Ecotoxicological assessment of flocculant modified soil for lake restoration using an integrated biotic toxicity index

Zhibin Wang, Honggang Zhang, Gang Pan*

Research Center for Eco-Environmental Sciences, Chinese Academy of Sciences, Beijing, 100085, China

*Corresponding author: Tel.: +86 10 62849686; Fax: +86 10 62943436; E-mail address: gpan@rcees.ac.cn

Abstract

Flocculant modified soils/clays are being increasingly studied as geo-engineering materials for lake restoration and harmful algal bloom control. However, the potential impacts of adding these materials in aquatic ecological systems remain unclear. This study investigated the potential effects of chitosan, cationic starch, chitosan modified soils (MS-C) and cationic starch modified soils (MS-S) on the aquatic organisms by using a bioassay battery. The toxicity potential of these four flocculants was quantitatively assessed using an integrated biotic toxicity index (BTI). The test
system includes four aquatic species, namely *Chlorella vulgaris*, *Daphnia magna*, *Cyprinus carpio* and *Limnodrilus hoffmeisteri*, which represent four trophic levels in the freshwater ecosystem. Results showed that median effect concentrations (EC50) of the MS-C and MS-S were 31 to 124 times higher than chitosan and cationic starch, respectively. *D. magna* was the most sensitive species to the four flocculants. Histological examination of *C. carpio* showed that significant pathological changes were found in gills. Different from chitosan and cationic starch, MS-C and MS-S did not apparently alter the solution viscosity but significantly alleviated the acute toxicities of chitosan and cationic starch. The toxicity order of the four flocculants based on BTI were cationic starch>chitosan>MS-S>MS-C. The results suggested that BTI can be used as a quantitative and comparable indicator to assess biotic toxicity for aquatic geo-engineering materials. Chitosan or cationic starch modified soil/clay materials can be used at their optimal dosage without causing substantial adverse effects to the bioassay battery in aquatic ecosystem.

**Keywords**

Chitosan, Cationic starch, Modified soil, Ecotoxicity, Aquatic organisms

1. **Introduction**

Over the past several decades, harmful algae blooms (HABs) have frequently occurred worldwide, causing serious ecological and economic impacts to aquatic ecosystems and human health (Akyuz et al., 2014; Paerl and Huisman, 2008). Several
chemical (Burson et al., 2014; Fan et al., 2013), mechanical (Li et al., 2014) and biological techniques (Kim et al., 2007; Nan et al., 2008) have been developed to reduce these impacts. Recently, lake geo-engineering techniques are discussed in solving this problem. The term “geo-engineering”, defined as achieving a desired chemical or ecological response by adding materials such as a modified clay or metal compound to a lake (Mackay et al., 2014). The range of materials used is growing and includes engineered materials, commercially available salts, flocculants, clay/soils and industrial by-products (Spears et al., 2014).

Although these materials may be useful in controlling nutrient level, there is a need to evaluate the impacts of adding exogenous materials to the aquatic ecosystem. Reports indicate that some chemical materials exhibit toxicity to aquatic biota. The lanthanum-modified clay (Phoslock®) is promising in holding phosphorus in the sediment (Meis et al., 2013), but the population growth rates of daphnia are 6% and 20% lower than the control at 100 and 1000 μg La/L, respectively (Lürling and Tolman, 2010). Clearwater et al. (2014) demonstrate that fingernail clam survival is adversely affected by high dosage (344 g alum/m²) of alum application and some aluminium accumulation occurred in the crayfish and mussels (Clearwater et al., 2014). The aqueous Al can increase the risk of infection in the crayfish by impairing the ability of haemocytes to recognise and/or remove bacteria from the circulation (Ward et al., 2006). Recent studies indicate that toxic Al³⁺ could be released after alum application at low pH (<6.0), and sediment-capping with alum could inhibit microbial nitrification and denitrification under aerobic conditions (Gibbs and
Recently, natural flocculant materials, such as chitosan and cationic starch, were developed as environmental friendly materials to control harmful algal blooms because of their high flocculation efficiency (Anthony and Sims, 2013; Hansel et al., 2014; Letelier-Gordo et al., 2014; Xu et al., 2013). To improve the HABs removal using clays, chitosan is used to modify the local soils and applied to small natural waters to control both cyanobacteria blooms and sediment nutrient release, leading to recovery in submerged macrophytes (Li and Pan, 2015; Li and Pan, 2013; Pan et al., 2012). Anthony and Sims (2013) find that cationic starch can effectively flocculate algae cells and remove total phosphorus in wastewater with an upward trend of TP removal with increasing dosage. Cationic starches serve as substrates in anaerobic digestion or fermentation processes using the harvested biomass as feedstock and such biomass can be safely used as animal feed or fertilizer (Anthony and Sims, 2013). Cationic starch modified soil has been reported by Shi et al. (2015) as the effective algae flocculant with the loading of 0.11 g/L for a removal efficiency of 86%. Although chitosan and cationic starch have been used in wastewater treatment and the removal of HABs in aquatic system, there are little studies on their toxicity effects on aquatic ecological system when they are applied in field (Li and Pan, 2013). It is necessary to evaluate the biotic toxicity of chitosan and cationic starch by using appropriate test methods.

