EXPERIMENTAL OBSERVATION AND ANALYSIS ON THE MICRO ARCHITECTURE OF BIOFLOCCULATION SEDIMENT

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ABSTRACT

Sediment flocculation is very important for cohesive sediment dynamics. The bioflocculation of sediment induced by biological activity has attracted more and more attention over the last decades. In order to accurately understand the formation mechanism and structural characteristics of bioflocculation sediment, firstly, the micro-morphology of bioflocculation sediment in water environment should be visualized and its flocculating structure should be analyzed, so as to lay a foundation for further related research. Bioflocculation sediment has a small particle size in the order of micron, and there is organic matter-biofilms on the surface and between the particles. Therefore, the characteristics of sediment, microorganisms and their metabolites should be taken into account. Appropriate instruments and equipment should be used to observe bioflocculation sediment in situ, which couldn't be dried, carbon plated, etc. In this paper, the flocculation structure of bioflocculation sediment was observed by means of environmental scanning electron microscopy and confocal laser scanning microscopy. A more intuitive understanding of its internal micro-environment and external behavior characteristics was obtained, and the great role of microbial activities and their metabolites in the structure and adhesion characteristics of bioflocculation sediment was revealed.

Keywords: bio-flocculation sediment; micro architecture; biofilm

1. INTRODUCTION

Flocculation refers to the particle interaction terms of consequence result from collisions due to all kinds of reasons, such as Brownian motion, fluid shear, and differential settling, and so on (Ernestet al., 1995). Flocculation is a non-negligible phenomenon in nature, which has been paid much attention in many fields. From the macroscopic view, it could be classified into the three classes of organic-flocculation, inorganic-flocculation and bioflocculation. Bioflocculation is different from conventional flocculation, in which microorganism plays a key role. As a life, it has a great difference with the general inert material because of the association of life activities. There have been many mechanisms of bioflocculation, such as charge-

neutralization theory, capsula theory, cellulose fibrils outside the cell theory, EPS bridging theory, and so on (Deng et al., 1998). The flocculation of cohesive sediment is also an important and implicated subject in the sediment dynamics study (Tang et al., 2008; Lee et al., 2002; Corpart and Cardau, 1993), and nowadays most related research have focused on the aspects such as formation mechanism, influencing factors and architecture characteristics of all kinds of organic-flocculation and inorganic-flocculation. The conception of "bioflocculation" is mostly applied in the environmental, chemical and biological fields, and there is little research concerning the phenomenon of sediment bioflocculation. Referred researches usually study the mutual interrelations between biological activity and physico-chemical properties from the macroscopic view, and nowadays most are limited to field sampling, data extracting, and then building the attachment of corresponding factors by some direct correlation analysis (Sabine et al., 2008). It is found that sedimentological factors and biological factors interact in a complex manner with the hydrodynamic regime both on a temporal as well as on a spatial scale. But most conclusions are qualitative illustrations. A complete theoretical system has not been formed and requires further study.

To better understand bioflocculation sediment' formation mechanism, architecture characteristics, and so on, it is necessary to make the micro-surface of bioflocculation sediment in water environment visualization and make study on its floc architecture, so as to establish a foundation for further research. The particles of bioflocculation sediment are of little size, which are in micrometer order, and the particle surface and interparticles all exit organic material-biofilm reduced by biological metabolic activities, the main component of which is extracellular polymeric substances (EPS), and 85~90% of biofilm is water (Zhao et al., 2011). As a result, it is necessary to give consideration to both the characteristics of sediment as well as microorganism and its metabolic products and introduce appropriate apparatus to make a direct observation of bioflocculation sediment without the dealing of drying, carbon-coating, and so on. This paper describes two multimicroscopic methods of environmental scanning electron microscopy (ESEM) and confocal laser scanning microscopy (CLSM) for observing the floc architecture of bioflocculation sediment. In doing so, a more realistic understanding of the internal microenvironment and outward behavior of bioflocculation sediment can be achieved, and it also reveals the enormous function of biological activities and the metabolic products on the architecture characteristics and collision properties. This will improve our knowledge of natural bioflocculation sediment dynamics and its environmental effect.

