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Granule Development in Anaerobic Baffled Reactors

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Abstract

The mechanism of biomass granulation in anaerobic reactors has been widely studied in the hope that this could be helpful in manupulating the rate and extent of granule formation, particularly in wastewaters that show little intrinsic propensity to granulate (e.g. fat and oil containing effluents). Similarly, in this study, sets of sludge samples were taken from Normal Fed Anaerobic Baffled Reactors (NFABRs) and Split-Fed Anaerobic Baffled Reactors (SFABRs) treating brewery and ice-cream wastewater in order to evaluate the development of granulation and compare the rate of compact granule formation in the reactors. The internal architecture of the granules and the role of extracellular polymers (ECPs) on granule formation were also investigated.

SFABRs proved to be a potential anaerobic reactor configuration for granule formation. It was revealed that the ability of granule formation in SFABRs can even extend to complex wastewaters, i.e. fat-bearing wastewaters, in a considerably short time. The mechanism governing stratification of bacterial population in granules was explained. The internal architecture of the granules and the role of ECPs on granule formation were speculated on with the changes observed on SEM pictures. The findings confirmed that ECPs are central to the process of granulation. Based on the granular growth course, the granulation process is categorized into three phases: (i) initiation (ii) adaptation and maturation and (iii) granulation.

Key words: Anaerobic baffled reactor (ABR), Split-fed anaerobic baffled reactor (SFABR), Granule development, Extracellular polymers (ECPs).

Introduction

Although Upflow Anaerobic Sludge Blanket (UASB) reactors have been built and operated since 1971, it was not until 1974-1976 that a particularly desirable variant of anaerobic sludge, granular sludge, was observed inside a pilot plant treating sugar beet factory wastewater. The superior settling characteristics of these sludge granules allowed higher sludge concentrations to be retained and consequently permitted the system to achieve much higher organic loading rates. Granular sludge development has now been observed in UASB reactors treating many different types of wastewater (de Zeeuw, 1987).

The phenomenon of granulation is a process in which a non-discrete flocculent biomass begins to form discrete well-defined pellets, or granules. These vary in dimension and appearance depending on the wastewater and reactor conditions, but generally have a flattened spherical geometry with a diameter of 1-3 mm.

Granule formation in an anaerobic baffled reactor (ABR), like any other anaerobic reactor is a complicated process involving microbiological and physicochemical reactions among cells, and their interactions with environmental parameters (i.e. nutrients, hydraulic forces due to flow and gas production, temperature and pH). Observation of the course of granule development expands our knowledge of the granulation process and may allow the possibility of obtaining appropriate levels of granulation in ABRs.

Materials and Methods

The reactors

Two identical reactors (except for the feeding regime), 200 mm wide, 600 mm long and 1000 mm deep, were constructed for the presented study. Figure 1 shows a schematic diagram of the Normal Fed ABR (NFABR) and the Split-Fed ABR (SFABR). Construction was from perspex, a transparent material, with the active reactor volume (100 l) divided into four equal 25 l compartments, each separated by vertical baffles. Within each compartment, downcomer and riser regions were created by a further staggered vertical baffle. The width of the downcomer was 4 cm, (associated wet volume of 6.67 l), and the riser was 11 cm, (associated wet volume of 18.33 l). The lower parts of the downcomer baffles were angled at 45 degrees in order to direct the flow evenly through the riser according to the general arrangement of Bachmann (Bachmann *et al.*, 1983 and 1985). This produced effective mixing and contact between the wastewater and anaerobic sludge at the base of each riser. Each compartment was equipped with sampling ports that allowed biological solids, gas, and liquid samples to be withdrawn. In addition, the sidewalls were enclosed within a water-jacket to maintain the reactor temperature at 35 °C.

Peristaltic pumps (Watson 100 series) were used to control the influent feed rate to the NFABR and to split the influent feed to each compartment in the SFABR.

Experiments were carried out with the NFABR and SFABR operating in parallel, each being set to the same increments in organic loading rate throughout the experiment. The influent to the NFABR was fed via a single point at the front of the reactor while the influent to the SFABR was divided and fed via a bottom inlet in each compartment. Details of the reactors are shown in Figure 1.

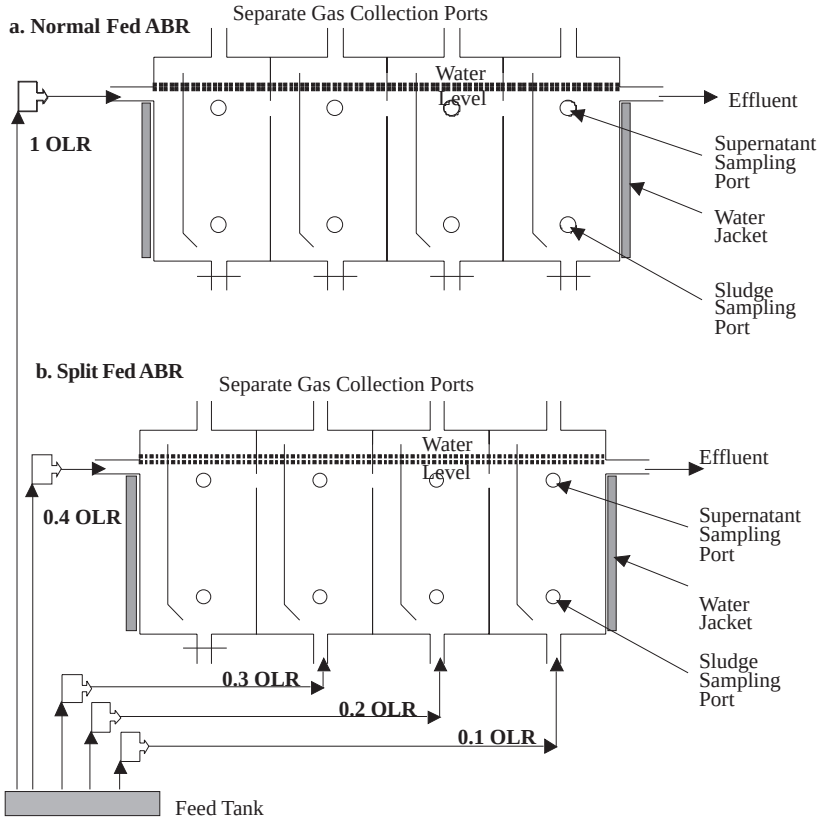

Figure 1. Schematic diagram of a Normally Fed Anaerobic Baffled Reactor (NFABR) and a Split-Fed Anaerobic Baffled Reactor (SFABR) showing an example of the split feed ratio as a fraction of the total organic loading rate (OLR).

