a non-significant variation among C, F, Z, and OZ (ANOVA, $P=0.186$). When comparing the Chao1 index within sediment samples, OZ treatment had significant lower Chao1 than control samples (Tukey's HSD, $P=0.02$).

Microbial community structure

There were no major taxa changes in community structure at the phylum level of the upper sediment (1-3 cm) samples among treatments (Figure 4.3). However, the percentage of *Chloroflexi* and *Acidobacteria* were found in fewer samples with zeolite/O₂ loaded zeolite-capping treatments than in the control and flocculation only treatment. *Firmicutes* was enriched after flocculation with zeolite/ O₂ loaded zeolite capping, which was 10 times as high as the percentage in control and flocculation only treatments, as well *Proteobacteria* was increased by 5%. Obvious shifts in community structure at phylum level were observed in flocculation-treated water samples (Figure 4.4). *Proteobacteria* remained at 40% over 10 days in the control, but increased after the first two days of treatments added and then decreased after day 7. *Actinobacteria* varied with different treatments and kept increasing to highest percentage in Z.

Table 4.2. Universal bacteria gene copy number of water samples

Sample ID	Mean	±	SD
Column initial	286,765	±	279,678
C1	710,694	±	88,434
C2	74,062	±	46,494
C4	83,616	±	43,084
C7	32,759	±	6,934
C10	13,948	±	4,442
F1	79,045	±	41,544
F2	346	±	3
F4	38,735	±	6,249
F7	16,298	±	9,121
F10	1,864	±	826
Z1	89,070	±	47,218
Z2	20,845	±	26,967
Z4	177,773	±	122,919
Z7	2,158	±	1,539
Z10	42	±	13
OZ1	40,885	±	35,890
OZ2	27,278,758	±	38,420,506
OZ4	81,525	±	16,589
OZ7	368	±	44
OZ10	109,790	±	56,570

* Numbers represent the mean value and one standard deviation (SD)

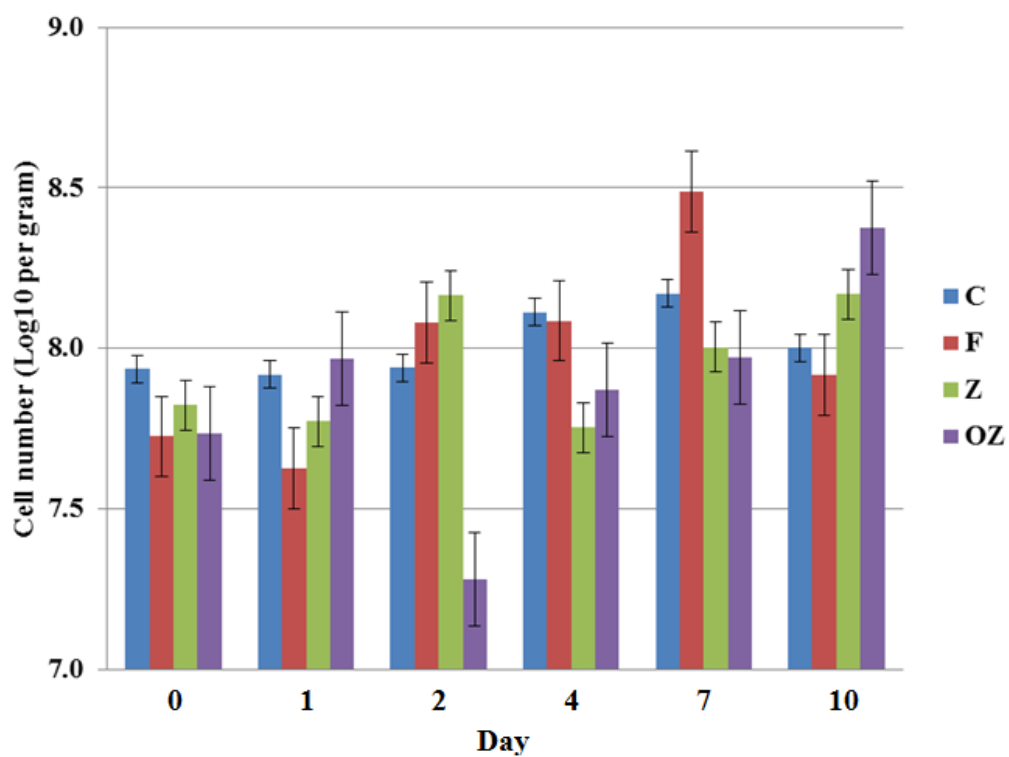


Figure 4.1 Estimated biomass of microcosm sediment samples. C represents control; F represents clay flocculation only; Z represents clay flocculation and zeolite capping; OZ represents clay flocculation and O₂ loaded zeolite capping.

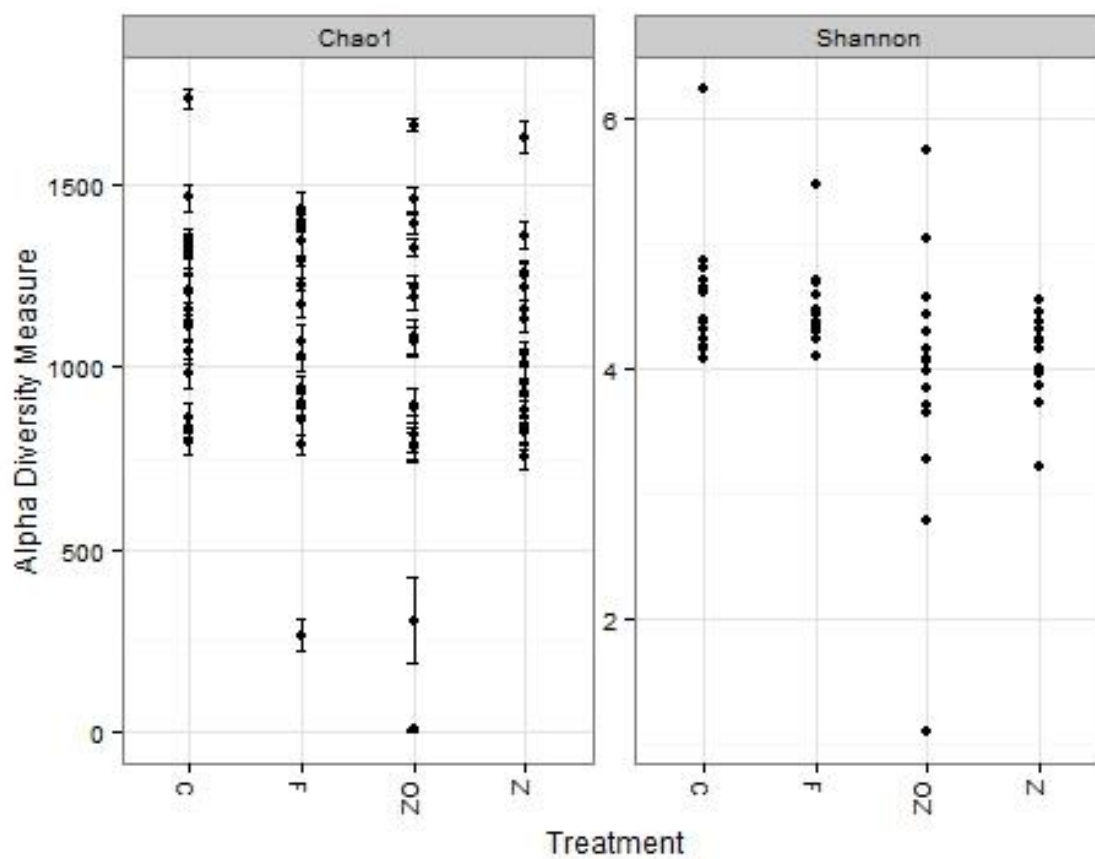


Figure 4.2 Alpha diversity of water samples. C represents control; F represents clay flocculation only; Z represents clay flocculation and zeolite capping; OZ represents clay flocculation and O₂ loaded zeolite capping.

