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子計畫一：污泥膠羽結構對精密分離處置程序之影響(1/3)

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行政院國家科學委員會專題研究計畫期中報告

精密固液分離在高科技產業之應用
子計劃一　污泥膠羽對精密分離處置之影響 (1/3)

計畫編號：NSC 91-2214-E-002-018
執行期限：91 年 8 月 1 日至 92 年 7 月 31 日
主持人：李篤中 教授
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執行機構及單位名稱：國立台灣大學化學工程學系

I. 中文摘要

本計畫擬以三年時間，藉由數種表面分析與分子生物學的方法，對污泥膠羽顆粒的內部結構作更進一步的探討與解析，以了解及其對後續消化與固液分離兩種操作間之關係。在第一年的報告中，我們比較以石蠟固定切片、冷凍切片、掃描式電子顯微鏡、穿透式電子顯微鏡等四種已廣為病理診斷醫學與生物組織研究的技術，來探討廢棄生物污泥從微觀至巨觀的結構。各種方法之適用觀察尺度與優缺點將在本報告中進行討論與比較。

關鍵詞：結構、膠羽、石蠟切片、電子顯微鏡、共軛焦雷射掃描顯微鏡

Abstract

This report summarized the experimental results of the first year in the three-year-project investigating the internal structure of sludge floc by surface analysis tools and molecular biological techniques. The target is to construct the correlation between the floc structure and the subsequent treatment of sludge. To continue our previous work on the light scattering and free settling, we will use the paraffin-embedded slicing, cryotome slicing, scanning and transmission electron microscopy to investigate the microscale and macroscale structure of the sludge floc. The feasibilities of individual techniques were compared in this report.

Keywords: Structure, flocs, slicing, electron microscope, confocal laser scanning microscopy

II. Introduction (計劃緣由與目的)

Compared with the wastewater biofilms and granules, *in situ* observation on the highly porous structure of sludge floc in aquatic environment is relatively difficult. Optical microscopes (OM) are usually used in observing the floc appearance and estimate the shape factor. Studies on fluid dynamics of flocs frequently used OM to detect the motion. However the low resolution of OM restricts the investigation on more detailed structure. Some techniques of pathological examination were widely applied. Mitani *et al.* (1983) embedded sludge flocs in the agar solution to keep its integrity without disturbing the internal structure. After solidifying in the room temperature, the embedded flocs were then cut into slices in thickness of 200~300 μm by stainless knife and then observed by optical microscopy. The results showed that a concentrated core (approximately 20% of floc diameter) of microbial

mass existed in the center of flocs. Li and Ganczarczyk (1990) applied different embedding and microtome slicing techniques to obtain thinner slices, and the slices were then stained for microscopic observation.

Microscopes with higher resolution than OM are applied to investigate the floc morphology. Confocal laser scanning microscopy (CLSM) accompanied with fluorescence *in situ* hybridization (FISH) can penetrate the flocs and scan at some given depth. The results showed that the mass distribution in floc is highly inhomogeneous and can be divided into many levels (Jorand *et al.*, 1995). Also the fractal dimensions obtained of each layer is different (Zartarian *et al.*, 1994). Investigating the smaller length scale required electron microscopy. Scanning electron microscopic test is often conducted to investigate the surface of morphology. After sliced into ultra-thin slices (< 0.1 μm), the sample can be observed by transmission electron microscopy. Detailed structure like the cell interior and distribution of ECPs can be investigated (Liss *et al.*, 1996). Cornelissen *et al.* (1997) applied atomic force microscopy to observe the microstructure in scale of less than 1 μm.

In this report, the feasibility of using aforementioned techniques to investigate the sludge floc structure would be evaluated.

III. Experimental (實驗方法)

Confocal laser scanning microscopy (CLSM)

Waste activated sludge was sampled from the wastewater treatment plant of Neili Bread Plant, Presidential Enterprise Co., Taoyuan, Taiwan on July, 2000. A particle sizer (LS230, Coulter, USA) estimated the volume average floc diameters as 73.6 μm. Confocal laser scanning microscope (CLSM) (Leica TCS SP2, Germany) was used to observe the internal floc structure. This microscope was equipped with an image processing software and the Argon laser source to stimulate the fluorescence. The microscope scanned the samples at fixed depth and digitized the image obtained. The sludge samples were first chemically fixed and then embedded in agarose for the fluorescence *in situ* hybridization (FISH). We used two different DNA probes, *Eub 338* (labeled by rhodamine) and *Arc 915* (labeled by tetrachlorofluorescein, TET). Eub 338 and Arc 915 were used for the detection of most eubacteria and methanogenic bacteria in anaerobic biosolids, respectively, with the following nucleotide sequences, respectively (Alm *et al.*, 1996):

Eub338: 5'-GCTGCCTCCCGTAGGAGT-3'

Arc915: 5'- GTGCTCCCCCGCCAATTCCT-3'

After the addition of probes, the hybridization was conducted at 50°C for one hour. The stained samples were washed three times (50°C for one hour) using hybridization buffer solution to remove extra probes.