2. EXPERIMENTAL METHODS AND OBSERVATION APPARATUS

2.1 Biofilm culture experiment

Several potential errors can arise when sampling bioflocculation sediment specimens directly from natural aquatic environment, because it is difficult to make a direct observation of bioflocculation sediment from natural environment without changing or disturbing its original characteristics, and also it is difficult to observe its whole changing process. So this paper would adopt some experimental methods to realize the forming process of bioflocculation sediment under a laboratory monitoring environment, which lays a foundation for the further study.

Sediment used in the experiment is fetched from Guanting Reservoir of Beijing, China. After sampling it is cleaned, dried and removed of the impurity and coarse sediment through 400-size sievet. The water of lotus pond in Tsinghua University is used as the cultivating water in the experiment. The mobility of the water body is poor, and it has some certain microbial species and quantity.

The concentration of nutrients in water is an important factor affecting the growth of biofilm. The water quality indicators of the lotus pond are shown in Table 1, which were determined in December. It showed that the nutritional degree of the experimental water sample is slightly lower. To maintain the optimal activity of microorganism, nutrient solution of the experimental water added with some carbon source, nitrogen source and inorganic factors is confected according to the confect scheme of Table 2, so as to provide a favourable environment for the biological activities such as metabolism and adsorption.

Table 1. The water quality indicators of the experimental water.

Indicator	**TN (mg/L)**	**TP (mg/L)**	**DO (mg/L)**	**COD_{Mn} (mg/L)**	**NH_4^+**
Value	0.60	<0.01	11.68	2.81	<0.05

Table 2. The confect scheme of nutrient solution.

Nutrient	**glucose**	**KH_2PQ_4**	**$NaHCO_3$**	**$MgSO_4$**	**NH_4Cl**	**$CaCl_2$**
Content ($mg \cdot L^{-1}$)	500	50	1000	50	100	15

Meanwhile static water is used as the hydrological condition, and the analysis room of sediment laboratory is selected as the experimental location. Therefore it could normalize all kinds of environmental factors and hydraulic factors in the culturing experiment and avoid as far as possible the complication of forming conditions under natural environment, which is favorable to illustrate the variation effected by a single factor.

The culturing manipulation of bioflocculation sediment is: Fetching some fresh water from the lotus pond at regular time everyday, strengthening the water nutrition according to Table 2 and confecting nutrient solution; Configuring 1L nutrient solution for every 6g sediment samples, eliminating 500ml supernatant liquid and adding another 500ml fresh nutrition solution every day, avoiding disturbance to the sediment samples when changing water.

2.2 Observation Apparatus

As mentioned above, observation instruments with high precision provide important techniqual supplies in the referred study. Environmental scanning electron microscopy (ESEM) and confocal laser scanning microscopy (CLSM) are the two apparatuses used in this paper.

2.2.1 Environmental Scanning Electron Microscopy (ESEM)

ESEM is a new type of large instrument with high precision used to observe the surface micro-, ultra-micro structure of observed objects in the wet or partially hydrated state, which has been increasingly applied in the fields of environment, microbiology and materials science. Its two key technologies (Gan et al., 2003; Tang and Dai, 2001) minimize sample damage of biofilm constriction on particle surface and EPS structure destruction on interparticles, which would be induced by the necessary treatments of conventional optical scanning electron microscopy (such as drying, dehydration, conduction, and so on). Firstly, multi-stage diaphragm pressure technology is adopted to form gradient vacuum, so the pressure of sample room can be maintained up to 2600 Pa and the temperature, the pressure and the relative humidity can be regulated; Secondly, the use of gas secondary electron detector can magnify the weak secondary electron signal of biological samples and eliminate the charge accumulation on the surface of samples. Specimens do not require extensive manipulation, fixation, dehydration, and air drying or critical point drying and metal coating for viewing purposes that a high vacuum SEM would require. As the result, bioflocculation sediment specimens, which are highly hydrated and with poor electrical conductivity, could be observed directly by ESEM, and the real microscopic images could be obtained.

In this paper the FEI Quanta 200 FEG ESEM is introduced, as shown in Figure 1 (a). As bioflocculation sediment requires being observed in a wet state, the 0℃ cold-platform observation model is selected. Therefore the original sample could be observed directly under the condition with a relative humidity without damaging its floc architecture. The maximum magnification of this ESEM would reach 1,00,000X under a high vacuum mode， which could meet the requirement of this experiment.