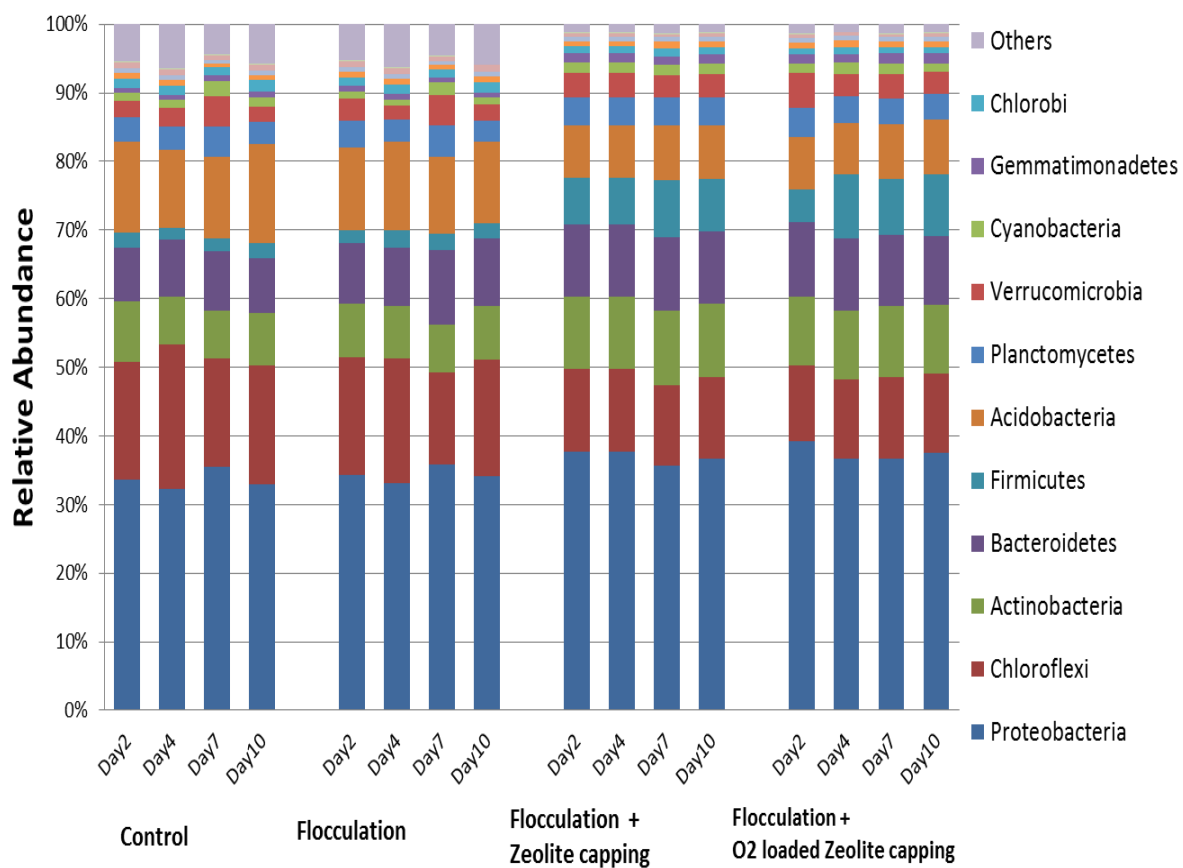


Figure 4.3 Relative abundance of dominant phyla observed in sediment samples. The legend shows the most abundant phyla in these samples.

A gradually decreasing trend was observed in the relative abundance of *Planctomycetes*. *Verrucomicrobia* was stable over 10 days.

Beta diversity was analyzed based on weighted unifracs distance matrix by non-metric multidimensional scaling (NMDS). Samples from C and F were clustered together, while samples from Z and OZ were clustered closely (Figure 4.4). The PERMANOVA analysis indicated distinct water microbial community structure between C and Z (Unique perms= 998, $P=0.001$), as well as C and OZ (Unique perms= 997, $P=0.005$). In addition, PERMANOVA test also indicated both treatment (Unique perms= 997, $P=0.001$) and time point (Unique perms= 998, $P=0.001$) were factors contributing to variability in microbial community structure.

Phospholipid Fatty Acid Analysis (PLFA)

To investigate different microbial community structure throughout time, the PLFAs were classified into 7 specific types of fatty acids based on lipid structural differences, which were normal saturated fatty acid (SATFA), terminal branched saturated fatty acid, mid chain branched saturated fatty acid, monounsaturated fatty acid, cyclopropyl fatty acid, epoxide fatty acid, and polyunsaturated fatty acid. The amount of each type of fatty acid was represented in mol percentage with the total bar adding up to 100 percent in Figure 4.5. Each of the sample structures appeared very similar in the early time points (day 1- 4). However, there was an intense change in lipid composition in the later time points (day 7- 10) of each of the samples. The biggest difference between the structure of the early time points and the later ones was the increase in monounsaturated lipids and the decrease in epoxide lipids. Essentially, they were the same type of lipid, the only difference between the two being a small structural change. Therefore, their inverse relationship in the sample structure was expected. The control and the flocculation only treatments had less than 10% monounsaturated lipids by day 10, and flocculation only treatments had 45% monounsaturated lipid composition. The zeolite and zeolite with added oxygen treatments had almost 40% monounsaturated lipid composition by day 10. The total change in epoxide levels in each treatment type by time point shows that each of the samples started out between 20-30% in epoxide composition. The control samples had 20% epoxide levels at day 10, and the flocculation only samples end with low percentage of epoxide

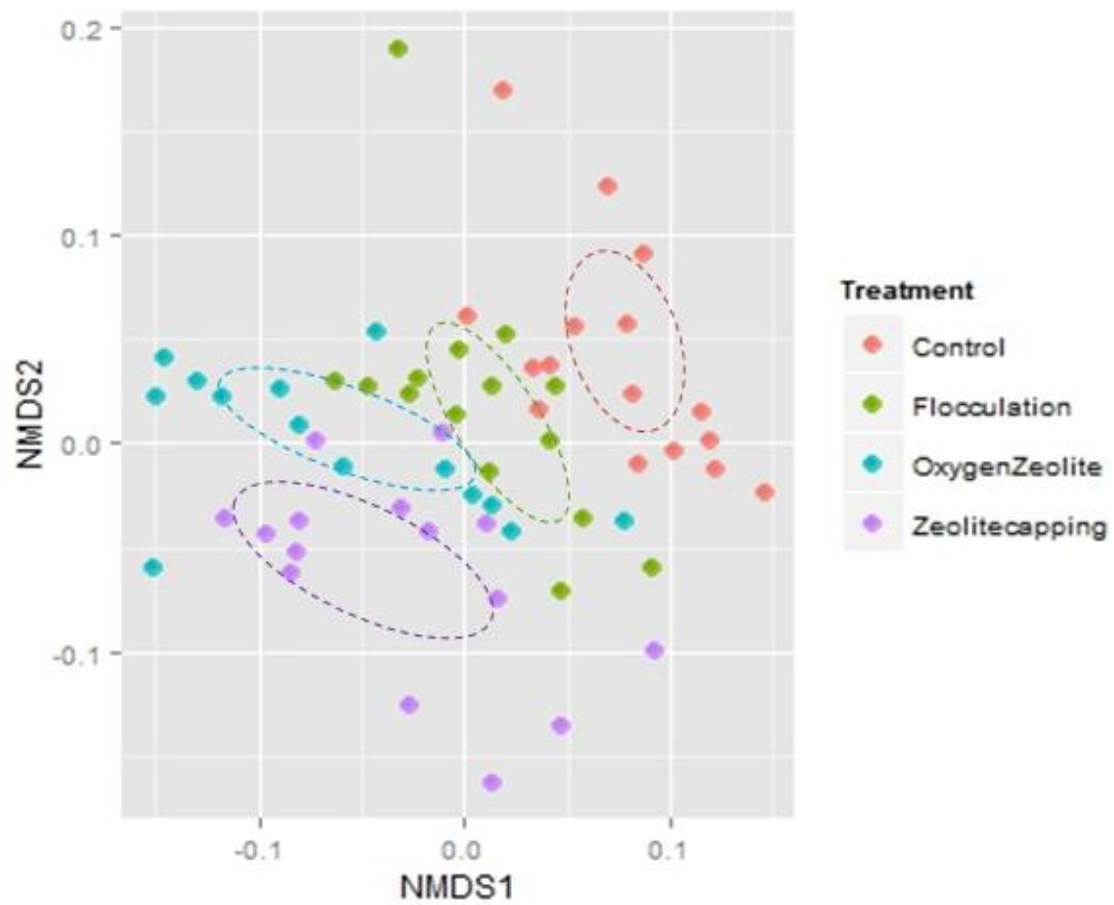


Figure 4.4 Non-metric Multidimensional scaling (NMDS) plot of weighted unifrac distance. Circle indicates the 95% confidence interval.

fatty acid level. The zeolite and zeolite with added oxygen treatment samples had approximately 5% epoxide levels at day 10.