Conventional methods of assessing toxicity effect of flocculants are to expose a single species to the flocculent solutions over a range of concentrations for a certain
period of time, but the results may be not sufficient because a single organism cannot represent an aquatic ecosystem. Therefore, the application of a battery of bioassay tests with organisms belonging to different trophic levels is recommended and developed (Hartwell, 1997; Nowell et al., 2014; Wei et al., 2011). Antunes et al. (2007) use a battery of bioassays (algae, crustaceans and dipterans) to screen the acute toxicity of water column and sediment from an abandoned uranium mine, and find that *Daphnia longispina* is the most sensitive organisms (Antunes et al., 2007). In order to evaluate the effects of human activities on the biosafety of water quality, Wei et al. (2008) develop an evaluation method using algae, daphnia and larval medaka (Wei et al., 2008). Tigini et al. (2011) study the toxicity of simulated textile and tannery wastewaters by using a battery of seven organism bioassays and find that the algae *Pseudokirchneriellasubcapitata* is the most sensitive organism (Tigini et al., 2011). While bioassay battery tests can provide more information than single species test to assess the toxicity of chemicals, it is still hard to quantitatively evaluate the biotic toxicity of biodegradable and/or non-degradable chemicals to the aquatic ecosystem and to the food chain.

Several integrated assessment toxicity models have been developed to evaluate the biotic toxicity in the field of pesticide and wastewater treatment. Potential ecotoxic effects probe (PEEP) index was developed to assess and compare the toxic potential of industrial effluents (Costan et al., 1993). Nowell et al. (2014) used Pesticide Toxic Index (PTI) to evaluate relationships between pesticide exposure and biological condition (Nowell et al., 2014). However, the information about the biotic toxicity of
flocculants to the aquatic organisms is very limited. There is an urgent need to develop an integrated biotic toxicity index to assess toxicological effects of chemicals on the aquatic organisms.

This paper aims to investigate the biotic toxicity of chitosan, cationic starch, chitosan modified soil (MS-C) and cationic starch modified soil (MS-S) to the aquatic organisms and elucidate the mechanism of the toxic effect by means of a battery of four bioassays that belong to different trophic levels. An integrated biotic toxicity index (BTI) was developed to make a comprehensive and comparable assessment on the biotic toxicity of the added flocculants on the aquatic organisms.

2. Materials and methods

2.1. Soil and Flocculants

The soils and chitosan used in this study were described in a previous study (Li and Pan 2013). Cationic starch was obtained from Minsheng Environmental Technology Co. Ltd, Dalian, China. The cationic starch was dissolved by adding 250 mg cationic starch to 100 mL deionized water. The molecular weights (MW) of chitosan and cationic starch are $5 \times 10^5$ g/mol and $1 \times 10^8$ g/mol, respectively. The chitosan modified soils (MS-C) and cationic starch modified soils (MS-S) were obtained by adding 100 mL chitosan solution (5 mg/mL) or 100 mL cationic starch solution (2.5 mg/mL) to 100 mL soil suspension (50 mg/mL), respectively. The mixture was well stirred and then ready for use in the toxicity experiment.
2.2. Test solution

BG11 medium was used for algae growth inhibition test only. The solution was adjusted to pH 8.2 by adding either 0.5 mol/L NaOH or 0.5 mol/L HCl solutions after autoclaving (Li and Pan, 2013). The artificial water with a pH of 7.8, a total hardness of 250 mg CaCO$_3$ /L was used for the other tests. The dissolved oxygen values were maintained at 8.0 mg/L.

2.3. Aquatic organisms

Chlorella vulgaris

The green algae *C. vulgaris* (FACHB-1227) were obtained from the FACHB, Institute of Hydrobiology, Chinese Academy of Sciences, and cultured in BG11 medium, at 25±1 °C and with a 12L: 12D h photoperiod in an illuminating incubator. At the start of new cultures, algae were harvested during the exponential growth phase and inoculated in fresh medium.

