Removal of micro-particles by microbial granules used for aerobic wastewater treatment

V. Ivanov, J.-H. Tay, S.T.-L. Tay and H.-L. Jiang

School of Civil and Environmental Engineering, Nanyang Technological University, Blk N1, 50 Nanyang Avenue, Singapore 639798. (E-mail: *cvivanov@ntu.edu.sg*)

Abstract Microbial granules with a diameter from 0.4 mm to 3.0 mm have been produced by fast sedimentation and retention of microbial aggregates in sequencing batch airlift reactors used for model wastewater treatment. The wastewater was with or without addition of calcium salt. The granules were able not only to degrade organic matter but to remove nano- and micro-particles from wastewater due to micro-channels and pores in the matrix of the granules. To detect the removal of 0.1 μm, 0.6 μm, 4.2 μm fluorescent microspheres, and cells of *Escherichia coli*, stained by permeable nucleic acid stain SYTO9™, the granules were incubated with these particles. The rate of particle removal and their accumulation in the granules was measured by a Fluoview300 confocal laser scanning microscope (CLSM) (Olympus, Japan); a FACSCalibur flow cytometer (Becton Dickinson, CA, USA), and a fluorescence spectrometer LS-50B (Perkin-Elmer, UK). The release or removal of biological and non-biological particles was analyzed by a flow cytometer after DNA staining. Total number of the particles bigger than 0.1 μm in the reactors was approximately 4×10^7 per ml, and 23% of these particles were bacterial cells. The 0.1 μm and 4.2. μm microbeads were accumulated within 250 μm in the upper layer of the microbial granule but externally added cells of *Escherichia coli* penetrated to the depth of approximately 800 μm in the granules without calcium addition. Microbial granules contained also attached ciliates but accumulation of the particles in protozoan cells was smaller than in the granule matrix. Kinetics of particle sorption was revealed by flow cytometry and fluorescence spectrometry. Almost half of the stained cells of *E. coli* can be removed by the granules for one hour. The ability of the microbial granules to remove the particles can enhance their function in aerobic treatment of wastewater.

Keywords Aerobic; microbial granules; particle removal; wastewater

Introduction

Quality and quantity of the particles in water play an important role in water quality. Since particles may be harmful themselves or due to accumulation of the hazardous pollutants at their surface, their removal from water or wastewater is essential for the treatment process (Kern *et al*., 1998). Physicochemical removal of the particles, such as precipitation and flocculation, has been traditionally employed in wastewater treatment. Biosorption of nano-and microparticles are not so effective but may be preferred due to low costs. Microbial biofilms, used in wastewater treatment, are capable of particle retention, due to the adsorption and deposition in the caverns and pores of the biofilm (Okabe *et al*., 1997; Eisenmann *et al*., 2001).

A goal of this research was to examine the ability of a specific kind of biofilm, microbial self-aggregated granules, to remove nano- and microparticles from wastewater. Microbial granules produced during aerobic wastewater treatment are aggregates having several advantages over conventional activated sludge flocs, such as a strong and compact structure, improved settling ability, and higher biomass retention (Morgenroth *et al*., 1997; Beun *et al*., 1999; Tay *et al*., 2001). Aerobic granules are spherical biofilm structures where microbes are attached to each other and embedded in an extracellular matrix. There are the layers of aerobic heterotrophic bacteria, nitrifying bacteria, anaerobic bacteria, and dead cells in microbial granules (Tay *et al*., 2001; Tay *et al*., 2002a,b; Tay *et al*., 2003; Toh *et al*.,

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2003). The pores and channels were also detected by measuring the penetration of 0.1 μm fluorescent beads into the granule interior (Tay *et al*., 2003).

Granule structure can be manipulated through changing the medium composition. For example, Ca^{2+}-fed granules were denser, more compact, and had higher polysaccharide contents (Jiang *et al*., 2003). These granules can treat wastewater with high organic loading rates (Moy *et al*., 2002). However, little is known about the ability of these Ca-enriched microbial granules to remove small particles. Divalent metal ions such as calcium are thought to play an important role in the self-immobilization of microbial biomass. Calcium ions can decrease negative cell surface charges (van Loosdrecht *et al*., 1987), and then enhanced microbial aggregation takes place (Mahoney *et al*., 1987; Yu *et al*., 2001). The main objective of this study was therefore to evaluate efficiency of particle removal from wastewater by aerobic microbial granules produced under different calcium concentrations.