DISCUSSIONS AND CONCLUSIONS

Geochemical characteristics of treatments

The geochemical data revealed that water columns treated by clay flocculation plus capping or O₂ loaded capping had significantly lower TN, TP, and NH₄⁺ concentration than samples from control. The pH decreased to a neutral range in later time points because the removal of algal cells from the water column. Although DO was not significantly different among the four treatments in this study, it is critical for shaping water microbial communities in natural systems (129). In addition, the reduction of TN in the Z and OZ was contributed by the NH₄⁺ decrease. NH₄⁺ can be absorbed and precipitated with clay particles. NH₄⁺ may also be trapped by zeolite due to its ability to reversibly bind alkaline-earth cations inside the framework structure of zeolite and can easily be exchanged by surrounding positive ions (114). NH₄⁺ can also be removed via biological pathways coupling nitrification and denitrification (130-132). The gradually increased nitrate concentration in Z and OZ treatments implied a possibility of nitrification processes on the zeolite biofilm. Compared with nitrogen, not all phosphorus in freshwater is bioavailable (133, 134). Internal phosphorus reduction resulted from inactivation agent binding the P in water column and sealing it in the sediments (63, 135, 136). The rebound of TN and TP in control and clay flocculation only treatments demonstrated that zeolite capping layer prevented TN and TP from be released back into the water column.

Microbial biomass and diversity were not affected.

The results in this study were consistent with reported research on sediment biomass (137). Bacteria biomass remained at similar levels as controls before and after clay flocculation and capping with zeolite. Alpha diversity was determined using Chao1 and Shannon metrics. The Chao1 estimator was calculated to predict the total number of OTUs (richness) present in each water and sediment sample (138). The Shannon index was measured to evaluate how equally abundant those dominant species are (139). The evenness of the water microbial community was slightly decreased after clay flocculation, indicating potential interaction between some bacteria

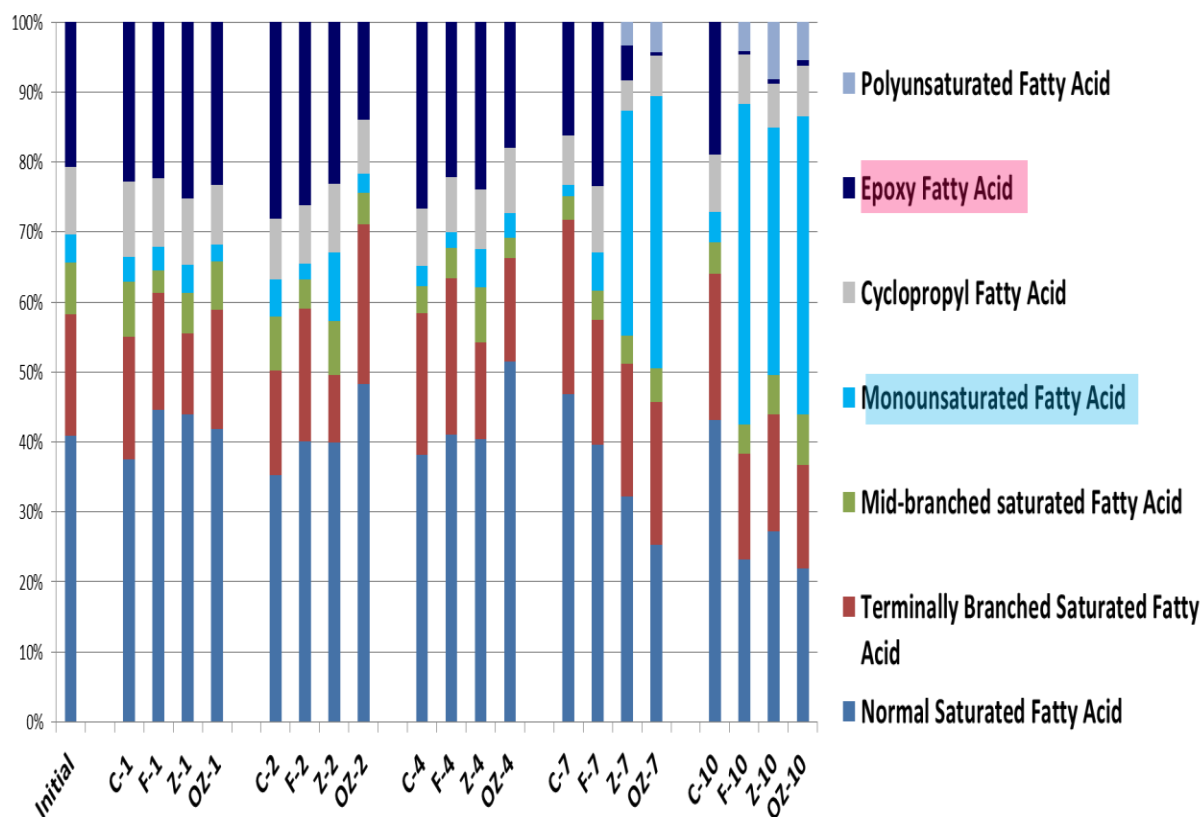


Figure 4.5 Distribution of 7 specific classes of fatty acids through out time in sediment. C represents control; F represents clay flocculation only; Z represents clay flocculation and zeolite capping; OZ represents clay flocculation and O₂ loaded zeolite capping.

cells and flocculent particles. Clay was modified by chitosan, which tends to attract negatively charged algal cells as well as specific bacterial cells (140). Overall, there was no significant difference in evenness among the four treatments, suggesting that evenness was not significantly impacted despite a small decline after clay flocculation. Supportive research was reported in the study of composition of freshwater bacterial communities associated with cyanobacterial blooms in four Swedish lakes (82). They found that richness of the bacterial community was not significantly affected either positively or negatively by the conditions created by the cyanobacterial bloom event (82). Thus, we suggested that microbial population and species richness were not susceptible to clay flocculation techniques because some species may take the place of, or outcompete other species.

Zeolite capping suggested ammonia oxidation in sediment samples

Some of the important subgroup members in *Proteobacteria* play essential roles in nitrogen cycling. For example, *Nitrosomonadales* (Figure 4.8 A) related to the ammonia oxidizing bacteria were enriched significantly in Z and OZ (ANOVA, $P < 0.0001$). The increased *Methylophilales* in Z and OZ were involved in denitrification in anoxic situations (141) (Figure 4.8 C). In addition, *Nitrososphaerales*, which were reported as cosmopolitan ammonia oxidizing *Archaea* in soil, were enriched in Z and OZ (142), even though they only comprised a small percentage in the community (Figure 4.8 A). *Nitrospirales* was also increased in Z and OZ (Figure 4.8 B); they related to chemolithoautotrophic nitrite-oxidizing bacteria involved with the second step of nitrification (143, 144). These findings indicate that the zeolite capping layer-absorbed ammonia from overlay water column and promoted sequential oxidation of ammonia via nitrite to nitrate at the water and sediment interface. The abundance of another *Archaea* member, *Euryarchaeota*, were significantly decreased in Z and OZ (ANOVA, $P = 0.03$), including *Methanosarcinales*, *Methanobacteriales*, and *Methanomicrobiales* (Figure 4.8 D). These are methanogens in an anoxic environment, and respectively produce methane generally by degradation of organic matters, using hydrogen to reduce carbon dioxide, and using only carbon dioxide (145-147). The possible explanation of methanogens reduction is that zeolite used as capping layer constantly contact with air and can elevate ORP level at the interface. The activities of methanogens were perhaps inhibited in the non-strictly anaerobic condition.

