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# Effect of the Food-to-Microorganism (F/M) Ratio on the Formation and Size of Aerobic Sludge Granules

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## Abstract

Laboratory experiments were carried out to investigate the effect of the sludge loading, or the food-to-microorganism (F/M) ratio, on the rate of aerobic granulation and the size of the granules in biological wastewater treatment. Four column batch reactors were used with a similar sludge suspended solids (SS) concentration of around 2000 mg/L. The reactors were fed with a glucose-based wastewater at different chemical oxygen demand (COD) concentrations, resulting in F/M ratios from 0.3 to 1.1 g COD/g SS-d. A higher F/M ratio appeared to promote faster formation of larger granules and a lower F/M ratio led to slower

formation of smaller granules. Upon complete granulation, the granules became rather stable in size, and the mean diameter of the granules in different reactors increased from 1.2 to 4.5 mm linearly with the F/M ratio applied. Molecular analysis of the sludge did not show the domination of any particular bacterial species during the granulation process. In is apparent that applying different F/M ratios in different granulation stages, e.g., a higher F/M in the early stage and a reduced F/M in the later stage, can be an effective start-up strategy to facilitate rapid granule formation and sustain small and healthy granules in bioreactors.

**Keywords**: Aerobic granulation; F/M ratio; activated sludge; microbial community; wastewater treatment.

## 1. Introduction

Aerobic sludge granulation is a new microbial immobilization technique that has the potential to fundamentally advance the biological wastewater treatment technology [1-3]. Compared to conventional activated sludge, the dense granule structure confers on granular sludge an excellent settling ability that allows for rapid sludge-effluent separation, a high level of biomass concentration, and a greater organic treatment loading capability [3-5]. Aerobic granules are considered as a special form of bacterial biofilm growth in suspension [5,6]. It has been demonstrated that granulation can be achieved by means of selective discharge of small and slow-settling sludge flocs [7]. However, the influences of process conditions on the quality and property of granules are still issues of investigation. For instance, the size of aerobic granules has a profound impact on the stability and treatment performance of granular sludge, and larger granules may become less stable in wastewater treatment. However, effective measures for controlling the size and improving the stability of aerobic granules in a bioreactor remain to be developed.

During the start-up of aerobic granulation, a short settling period is commonly adopted to force the discharge of small and loose sludge flocs from the reactors and hence to retain denser sludge [3,7]. Such an early washout of small and slow-settling sludge from the suspension leads to a loss of biomass, resulting in an increase in sludge loading rate [1,3]. A high food-to-microorganism (F/M) ratio would enhance microbial growth [8] and hence facilitate the aerobic granulation process [9,10]. In connection to biomass growth, the sludge loading rate, or the F/M ratio, in a bioreactor could be an essential parameter that regulates the size of granules. However, the correlations between the F/M ratio and the rate of aerobic granulation and the size of granules have not been well established.

F/M ratio is a process variable that can be easily adjusted in operating bioreactors. A suitable F/M can be favorable for both the progress of granulation and the size control of granules. In this study, laboratory experiments were conducted with batch reactors to investigate the effect of the F/M ratio on the formation, size, and stability of granules. The rates of sludge granulation and the stable size of granules formed under different F/M conditions were determined. The findings are essential to the development of an effective start-up strategy for the formation and maintenance of small and healthy granules in long-term biological wastewater treatment operation.

## 2. Materials and Methods

### *2.1. Experimental set-up and operation*

Four 0.4-L graduate cylinders (H 22 cm × D 5.2 cm) were used as column batch reactors for the experimental study on aerobic granulation. Activated sludge from a full-scale sewage treatment plant (Stanley Sewage Treatment Works, Hong Kong) was used as the seed sludge after one month of laboratory acclimation with a glucose-based synthetic wastewater. The seed sludge was well mixed before loading into the four reactors to have the same initial mixed

liquor suspended solids (MLSS) concentration of 2000 mg/L. The reactors were fed once a day with a synthetic wastewater consisting of glucose, $NH_4Cl$, $KH_2PO_4$ and $NaHPO_4 \cdot 6H2O$, and other nutrients that was prepared according to the formula given by Tay et al. [10].

The operating condition was the same for the four reactors, R1, R2, R3, and R4, except the feed substrate concentration. Different influent organic concentrations in terms of the chemical oxygen demand (COD) – 600, 1400, 2200, and 2200 mg/L – were used for the four reactors to have F/M ratios of 0.3, 0.7, 1.1, and 1.1 g COD/g SS-d for R1, R2, R3, and R4, respectively. For R4, however, the influent COD concentration changed from 2200 to 600 mg/L in the third week after its start-up, which reduced the F/M ratio from 1.1 to 0.3 g COD/g SS-d. The experiments were carried out at room temperature, and the water temperature was 20-22˚C. $NaHCO_3$ was dosed into the feed solution to maintain the pH of the reactors in the neutral range between 7.0 and 7.5. Aeration was conducted from the bottom of the reactors at an air flow rate of around 1.0 L/min during the aeration phase, and the dissolved oxygen (DO) concentration in the sludge suspension was in a range of 2-5 mg/L.

At the end of each 24-hr cycle, the sludge was allowed to settle in the column without aeration. During the early settling phase, a certain amount of the sludge suspension was withdrawn by siphon below the water surface. The slow-settling sludge flocs in the suspension were therefore removed from the reactors. The sludge concentration in each reactor and the amount of daily sludge discharge from the reactor were measured. Accordingly, the rate of the daily sludge discharge was then adjusted to maintain the MLSS concentration at about 2000 mg/L in the reactors. The purpose of this operation was to selectively remove the small and slow-settling sludge from the sludge mixture while keeping the sludge concentration and F/M ratio at the pre-determined levels in each reactor [7]. After another 30 min of sludge sedimentation, the supernatant was withdrawn from the reactors, and the wastewater influent was added into each reactor to restore its original water volume of 0.4 L.