*Daphnia magna* and *Limnodrilushoffmeisteri*

The *D. magna* and *L. hoffmeisteri* were isolated from Lake Taihu, China and were maintained in artificial water at 25 ± 1 °C, on a 16 h light and 8h darkness regimen. The average weight of the *L. hoffmeisteri* was 40 ± 10 mg, and the average body length was 10 ± 2 mm. *D. magna* were fed with *Scenedesmusobliquus* (10$^6$ cells/mL) and *L. hoffmeisteri* were fed with approximately 100 mg powder fish food every day.

*Cyprinus carpio*

*C. carpio*, were obtained from a fish farm and acclimated for a month to lab
conditions in 100 L tank filled with artificial water prior to the tests. The average mass/size of *C. carpio* used in the test was 0.5±0.1g/3.0±0.2cm. The fish were fed with commercial carp food at a rate of 1.5% of body weight. The tank water was changed weekly. Ammonia, nitrate and nitrite levels were kept below toxic concentrations (<0.1 mg/L) (Eyckmans et al., 2012).

2.4. Experiment design

**Soil leachate and toxicity tests**

Soil materials may potentially release heavy metals into water phase under a variety of conditions. The toxicity characteristic leaching procedure (TCLP) was carried out to determine the mobility of metal elements in soil (USEPA, 1992). The metal elements leached from the soil by three different extraction fluids were analyzed using Inductively Coupled Plasma Emission Spectrometry (ICP-OES, Optima 8300, PerkinElmer, USA). As a complementary test, the effects of soil on four species were determined. Following a static design, the organisms were exposed to five concentrations soil (62.5, 125, 250, 500 and 1000 mg/L) in BG11 medium or artificial water.

**C. vulgaris growth inhibition test**

The tests were conducted using a 72 h growth inhibition bioassay. The algae were exposed to 9 dilutions of four flocculant materials. Each treatment had three replicates and was kept in 125mL erlenmeyer flask which contained 50 mL test solution. The initial algae cell density of each treatment was $1 \times 10^4$ cells/mL. The cell
density was determined using a Neubauer hemocytometer. The flasks were incubated under cool white fluorescent light of 2000 lx on a 12 h light and 12 h darkness regimen. The *C. vulgaris* suspension in each flask was thoroughly mixed by shaken every 8 h to prevent cell aggregating. The yield in each individual treatment was calculated as the difference between the cell densities at the end and at the beginning of the test. The inhibition in yield (*I*<sub>y</sub>) was expressed as (Costa et al. 2014):

\[ I_y = 100 \frac{(Y_C - Y_T)}{Y_C} \]  

where *Y*<sub>C</sub> and *Y*<sub>T</sub> represent the yield for the controls and each replicated treatment, respectively.

**D. magna immobilization test**

Acute toxicity to *D. magna* was examined with the 48h *Daphnia magna* immobilization test. The acute immobilization tests were conducted in accordance with the USEPA guidelines. Neonates aged less than 24 h and born within the 3rd to 5<sup>th</sup> culture broods were used in the test. For each treatment, 10 offsprings were used by 100mL flask which contained 50mL solution, test in triplicate. The details of concentration setting were provided in (Table S1 in supplementary information). Immobilized organisms were counted after a 48 h exposure period and the daphnias were not fed during the test.

**L. hoffmeisteri acute toxicity test**

*L. hoffmeisteri* were exposed to 50 mL test solutions in 10cm Petri dishes for 96h. The test solution was renewed every 24 h. Each dish contained 5 worms, tested in triplicate. Immobilized organisms were counted after a 96 h exposure period and the
worms were not fed during the test. The details of concentration setting are provided in (Table S1).

**C. carpio acute toxicity test**

The acute toxicity of the four flocculants to *C. carpio* was evaluated in 96h static tests where fish were placed in 1.5 L of exposure solution in 2 L glass beakers. The test protocol followed Chemicals-Fish acute toxicity test (GBT/27861-2011). There were six treatment concentrations with three replicates (Table S1). Each beaker contained 5 fish. Survival was assessed daily and dead organisms removed when found. Survival and changes in gill histology at 96 h were the primary endpoints.

**Histopathology**

The morphological changes of *D. magna, L. hoffmeisteri* and *C. carpio* were observed using a dissecting microscope and imaging software (Image Analysis System 13.0). The algae were observed by Axio Scope A1 microscope (Zeiss, Germany) at 400× magnification.

In the fish acute toxicity test, the live fish were anaesthetized with MS-222, fixed in Bouin’s fluid for 24h, and then processed for histology where 6μm sections per fish per slide were stained with hematoxylin/eosin (H&E). Gill, liver, kidney, gut, skin and heart histopathology were evaluated using an Axio Scope A1 microscope (Zeiss, Germany) at 400× magnification.