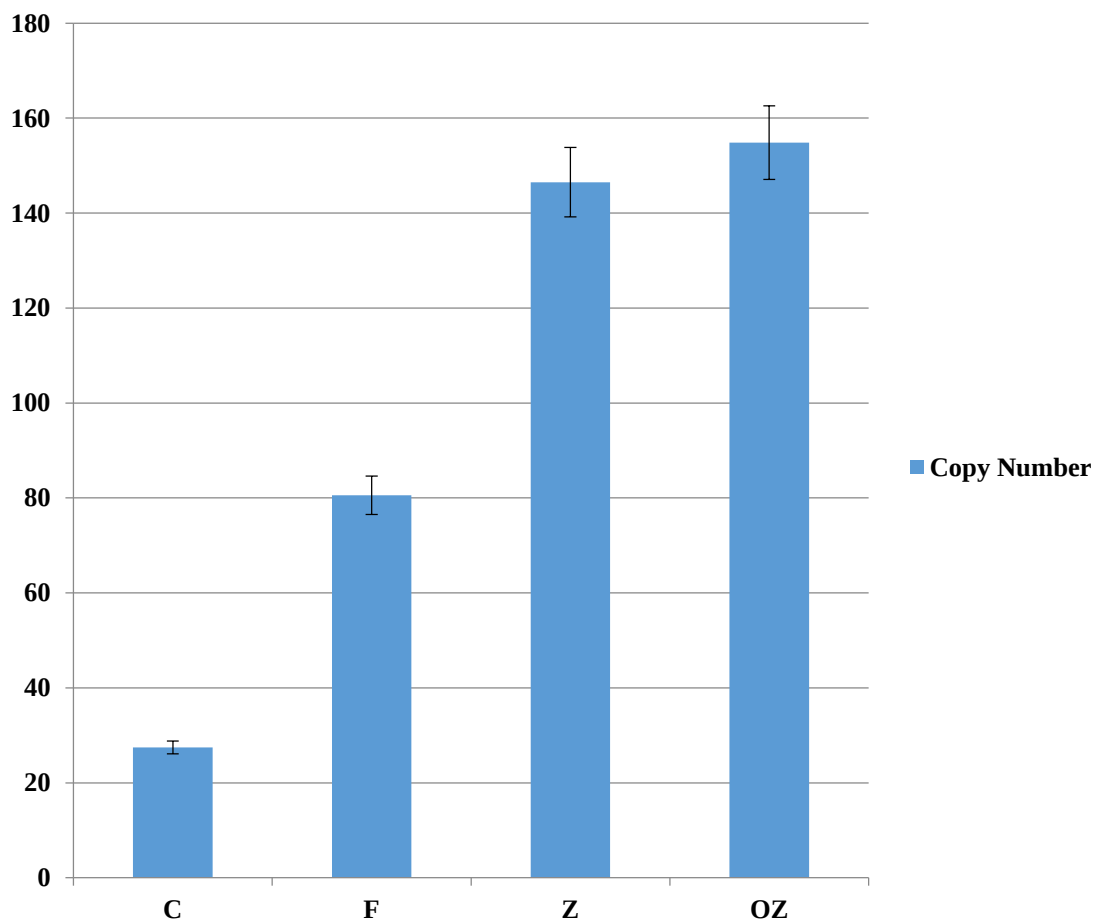


Figure 4.6 Ammonia oxidizing archaea gene copy number per nano-gram. C represents control; F represents clay flocculation only; Z represents clay flocculation and zeolite capping; OZ represents clay flocculation and O₂ loaded zeolite capping.

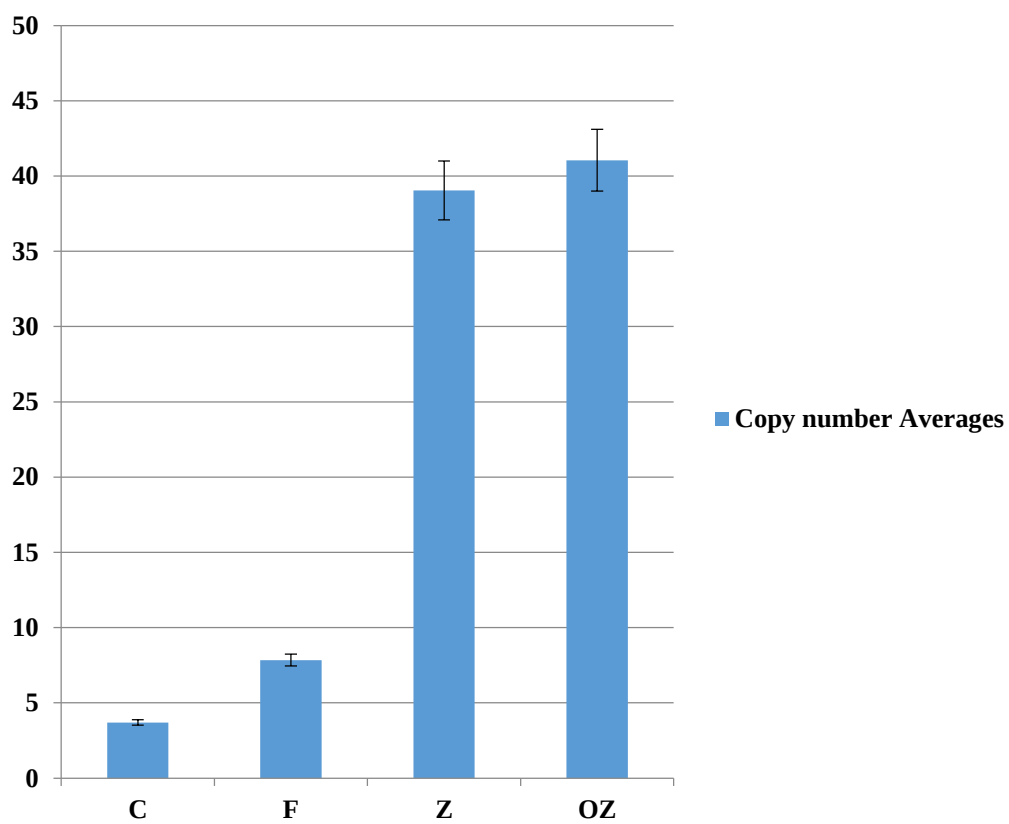


Figure 4.7 Ammonia oxidizing bacteria gene copy number per nano-gram. C represents control; F represents clay flocculation only; Z represents clay flocculation and zeolite capping; OZ represents clay flocculation and O₂ loaded zeolite capping.