Material and methods

Two identical laboratory-scale sequencing batch reactors (SBRs) were operated in parallel for the experiments. R1 was the control reactor, with no calcium supplement. R2 was a reactor with calcium concentration of 100 mg l^{-1} (Jiang *et al*., 2003). Each reactor had a working volume of 2.0 l. The internal diameter of a column was 5.0 cm. The experiments were performed in a temperature control room at 25°C. Aerobic sludge from Jurong Water Reclamation Plant (Jurong, Singapore) was used as inoculum. The inoculum concentration was 3.0 g SS l^{-1} in each reactor. Air was supplied by a fine bubble aerator at the bottom of the columns at a superficial air velocity of 0.03 m s^{-1}. A synthetic wastewater was used. The composition of the concentrated medium was as follows: ethanol 0.46 g l^{-1}, sodium acetate 0.547 g l^{-1}, NH_4Cl 0.15 g l^{-1}, KH_2HPO_4 0.168 g l^{-1}, KH_2PO_4 0.066 g l^{-1}, $MgSO_4 \cdot 7H_2$ 0.06 g l^{-1}, trace solution 1 ml l^{-1} (Beun *et al*., 1999). Each reactor was operated with a cycle length of 4 hours. One cycle consisted of 2 min influent addition, 233 min aeration, 2 min settling and 3 min effluent withdrawal. The HRT was 8.0 hours and the COD loading was kept constant at 4 g COD l^{-1} d^{-1}. The pH value was from 7.0 to 8.0.

Viewing of the aerobic granules was accomplished using a scanning electron microscope (Stereoscan 420, Leica Cambridge Instruments). The aerobic granule samples were soaked in separate aluminum dishes containing 2% glutaraldehyde for 2 hours. This was followed by three consecutive washes of 20 minutes each in 0.1 mol l^{-1} sodium cacodylate buffer solution. The samples were then dehydrated in a series of 10 minutes washes in 50, 70, 85 and 95% ethanol. For the storage of samples, 100% ethanol was used. The granules were dried to the critical point with carbon dioxide. As the SEM illuminates the granule samples with electrons, they also have to be made to conduct electricity. The dried samples were coated with a very thin layer of gold by a sputter coater at 20 mA in high vacuum.

To detect the consumption of the particles five intact granules were suspended in 0.2 ml of phosphate-buffered saline (PBS) with the addition of the particles of different size. The granules were incubated for 15, 30 and 60 min in 0.2 ml Eppendorf tubes in the shaker at 100 rpm in the suspension of different particles: (a) in the suspension of 0.1 μm microspheres from TetraSpec Fluorescent Microsphere Standards® (Molecular Probes, OR, USA); (b) in the suspension of 4.2 μm microspheres from TetraSpec Fluorescent Microsphere Standards® (Molecular Probes, OR, USA); and (c) in the suspension of the cells of *Escherichia coli* stained by permeable nucleic acid stain SYTO9 (Molecular Probes, OR, USA). The staining of the cells was performed at a final concentration of SYTO 2 μmol l^{-1} for 1 hr in the dark at room temperature. The cells were washed out from the excess of the stain twice by the incubation with 0.2 ml PBS for 30 min and centrifugation.

The images of the granules were acquired by a Fluoview300 confocal laser scanning microscope (CLSM) (Olympus, Japan). Green and red fluorescence was excited by a 10 mW argon laser at 488 nm, and was measured by channel 1 with longpass filter 510 nm, and channel 2 with bandpass filter 580 nm – 640 nm after the splitting filter 570 nm. An Olympus UPLAPO 10× objective with a numerical aperture of 0.3 was used to study the samples. Sets of XY profiles (plain view profile) and XZ profiles (cross sectional profile) were produced along with relevant relative fluorescence intensity graphs in the Z direction. The relative fluorescence intensity graphs are for the purpose of quantification of the fluorescence of the profiles. The photo detector sensitivity for the measurement of green and red fluorescence was adjusted manually to ensure maximum fluorescence of the microbial aggregate and minimum background noise.