**Biotic toxicity index (BTI)**

In order to comprehensively and quantitatively assess the toxicological effect on the aquatic organisms after adding the geo-engineering materials, a biotic toxicity
index (BTI) was established by means of a battery of four bioassays, using organisms that belong to different trophic levels. The BTI was determined according to the equation:

$$\text{BTI} = X_A^N \sum_{i=1}^{n} \left( \frac{1}{\text{EC}_{50i}} \right)$$  \hspace{1cm} (2)

Where $X_A$ is the practical dosage of material A (mg/L), $n$ is the number of species in the bioassay battery. $\text{EC}_{50i}$ is the median effect concentration of the material for the separate species (mg/L). $X_A$ and $\text{EC}_{50i}$ are expressed in the same units. The value of $N$ is calculated according to the following three scenarios:

**Scenario 1:** when the material A is biodegradable, then $N=1$;

**Scenario 2:** when the material A is non-biodegradable, and the selected test organisms do not have a food chain relationship, then $N = \sum_{i=1}^{n} (\text{BCF}_i) / n$, and $\text{BCF}_i = \frac{C_{si}}{C_w}$, where $\text{BCF}_i$ is the bioconcentration factor (McGeer et al., 2003), $C_{si}$ is the material concentration in each kind of test organism at steady state ($\mu g.g^{-1}$ dry weight), and $C_w$ is the material concentration in water (mg. mL$^{-1}$). If $X_A=0$ mg/L, then $\text{BTI}=0$;

**Scenario 3:** when the material A is non-biodegradable, and the test organisms in the bioassay battery are from the same aquatic ecosystem, then $N = \text{BMF} = \left( \frac{F_n/F_L}{T_{Ln}/T_{Li}} \right)$, where $\text{BMF}$ is the biomagnification factor (Hoekstra et al., 2003). $F_n$ and $F_L$ are material concentrations of the highest and lowest trophic level species, respectively. $T_{Ln}$ and $T_{Li}$ are the trophic level of the highest and trophic levelspecies which can be determined by stable isotope ratios of $\delta^{15}$N and $\delta^{13}$C. If $X_A=0$ mg/L, then $\text{BTI}=0$.

A higher BTI implies that the material has higher risk to the aquatic organisms or...
aquatic food chain.

2.5. Data analysis

EC$_{50}$ and general statistical analysis of the data are estimated using PASW statistics 18.0 (SPSS software, IBM, http://www-01.ibm.com/software/analytics/spss/). A significance level of 0.05 is used in all statistical analyses.

3. Results

3.1. Toxicity of metals in the soil

The metal concentrations leached from the soil at pH 2.88 were higher than those at pH 4.93 and pH 7.85. Acidic condition was used to test the maximum potential of heavy metal risk from the soil. Table 1 showed that the metal concentration leached under simulated environmental conditions from the tested soil materials did not exceed the acute 48 h EC$_{50}$ values to *D. magna* (Biesinge. and Christen., 1972).

**Table 1- The concentration of metal elements leached from the soil by three different extraction fluids (mg/L)**

<table>
<thead>
<tr>
<th>Elements a</th>
<th>Concentration of metal elements</th>
<th>D. magna 48-h EC$_{50}$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>A leachate b</td>
<td>B leachate c</td>
</tr>
<tr>
<td>Aluminum</td>
<td>--</td>
<td>0.06</td>
</tr>
<tr>
<td>Arsenic</td>
<td>0.10</td>
<td>0.08</td>
</tr>
<tr>
<td>Barium</td>
<td>--</td>
<td>0.31</td>
</tr>
<tr>
<td>Copper</td>
<td>--</td>
<td>0.02</td>
</tr>
<tr>
<td>Iron</td>
<td>--</td>
<td>0.07</td>
</tr>
<tr>
<td>Magnesium</td>
<td>2.30</td>
<td>4.99</td>
</tr>
<tr>
<td>Manganese</td>
<td>0.14</td>
<td>1.57</td>
</tr>
<tr>
<td>Plumbum</td>
<td>--</td>
<td>0.04</td>
</tr>
<tr>
<td>Stannum</td>
<td>0.20</td>
<td>0.19</td>
</tr>
<tr>
<td>Zinc</td>
<td>--</td>
<td>0.18</td>
</tr>
</tbody>
</table>

a The following elements were below the detection limit (<0.01mg/L): Ag, Be, Bi, Cd, Ce,
Co, Cr, Se, Sb, Sn, U.

b Deionized water, pH=7.85. c Acetic acid solution, pH=4.93. d Acetic acid solution, pH=2.88.