Wastewater characteristics and operational variables

The study was carried out in phases, depending on the requirements and findings of both. The first and the third phases were carried out with ice-cream wastewaters and the second with brewery wastewater. The reason for this was that some problems were encountered in the first phase due to the complexity of the wastewater used, i.e. ice-cream wastewater, and due to a potential problem in the conventional ABR during the start-up period resulting from the near plug-flow characteristics of this configuration. Therefore, the second phase focused on the development of a novel anaerobic baffled reactor configuration to eliminate the problems, and the findings could be applied to the third phase to assess if any improvements could be achieved. Therefore, in the second phase a less complex wastewater, i.e. brewery wastewater, was preferred.

The first phase, which required 190 days, used two NFABRs one of which was dosed with polymer after the start-up period to investigate and compare the effect of polymer amendment.

The second phase, which required 140 days, consisted of two runs, each with two reactors. The first run of the second phase was to compare the effect of feed splitting. After this run, the reactor giving the better results was selected and applied to the second run, which investigated the effect of polymer-conditioned sludge and granular sludge usage as seed. At the end of the second phase, comparisons were made to select the best two of four reactors used in the second phase. The selected reactor configurations were used in the third phase.

The third phase, which required 70 days of continuous operations, investigated the competence of the selected reactor configurations on the treatment of ice-cream wastewater.

Operational variables applied in the study are in Table 1. In the first phase, the strength of the feed was kept constant and the flow rate was increased in order to increase the organic loading rate (OLR). In the following phases, the flow rates was kept constant and the strength of the feed was increase to raise the OLR. The main reason was that the size of the reactors (100 l) made feed preparations difficult for large volumes of feed requirements in the first phase for the OLRs in excess of 5 kg $COD.m^{-3}.d^{-1}$.

The feed was supplemented with a number of nutrients and trace elements in order to provide a balanced feed to the reactors (COD: N: P, 250: 7: 1). The micro-nutrient deficiency was corrected according to Kasapgil (1994).

Seed sludge

The reactors were seeded with anaerobically digested sewage sludge taken from a completely mixed anaerobic digester at a local municipal sewage treatment works. It was first sieved (< 3 mm) to remove any debris and large particles and was then introduced into all four compartments of each reactor. Each 25 l compartment contained 7.2 l sludge with a suspended solids composition of 36 600 mg SS/l and 22 300 mg VSS /l giving a total of 644 g VSS in each reactor. This value (6.44 g VSS/l) is in accordance with the initial VSS values used in other studies on ABRs (Barber and Stuckey, 1999). The remaining parts of each compartment were filled with tap water. After seeding the reactors, the lids were sealed, and the head space above each compartment was flushed with oxygen-free nitrogen gas in order to displace residual air from the system. The reactors were then allowed to stabilize for 24 h without further modification before starting the experiments.

Sampling and analysis

Throughout the experiment sludge samples were taken from each compartment of all reactors at 15-day intervals and the biomasses were examined by scanning electron emicroscopy (SEM). Samples were first fixed for 4 h at room temperature with 2% (w/v) glutaraldehyde in Sorenson's phosphate buffer, and dehydrated through a graded series of water-ethanol mixtures (10%, 25%, 50%, 75%, 90%, and 100%). The samples were brought to equilibrium in each mixture for 10 min, and finally dried by the critical-point drying method before sputter-coating with gold particles. The samples were then examined in a scanning electron microscope at $4 \sim 8$ kV (Cambridge). Micrographs were produced at magnifications between 10x and 15,000x.

Volatile fatty acid (VFA) was analysed using an Unicam 610 series gas liquid chromatograph with auto injector and PU 4811 computing integrator. The operating conditions were as follows: column: 2000 mm x 2 mm I.D. glass packed with 10% AT-1000 on 80/100 chromosorb W-AW, detector temperature: 180 °C, column temperature: 140 °C, carrier gas: nitrogen at 20 ml/min.

Table 1. Operational variables and characteristics

	Run		OLR	HRT	COD	Operation
Phase	No	Feed				Time
			(kgCOD/m^3.day)	(Days)	(mg/l)	(Days)
I	I.1	Ice-cream	0.62	10	6200	43
			0.95	6.5	6200	25
			1.83	3.4	6200	26
			3.04	2.04	6200	48
			5.2	1.2	6200	27
			10	0.62	6200	9
			14.4	0.43	6200	12
II	II.1	Brewery	0.9	2	1800	15
			1.5	2	3000	15
			2.75	2	5500	15
			5.5	2	11000	15
			10.5	2	21000	10
	II.2	Brewery	0.9	2	1800	15
			1.5	2	3000	15
			2.75	2	5500	15
			5.5	2	11000	15
			10.5	2	21000	10
III	III.1	Ice-cream	0.9	2	1800	15
			1.5	2	3000	15
			2.75	2	5500	15
			5.5	2	11000	15
			10.5	2	21000	10

The sludge surface charge of the biomass samples from each compartment was measured by the colloid titration technique (Kawamura and Tanaka, 1966, Morgan *et al.*, 1990, Jia *et al.*, 1996). In this method, a known amount of positive polymer was added to the sample, and then the excess positive charge was back-titrated with a negatively charged polymer, allowing the surface charge to be established by difference. 0.001 N Potassium salt of polyvinyl sulfate (PVSK) (Sigma) and 0.001N polybren (Sigma) were used as standard anionic and cationic colloids, respectively. Toluidine blue (Sigma) was used as an indicator to show the electrical end-point of the titration.