84

85 *2.2. Determination of the organic uptake capability and settling behavior of the granules*

86 After the completion of aerobic granulation, the granular sludge was characterized for its
87 organic uptake rate and settling velocity. For each sludge sample from a reactor, the organic
88 uptake test was performed in a 250-mL glass beaker, and the sludge and glucose concentrations
89 were 1000 mg MLSS/L and 300 mg/L, respectively. The sludge mixture was sampled at
90 various time intervals and the glucose concentration in the liquid phase of the sludge was
91 measured. The mass-balance equation for organic in the reactor may be written as
92 $\frac{dS}{dt}V = QS_0 - QS + rV$, where $S$ is the substrate concentration and $S_0$ is its initial concentration,
93 $t$ is time and $r$ the rate of the substrate removal. Since $Q = 0$ for a batch reactor and a
94 first-order correlation $r = -(kX)S$ may be assumed for the early phase of substrate uptake,
95 where $k$ is a specific rate coefficient and $X$ the biomass concentration in the batch reactor, the
96 rate of organic removal may be approximated by $\frac{dS}{dt} = -kXS$. By linear regression of $\ln(S_0/S)$
97 versus $Xt$, the apparent specific organic uptake rate coefficient of the sludge can be estimated.
98 The settling experiments were conducted for individual mature granules following the
99 procedure described by Xiao et al. [11] in water column. The acrylic settling column was 90
100 cm in height and 8.1 cm in diameter with a corn bottom and a valve. For each setting test, a
101 granule was placed at the top of the water column, and the settling velocity of the granule
102 through the lower 60 cm was measured. The granule reaching the bottom was then released
103 and retrieved. The granule was placed on a stereomicroscope (S8 APO, Leica, Germany)
104 equipped with a digital camera (EC3, Leica, Germany), and the granule was sized according
105 to its projected area, $A$, and expressed by the equivalent diameter of $d = \sqrt{4A/\pi}$ [12].

106

107 *2.3. Water and sludge analysis*

The COD and SS concentrations and the sludge volume index after 5 min ($SVI_5$) were measured following the Standard Methods [13]. The total organic carbon (TOC) concentration was measured using a TOC analyzer (IL550, HACH-Lachat, USA). The glucose concentration was determined by a UV/VIS spectrophotometer (Lambda 25, Perkin Elmer, USA) according to the phenol-sulphuric acid method [14]. The morphology of the aerobic granules was observed under a stereomicroscope (S8 APO, Leica, Germany). The particle size distributions (PSD) of the sludge samples (< 2000 μm) were measured using a laser diffraction particle counter (LS13 320, Beckman Coulter, USA). When granules grew larger, photographs of the granules in a sludge sample were taken by a digital camera with the stereomicroscope. The photo images of the granules were analyzed by an image analysis system (analySIS 3.1) for PSD of the granular sludge.

A heat extraction method was modified to extract extracellular polymeric substances (EPS) from activated sludge and granules [15]. The sludge was first washed three times and dewatered by centrifugation (5810R, Eppendorf, Germany) in a 25-mL tube at 4000 g for 5 min. The sludge pellet in the tube was then homogenized into 2.5 mL of 0.05% NaCl solution by a beadbeater (Mini-beadbeater$^{TM}$, Biospec, USA) without beads. The sludge mixture was then diluted with the NaCl solution to its original volume of 25 mL. The sludge suspension was heated to 60˚C in a water bath for 30 min, and the sludge mixture was then centrifuged at 4000 g for 15 min. The supernatant collected was regarded as the EPS extract of the sludge, which was analyzed for TOC, polysaccharides (PS), proteins, and humic-like substances (HS). The PS content was determined using the phenol-sulphuric acid method [14] with glucose as the standard. Proteins and HS were analyzed by a UV/VIS spectrophotometer (Lambda 25, Perkin Elmer, USA) following the modified Lowry method [16] using bovine serum albumin (Sigma) and humic acid (Fluka) as the standards, respectively.

### *2.4. DNA extraction and denaturing gradient gel electrophoresis (DGGE) analysis of the sludge*

DGGE band profiles were used to reveal the most abundant DNA types among the microbial species in a sludge sample [17]. The genomic DNA of the biomass was extracted following the protocol described by Zhuang et al. [18] using a beadbeater (Mini-beadbeaterTM, Biospec, USA) and a microcentrifuge (MiniSpin plus®, Eppendorf, Germany). Subsequently, the variable V3 region of the bacterial 16S rDNA gene sequence was amplified by polymerase chain reaction (PCR) [19] with a DNA Engine® Peltier Thermal Cycler (PTC-200, MJ Research, USA). A touchdown thermal profile technique was used for the PCR procedure [20]. As described by Li et al. [21], the PCR-amplified DNA products were then separated by DGGE, and the DGGE gel images were acquired using the ChemiDoc (Bio-Rad, USA) gel documentation system. The DGGE band patterns were then used to calculate the Shannon–Weaver index for the species diversity of different sludge samples [21].

## 3. Results and Discussion

### *3.1. Formation and physical properties of the aerobic granules*

Aerobic sludge granulation was well achieved in all four batch reactors operated at different F/M ratios (Figs. 1 and 2). The sludge MLSS concentrations were kept largely comparable between the four reactors during the experimental study. Within the range tested, the F/M ratio, or the biomass loading rate, did not appear to be the crucial factor for granule formation. As indicated previously [7], selective discharge of small and slow-settling sludge flocs from the sludge suspension was the determining factor for aerobic granulation. However, the F/M ratio displayed a profound effect on the rate of granulation and the morphological property of the granules. In general, a higher F/M ratio brought about faster formation of larger