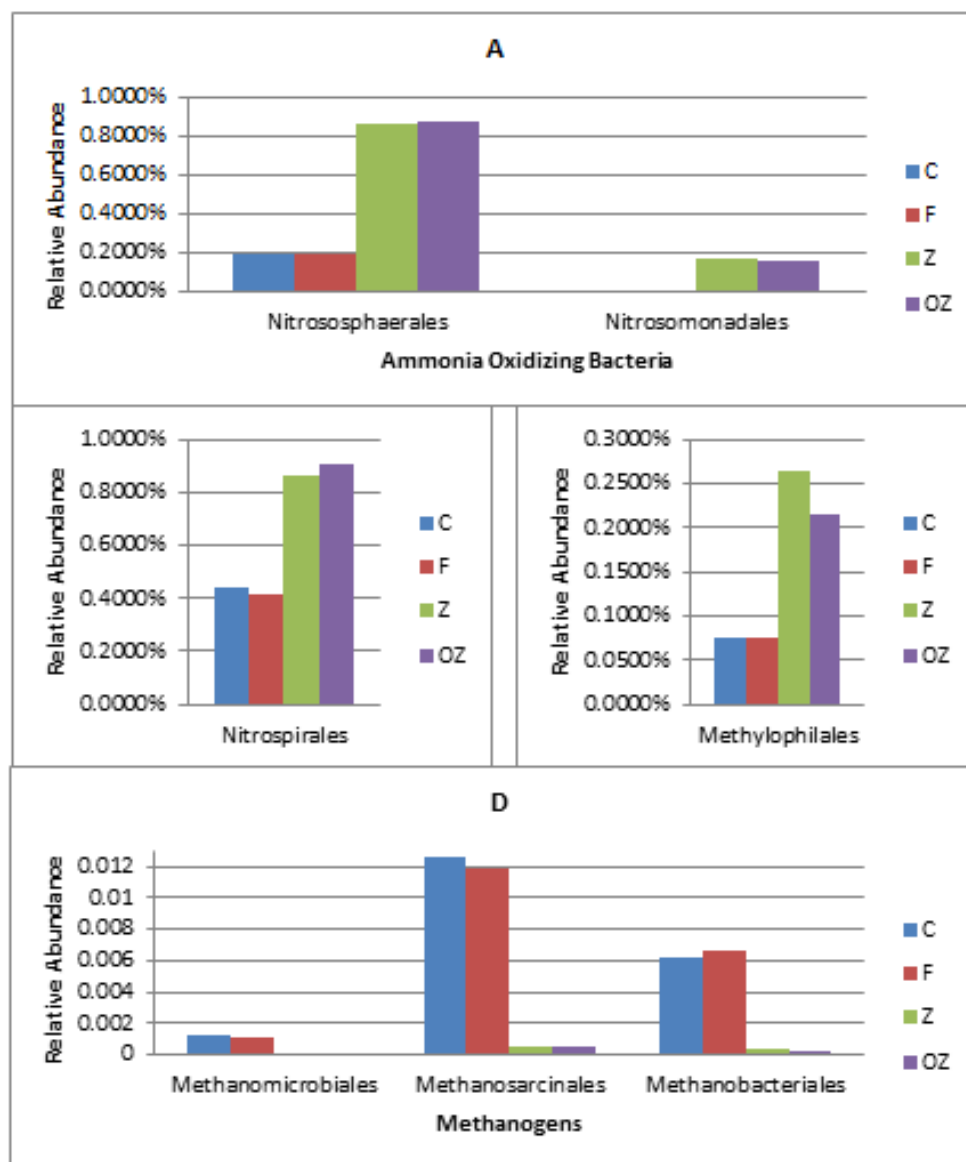


Figure 4.8 Changes in relative abundance of taxa involved with ammonia oxidization (A), nitrite oxidization (B), denitrification (C) and methane producing (D). C represent control; F represents clay flocculation only; Z represents clay flocculation and zeolite capping; OZ represents clay flocculation and O₂ loaded zeolite capping.

Distinct community structure in water samples

Deltaproteobacteria, related with sulfate-reducing bacteria, were significantly enriched after flocculation (Tukey's HSD, $P=0.006$). Elevated levels of *Verrucomicrobiae* were also a defining factor of flocculation-treated water. The abundance of this cluster was negatively correlated with pH, and positively correlated with hydraulic retention time and temperature (148). Members of the *Verrucomicrobia* have also been observed in both surface and hypolimnetic waters, suggesting a variety of metabolic strategies within the treatment (109). Rather, *Cytophagia* affiliated with *Bacteroidetes* was found significantly reduced in water samples after clay flocculation (Tukey's HSD, $P=0.03$), and it has been reported that representatives of the classes *Cytophagia* and *Flavobacteria* played a central role in the degradation of biopolymers in marine and freshwater environment (149) (Figure 4.9). In this study, limited nutrients, such as lower level of ammonia and absence of organic compounds, may inhibit growth of these bacteria after flocculation.

Environmental parameters were fitted using NMDS (Table 4.3). The microbial community in C and F was strongly affected by total nitrogen, ammonia, and phosphate concentration, and Z and OZ were more shaped by both nitrate concentrations and pH. Nutrients may shape bacterial community structure directly and/or indirectly. Previous study reported TP concentrated influenced the number of phosphate-decomposing and phosphate-dissolving bacteria (150). Additionally, nitrogen and phosphorus concentration usually regulate the growth of algae. Algal exudates are highly bioavailable to bacteria compare with other source of dissolved organic matter (151). The species composition, physiological state, and biomass of phytoplankton seem to regulate the photosynthetic extracellular release, which, in turn, may influence the composition of bacteria communities (152). In conclusion, microbial community structures in control and clay flocculation only treatments were different from flocculation plus capping treatments. Nutrient concentrations are the main factors structuring community structures and zeolite capping prevented nutrients from being resuspended into the water column.

To identify which taxa were differentially abundant in each sample, heatmap combined with hierarchical clustering were plotted (Figure 4.10). Relative abundance at the genus level with

triplicates in each sample were averaged and then the most dominant 25 genera were depicted on the heatmap. The dendrogram on the top of heatmap showed the clusters of water samples, specifically showing samples in Z and OZ were separated from samples in C and F.

Polynucleobacter, *unclassified Comamonadaceae*, *unclassified Actinomycineae* were present at a higher proportion in Z and OZ; while *Planctomyces*, *unclassified Pirellulaceae*, *unclassified Rhizobiale*, *Devosia* and *Hydrogenophage* were not detected in Z and OZ.

Flocculation with zeolite/O₂ loaded capping resulted in a healthier water body

In-depth 16S rRNA gene sequencing information was explored to visualize the occurrence of water bacteria. They were associated with environmental conditions created by clay flocculation techniques. The top 23 abundant genera and environmental parameters were subject to Spearman correlation analysis. Spearman correlation (rho value) shown in Table 4.4 indicated that 18 genera were moderately or strongly correlated with physical and geochemical factors. Green color filled rho values represent significant correlations. Some of the important taxa were correlated with treatment type as well as nutrient condition. *Polyuncleobacter*, *Unc.Comamonadaceae*, *Unc.Actinomycineae*, *Unc.Alcaligenaceae*, and *Novosphingobium* were increased in flocculation with capping layer added samples, in which eutrophic condition was alleviated. *Polyuncleobacter* is a lineage of *Betaproteobacteria* and it is able to grow in the absence of oxygen (153). The positive correlation of minor freshwater taxa (such as *Planctomycetes*, *Acidobacteria*, *Chloroflexi*) (154) with nutrient concentrations implied that members of minor freshwater taxa are warning signs of unhealthy water environment. For example, the decreased family of *Xanthomonadaceae* in water sample (Tukey's HSD, $P=0.02$) including the two plant-pathogenic genera *Xanthomonas* and *Xylella* are increasingly recognized as an important cause of hospital-acquired infections (155). *Planctomyces* and *Unc.Pirellulaceae* are members of *Planctomycetes*, related to microbes capable of anaerobic ammonia oxidation, are commonly found in eutrophic and polluted environments (156). Supporting results were reported that the number of *Planctomycetes* increased under high pH values, ranging from 6.8 to 9.4, high conductivity, and during the summer, usually associated with algal blooms (157).

Table 4.3 The fit of environmental variables to the non-metric multidimensional scaling

	NMDS1	NMDS2	P-value
TN	0.97739	0.21147	0.001*
NO₃⁻	-0.98162	0.19082	0.001*
NH₄⁺	0.95805	-0.28659	0.001*
TP	0.52835	0.84903	0.001*
PO₄³⁻	0.82912	-0.55907	0.001*
NO₂⁻	0.91178	-0.41069	0.497
pH	-0.11139	0.99378	0.053
DO	0.49337	0.3681	0.001*