Counts of the particles were performed with a FACSCalibur flow cytometer (Becton Dickinson, CA, USA) equipped with an air-cooled argon laser (488 nm, 15 mW) and red diode laser (635 nm). The impulses from labeled microbial cells were discriminated from other events by comparing the dot plots of FL3 (red fluorescence of the cells selected by longpass filter 670 nm) and FSC (forward scatter of the light) in the experimental samples and in negative non-stained controls. Consumption of the fluorescent particles was evaluated also by luminescence spectrometer LS-50B (Perkin-Elmer, USA) by measuring the synchronous scan of fluorescence distance between excitation and emission of 20 nm.

Results and discussion

Microbial granules in different reactors

The oxidation activity of the granules in R1 was higher than that in R2. The SOUR of R1 was calculated to be 550 mg DO g SS^{-1} h^{-1} as compared to 82 mg DO g SS^{-1} h^{-1} for R2. Probably, the cause is that the granules in R1 were smaller and the surface to volume ratio was higher than that for the granules in R2. Average granule diameters for R1 and R2 were 272 μm and 750 μm, respectively (Figure 1).

Concentration of particles within the liquid phase in reactors 1 and 2

The numbers of inorganic and biological particles with different sizes within the liquid phase in R1 and R2 were evaluated by flow cytometry (Figure 2). Although the total concentration of the particles in the two reactors was almost the same, the concentrations of both biological particles and non-biological particles were different. The concentration of biological particles in R1 was 22.8×10^6 ml^{-1} and higher than that in R2, while the concentration of inorganic particles in R2 reached 29.6×10^6 ml^{-1} and was higher than that in R1 (Table 1). The results show that the addition of calcium can enhance the removal of biological but not non-biological particles from wastewater. In fact, calcium ions can decrease

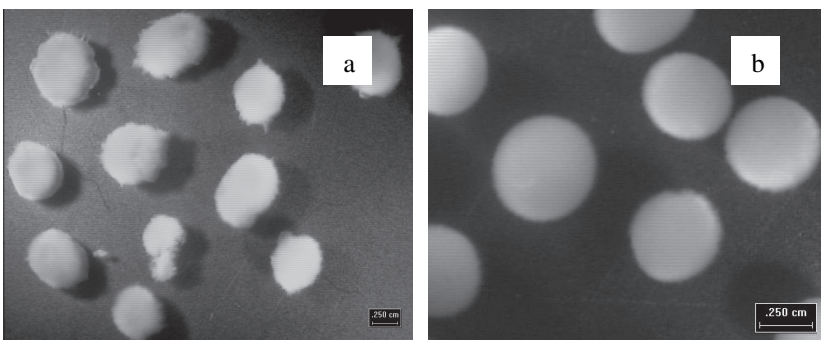

Figure 1 Images of granules in R1 (a) and R3 (b)

Table 1 Concentration of biological and non-biological particles bigger than 01. μm in the reactors R1 and R2

Particles	Concentration in R1 (10^6 ml^{-1})	Concentration in R2 (10^6 ml^{-1})
Total number of particles bigger than 0.1 μm	40.4	38.8
Biological particles	22.8	9.2
Biological particles smaller than bacteria	13.3	7.1
Biological particles of bacterial size	9.5	2.1
Non-biological particles	17.6	29.6

negative cell surface charges (van Loosdrecht *et al*., 1987) and stimulate microorganisms to attach to each other (Mahoney *et al*., 1987; Yu *et al*., 2001). Therefore, augmentation of calcium may increase the possibility of attachment of microbial cells to aerobic granules and then decrease the number of biological particles in the effluent.

Accumulation of the particles in the granules

The CLSM was used to determine the sorption capability of the granules in R1 and R2. In the series of graphs shown in Figure 3 (data for reactor R1) and Figure 4 (data for reactor R2), the 0.1 μm and 4.2 μm microbeads were concentrated in the upper layer of the granules to a depth 250 to 300 μm from the edge of the granule. Cells of *E. coli* penetrated to a depth of approximately 800 μm in the granules from R1 and only to a depth of 250 μm in the granules from R2 (calcium-enhanced granules).