Every week 0.02g sediment sample with free water was withdrawn from the culturing vessel at regular time and placed into a sealed plastic bottle of 0.5ml, and then sent to be observed with ESEM. It would not last for more than 10min from sampling until observation, so the sample could be maintained in a original state with water. During observation firstly the sample was placed on the special sample platform of ESEM, and then a few drops of distilled water were dropped into the pits in the sample room to maintain the humidity of the observing environment. Cold-platform ESEM environment model was chosen, and the observation took place in a relative humidity of 100%. Therefore the real ultrastructure of samples could be obtained without damaging the architecture of bioflocculation sediment.

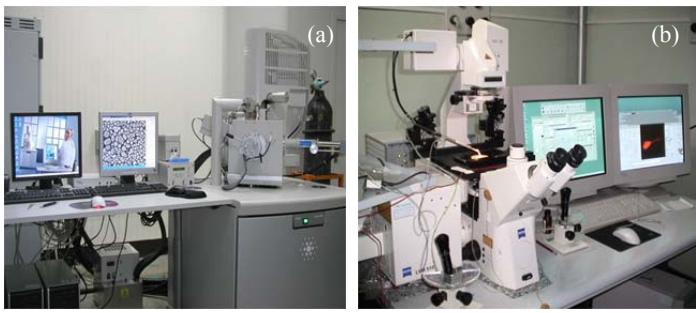

Figure 1. Observation Apparatus: (a) FEI Quanta200 FEG ESEM; (b) Olympus BX61 CLSM.

2.2.2 Confocal Laser Scanning Microscopy (CLSM)

CLSM is an observing and analyzing system using laser as its light source, adopting confocal theory and the corresponding equipment on the basis of conventional optical microscopy, and using computers for digital

image processing (Chen et al., 2007). The observed samples are usually stained by all kinds of fluorescent probes to mark its subcellular structure or molecule before being observed. On the basis of fluorescent microscopy imaging, UV-visible laser is used to excite fluorescent materials, and fluorescent images of sample ultrastructure could be acquired. Different architectures or components have their own fluorescent probes (Xi et al., 1996). CLSM could carry out optical sectional scanning, and could be used meanwhile maintaining the original water of biological samples and its adsorption to the matrix. As it has many advantages such as simple manipulation of observation, implementation of 3D reconstruction, application of multiple fluorescent probes, recording many aspects of the image digital information, and so on, CLSM is widely used in many studies of related fields, and it provides a possible technical method for studying the spatial architecture of bioflocculation sediment and biofilm growth on particle surface and porosity.

In this paper Olympus BX61 CLSM (as shown in Figure 1 (b)) is introduced to observe the spatial architecture of bioflocculation sediment. Fluorescein isothiocyanate (FITC) is chosen as the fluorescent dye, which could combine with the main component of EPS-polysaccharides and be excited green fluorescence received by confocal laser scanning microscopy.

3. RESULTS AND DISCUSSION

3.1 ESEM Observation Analysis

The ESEM images are shown in Figure 2, of which, Figure 2 (a) illustrates an ESEM image of micro surface of sediment particles when bioflocculation sediment has not formed in the prime of the experiment, while Figure 2 (b) shows the micro architecture of bioflocculation sediment obtained in the culturing experiment for 60 days. From the images it could be found that the implication on sediment from microorganism is pronounced, the unattached particles become adhesive to each other due to the metabolic products of microorganism. Abundant biofilm embeds particles and permeates the void space. The interplay between sediment and biofilm leads to a floc or reticulate structure. The honeycomb structure resembles with the original flocculation sediment in shape, and its forming is due to biological activies, therefore it is defined as bioflocculation sediment, which refers to the flocculation-similar structure of sediment particles induced by adsorption and collision of microorganism metabolic products. It has revealed that biofilm would influence greatly on the structure and morphology of sediment in aquatic environment, and might further affect the mechanical properties such as settling velocity, incipient motion, and so on.

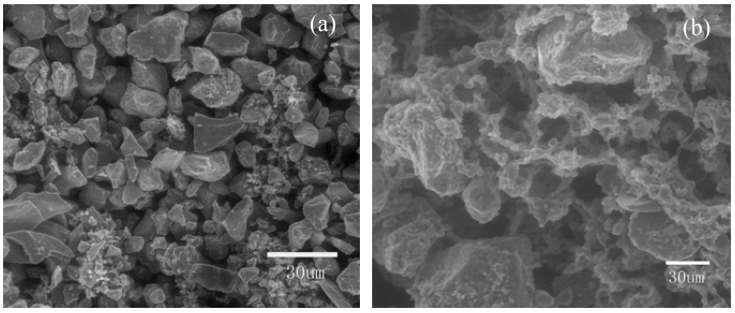

Figure 2. ESEM images: (a) the original sediment particles without biofilm; (b) bioflocculation sediment with abundant biofilm.