158 granules, and a lower F/M ratio led to slower formation of smaller granules (Figs. 2 and 3). In
159 R1 at a low F/M of 0.3 g COD/g SS-d, small granules were observed after 25 d and granulation
160 was fully achieved throughout the column reactor after 40 d. In R2 at a medium F/M of 0.7 g
161 COD/g SS-d, granules appeared after 16 d and granulation was completed after 20 d. In R3 and
162 R4 at a high F/M of 1.1 g COD/g SS-d, granules were observed after only 7 d and granulation
163 was completed in about 14 d. A comparison between the different column reactors suggests
164 that faster biomass growth under a higher F/M condition would facilitate a rapid granule
165 formation and growth. Nonetheless, a high biomass loading, e.g. F/M > 0.5 g COD/g SS-d [22],
166 may not be a necessity for complete sludge granulation.
167 As described previously, upon complete granulation, the F/M ratio for R4 was reduced
168 from 1.1 to 0.3 g COD/g SS-d after 20 d of the start-up. With the decrease in F/M, breakage
169 of large granules occurred, resulting in losses of the biomass. However, the aerobic granules
170 in R4 became stabilized eventually at smaller sizes after about 10 d, and the granules
171 appeared to be comparable to those formed in R1 at the low F/M of 0.3 g COD/g SS-d (Figs.
172 2 and 3). Based on the rate of sludge discharge from the four reactors, the sludge retention time
173 (SRT) was kept at 15, 8, and 5 d for R1, R2, and R3, respectively. The SRT of R4 changed from
174 5 to 15 d after running 20 d. The $SVI_5$ of the mature granules were 27.0±2.4, 36.8±3.7,
175 42.5±5.1, and 29.2±2.2 mL/g for R1, R2, R3, and R4, respectively. A lower F/M ratio produced
176 smaller granules with a better sludge compressibility.
177 Different from the loose and irregular activated sludge flocs, all of the aerobic granules
178 produced in the four reactors were round with a clear boundary and smooth surface (Fig. 2).
179 However, the morphology and structural features were different for the granules produced at
180 different F/M ratios. The slowly-forming granules in R1 were smaller and more tightly-packed
181 than the fast-forming granules in R2 and R3. The mature granules in R1 had an average
182 diameter of around 1.5 mm, whilst the granules grew to 2.8 mm in R2 and 4.5 mm in R3 (Fig.

3). Interestingly, the granules in R4 grew rapidly to 3 mm at the high F/M of 1.1 g COD/g SS-d and decreased gradually from 3 mm to 1.2 mm after the F/M was reduced to 0.3 g COD/g SS-d. Upon complete granulation, the granules appeared to be rather stable in size, and the mean diameter of the mature granules could be correlated nearly linearly with the F/M ratio ($R^2$ = 0.99) applied to the different reactors (Fig. 3b). The result indicates that the sludge loading rate can be an effective operating parameter to regulate the size of granules in a bioreactor.

*3.2. Organic substrate uptake capability and settling behavior of mature granules*

All of the four reactors after sludge granulation performed well in organic removal with an effluent COD of lower than 50 mg/L. The specific laboratory test however showed different organic substrate uptake capabilities of the mature granules formed in different reactors. For the same initial glucose concentration and the same biomass concentration, the glucose concentration decreased rapidly from 300 to below 10 mg/L after 30 min for R1 and R4 granules, whilst the glucose concentration decreased from 300 to 20 mg/L after 60 min for R2 granules and after 200 min for R3 granules (Fig. 4a). The small granules from R1 and R4 showed a considerably faster glucose uptake rate than did the larger granules from R2 and R3.

The comparison between different granules in the four reactors proved that small granules had a clear advantage over larger granules in the uptake of organic substrates and nutrients. The apparent specific glucose uptake rate coefficient of the granular sludge decreased nearly linearly ($R^2$ = 0.93) with the mean size of the granules from 8.4/g SS-h for R4 granules to 0.78/g SS-h for R3 granules (Fig. 4b). The specific surface area of granules increased as the granules became smaller in size. Hence, in comparison to larger granules at the same concentration, smaller granules had more surface areas that would allow faster substrate uptake and utilization. Assuming $\rho_c$ =1.06 g/cm$^3$ for the density of the (wet) bacterial cells and a factor $f$ = 3.45 for the ratio between the wet mass and the dry mass of the cells in the granules [11],

using the mean size to calculate the surface area of mature granules from each reactor and assuming a constant porosity of 0.8 for aerobic granules, the surface area-based specific glucose uptake rate coefficient can be estimated as 0.135, 0.087, 0.046, and 0.128 /$cm^2$-h for the granules from R1, R2, R3, and R4, respectively. Apart from the difference in surface area between granules of the same weight but different sizes, smaller granules apparently had a faster substrate uptake capability per unit surface area. Compared to larger granules, smaller granules would impose a less degree of mass transport limitation for the substrates and dissolved oxygen into the granule interior [11]. Thus, from the point of view of biological wastewater treatment, small granules are more preferred to large granules for more effective organic degradation.

Granular sludge settled much faster in water than activated sludge flocs, and the settling velocity generally increased with the size of granules. The settling velocities ranged from 0.42 to 0.89 cm/s for individual mature granules from R1 and R4, from 0.64 to 1.84 cm/s for the granules from R2 and from 1.37 to 4.12 cm/s for the granules from R3 (Fig. 5). These values are similar to the settling velocities of aerobic granules reported in previous studies [9,11]. In comparison, activated sludge flocs were found to have a much slower settling velocity ranging from 0.16 to 0.49 cm/s [23]. The slope of the linear regression between the granule size and settling velocity after log-log transformation is 0.64, 0.80, and 0.76 for the granules cultivated in R1, R2, and R3, respectively. It is apparent that the granules became denser and the settling velocity increased more with the size for the large granules from R2 and R3 in comparison to that for the smaller granules from R1.

### *3.3. Sludge EPS during the granulation*

The EPS of the sludge in the four reactors had a similar trend of change during the granulation process (Fig. 6). The EPS content decreased initially for the sludge in all reactors

and then increased to different levels with the granule formation. The total EPS in R1 and R2 sludge after 40 d were about 85 and 90 mg TOC/g SS, respectively. The EPS in R3 and R4 at high F/M ratios reached 120 mg TOC/g SS after 20 d. The granules cultivated in R3 and R4 contained more EPS than the granules formed in R1 and R2 at lower F/M ratios. After the completion of granulation in the four reactors, the EPS contents of the granular sludge all decreased and fluctuated at a lower level of around 80 mg TOC/g SS. The sludge EPS consisted of more proteins than polysaccharides and humic-like substances. Large granules formed at a high F/M ratio had a higher protein proportion in EPS than that of the smaller granules formed at a low F/M. This result is consistent with the previous findings for the EPS composition in aerobic granules [24]. Proteins have been reported as the core EPS constitutes of the aerobic granules, which are believed to be the important building materials for the internal structure of granules [24].