3.2. Toxic effects of flocculant materials

Table 2 indicated that the chitosan appeared to be less acutely toxic than cationic starch, with the $EC_{50}$ for the four organisms being around two times higher than the cationic starch. $EC_{50}$ of the MS-C and MS-S were 31 to 124 times higher than chitosan and cationic starch. D. magna was the most sensitive species to the four flocculants. The order of sensitivity (from highest to lowest based on $EC_{50}$ values) of the four species assessed to the four flocculants was $D. magna>C. vulgaris>C. carpio>L. hoffmeisteri. $EC_{50}$ was not obtained for the soil because only 1.5% growth inhibition of algae and no immobilization or mortality of daphnia, tubificidae and fish were found at the highest soil concentration (1000 mg/L) tested. The soil did not show the acute toxicity to the four aquatic organisms.

Table 2- $EC_{50}$ for flocculant materials to the four species.

<table>
<thead>
<tr>
<th>Test endpoint</th>
<th>Chitosan</th>
<th>MS-C</th>
<th>Cationic starch</th>
<th>MS-S</th>
<th>soil</th>
</tr>
</thead>
<tbody>
<tr>
<td>72-h algae yield inhibition</td>
<td>3.5 (2.3-4.5)</td>
<td>110.2 (99.9-122.1)</td>
<td>1.8 (1.2-2.5)</td>
<td>113.2 (94.8-137.5)</td>
<td>&gt;500</td>
</tr>
<tr>
<td>48-h daphnia immobilization</td>
<td>2.2 (1.6-2.9)</td>
<td>102.0 (84.0-126.9)</td>
<td>0.9 (0.6-1.4)</td>
<td>90.2 (72.4-114.4)</td>
<td>&gt;500</td>
</tr>
<tr>
<td>96-h tubificidae immobilization</td>
<td>6.9 (5.4-8.1)</td>
<td>323.2 (248.7-443.7)</td>
<td>3.7 (2.9-4.6)</td>
<td>248.7 (192.9-330.3)</td>
<td>&gt;1000</td>
</tr>
<tr>
<td>96-h fish mortality</td>
<td>3.0 (2.3-3.6)</td>
<td>165.7 (125.0-232.0)</td>
<td>1.4 (0.8-2.1)</td>
<td>173.1 (124.6-268.1)</td>
<td>&gt;1000</td>
</tr>
</tbody>
</table>

3.3. Morphology and Histopathology

The micrographs of the four species exposed to different concentrations of flocculant materials were used to provide an intuitive interpretation of the interaction
between the flocculants and biological surfaces. Algal floc micrographs showed that the four kinds of flocculants could wrap and hold *C. vulgaris* cells and aggregated them into large and complex flocs. Although the *C. vulgaris* cells were thoroughly mixed by shaken to prevent cell clumping, most of the algae cells sink to the bottom compared to the control (Fig. 1 a-2 to a-5). Lots of flocs adhered to the surface of *L. hoffmeisteri, D. magna* and the gill tissue of *C. carpio* (Fig. 1 b-2 to b-5, c-2 to c-5 and d-2 to d-5).

**Fig.1**- The morphological changes of *C. vulgaris, L. hoffmeisteri, D. magna* and *C. carpio* exposed to different concentration of flocculants. (a-1), *C. vulgaris* control. (a-2), *C. vulgaris* exposed to 2.4 mg/L chitosan. (a-3), *C. vulgaris* exposed to 1.2 mg/L cationic starch. (a-4), *C. vulgaris* exposed to 36.0 mg/L chitosan modified soils. (a-5), *C. vulgaris* exposed to...
25.4 mg/L cationic starch modified soils. (b-1), L. hoffmeisteri control. (b-2), L. hoffmeisteri exposed to 4.8mg/L chitosan. (b-3), L. hoffmeisteri exposed to 2.0 mg/L cationic starch. (b-4), L. hoffmeisteri exposed to 131.1 mg/L chitosan modified soils. (b-5), L. hoffmeisteri exposed to 87.5 mg/L cationic starch modified soils. (b-6), L. hoffmeisteri exposed to 2.0 mg/L chitosan. (c-1), D. magna control. (c-2), D. magna exposed to 2.0 mg/L chitosan. (c-3), D. magna exposed to 0.8mg/L cationic starch. (c-4), D. magna exposed to 74.0 mg/L chitosan modified soils. (c-5), D. magna exposed to 39.3mg/L cationic starch modified soils. (d-1), Gill of C. carpio control. (d-2), Gill of C. carpio exposed to 2.0mg/L chitosan. (d-3), Gill of C. carpio exposed to 0.8mg/L cationic starch. (d-4), Gill of C. carpio exposed to 91.9mg/L chitosan modified soils. (d-5), Gill of C. carpio exposed to 70.0mg/L cationic starch modified soils.