Kinetics of sorption and desorption of the particles

The kinetics of the sorption and desorption of particles during incubation of the granules in buffer solution was studied using fluorescence spectrometry. Fluorescence of stained *E.*

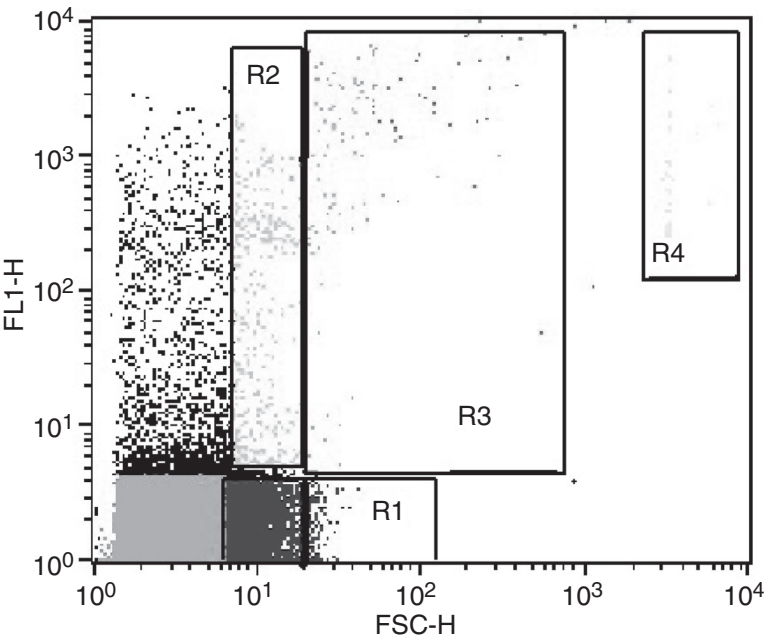

Figure 2 The regions on dot plot used for the enumeration of the particles in the reactors. FSC-H and FL1-H axes are the heights of the peaks of forward scattered light and green fluorescence, respectively. The regions are as follows: R1, inorganic particles; R2, biological particles smaller than bacteria; R3, biological particles of bacterial size; R4, the beads of 6 μm and their pairs used for the enumeration of the particles. The data related to the particles from reactor 1