The contents of polysaccharides and humic substance in EPS were similar for the granules in the four reactors, ranging from 20 to 40 mg/g SS. Between polysaccharides and humic matter, the fraction of humic materials was somewhat higher than that of polysaccharides. The humic substances in EPS are expected to play an important role in immobilizing exoenzymes through their reversible complexation with the enzymes [25]. It is generally considered that microbial EPS play an essential role in microbial granulation [26]. EPS are expected to bind cells closely for the formation and the structure stability of granules [27]. However, the results of this experimental study suggest that the roles of EPS in sludge granulation are complex. During the phase of granule formation, the EPS abundance increased with time; while upon granulation the EPS content decreased from the peak levels. Wang et al. [28] also observed that the amount of EPS increased during the early phase of aerobic sludge granulation, but changed little after the granules had matured.

### *3.4. Microbial population dynamics during aerobic granulation at different F/M ratios*

Well-resolved DGGE bands were obtained that show the changes of bacterial communities during sludge granulation in the four batch reactors (Fig. 7). The DGGE banding patterns showed the positions of major bands shifting during the course of granulation in the four reactors, which suggests changes in the dominant species with the granule formation and growth. Nonetheless, for the reactors under different F/M conditions, the bacterial populations changed differently, producing different trends of DGGE banding profiles. In the early phase of granule growth, or for the precursor granules in R1 (25 d), R2 (16 d), R3 (7 d), and R4 (30 d), there were not the same dominant species that could be found in all four reactors. The comparison suggests that aerobic granulation did not request the domination of one or more particular microbial species. The mature granules in all of the four reactors after 100 d also had different population structures.

The difference in bacterial community among the four reactors probably was related to the operating condition, such as the F/M ratio. According to the Shannon–Weaver index, the population diversity changed in different ways for the biomass in the four reactors with different F/M ratios (Fig. 8). During the start-up of sludge granulation, it is apparent that a higher F/M ratio would facilitate the change of the bacterial community and lead to a lower species diversity in the bioreactors (R3 and R4). Upon the completion of granulation, the F/M ratio became less important to the microbial structure, and the population diversities of the sludge approached a similar level in the four reactors. It also has been recognized in ecology that competition for growth-limiting resources would bring about chaotic fluctuations in species abundances [29].

The biomass loading rate, or F/M ratio, exhibited a profound effect on the aerobic granulation process. An F/M ratio as high as 1.1 g COD/g SS-d facilitated rapid formation of large granules in about 15 d. In comparison, a low F/M ratio of 0.3 g COD/g SS-d in R1 would

result in a slow granulation process of more than 40 d (Fig. 3a). However, although large granules can be formed more quickly at a higher F/M, the large sizes of granules are not desirable for the wastewater treatment purpose. It has been found that large granules usually are less stable and have more problems in long-term operation, such as breakage, erosion, floating, and fungal contamination [30,31]. In comparison, small and healthy granules are more favorable for use in biological wastewater treatment. Under a low F/M condition, as shown in R1 and R4, small granules could be formed and stabilized with a mean size of 1.5 mm or smaller. Because of the less degree of mass transport limitation and a higher level of stability, small granules are much more preferred to large granules during aerobic granulation in bioreactors.

The present study demonstrated that the size of granules can be controlled by the sludge F/M ratio, together with the selective sludge discharge technique, during the granulation process. A low F/M of 0.3 g COD/g SS-d led to the formation and stabilization of small granules after about 40 d. Moreover, adjusting the F/M ratio at different stages of granulation can be a more effective start-up strategy, as shown by the operation of R4. A high F/M ratio could be applied in the early stage, which would bring about fast granule formation and growth. Afterwards, the F/M ratio could be reduced to a lower level to allow the formation and stabilization of smaller granules. The effective operating strategy for the control of the size of aerobic granules is of great importance to the development and actual application of the granulation technology in biological wastewater treatment.

## Conclusions

- Aerobic granules were cultivated with selective discharge of small and slow-settling sludge flocs in four batch column reactors at different F/M ratios from 0.3 to 1.1 g COD/g

SS-d. A higher F/M ratio promoted faster formation of larger granules and a lower F/M ratio led to slower formation of smaller granules.

- Upon complete granulation, the mature granules became rather stable in size and EPS content. The mean size of the granules in different reactors increased from 1.2 to 4.5 mm nearly linearly with the F/M ratio applied to the reactors, and the specific organic uptake rate of the granular sludge decreased nearly linearly with the mean size of the granules.
- Adjusting the F/M ratio in different granulation stages, e.g., >1.1 g COD/g SS-d in the early stage and < 0.3 g COD/g SS-d in the later stage, can be a simple and effective start-up strategy to facilitate rapid granule formation and sustain small and healthy granules in bioreactors.

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401 **Figure captions**

402

403 **Fig. 1.** (a) Sludge MLSS and (b) the F/M ratio in the four batch column reactors during the
404 experiment.

405 **Fig. 2.** Photographs of the mature granules produced in the four reactors after 120 d: (a) R1, (b)
406 R2, (c) R3, and (d) R4.

407 **Fig. 3.** (a) Change of the mean size of the sludge in the four reactors during the granulation
408 process; and (b) the correlation between the mean size of mature granules from
409 different reactors and the F/M ratio applied for granulation.

410 **Fig. 4.** (a) Reduction in glucose concentration during the glucose uptake tests on the granular
411 sludge from the four reactors; and (b) the correlation between the apparent specific
412 glucose uptake rate coefficient and the mean size of granules from different reactors.