Results and Discussion

Granule Development

The photomicrographs of floc and granule samples taken from the NFABR and SFABR treating brewery wastewater are shown in Figures 2 and 3, respectively.

Figures 2a, 2b, 3a and 3b show that a mixed population of anaerobic bacteria and inert material forms the nuclei at the initial stage of granule formation, and after 45-52 days the flocs develop into granules. Although flocs generally showed a mixed population, Figure 2c reveals a predominance of filamentous- type bacterial cells. This may have resulted from the specific substrates prevailing in Compartment 3 of the NFABR. It was established clearly that the composition of the carbon source in the wastewater has a strong influence on the characteristics of granular sludge and population dynamics (Grotenhuis *et al.*, 1988, Yang and Anderson, 1993, Syutsubo *et al.*, 2001). It was established that the NFABR had partial phase separation such that initial compartments received high concentrations of acetate, whilst the final compartments received low concentrations of acetate, the latter favoring filamentous- type bacteria (Uyanik *et al.,* 2002 a and b). For example, Compartments 1 and 2 had acetate concentrations of around 465 mg/l and 1450 mg/l, respectively, on day 45, while Compartments 3 and 4 had acetate concentrations of around 158 mg/l and 63 mg/l, respectively on Day 45 in the NFABR.

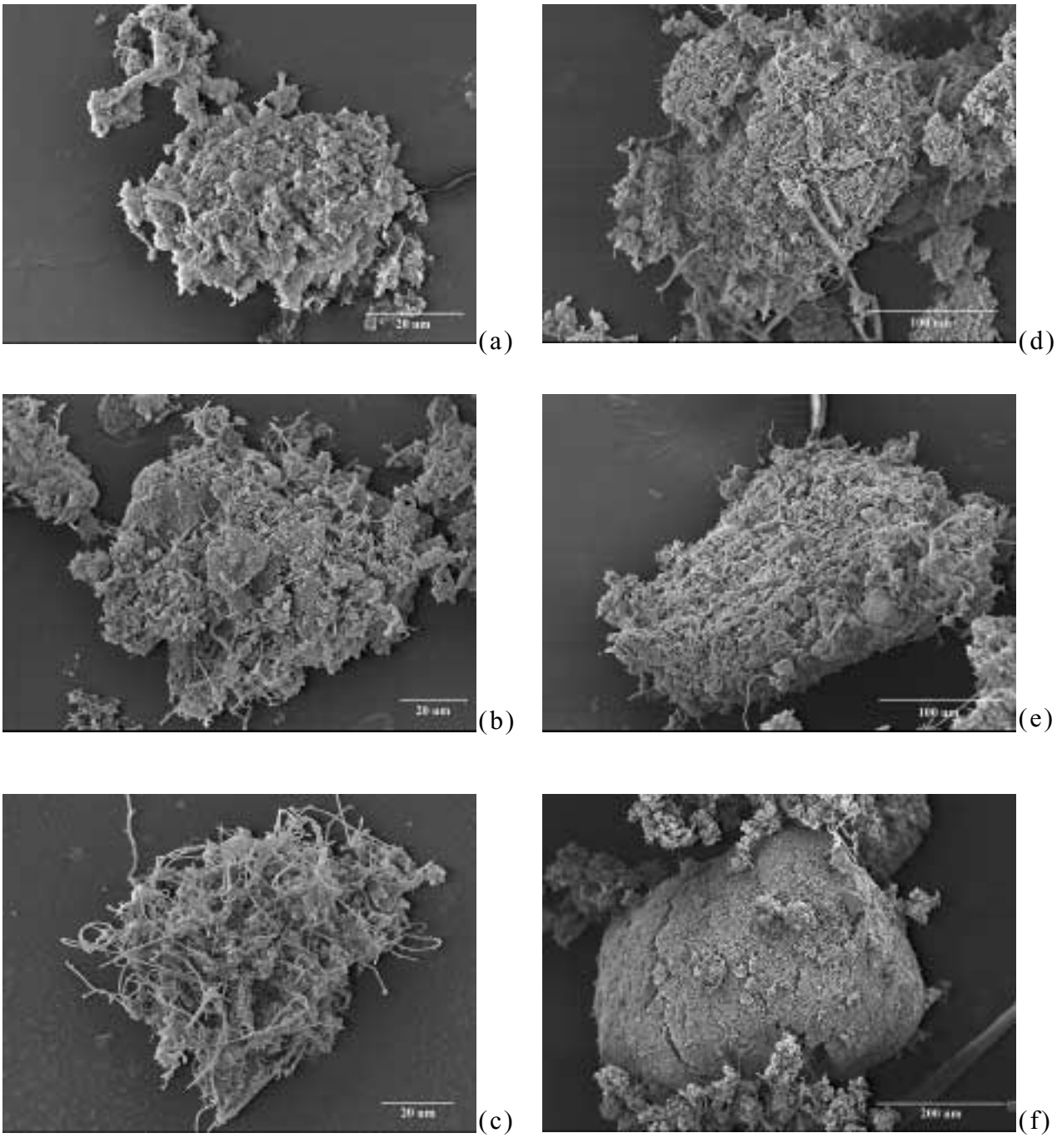

Figure 2. Scanning electron photomicrographs of granule development in Compartment 3 of the NFABR treating brewery wastewater: after (a) 15 days, (b) 30 days, (c) 45 days, (d) 52 days, (e) 60 days and (f) 70 days.