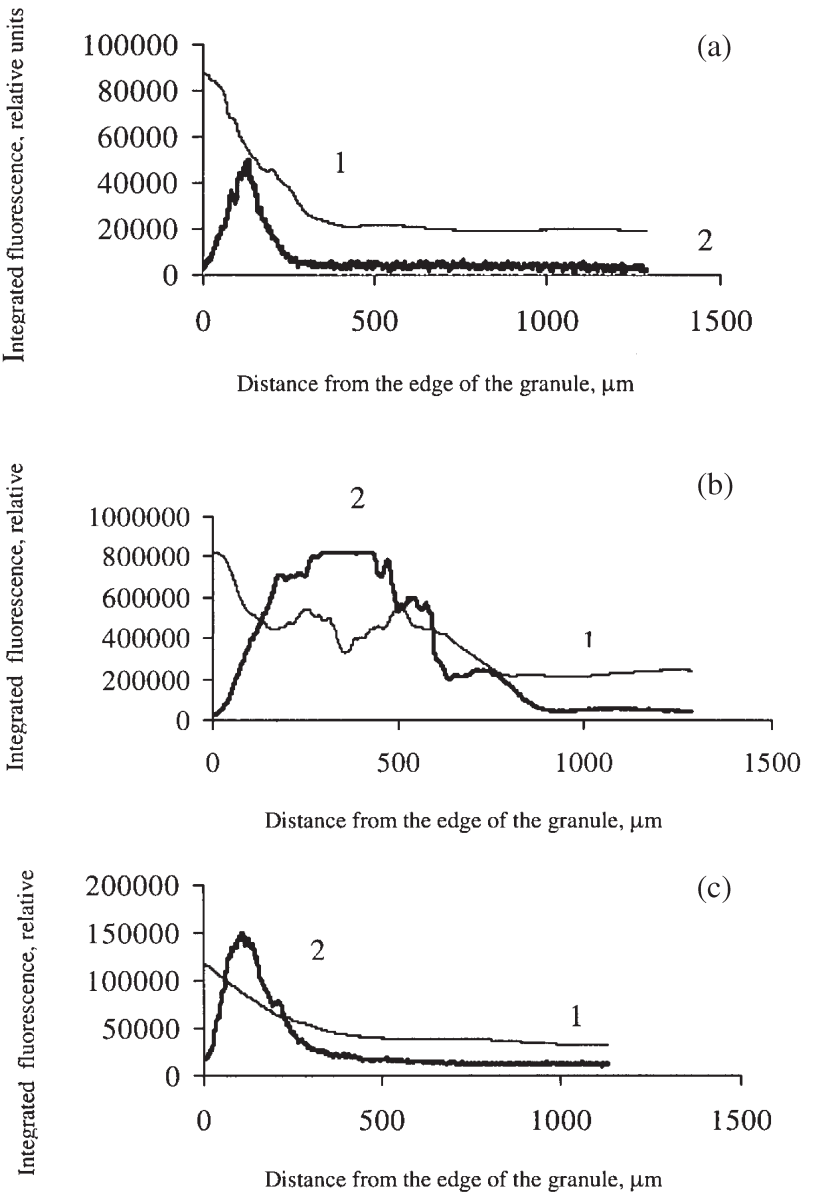

Figure 3 Penetration of 0.1 μm beads (a), cells of *E. coli* (b), and 4.2 μm beads into the granule from R1. Curve 1 shows intensity of transparent light

coli cells dropped to 53% (granules from R1) or 61% (granules from R2) of the initial level after 1 h of incubation of the granules with bacterial cells. Bigger particles, 4.2 μm fluorescent beads, were not adsorbed by the granules. Adsorption of smaller fluorescent beads cannot be studied by fluorescent spectrometry because fluorescence was increased during incubation, probably, due to the release of some fluorescence-enhancing substances from the granules.

Flow cytometry demonstrated that it was a balance of released and consumed bacterial cells during incubation of the granules from R1 (Table 2). However, bacterial cells detached actively from the granules grown with addition of calcium (Table 2). The balance of small particles, with size between 0.1 to 0.6 μm, was almost zero for the granules from R1, but more particles were detached from the granules grown with the addition of calcium