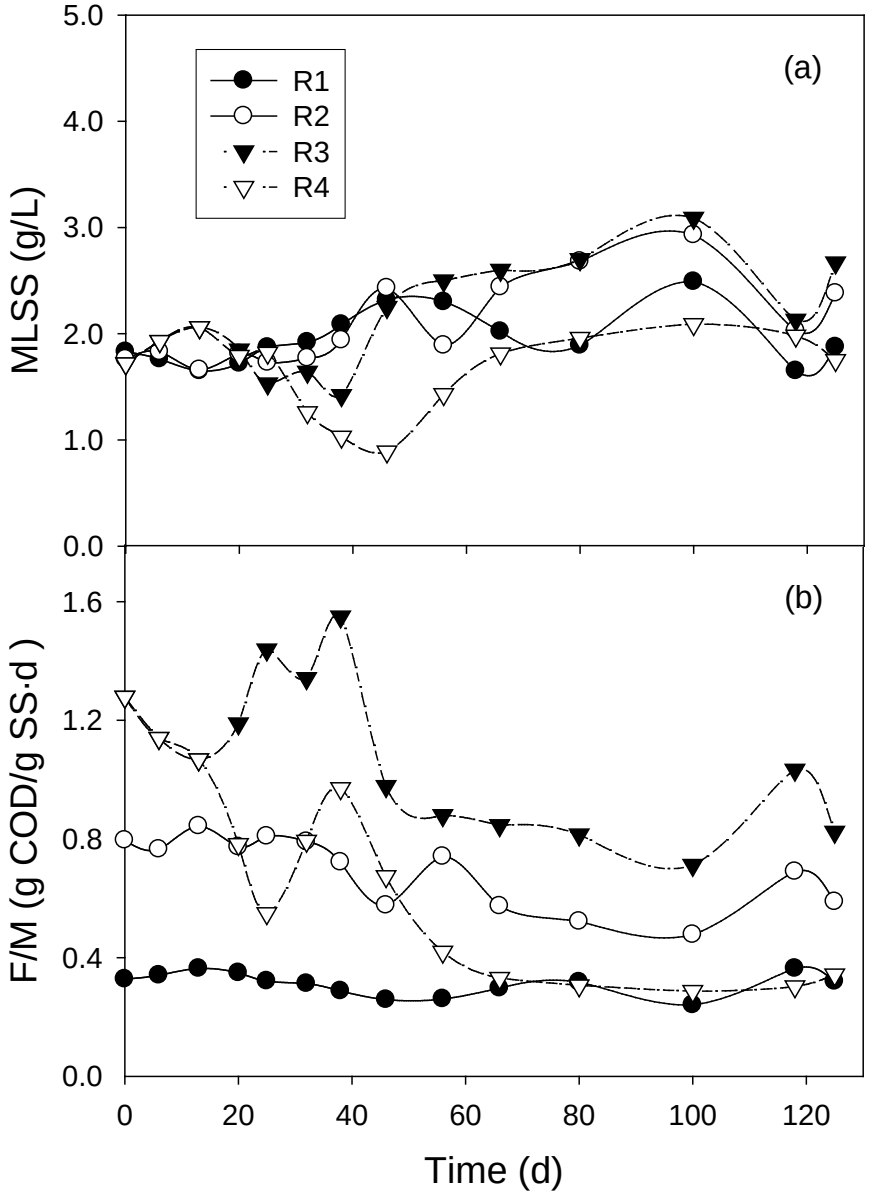
413 **Fig. 5.** Settling velocities of individual granules formed different reactors.

414 **Fig. 6.** Analysis of the EPS contents, including TOC, proteins, polysaccharides, and
415 humic-like substances, for the sludge from different reactors during the granulation
416 process.

417 **Fig. 7.** DGGE profiles of the bacterial communities in the four reactors during the sludge
418 granulation process. AS: seed activated sludge, m-n: sludge from reactor m (R1, R2, R3
419 or R4) after n days of the start-up operation, e.g., 2-16: sludge from R2 after 16 d (top:
420 image; bottom: schematic).

421 **Fig. 8.** Shannon-Weaver index (H) for the bacterial species diversity calculated from the
422 DGGE band profiles for the four bioreactors.

423

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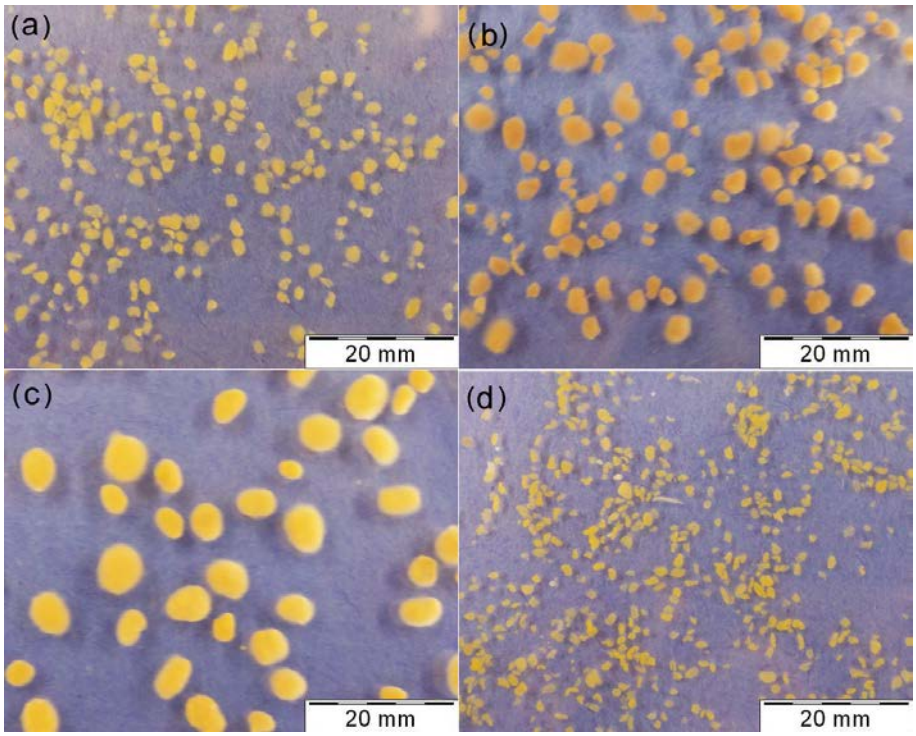



**Fig. 2.** Photographs of the mature granules produced in the four reactors after 120 d: (a) R1, (b) R2, (c) R3, and (d) R4.