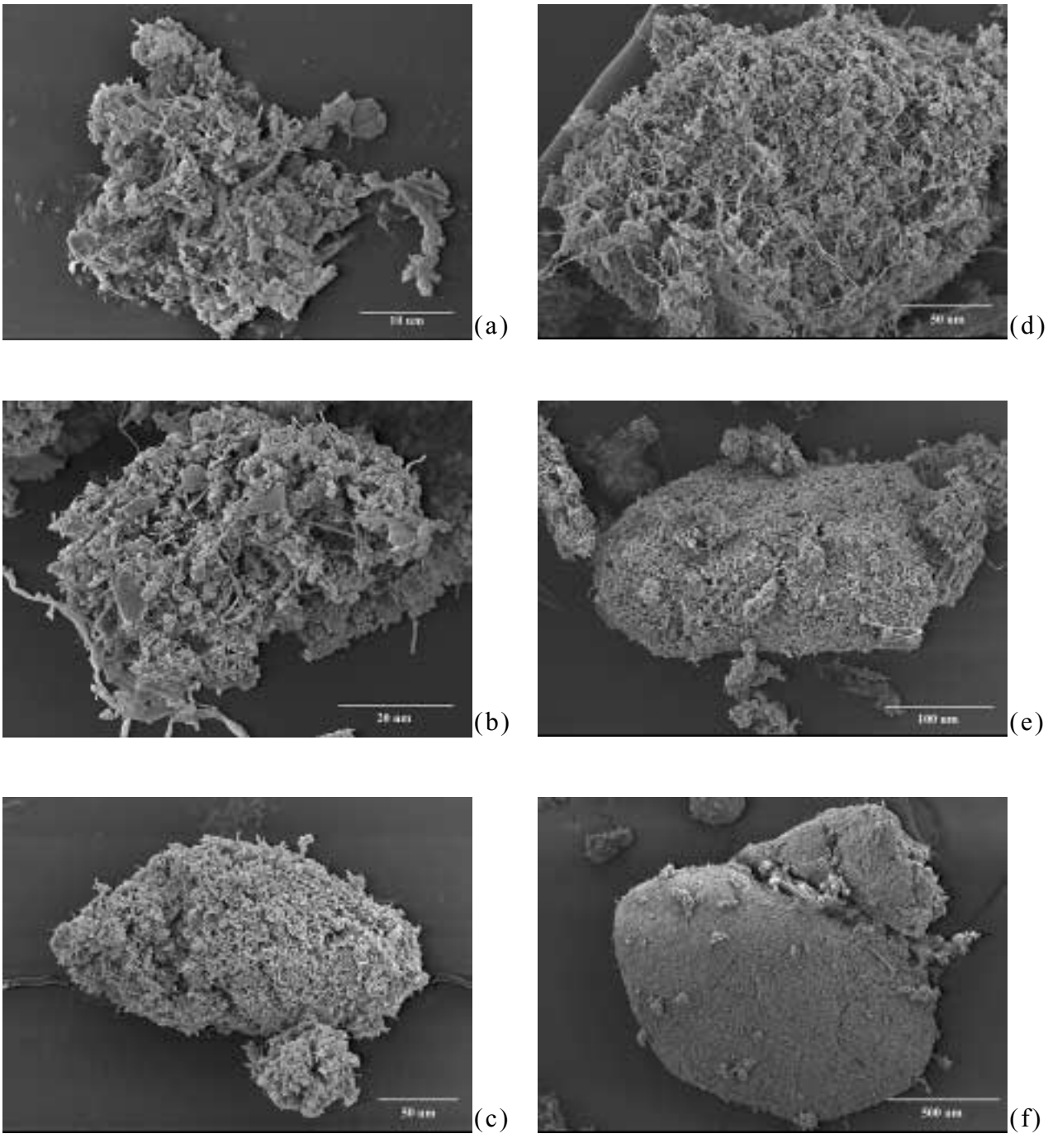

Figure 3. Scanning electron photomicrographs of granule development in Compartment 3 of the SFABR treating brewery wastewater: after (a) 0 days (seed), (b) 15 days, (c) 45 days, (d) 52 days, (e) 60 days and (f) 70 days.