The ESEM images of bioflocculation sediment have shown that sediment particles are bridging connected by biofilm. It is these “bridges” that collect the original discrete sediment particles and induce complicated floc architecture. It coincides with the EPS bridging theory which has been more generally accepted in all the bioflocculation sediment forming mechanisms nowadays. It is believed in this theory that the material foundation of bioflocculation is extracellular polymeric substances (EPS), which plays its effect on paticle surface in form of “bridging” and induces flocculation (Smith et al., 1992; Xing et al., 2003). Some researchers (Sabine et al., 2008; Zhao et al., 2011) have measured and analyzed the corresponding biological and sedimentological indicators and found that metabolic activities of microorganism interact with sedimentological factors to influence the sediment properties by binding fine-grained sediment, changing water content and enhancing the organic content through secretion products; the colloidal and bound EPS moieties showed strong correlation with the critical shear stress for erosion over sediment depth. It was suggested that the cohesive strength of the sediment was controlled by a high number of active adsorption sites and higher charge densities in fine grained sediments. The EPS network might significantly enhance this by embedding particles and permeating the void space but also in offering additional ionic binding sites and cross-linkages. All these conclusions could be further confirmed by the ESEM observation in this paper.

3.2 CLSM Observation Analysis

The observation and analysis above reveal that sediment in water environment is influenced significantly by bioflocculation. Biofilm secreted by microorganism gradually embeds sediment particles and permeates the void space to a stable state, and induces bioflocculation sediment. The spatial structure characteristics of bioflocculation sediment would become different, as well as its porosity would change gradually because of the colonizing of biofilm. The spatial structure interrelation between biofilm and sediment particles would be obtained through sectional scanning of bioflocculation sediment using CLSM.

The obtained image of bioflocculation sediment obtained in the culturing experiment for 60 days is shown in Figure 3 (a), in which, the green part is the biofilm which was excited fluorescence because of the combination of its component polysaccharides and the fluorescent probe FITC, and the black part is the pores and the sediment particle section which is colorless due to its opacity. The image obtained by fluorescence staining and CLSM observation is a RGB color intensity image, in which every pixel includes three color information of Red, Green and Blue. Fluorescence staining uses the luminance of excited fluorescence with good monochromaticity to reflect the spatial distribution density of materials, and the spatial distribution information of fluorescent staining materials could be obtained through extracting alone the luminance values of the RGB color intensity image (Zhao et al., 2011). For this purpose, a color space algorithm built by NTSC (Blinn, 1993) is chosen to separate the luminance and chrominance so as to realize the transform from a color image to a gray image. Figure 3 (b) shows the gray image of bioflocculation sediment transformed from Figure 3 (a). Then the Otsu method is adopted to calculate the gray value threshold of a gray image for the black-white binarization, that is to set all of the pixel gray values below the optimal threshold as 0 (illustrating the part without biofilm) and all of the pixel gray values greater than the optimal threshold as 1 (illustrating the part with biofilm). The result is shown in Figure 3 (c). It could be found that the black-white image after the above treatment well preserved and highlighted the structure characteristics of bioflocculation sediment. The biofilm proportion in the graphic section could be obtained by calculating the ratio of 1-value pixels to all the pixels in the black-white image. As mentioned above, the value of fluorescence luminance could demonstrate the quantity and quality of biofilm, and the distribution of fluorescence luminance could illustrate the distribution condition of biofilm. The histograms of pixel numbers and the probability density distributing in different fluorescence luminance areas are calculated (Figure 4), and the result showed that the distribution area of biofilm decreases gradually from low fluorescence luminance interval to high fluorescence luminance interval, and the decreasement present a uniform trend expect the minimum interval of fluorescence luminance [1～12], in which the fluorescence luminance probability density is a little greater. The skewness S is greater than 0, illustrating that the fluorescence luminance distributes more on the right of average value, which is the area with high fluorescence luminance. It reveals that the biofilm of the bioflocculation sediment shown in the figure is abundant and would play a tremendous impact on the bulk density, cohesive force between particles, and so on.