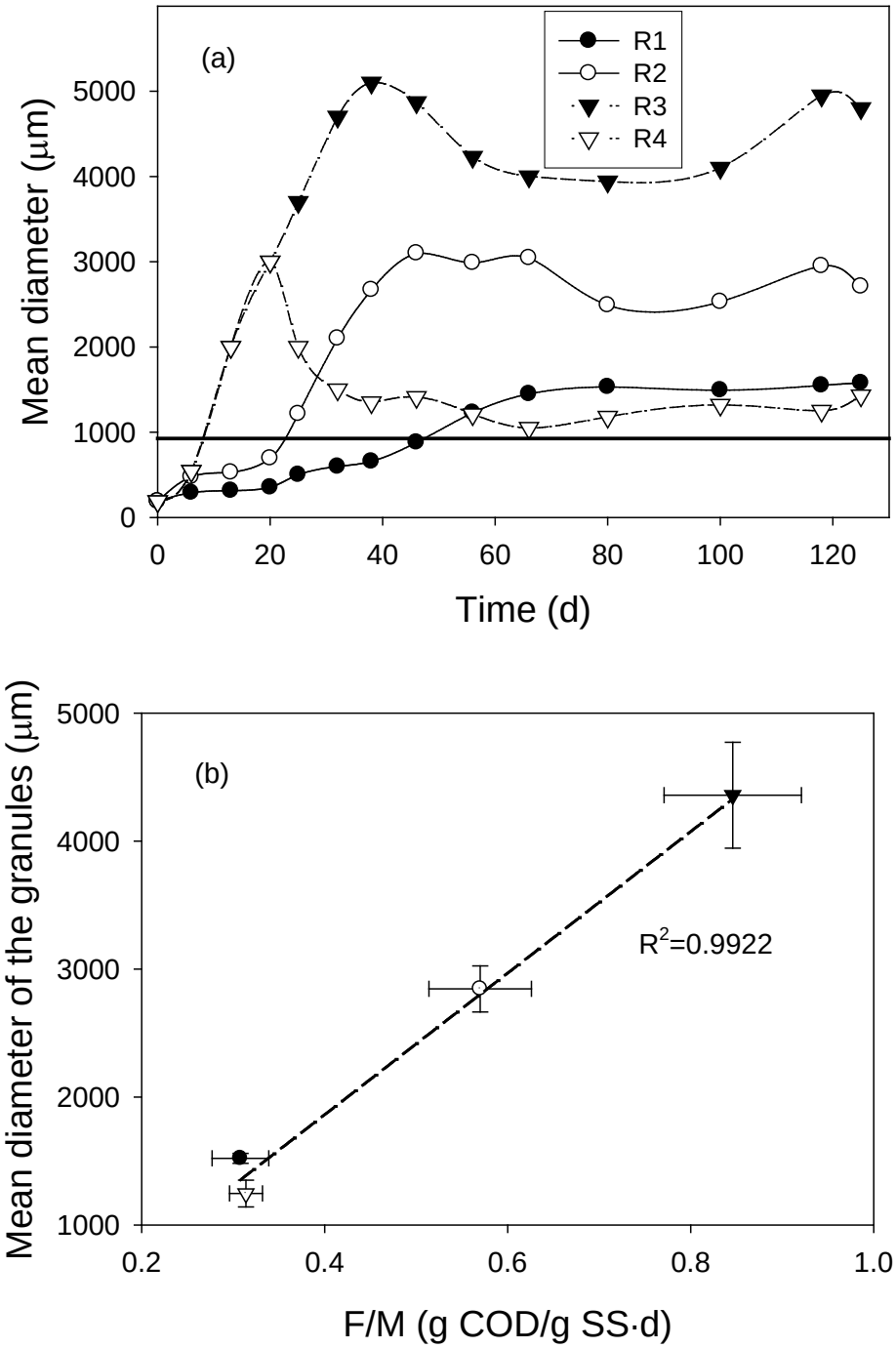
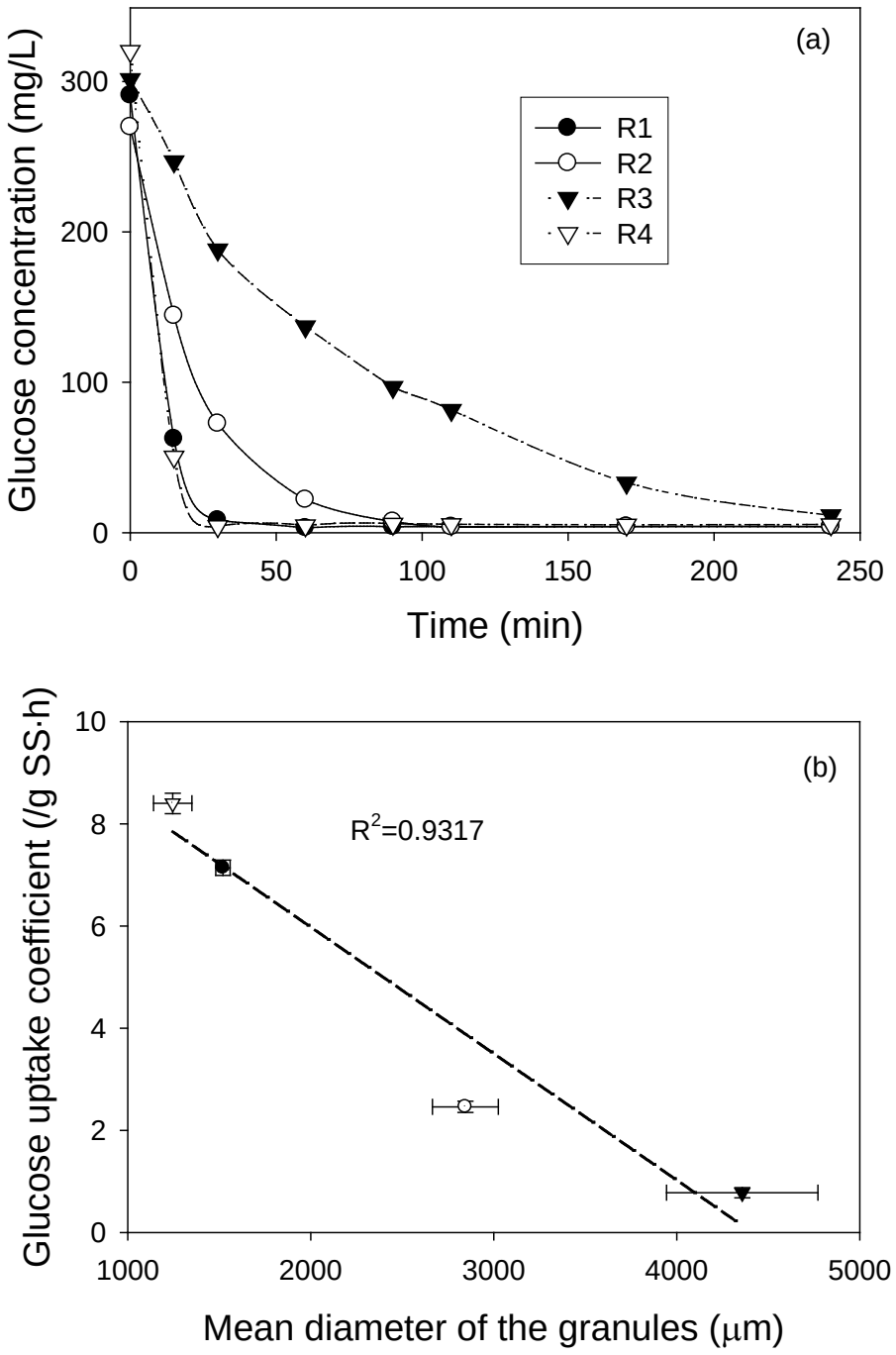