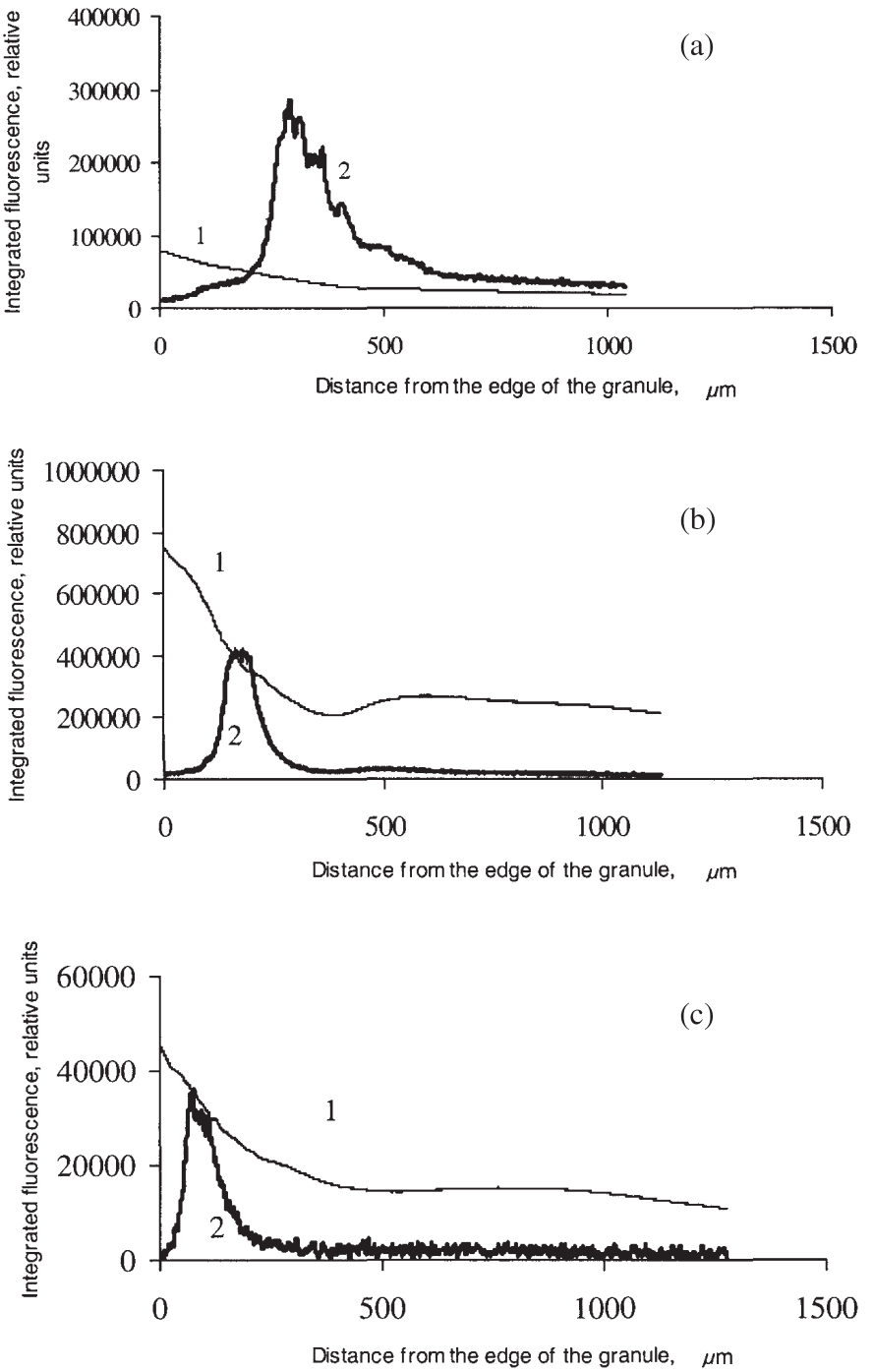

Figure 4 Penetration of 0.1 μm beads (a), cells of *E. coli* (b), and 4.2 μm beads into the granule from R2. Curve 1 shows intensity of transparent light

(Table 2). Therefore, calcium is not a favorable element for the removal/adsorption of bacterial cells by the microbial granules used in wastewater treatment.

Role of protozoa in the consumption of the particles

Addition of calcium did not affect the colonization of the granule surface by protozoa

Table 2 Changes of the concentration of the particles (10^6 ml^{-1}) in water during incubation of the granules from R1 (without calcium) and R2 (with calcium)

Time of incubation (min)	Sum of 0.1 μm and 0.6 μm particles		Bacterial cells	
	R1	R2	R1	R2
0	11.0	15.6	5.4	4.5
15	10.6	16.2	5.3	10.9
30	n.d.	19.0	5.2	10.8
60	9.9	20.3	5.1	15.9

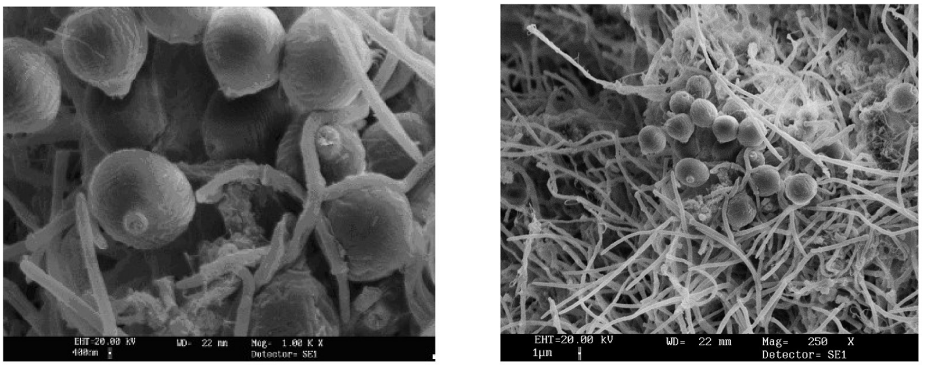

Figure 5 Cells of protozoa on the surface of microbial granule from R2 (different magnifications are shown)

shown in Figure 5. It was 63.6 ± 11.3 and 67.4 ± 10.1 cells of protozoa mm^{-2} of granule surface for the granules from R1 and R2, respectively. This low level colonization and equal distribution of adsorbed cells of *E. coli* in the upper layer on the granule to the depth 800 μm showed that consumption of bacterial cells by protozoa did not play a significant role in the removal of bacterial cells from wastewater by microbial granules.

Conclusions

The microbial granules can remove bacterial cells and smaller particles from wastewater. Accumulation of these particles must be accounted for in the design of wastewater treatment by microbial granules and their disposal or re-utilization. Addition of calcium does not enhance the removal of cells and non-biological particles from wastewater treated by microbial granules. The microbial granules contain protozoa but their role in the removal of suspended bacterial cells from wastewater is not significant.

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