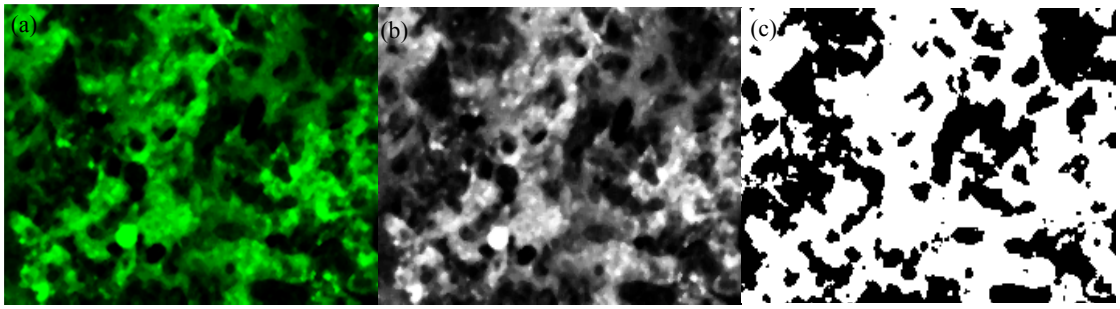

Figure 3. CLSM image of bioflocculation sediment: (a) The color fluorescent image; (b) The gray image of 256 color; (c) The blace-white image.

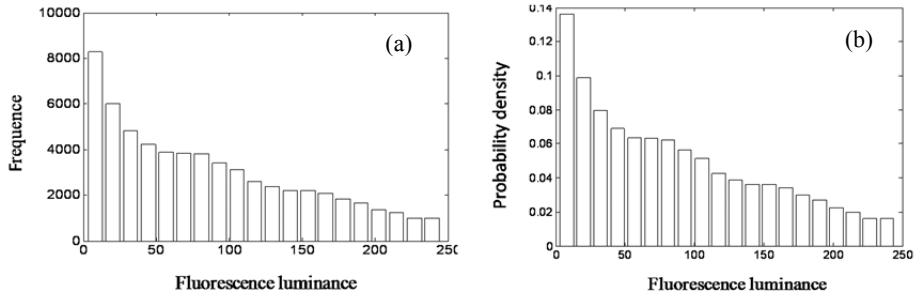

Figure 4. Distribution of fluorescence luminance: (a) Frequence distribution of fluorescence luminance; (b) Probability density distribution of fluorescence luminance

From the observation result of biofilm culture experiment it is found that microorganisms can form a thick layer of biofilm on the surface of sediment particles, having a certain impact on the apparent particle size. Figure 5 (a) shows the optical tomography of one single sediment particle covered with biofilm by CLSM, in which the green part is the biofilm and the black part is the sediment particle section. Defining the ratio of biofilm thickness δ to sediment particle diameter D as the "biofilm thickness ratio" λ of the biofilm sediment (Figure 5 (b)), it reflects the effect of biofilm on the particle size of sediment, and also reflects indirectly the bulk density of biofilm sediment particles. The biofilm thickness ratio of the biofilm sediment particle in Figure 5 (a) is about 1:6, showing that biofilm has a great influence on sediment particles. Yet biofilm growth is a random process, and its growth situation is related to many factors including the microorganism species in water, the nutritional status of water, temperature, hydraulic condition, and so on, so the biofilm thickness formed on sediment surface is not a fixed value.

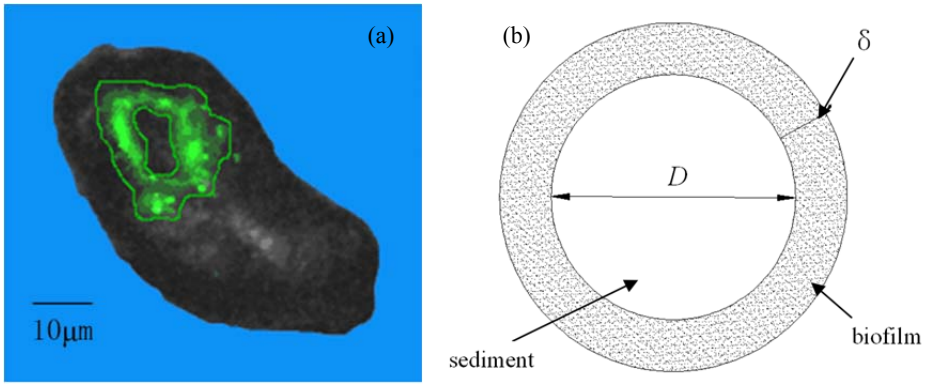

Figure 5. (a) CLSM image of one single sediment particle covered with biofilm; (b) The diagrammatic sketch of biofilm thickness.