**Fig. 3.** (a) Change of the mean size of the sludge in the four reactors during the granulation process; and (b) the correlation between the mean size of mature granules from different reactors and the F/M ratio applied for granulation.

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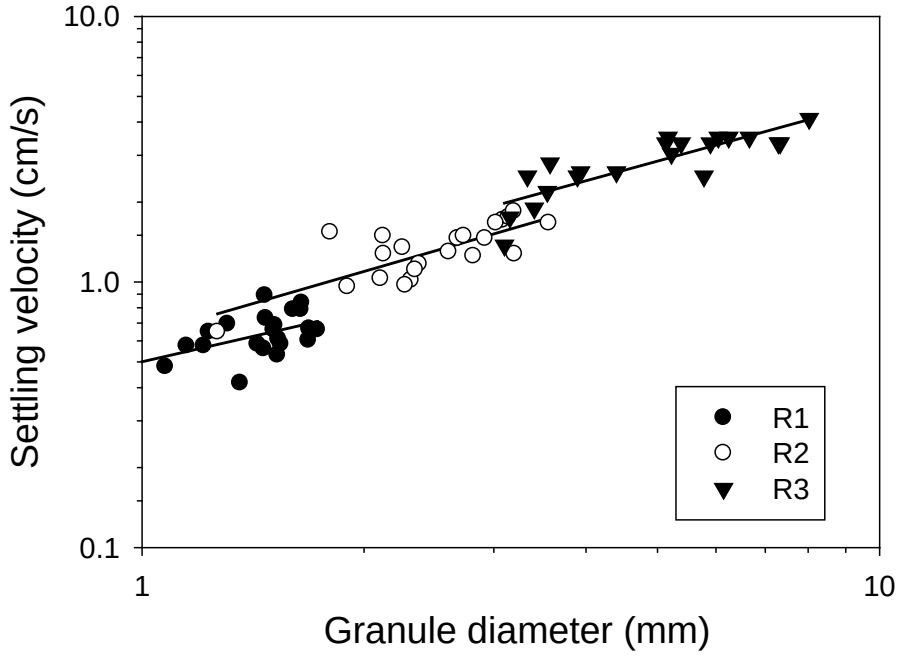


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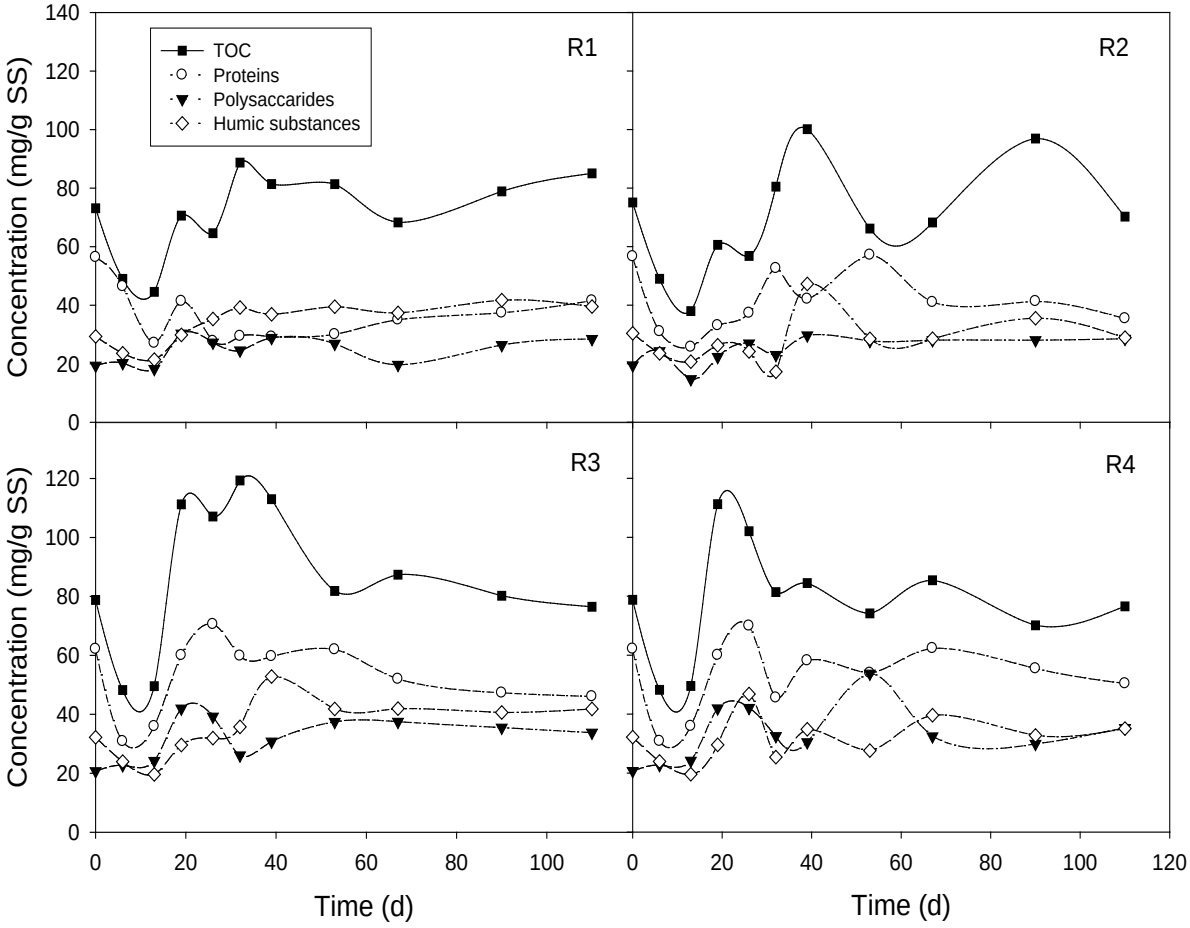