* indicates P value at $\alpha=0.05$ is significant. Number of permutations: 999

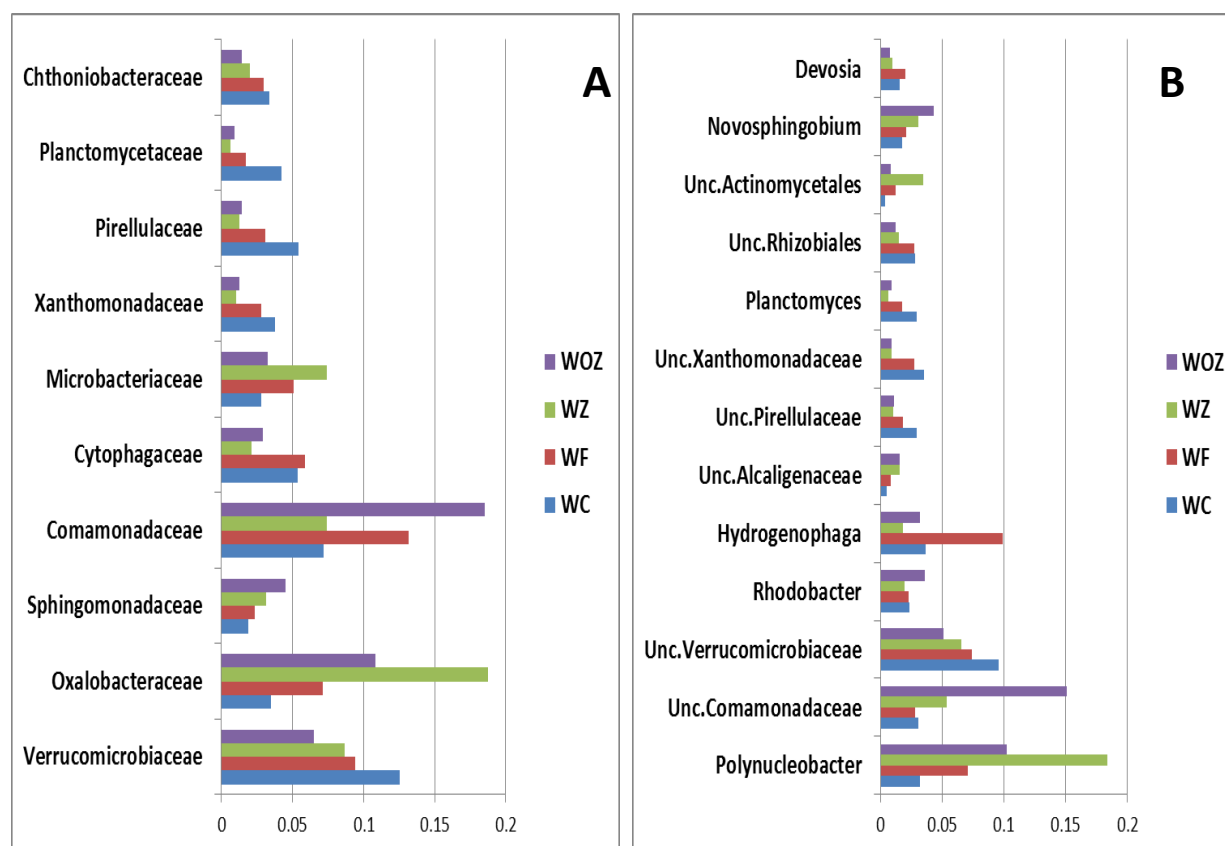


Figure 4.9 Taxa with visible abundance variations at family level (A), and genus level (B) in water samples. WC represents control; WF represents clay flocculation only; WZ represents clay flocculation and zeolite capping; WOZ represents clay flocculation and O₂ loaded zeolite capping.

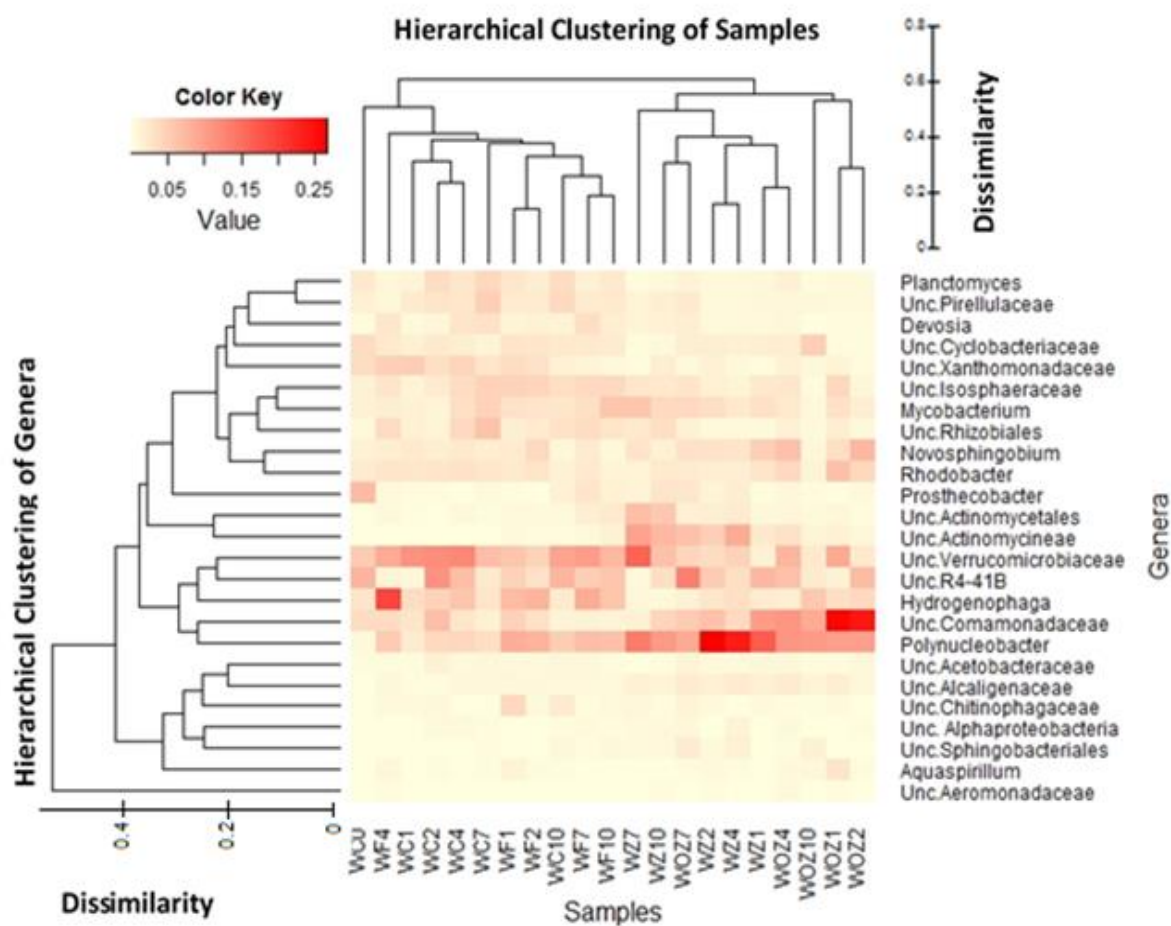


Figure 4.10 Heatmap combined hierarch clustering of sample types and top 25 genera in water samples. WC represents control; WF represents clay flocculation only; WZ represents clay flocculation and zeolite capping; WOZ represents clay flocculation and O₂ loaded zeolite capping. Numbers (0, 1, 2, 4,7 and 10) indicate sampling date.

Table 4.4 Spearman rank correlation of top 23 abundant genera and environmental parameters

	DO	pH	TN	NO ₃ ⁻	NH ₄ ⁺	TP	PO ₄ ³⁻
<i>Polynucleobacter</i>	0.12	-0.20	-.729**	.480*	-.551**	-.683**	-.457*
<i>Unc.Comamonadaceae</i>	-0.34	0.28	-.718**	.634**	-.761**	-0.38	-.690**
<i>Unc.Verrucomicrobiaceae</i>	-0.12	-0.13	.527*	-0.30	.519*	.513*	0.43
<i>Unc.Actinomycineae</i>	0.39	-0.38	-.476*	0.35	-0.37	-.666**	-0.33
<i>Mycobacterium</i>	.440*	-.514*	0.05	-0.41	0.36	-0.10	0.29
<i>Unc.Isosphaeraceae</i>	-0.05	-0.13	0.14	-.613**	.495*	0.20	0.38
<i>Hydrogenophaga</i>	-.437*	0.08	0.08	-0.30	0.22	0.29	0.31
<i>Aquaspirillum</i>	-0.35	0.04	-.570**	0.24	-.469*	-0.13	-.443*
<i>Unc.Alcaligenaceae</i>	0.01	-0.26	-.695**	.507*	-.582**	-.685**	-.573**
<i>Unc.Pirellulaceae</i>	0.15	-0.13	.662**	-.480*	.641**	0.32	.616**
<i>Unc.Xanthomonadaceae</i>	-.480*	0.34	0.42	-0.33	0.41	0.37	0.37
<i>Planctomyces</i>	-0.14	0.16	.434*	-.489*	.453*	.440*	0.39
<i>Unc.Sphingobacteriales</i>	.754**	-.491*	0.05	0.10	-0.03	-0.41	0.06
<i>Unc. Alphaproteobacteria</i>	0.19	-.514*	0.06	0.07	0.03	-0.38	0.04
<i>Unc.Rhizobiales</i>	0.13	-0.42	0.41	-.715**	.755**	0.21	.669**
<i>Unc.Actinomycetales</i>	0.40	-.452*	-0.17	-0.07	0.08	-0.42	0.06
<i>Novosphingobium</i>	-.506*	0.08	-.759**	.481*	-.580**	-0.37	-.649**
<i>Devosia</i>	0.18	-.564**	0.36	-.635**	.680**	-0.02	.627**

*. Correlation is significant at the 0.05 level (2-tailed).