Microtome slicing

The sludge samples was first chemically fixed by formalin buffer, embedded by agarose in the cassette, and then waxed in the paraffin. The block was then sliced into sections in thickness of 5 μm by microtome (Leitz Model 1400). The thin paraffin section was float in a water bath and, afterward, transferred to a glass slide. The slide was dried in air. Then the slice was put in an oven at 70°C for 10 minutes to melt the paraffin and dewaxed with xylene. Finally the slice was stained by hematoxylin and eosin (H&E) (Carson, 1990). For some non-biological materials (inorganic aggregates), the samples were embedded in OCT compounds (the composition is listed in **Table 2.5**), quenched by liquid nitrogen to –195°C, curing for 3 minutes, and then transferred to the working chamber of cryotome at –40°C. The impact of quenching on the floc morphology is quite minor and can be ignored. Cryotome

(CRYOTOME E, Shandon, UK) were used to section the samples into the slices of same thickness.

Scanning electron microscopy (SEM)

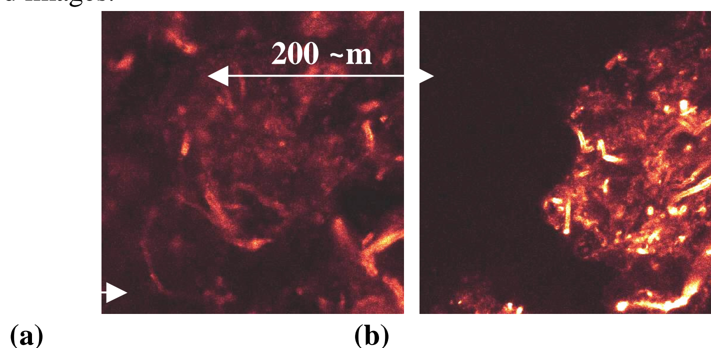
The sludge samples were first chemically fixed, dehydrated by ethanol, dried by critical-point CO_2, and coated by gold (SPISUPPLIES ION SUPTTER). The gold-coated flocs were ready for the observation of scanning electron microscopy (JSM-5600, JEOL, Japan). The voltage of the electron beams was set as 15 kV. The pre-treating procedure of sludge cakes was slightly different from the flocs. The dewatered cakes (obtained from the filter) were first embedded in high-melting-point agarose to keep the entire integrity. Some clump of agarose near the surface was taken away carefully and then immersed in the glutaraldehyde and OsO_4. The subsequent procedures were then completely the same as the sludge flocs. Remained agarose sticking on the cake (white portion) should be carefully removed to ensure the cake surface could be observed by electron beam.

Transmission electron microscopy (TEM)

The sludge samples were first chemically fixed, dehydrated by ethanol, and embedded in Spurr's resin. After block trimming, a rough sectioning was performed to obtain a section in thickness of 5 μm and stain by toluidine blue. This is to confirm if the sample was well embedded. An ultramicrotome was then used to slice the resin-fixed flocs into very thin sections in thickness of 0.06 μm. The sections were placed on copper grids (300 mesh size) for staining, using 5% uranyl acetate in methanol (to stain the proteins and nucleic acid) and 10% lead citrate in RO water (to stain the cell membrane), and then washed to remove the residual dyes. The stained flocs were then observed by transmission electron microscope (TEM, ZEISS EM109, Germany). The voltage of electron beam was set at 80 kV.

IV. Results and Discussion (結果與討論)

CLSM images of original and frozen/thawed sludge flocs are demonstrated in **Figures 1a** to **1b**, respectively. In the case of activated sludge, flocs are mainly composed by aerobes (eubacteria) and only the Eub338-staining can be noticed. For original sludge flocs, a porous structure surrounded by filamentous bacteria can be noticed. After freezing/thawing, the whole integrity became compact and the filamentous disappeared from the images (or compressed into the compact). Though the frozen/thawed pretreatments were reported to be effective in reducing microbial density in sludge, no significant decrease of the luminescence intensity was noticed. It might be inferred that those bacteria might be temporarily inactivated and still can be "detected" by FISH and CLSM. **Figure 2** showed the three-dimensionally reconstructed images.



(a) **(b)**

Fig. 1 CLSM images of sludge flocs: (a) Original; (b) Frozen/thawed

A proper staining is essential to visualize some specific groups of bacteria. For the sludge, only 70-90% of the suspended masses are composed of microbial cells. Thus not all materials can be well observed. Also due to the unexpected excitation of non-stained matters by the laser, some ghost images might occur which lower down the quality of images.

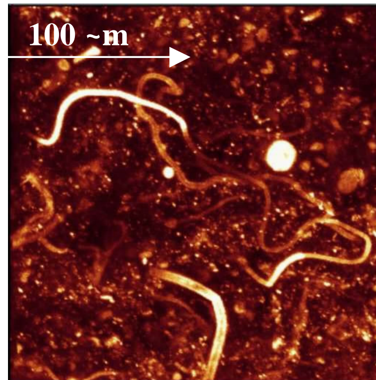


Fig. 2 Tomograph images reconstructed from CLSM images