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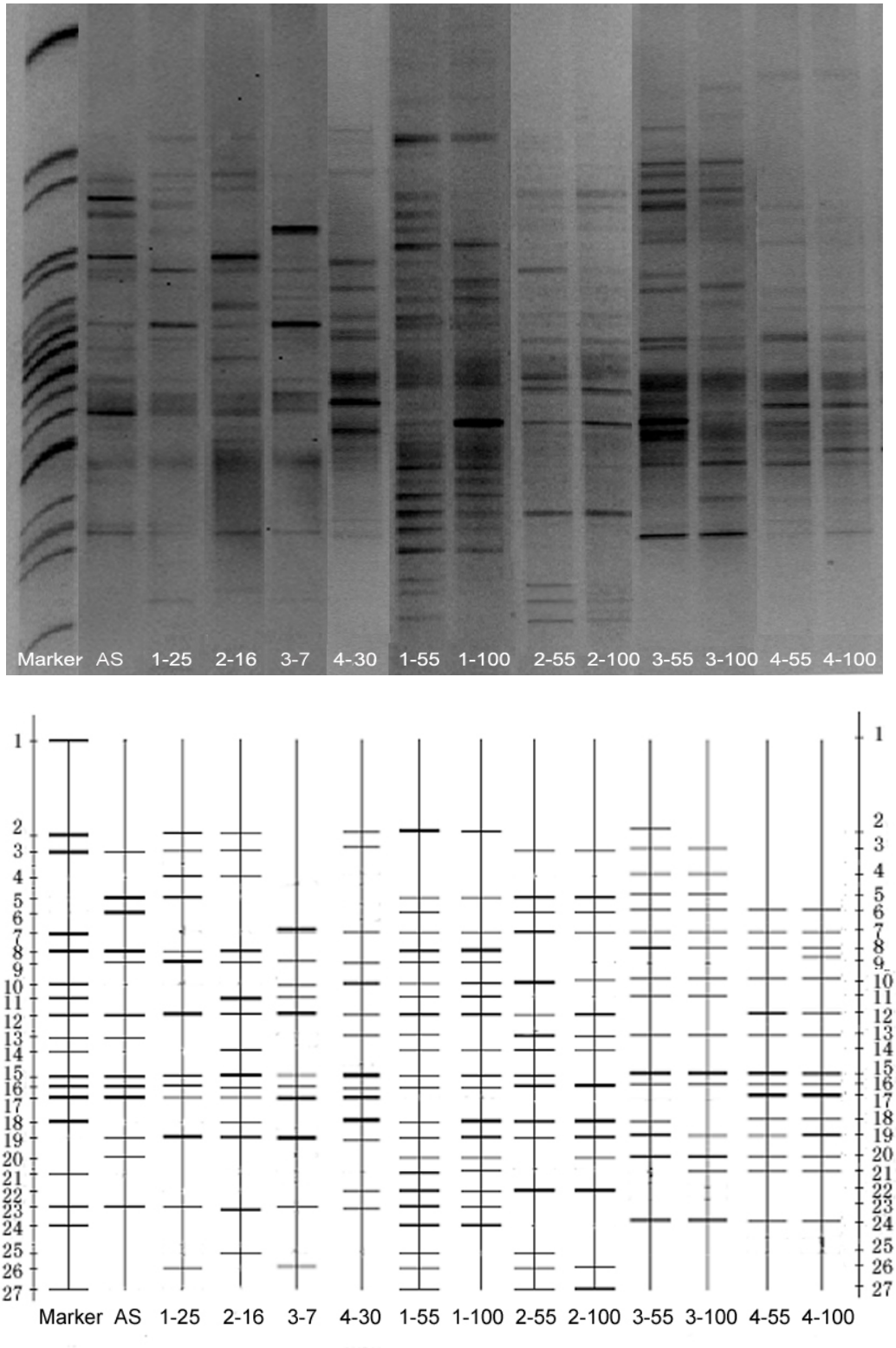


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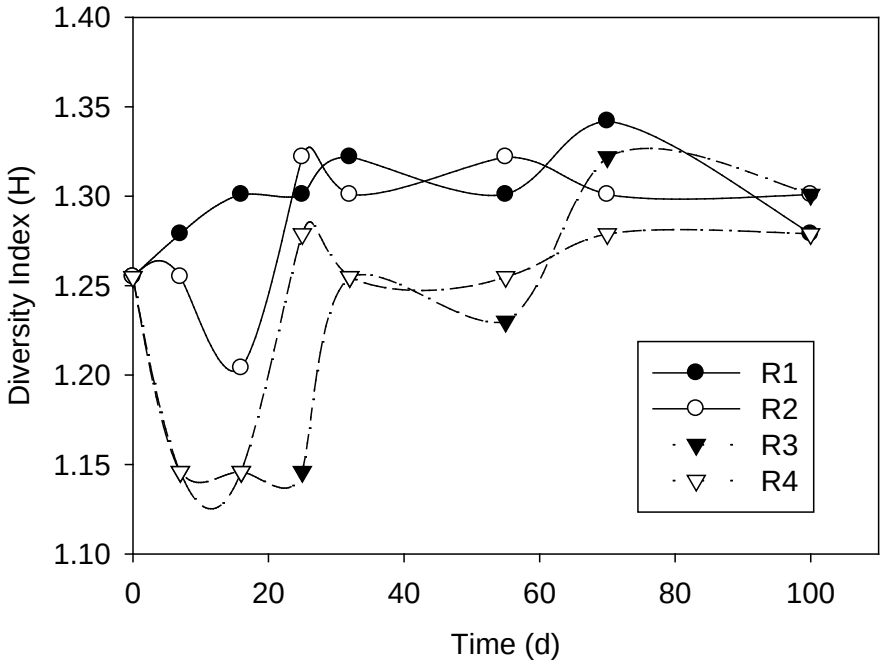


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