Gill, liver, kidney, heart, gut and muscle histopathology were monitored in common carp exposed for 96h to the four flocculant materials. Fish acute toxicity tests indicated the histopathological changes were only happened in gill tissue. Gill tissues of fish sampled from the control (Fig. 2a) and the soil control (Fig. 3a) were normal with blood spaces of the lamellae obvious and uniform in size. The gill of fish showed a significant increase in the number of red blood cells compared to the control when they were exposed to 91.9 mg/L MS-C (chitosan content 8.4 mg/L) or 2.0 mg/L chitosan for 96 h (Fig. 2c and e). Exposure to 70.0 mg/L MS-S (cationic starch content 3.3 mg/L) or 0.8 mg/L cationic starch for 96 h also caused a significant increase in the number of gill cells. More seriously, large areas of adjacent lamellas were fused when they were exposed to higher concentrations of modified soil, chitosan and cationic starch (Fig. 2 d and f, Fig. 3 d and f).
The bottoms of the lamellae engorged with red blood cells and significantly increased gill lamellar thickness for the carp exposed to 2.0 mg/L chitosan (Fig. 2e) and 0.8 mg/L cationic starch (Fig. 3e), however, the similar pathological symptoms were not found in the fish exposure to 31.8 mg/L MS-C (chitosan content 2.9 mg/L, Fig. 2b) and 21.6 mg/L MS-S (cationic starch content 1.0 mg/L, Fig. 3b). The height of the lamellae decreased while the gill lamellas were thickened. (Fig. 2c and e, Fig. 3c and e).
Fig. 3- Histological sections of gill tissues of *C. carpio*. (a) *C. carpio* exposed to 1000 mg/L soil. (b) *C. carpio* exposed to 21.6 mg/L cationic starch modified soils. (c) *C. carpio* exposed to 70.0 mg/L cationic starch modified soils. (d) *C. carpio* exposed to 226.7 mg/L cationic starch modified soils. (e) *C. carpio* exposed to 0.8 mg/L cationic starch. (f) *C. carpio* exposed to 3.2 mg/L cationic starch.

3.4. Biotic toxicity index (BTI)

Chitosan and cationic starch are easily biodegradable which can hardly accumulate and transfer through food chain (Bloto et al., 2007). In this scenario, parameter $N=1$. Based on the $EC_{50}$ of the flocculants to the bioassay battery (Table 2), the functional relation between $BTI$ and dosage ($X_A$) of the flocculants was calculated by using formula (2), as shown in Fig.4. The order of biotic toxicity of the four flocculants to the bioassay battery was cationic starch $>$ chitosan $>$ MS-$S$ $>$ MS-$C$. The BTI of cationic starch and chitosan was found to increase as the dosage of the flocculants increased. However, the BTI for MS-$C$ and MS-$S$ remained very low which did not have significant change as the dosage increased (Fig.4).
Fig. 4. The functional relation between $BTI$ and dosage ($X_A$) of the four flocculants.

Based on the practical dosage ($X_A$) of flocculants in the published literatures, we calculated the BTI of Modified soil (MS) and corresponding modifiers, respectively. Table 3 showed that the BTI of chitosan or cationic starch was higher than modified soil which contained the same amount of modifier. Soil could reduce the biotic toxicity of chitosan and cationic starch.

**Table 3** - BTI for MS-C, MS-S according to the practical dosage ($X_A$) of flocculants in the published literatures.

<table>
<thead>
<tr>
<th>Modified soil/clay flocculant Type</th>
<th>BTI $X_A$ (mg/L)</th>
<th>Chitosan in MS (mg/L)</th>
<th>Cationic starch in MS (mg/L)</th>
<th>BTI</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>MS-C</td>
<td>11</td>
<td>0.3</td>
<td>1</td>
<td>1.2</td>
<td>--</td>
</tr>
<tr>
<td></td>
<td>25</td>
<td>0.7</td>
<td>2.5</td>
<td>3.1</td>
<td>--</td>
</tr>
<tr>
<td></td>
<td>77 $^a$</td>
<td>&lt;0.6</td>
<td>2</td>
<td>2.4</td>
<td>--</td>
</tr>
<tr>
<td></td>
<td>102 $^b$</td>
<td>&lt;0.6</td>
<td>2</td>
<td>2.4</td>
<td>--</td>
</tr>
<tr>
<td>MS-S</td>
<td>110</td>
<td>3.3</td>
<td>--</td>
<td>--</td>
<td>10</td>
</tr>
</tbody>
</table>

$^a$, chitosan: soil(w/w)=1:17.5; $^b$, chitosan: soil(w/w)=1:50
4. Discussion

4.1. Toxic effects of chitosan and cationic starch on aquatic organisms

Natural flocculant materials are widely studied as geo-engineering materials for controlling harmful algal blooms or nutrient levels (Li and Pan 2013; Wang et al., 2013). Among these flocculants materials such as chitosan and cationic starch are the most promising ones for application due to abundant source, easy availability and biodegradation with less secondary pollution (Hansel et al., 2014; Letelier-Gordo et al., 2014). However, cationic polymers are often toxic to the aquatic organisms (Lee et al., 2014) and direct application of these materials in aquatic environment may pose adverse effects (Bullock et al., 2000; Rizzo et al., 2008).