**. Correlation is significant at the 0.01 level (2-tailed).

PLFA composition change in sediment samples with zeolite capping

Lipid composition was analyzed using GC/MS resulting in FAME profile for each sample. The differences in fatty acid methyl esters (FAME) composition between samples are shown in the heatmap (Figure 4.11). Two prominent heatmap patterns were observed showing distinct FAME profile between early (day 0 -4) and late (day 7-10) time points. Major distinct is the early time points had the stress biomarker epoxide FMAEs and the late time points had traditional monounsaturated and polyunsaturated FAMEs (Figure 4.12B). The early time point samples were enriched in 10-Me-16:0, 7, 8-epoxide-16:0, cy17:0, 10-me-17:0, 9, 10-epoxide-18:0, and depleted in 15:0, 15:1, me-16:1, 18:2 ω 6, 18:3 ω 3, 18:1 ω 9c/t, 20:4 ω 6, and 20:5 ω 3. The epoxide FAMEs comprised relatively higher concentration, ranging from 12.77 to 19.22 pmol PLFA g⁻¹. However, there is an intense change in lipid composition in the later time points of each sample, enriched in 15:1, 16:1 ω 7, 16:1 ω 5, and 17:1 which are affiliated to monounsaturated fatty acid (158). Principal component analysis (Figure 4.12B) revealed that sampling time explained 54.4% of the variation in lipid composition. In addition, differences in early time points show heavy weight FAMEs, such as mid-chain branched saturated fatty acids (10 Me 18:0) in the top right quadrant of Figure 4.12 B), terminally branched saturated fatty acids (16:0) in the bottom right quadrant of Figure 4.12 B. Furthermore, late time points revealed top left quadrant polyunsaturated fatty acids (20:5 ω 3 and 18:1 ω 7), versus the bottom left quadrant which had terminally branched saturated fatty acid (a15:0).

Variations in microbial community structure indicated bioremediation processes

Lipid composition of the samples changed dramatically in the later time points of the treatment. Essentially, monounsaturated and epoxide lipids are various configurations of the same type of lipid. The presence of epoxide fatty acids in bacteria is abnormal, because monounsaturated fatty acids normally make up most of the bacterial membrane. The epoxide lipids are formed at the expense of monounsaturated lipids in order to respond to environmental stress. Bacteria change membrane lipid to maintain integrity. Previous studies showed the decrease of monounsaturated fatty acids and increase of epoxide fatty acids is strongly indicative of a bacterial stress response to chlorine exposure, which are commonly used to disinfect fishpond and other aquatic environment. Likewise, our results showed epoxide fatty acids at a highly

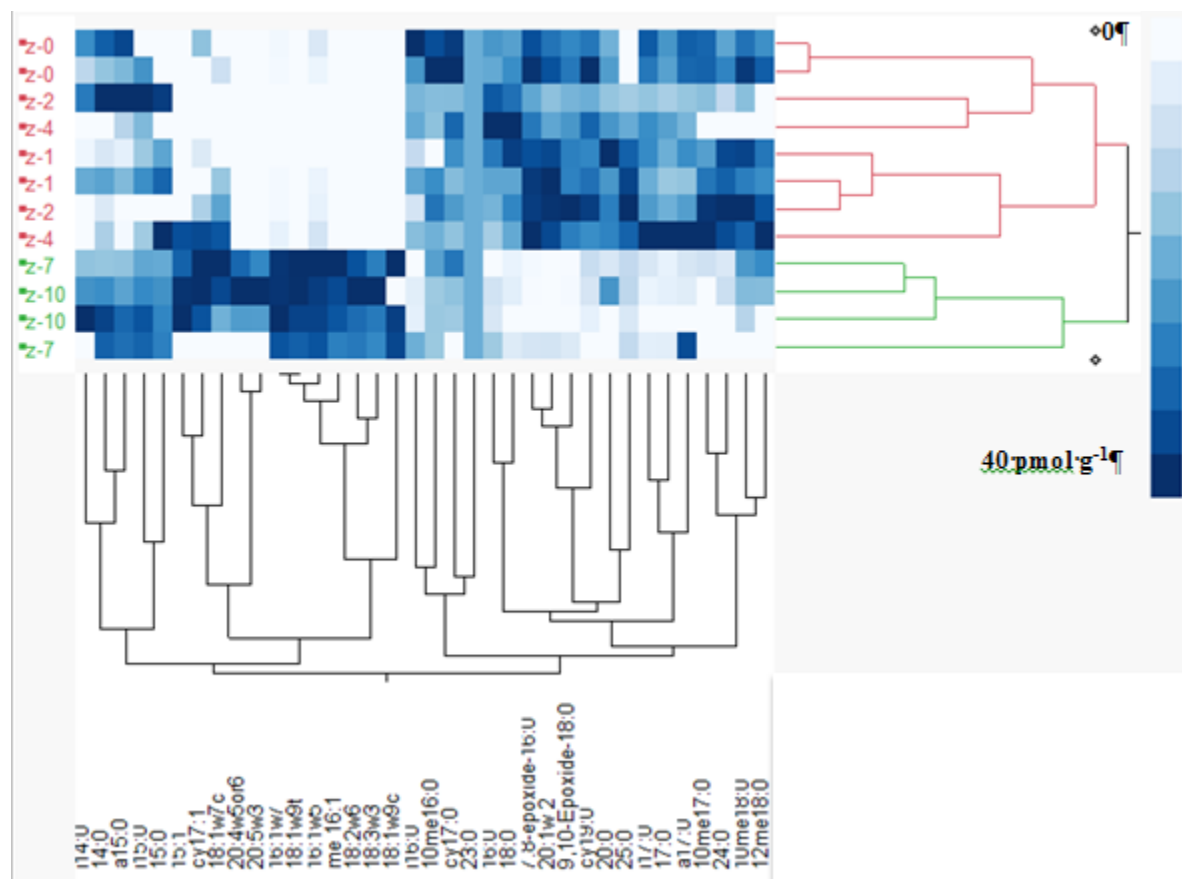


Figure 4.11 Heatmap of phospholipid fatty acid composition in each time point. Z represents samples with zeolite capping. Numbers (0, 1, 2, 4, 7 and 10) indicate sampling date.

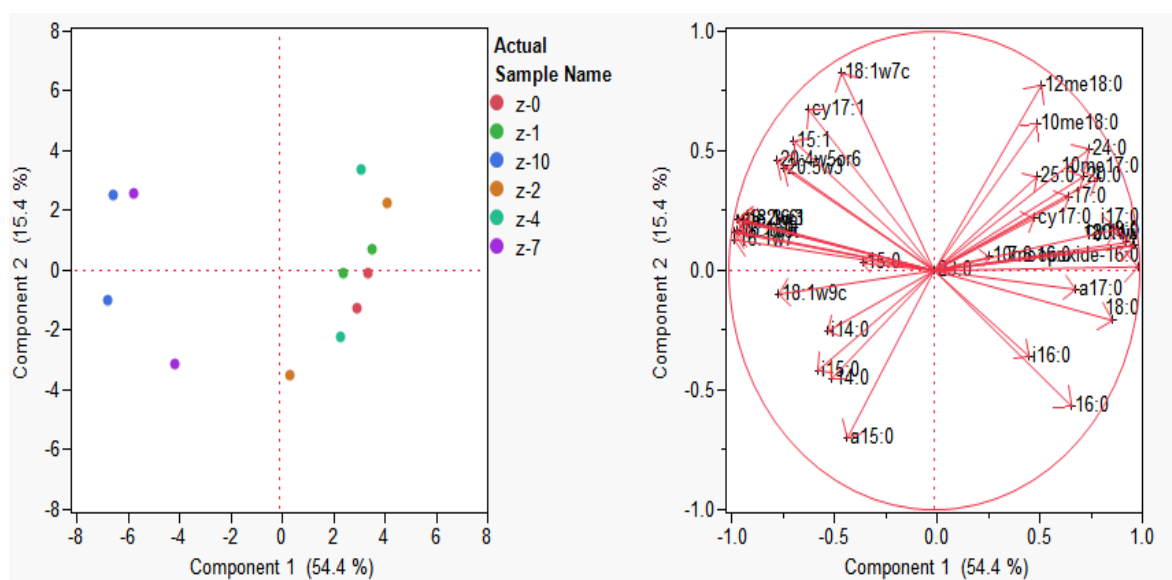


Figure 4.12 Principal component analysis of the PLFA profile of 12 sediment samples used 36 PLFA gradients as species. Z represents samples with zeolite capping. Numbers (0, 1, 2, 4, 7 and 10) indicate sampling date.