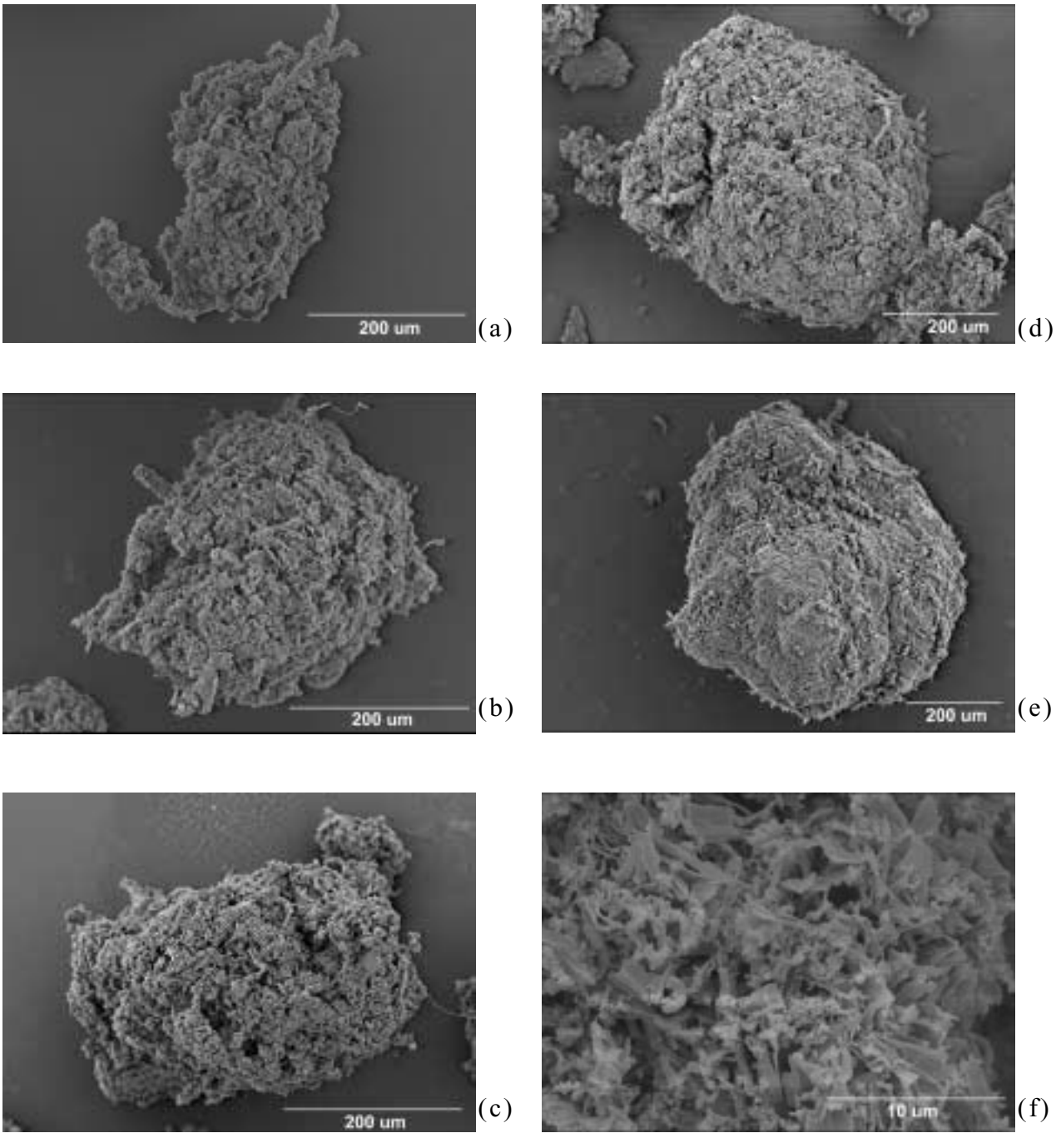

Figure 4. Scanning electron photomicrographs of granule development in Compartment 3 of the polymer-amended SFABR treating ice cream wastewater: after (a) 15 days, (b) 30 days, (c) 45 days, (d) 60 days and (e) 70 days. Figure 3f highlights the effect of inert material on granule formation; the sludge sample for this figure was taken from Compartment 1 of the polymer-amended SFABR treating ice cream wastewater on day 30.

On the other hand, the SFABR is believed to provide a well-adjusted substrate composition for each compartment and the finding of mixed populations in all compartments of the SFABR was expected. For example, Compartments 1 and 2 had acetate concentrations of around 290 mg/l and 266 mg/l, respectively, on day 45, while Compartments 3 and 4 had acetate concentrations of around 129 mg/l and 83 mg/l, respectively, on day 45 in the NFABR. After 45 days, maturation of granules in both reactors occurred as granule dimensions increased substantially (Figures 2, 3d,e, f).

The photomicrographs of the sludge samples taken from Compartment 3 of the polymer amended SFABR treating ice-cream wastewater can be seen in Figures 4a,b,c,d and e, and shows a similar course of development to the other reactors. However, the size of the initial flocs and granules was much larger than that in the other reactors. It is thought that this was a direct result of polymer amendments in the seed, and not the ice-cream wastewater, as the latter contains lipids, which are known to be inhibitory towards granulation (Petruy and Lettinga, 1997).

Granule structure

The observation of mixed culture in the initial stage of granule formation appears to contradict the widely held belief that filamentous *Methanosaeta* form the initial nuclei and promote granule formation i.e. spaghetti theory (Wiegant, 1987). The observation that a diverse bacterial population was associated with inert material of granular nuclei also contradicts the idea that a relatively homogeneous population of acidogenic bacteria or other specific bacteria is responsible for the initial stage of granule formation (Macleod *et al.*, 1990, Vanderhaegen *et al.*, 1992, El-Mamouni *et al.*, 1997).

One reason why a filamentous type of anaerobic bacteria has previously been considered to initiate granule formation is the observation of a multilayered structure in mature granules (Quarmby and Forster, 1995). A similar stratification of bacterial populations was also observed in this study in mature granules taken from Compartment 3 of the NFABR treating brewery wastewater in Phase I (Figures 5a,b and c), Compartment 3 of the polymer-amended NFABR treating brewery wastewater in Phase I (Figures 5d,e and f), and Compartment 4 of the SFABR treating ice cream wastewater (Figures 6a,b and c). Having looked at the naturally occurring cavities on the granules before and/or after dissection, these granules showed a morphologically distinct population of bacteria in the deep section compared with those at the surface, with a considerable part of the granule interior consisting of filamentous and long rod-shaped bacteria, whilst the outer layer comprised short rod-shaped and coccoid bacteria. This may have led some researchers to believe that *Methanosaeta* ssp. are the primary initiator of granule formation. However, it is our belief that the predominance of *Methanosaeta* within granules takes place not at the initial stage of granule formation but subsequently, when the flow of substrates through the granule matrix leads to the development of an internal growth environment substantially different from that at the surface of the granule (in the bulk phase of the reactor). *Methanosaeta* are known to grow on only acetate and are also favored by low acetate concentrations (Schmidt and Ahring, 1996). Consequently, granules possessing a surface horizon of highly active acetogenic bacteria would be more likely to contain acetate as the major metabolite in the interior horizon, allowing *Methanosaeta* to predominate within the interior of most granules.

The internal architecture of granules may also depend on the VFA concentration. When substrate diffuses into a granule, a breakdown of complex VFA happens at the surface horizon with the production of hydrogen and acetate, which are later utilized in the interior region of the granule by low acetate utilizers and hence predominated by them. However, if the external concentration of VFA is high, complex VFA may also diffuse into the center of the granule. Consequently, the presence of low acetate as the sole substrate may not take place, leading to the possibility of a greater diversity existing in the center of these granules. This is supported by the SEM of a sectioned mature granule sample taken from Compartment 2 of the NFABR treating brewery wastewater in Phase II in which a relatively similar microbial population can be seen in both the internal and surface regions (Figures 6d,e and f). Since high concentrations of complex VFA were generally observed in Compartments 1 and 2 of the NFABR, favorable conditions are unlikely to exist here for the establishment of stratified granules.