4. CONCLUSIONS

A detailed study using correlative microscopy (ESEM, CLSM) allowing immediate visualization of specimens after sampling and in-situ manipulation has provided important structural observations of the floc matrix of bioflocculation sediment. As demonstrated here, the use of multiple microscopes with overlapping resolution limits can provide unique insight into the structure of a floc.

A floc architecture study of bioflocculation sediment revealed the significance of microorganism metabolic products-biofilm, which served to bind or bridge the particles together and provide structural support of an apparent plastic with thixotropy nature. Bioflocculation sediment samples were found to contain dense matrices of polymeric material biofilm. These dense biofilm networks will significantly change the floc ultrastructure as well as potentially resulting in implications on physico-chemical properties properties of sediment. It may enhance sediment stability by permeating the void space and strengthening the interparticles forces. By viewing the structural detail of the floc matrix in bioflocculation sediment, we may be able to obtain a better assessment of the outward behavior of flocs (i.e., transport and settling) and, potentially, learn how to better manipulate the physical, chemical, and/or biological characteristics of flocs for the benefit of cohesive sediment dynamics.

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REFERENCES

Blinn J.F. (1993). Ntsc: Nice technology, super color. IEEE Computer Graphics and Applications, 3(2):17-23.

Chen Jingxiang, Sun Fengjun, Liu Songqing, et al. (2007). Study on Method for Observation of Bacterial Biofilm Formation under Confocal Laser Scanning Microscope. *Progress in Modern Biomedicine,* 7(5):653-655.

Corpart J.M., Cardau F. (1993). Formulation and polymerization of microemulsion containing a mixture of cationic and anionic monomers. *Colloid & Polymer Science*, 271(11):10-25.

Deng Shubo, Hu Youmin, Luo Qian. (1998). Research and application of microorganism flocculant. *Metallic Ore Dressing Abroad,* 1:15-27.
Ernest, A. N., Bonner, J. S., Aulenrieth, R. L. (1995). Determination of particle collision efficiencies for flocculent transport models. *Journal of Environmental Engineering,* 121(4):320-329.
Gan Shuyi; Chen Changqi; Zhu Wu, et al. (2003). Principle and Realization of the Environmental Scanning Electron Microscope. *Vacuum Electronics,* 6:29-32.
Lee D.G., Bonner J.S., Gallon L.S., et al. (2002). Modeling coagulation kinetics incorporating fractal theories: comparison with observed data. *Water Research,* 36:1056-10661.
Sabine U.G., Thomas J., Westrich B., et al. (). Microbial stabilization of riverine sediments by extracellular polymeric substances. *Geobiology,* 2008, 6(13):57-69.
Smith G., Straver M.H., Lugtenberg B.J., Kijne J.W. (1992). Flocculence of Saccharomyces cerevisiae cells is induced by nutrient limitation, with cell surface hydrophobicity as a major determinant. *Applied and Environmental Microbiology,* 53(11):3709-3714.
Tang Jianhua, He Qing, Wang Yuanye, et al. (2008). Study on in-situ flocs size in turbidity maximum of the Changjiang Estuary. *Journal of Sediment Research,* 2:27-33.
Tang Xueming, Dai Shuwen. (2001). ESEM observation for biological samples. *Journal of Chinese Electron Microscopy Society,* 20(3):217-223.
Xi Xin, Jiang Sichang, Fang yaoyun. (1996). Principles and biological applications of laser scanning confocal microscopy. *Chinese Journal of stereology and image analysis,* 1(3,4):74-79.
Xing Lizhen, Kong Ji, Chen Wenbing. (2003). Characteristics of bioflocculants and their application in wastewater treatment. *Industrial Water Treatment,* 23(4):10-13.
Zhao, H., Fang, H., Chen, M. (2011). Floc Architecture of Bioflocculation Sediment by ESEM and CLSM. *Scanning,* 33, 437-445.