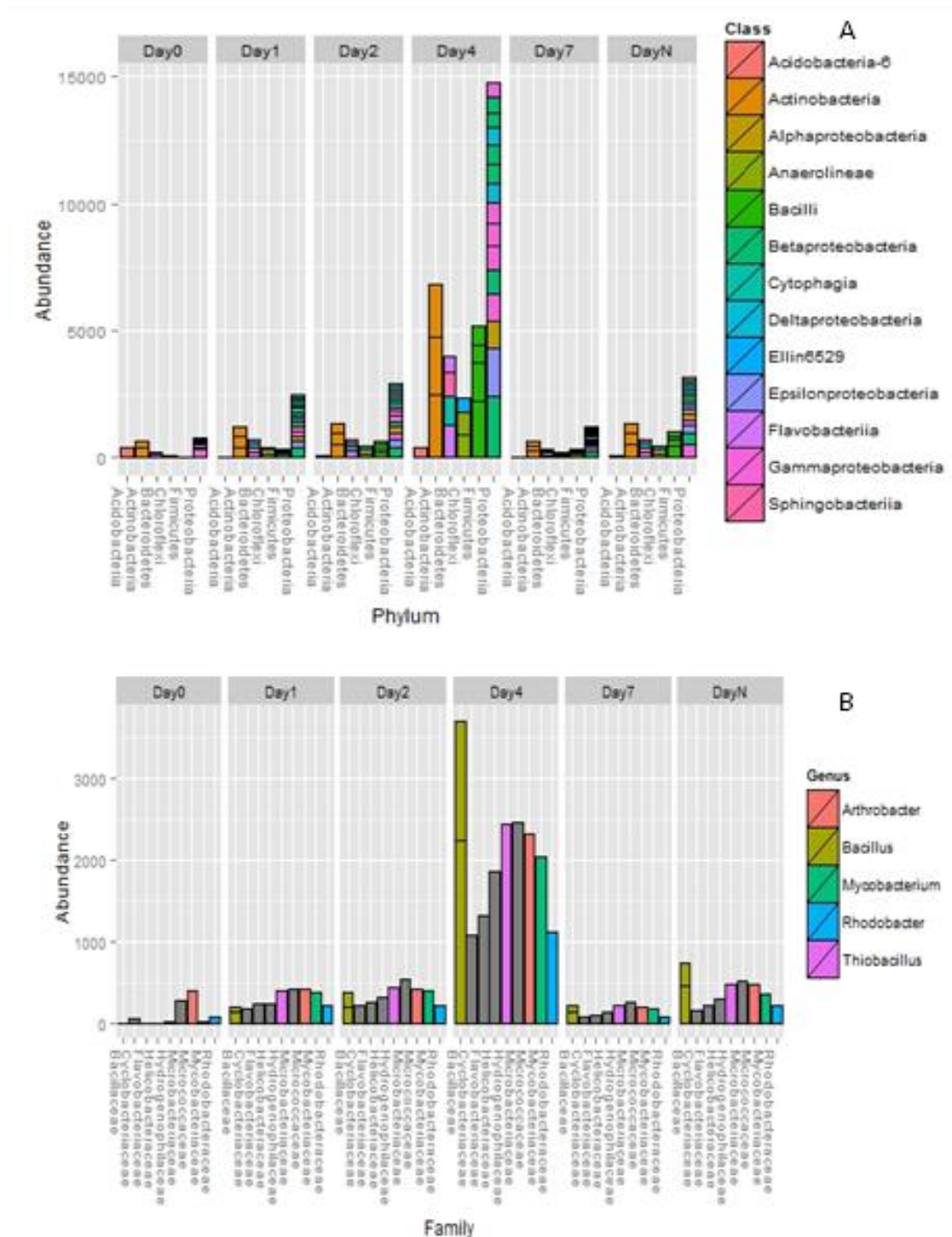


Figure 4.13 Most abundant Phyla (A) and Families (B) change of top 50 OTUs in zeolite capping sediment samples.

relative abundance in early time points compared to late time points during the treatment. This suggested that the initial samples may be polluted by chlorine contamination, and the bacterial community physically altered their membrane structure in order to respond to a chemical pollutant in the contaminated water.

The mid-chain branched fatty acids (10Me 16:0, 10Me17:0, and 10Me 18:0) decreased from 8% to 5% after day 4. The presence of these FAMES was indicative of metal contaminated (Cr and Pb) soil (159, 160), and they may be correlated with metal concentrations. The methyl branched PLFAs are indicative of *Actinobacteria*, and *Mycobacteria*. This finding was supported by 16S rRNA gene sequencing results in Figure 4.13 B. *Mycobacteriaceae* was present with a trend of increase in earlier time and decreasing after day 4. Many *Mycobacterium* species readily grow with ammonia or amino acids as nitrogen sources and glycerol as a carbon source in the presence of mineral salts. This might be a possible explanation of elevated *Mycobacterium* immediately after flocculation with zeolite capping. The ammonia absorbed by zeolite can be utilized by *Mycobacterium* and lead to the bloom of *Mycobacterium* in earlier days and correspondingly decrease in abundant when ammonia was depleted. *Alcaligenes eutrophus*, a member of *Bacillus* is often found in soils and sediments containing high contents of heavy metals in various geographical locations. Cyclopropyl fatty acids are mainly characteristic of Gram-negative bacteria (*Rhodospirillum*, *Cromatium*, and *Legionella*) and Gram-positive bacteria (*Clostridium* and *Bifidobacterium*). Members from *Desulfovibrio* were found contain high i15:0 or a15:0 or both, and they were related to sulfate-reducing bacteria (161, 162). Moreover, cyclopropyl fatty acids were significantly present (8%-10%) implying that bacteria in sedimentary community adapted to the algal bloom environment. Iso and anteiso fatty acids, mainly a15:0, i15:0, i16:0, and i17:0 comprised approximately 15% in sediment sample, are indicative of Gram positive bacteria and some Gram-negative anaerobic bacteria such as *Cytophaga*, *Acetobacter*, and *Flavobacterium*. The constant composition might reflect the whole experimental period was subject to anaerobic environment. The DO values were maintained in a range of 0.2 to 3.18 mg/L.

This study was the first to investigate the impact of HAB control techniques on microbial communities in microcosm scale experiments using both PLFA analysis and 16S rRNA gene sequencing. High throughput 16S rRNA gene sequencing provided insight into this understudied topic. Our results revealed that distinct water community structures were observed with and without zeolite capping treatments. The differences resulted from significant reduction of TN, TP and NH_4^+ concentration and increase of NO_3^- concentration in zeolite-capping and O_2 loaded zeolite-capping treatment. The enriched *Nitrososphaerales*, *Methylophilale*, *Nitrososphaerales*, and *Nitrospirales* in sediments indicated ammonia oxidization via nitrite to nitrate in flocculation with zeolite/ O_2 loaded zeolite capping. Bacterial biomass was not altered by clay flocculation. *Planctomyces*, *Unc.Pirellulaceae*, and *Xanthomonadaceae* known affiliated to pathogen adapted to eutrophic water bodies were reduced as reduction of nutrients concentration. Based on this study, algal sedimentation resulted in reallocating the abundance of various species in the sedimentary microbial community. Clustering and PCA analysis showed the PLFA profiles varied along time after algal sedimentation with zeolite capping. Coupling 16S rRNA gene sequencing with PLFA analysis confirmed the presence of ammonia oxidizers, metal reducers, and sulfate reducers. Results suggest the microbial community is able to withstand the chemical changes brought on by clay flocculation. Overall, flocculation seems to be a successful treatment method with low negative impact on microbial community. Future functional meta-omics studies should focus on describing the response of nitrification and denitrification genes to algal bloom control techniques. In addition, pilot-scale testing will help describe this issue in a more practical and meaningful manner.

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VITA

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