Extracellular polymers

Extracellular polymers (ECPs) are known to have wide ranging implications in cell-surface adhesion and to be essential for the initiation, formation and stability of anaerobic reactor granules (Quarmby and

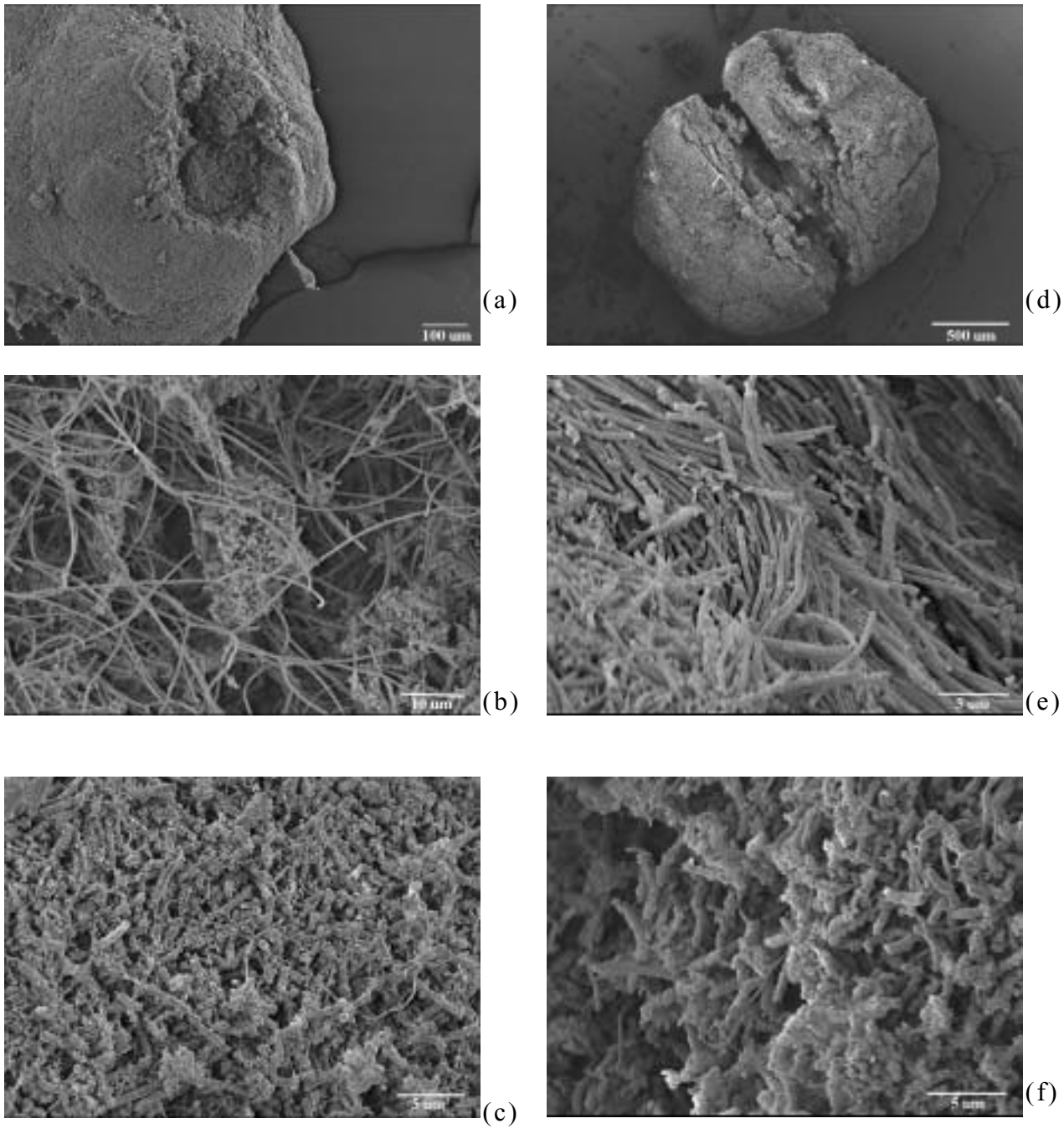

Figure 5. Scanning electron photomicrographs showing the external (b,e) and internal (c,f) surfaces of granule samples taken from Compartment 3 of the control NFABR treating ice-cream wastewater after 190 days (a, b, c) and Compartment 3 of the polymer amended NFABR treating ice cream wastewater after 190 days (d, e, f).