Cationic flocculants maybe toxic to zooplankton and fish because the surfaces of aquatic organisms often carry with net negative charge (Lee et al., 2014). Dissolved chitosan is cationic polymer with high charge density (Rinaudo, 2006). Hence, the chitosan and cationic starch can readily bind to the surface of aquatic organisms. This ultimately can result in toxicity to the aquatic organism due to the reduction of oxygen transfer through damaged cell surfaces or by effects on the ionic balance.

C. vulgaris cells were agglomerated and sedimented to the bottom at different concentrations of chitosan and cationic starch. Compared to the control, the chitosan and cationic starch do not exhibit a detrimental impact on C. vulgaris cell integrity in 72h (Fig. 1 a-1 to a-3). However, C. vulgaris growth inhibition occurred (Table 2).
Costa et al. (2014) found that cationic polymers could cause physiological damage to the green microalgae due to the especially strong affinity of the flocculants to the algal cellular surface and further inhibit the proliferation of the cells (Costa et al., 2014). We found lots of white flocs adhere to the surface of *D. magna* (Fig. 1 b) and *L. hoffmeisteri*, even some cladoceras were stuck together by the cationic starch (Fig. 1 c-2). The toxicity effects of the chitosan and cationic starch to the zooplankton may result from mechanical impairment, including locomotion inhibition and disturbance of predation mechanisms (Costa et al., 2014).

Fig. 1d indicated that the flocs of chitosan and cationic starch could adhere to the surface of the gill of the carp and cause thickening and shortening of common carp gill filaments leading to destruction of the filament structure. Large areas of adjacent lamellae are fused when the carp exposure to high concentrations of chitosan and cationic starch (Fig. 2 f and Fig. 3 f), which is similar to that observed previously with cationic polymer exposure in lake trout fry (Liber et al., 2005). Since the chitosan and cationic starch with long-chain structure are difficult to pass through the cell membranes (Goodrich et al., 1991), the most likely mechanism of pathological changes of gill tissue is flocculants adsorption onto the organ surfaces. Hence the microenvironment surrounding the gill cells will be altered and transport mechanisms between the cells and the water are disrupted, with further impacts on respiratory and ion regulation processes (Rowland et al., 2000).
4.2. Toxic effects of chitosan and cationic starch modified soil

The biotic toxicity of MS-C and MS-S to the bioassay battery was much less than chitosan and cationic starch (Table 2). The adding of soil could reduce toxicities of chitosan and cationic starch by one to two orders of magnitude. Some reports indicated that clays could effectively reduce the acute toxicities of cationic polymers to aquatic organisms (de Rosemond and Liber, 2004). Goodrich et al. (1991) also found that the biotic toxicity of cationic polymer was reduced 33- to 75-fold at higher humic acid concentrations (Goodrich et al., 1991). The adsorption and neutralization of the positive charge of cationic polymers to the surface of clays is well documented (Cary et al., 1987). Soil particles could reduce the toxicity of chitosan and cationic starch to the aquatic organism by adsorbing much of the flocculants onto soil surfaces. The flocculant modified/adsorbed soil particles are not only less toxic but also more effective in flocculating algae cells especially at high particle concentration where collision between the modified soil particles and the algae cells can be effectively increased (Li and Pan, 2013).

Suspended particles (SP) are ubiquitous in natural waters. The mean SP concentration can range from 2 - 200 mg/L (Bolto and Gregory, 2007) to as high as 65 g/L in the Yellow River (Pan et al., 2013). The application dosage of soil is generally comparable to the SP concentration in many nature waters (Li and Pan, 2013; Zou et al., 2006). SP (>500 mg/L) itself showed no toxic effect to the four aquatic species (Table 2). The concentrations of metal leached from the soil at pH 7.85 and 4.93 are far below the EC50 of these metals to D. magna (Table 1). It can be confirmed
that chitosan and cationic starch is the main toxic components in the modified soil.

Since the toxicity of these modifiers can be reduced after combining them with soil/clay particles, flocculants modified soil or clay provide an approach to improve the ecological safety of the cationic polymers for HABs control.