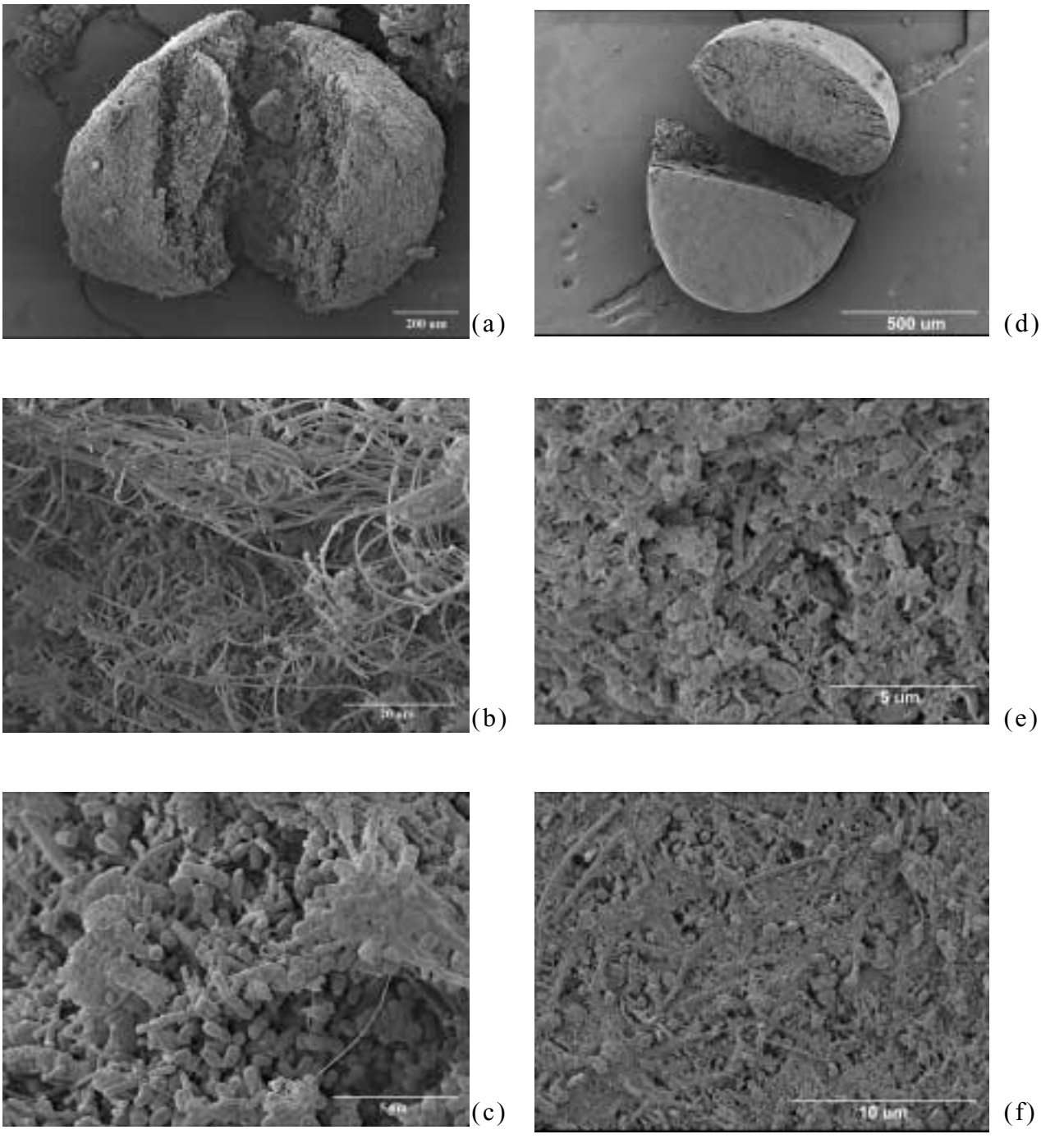

Figure 6. Scanning electron photomicrographs showing the external (b,e) and internal (c,f) surfaces of granule samples taken from Compartment 4 of the SFABR treating brewery wastewater after 70 days (a,b,c) and Compartment 2 of the NFABR treating brewery wastewater after 70 days (d,e,f).

Forster, 1995, Wolfaardt *et al.,* 1998, Veiga *et al.*, 1997). Scanning electron microscopy has been used to detect the presence and place of ECPs in granules (Dolfing *et al.*, 1985). Evidence from the series of photomicrographs taken during granule development in this study (Figure 8) confirms that ECPs, shown as the thin fibrous matrices around cells, were present within granule structures regardless of reactor configuration, granule age or wastewater characteristics.

It is thought that granulation is initiated by ionic bonds formed between positively charged cations (Ca^{2+} and Mg^{2+}) and the negatively charged surfaces of anaerobic bacteria (Mahoney *et al.*, 1987, Yu *et al.*, 2001) (Figure 4f). The subsequent bridging that occurs between additional bacterial cells and other particulate materials forms a heterogeneous matrix of such a magnitude as to allow the formation of a discrete floc aggregate (Morgan *et al.*, 1990). ECPs may therefore have two roles. First, they impart a greater negative charge to the surface of anaerobic bacteria (Morgan *et al.*, 1990), and second, once cells have formed an initial attachment, ECPs render the bridging irreversible by forming ECP matrices around the nuclei. ECPs therefore play a pivotal role in the initial adhesion of cells during nucleation and probably maintain much of the mechanical strength in mature granules, particularly between the acidogenic populations of the surface layer. As granules mature, we believe that selection pressures, based on the substrate specificity of bacterial cells and the environmental conditions, seem to be the most probable explanation for the eventual development of relatively restricted bacterial diversity, commonly seen in mature granules (Figures 5c and f and 6c and f).

Work by Jia *et al.* (1996), Schmidt and Ahring (1996) and Morgan *et al.*, (1990) has established direct relationship between ECPs and sludge surface charge, and concludes that as ECPs have a negative charge, higher concentrations of ECPs will tend to increase the overall negative charge of the sludge surface. Our results show that the negative charge increases up to the point of granulation (30 days, Figure 7) and thereafter declines as granules mature and their internal structure becomes dominated more by filamentous methanogens. This finding is further evidence that ECPs are central to the process of granulation.

Conclusion

Scanning electron photomicrographs revealed that when the concentration of complex VFA was maintained at a low level, granules generally exhibited stratification of their bacterial populations. However, stratification was unlikely to occur when high concentrations of complex VFA were present, with granules tending to show a homogeneous microbial composition. It is believed that stratification does not arise simply from the selection of microbial populations during the initial (nucleation) stage of granule development. Rather, it develops when specific internal environments are created under certain conditions of substrate flow through the granule matrix and this exerts selection pressures that favor the growth of a limited range of bacterial species in each of the specific regions of the granule.