4.3. Biotic toxicity index

As a toxicity assessment and screening tool for the lake geoengineering materials, BTI could be used to assess the toxicity of flocculants on aquatic organisms in three scenarios. In this work, we calculated the BTI of chitosan and cationic starch in scenario 1 since both chitosan and cationic starch are biodegradable in the aquatic ecosystem. There is a single linear regress relationship between BTI and the dosage of flocculants ($X_A$) when $N=1$. The contents of modifier in MS were often below 10% in the published literatures and the MS usually shows higher flocculation efficiency (Table 3). Some clays can flocculate algae cells without being modified by flocculants (Lewis et al., 2003; Pan et al., 2006). The flocculation ability of soil/clay was improved by adding chitosan or cationic starch, however, the toxicity of modifier was correspondingly reduced.

In lake geo-engineering, mineral-based byproducts and inorganic flocculant have been used widely. The application of non-degradable inorganic salts may increase the metal (e.g. aluminum, iron, lanthanum) concentration in natural waters. The metals may be ingested and accumulated in biological bodies or transport to a higher trophic level through aquatic food chain (Cui et al., 2011) and produce adverse impacts such
as deformities and death on aquatic organisms (Bird et al., 2008). We can use formula (2) to calculate the BTI of non-biodegradable flocclant in scenario 2 if there is not food chain relationship among the test organisms. In this situation, the bioconcentration factor (BCF) was introduced into formula (2). So the BTI has exponential relationship to the practical dosage \( X_A \), and the BTI of non-biodegradable floculant grew more faster than biodegradable floculants with the increased of \( X_A \).

In practical applications, lake managers are more concerned about the impacts of flocculants on actual aquatic ecosystem than standardized laboratory toxicity tests (the latter are more replicable which is important for experiments). If the organisms in the bioassay battery are from the same aquatic ecosystem, they can form an actual food chain relationship. In this scenario, the trophic level and biomagnification action were considered and biomagnification factor (BMF) was introduced. Due to the biomagnification, the higher trophic level may suffer from more damage than the lower one. Under this scenario, the BTI also has exponential relationship to the practical dosage \( X_A \). It is possible to obtain the toxicity effects of the non-biodegradable flocculants to the aquatic food chain. With toxicity data of metal salts and with well established methods for obtaining the battery in the same system, scenario 2 and 3 can be measured in separate studies. Nevertheless, the BTI provided here could provide useful information for the lake manager to screen and rank the toxicity of flocculants for the lake geo-engineering.

Although the BTI can be used to reveal the biotic toxicity of flocculants, it still has several limitations which must be further studied. Firstly, toxicity values are based on
short-term laboratory experiments with acute $EC_{50}$ endpoints; the BTIdoes not reflect long-term/chronic exposure or incorporate sublethal endpoints. Secondly, the BTI does not account for many environmental factors, which can affect the toxicity and bioavailability of the flocculants. More comprehensive studies on ecotoxicological effect of geo-engineering materials are needed before they can be widely applied in natural waters at large scale.

4.4. Implication for lake geo-engineering

Although natural flocculants have the potential to be more biodegradable and environmental friendly than non-degradable chemical salts (Bolto and Gregory, 2007), it does not necessarily imply that they are ecologically safe for the aquatic system especially when they are modified by chemical reactions. Before these materials can be used in field at large scale, their ecological safety and ecotoxicology should be comprehensively studied. Our results demonstrated that using chitosan or cationic starch alone may cause some toxic effects to the aquatic biota (Table 2). The aquatic organisms may suffer from movement inhibition or pathological changes of tissues at low concentration of chitosan or cationic starch (Fig. 2, Fig.3). By modifying with the soil/clay particles, the acute toxicity of chitosan and cationic starch can be largely decreased while the flocculation efficiency is substantially enhanced (Li and Pan 2013; Zou et al., 2006). A preliminary toxicity test is necessary to screen the toxicity risk of flocculants before practical application. The BTI method proposed here is a comparable and quantitative method which can reflect the toxicity of flocculant to the
5. Conclusion

Biotic toxicity index (BTI) were used to assess the toxicity potential of four representative geo-engineering materials including chitosan, cationic starch, chitosan modified soil, cationic starch modified soil to the aquatic organisms. The fact that EC$_{50}$ values of chitosan and cationic starch are much lower than that of chitosan or cationic starch modified soil/clay materials indicates that direct use of chitosan or cationic starch alone as flocculants has a much higher toxic risk than the modified soil/clay materials. When MS-C and/or MS-S are used at the optimized dosage of 11-110 mg/L, it may not cause substantial adverse effects to the four representative organisms in aquatic ecosystem. The mainly acute toxic effect of flocculants on the fish is pathological changes of gill tissues caused by the affinity of flocs to the biological surface. The results of BTI indicated that the potential impact of flocculants on the aquatic organisms was in order: cationic starch > chitosan > MS-C > MS-S. The BTI can be used to describe the toxic effects of biodegradable or non-biodegradable flocculants on the aquatic organisms or food chain.

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