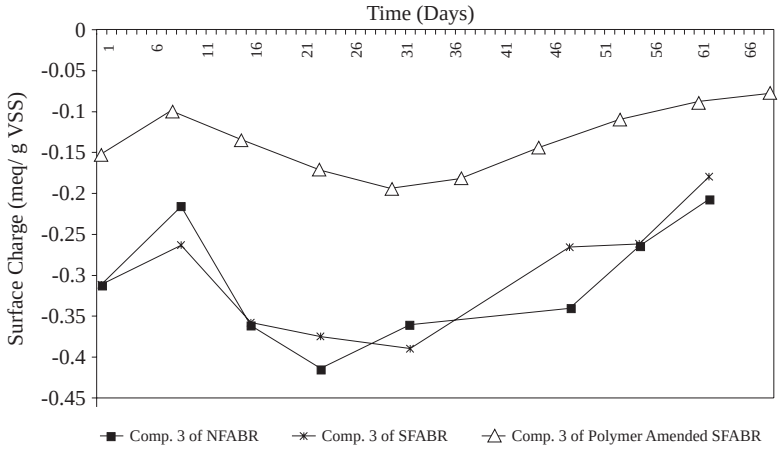

Figure 7. Sludge surface charge of compartments monitored for granule development.

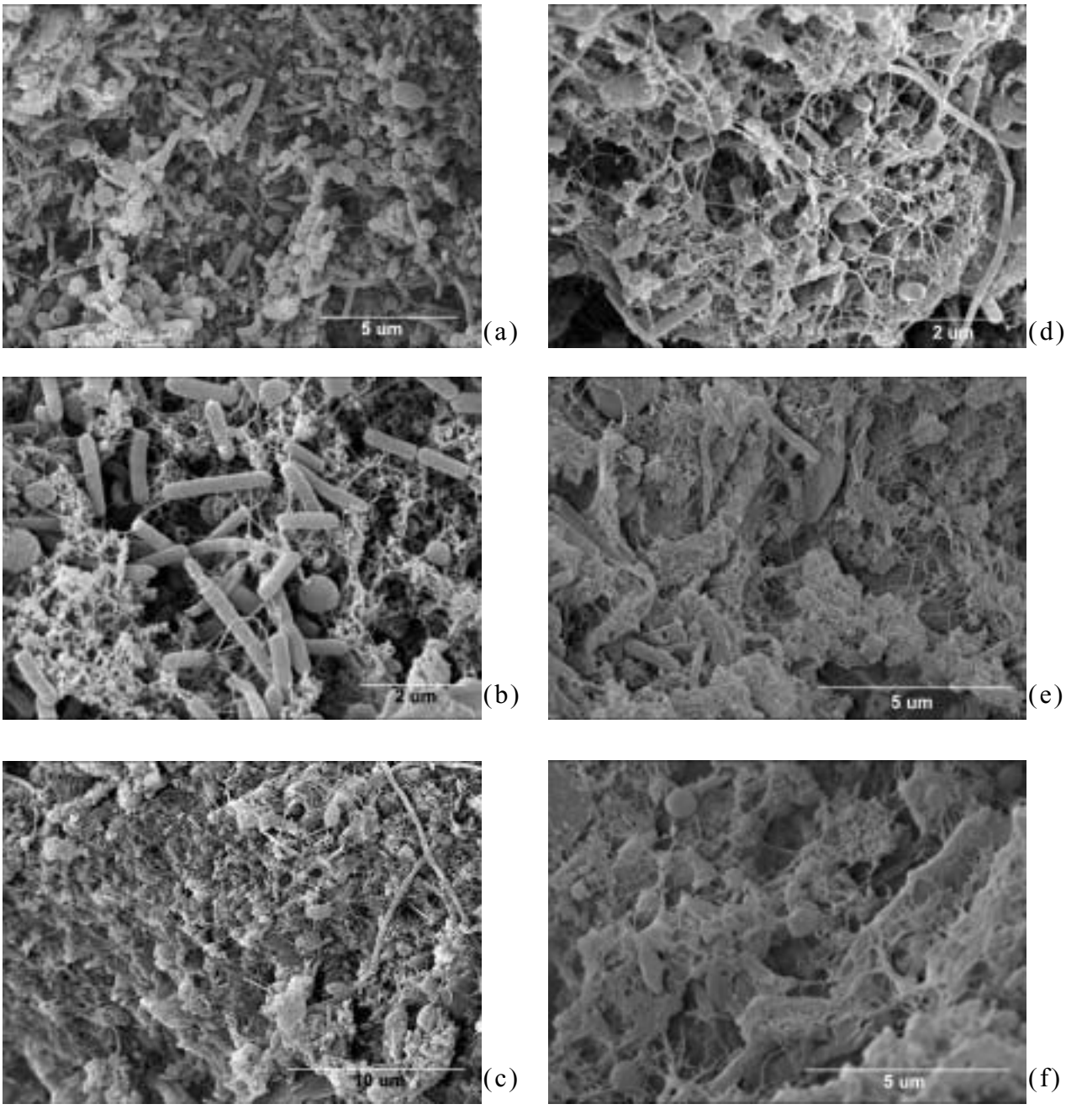

Figure 8. Scanning electron photomicrographs highlighting the effect of ECPs. Samples taken from (a) Compartment 2 of the NFABR treating brewery wastewater on day 70, (b) Compartment 1 of the NFABR treating brewery wastewater on day 70, (c) Compartment 3 the of SFABR treating brewery wastewater on day 15, (d) Compartment 4 of the SFABR treating brewery wastewater on day 30, (e) Compartment 3 of the polymer-amended SFABR treating ice cream wastewater on day 30, and (f) Compartment 1 of the polymer-amended SFABR treating ice cream wastewater on day 45.

SEM photomicrographs support the widely held view that biochemical factors, such as ECPs, sludge surface charges and ionic bonds between positively charged inert material and microbial species, are instrumental in both the initial formation and mechanical stability of anaerobic granules.

Based on the course of granular growth the granulation process can be categorized into three phases: (i) initiation, (ii) adaptation and maturation, and (iii) granulation. Initiation is a state in which a random process of adhesion of microorganism between cells and/or between cell and inert materials occurs. Adaptation and maturation is a state in which population dynamics are driven purely by substrate composition. In this way bioparticles grow and dominant microorganisms in the bioparticles emerge, and, depending on substrate composition, stratification of microorganisms through the granule matrix begins. Finally, granulation is the last stage of granule formation in which bioparticles grow larger due to cell division and/or new cell adhesion, and become compacters due to ECP.

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