**Microbial diversity, and biofilm growth, in size-resolved anaerobic granules**


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Abstract
An anaerobic granular biofilm in upflow anaerobic digesters underpin efficient organic removal and biogas production from wastewaters. A single digester contains millions of individual granules – but not all granules are identical. Anaerobic sludge from a full-scale digester was size-separated and found to comprise aggregates from across a range of size fractions, each with distinct physico-chemical properties across parameters including volatile solids concentration; density; structural integrity; and methanogenic activity. Clear gradients in microbial diversity across the size fractions indicated that as the size of granules increases the microbial community converges toward a core microbiome. Over a subsequent, short, laboratory-scale, bioreactor trial, shifts in the distribution of granule sizes were observed in 12 upflow bioreactors and an hypothesis is presented on the growth and biofilm life-cycle of anaerobic sludge granules.

**Keywords (max 3 keywords)**
Sludge Granules; Microbial Diversity; Biofilm Life-cycle

**Topic:** Microbiology of Anaerobic Digestion

**INTRODUCTION: ANAEROBIC GRANULAR BIOFILMS**
Anaerobic granular biofilms form spontaneously, and are exclusively found, in upflow anaerobic digesters. Each granule comprises a diverse microbial consortium, providing the community necessary for complete mineralisation of complex organic feedstocks to methane and carbon dioxide (Hulshoff Pol et al., 2004). **BUT, not all granules are the same.** Diaz et al. (2006) observed differences in the size and colour of anaerobic granules, and Ahn (2000) found the typical range of granule diameters was 0.1-5 mm. Other studies have observed size-stratification of the sludge bed where large granules occupy the bottom of the digester. We hypothesise that differently sized granules represent different stages of biofilm growth, and that granules sampled from a single digester at a single point in time, having survived the same environmental conditions, may, in fact, represent different stages of growth over a biofilm life-cycle. The objectives of this study were twofold; to: (1) intensively characterise morphological, physico-chemical, physiological and ecological differences across a highly-resolved set of granule size fractions from a full-scale bioreactor, and (2) explore the growth of granules in a laboratory-scale bioreactor trial.

**MATERIALS AND METHODS**
Granules were sourced from a full-scale digester. The size distribution was determined by passing sludge through a series of sieves yielding 10 discrete fractions. For each fraction, the settled volume, volatile solids concentration (APHA, 1989), density, settling velocities, extracellular polymeric substance (EPS) component concentrations were determined, along with specific methanogenic activity (SMA) against key substrates (Colleran et al., 1992). Additionally, SEM was used to visualise the ultrastructure. Finally, high-throughput sequencing of the 16S rRNA gene was used to describe community structure of granules from each size fraction.

**RESULTS AND CONCLUSIONS**
Granular sludge characterisation
Granules from Fractions D–G contributed a volumetric majority of the VS of the sample (Fig. 1). A gradient was apparent across the size fractions in surface structural features (Fig. 1). Smallest
granules (Fractions A–C) were not spherical but instead presented as ‘flakes’, whilst medium-sized granules were ‘flatter’ (i.e. less spherical; Fractions D–F), and largest granules appeared more spherical. In fraction J, large cracks and void spaces were apparent and the granules appeared to be breaking apart and losing structural integrity. Smaller granules were denser, but much less settleable (Fig. 1). Previous observations indicate that large granules are found toward the bottom of the sludge bed of bioreactors, whilst smaller granules dominate toward the top. It therefore appears that stratification, and localisation, of granules in bioreactors is influenced primarily by settling velocity rather than density. Mid-range granules (Fractions D–F) were generally most active, indicating that these may be the most important for methane production and wastewater treatment in AD systems, and also pointing to potential management strategies.

**Figure 1.** Physico-chemical and physiological data from granule size fractions, A – J. (a) Bar plot indicating the size ranges and relative volumetric contributions to the sludge; (b) VS proportions of TS; (c) typical SEM micrographs of selected granules; (d) scatter plot illustrating density, and settling velocity, of granules (n=10) from each size fraction; (e) heat map depicting specific methanogenic activity (SMA) of sludge samples (n=3) from each size fraction (except fraction J) against acetate (Ace), propionate (Prop), butyrate (Buty) and H2/CO2 (Hyd); and (f) stacked bar charts showing relative concentrations of proteins, humic-like substances (HLS) and polysaccharides components in loosely-bound and tightly-bound-EPS extracted from each size fraction (except fraction J).

**Diversity shifts**

Alpha diversity analysis indicated a strong, linear diversity gradient across the size fractions with a significantly higher rarefied richness in the smaller granules (Fig 2; between Fractions A and J, p=0.00019) and with significant differences between nearly each fraction. An identical trend was observed for Shannon entropy (Fig 2; between Fractions A and J, p=0.00012).

**Figure 2.** Microbial diversity, and community structure, in samples (n=3) from across the 10 size fractions, A-J. Alpha diversity: box plot of (a) rarefied species richness and (b) Shannon Entropy. Beta diversity: Non-Metric Multidimensional Scaling (NMDS) using (c) Bray-Curtis dissimilarity and (d) weighted UniFrac distances, where each point corresponds to the community structure of a sample; size fractions are indicated by colour, and ellipses are drawn at a 95% CI.
Beta diversity analysis revealed a highly significant differentiation pattern and gradient between fractions using the Bray-Curtis (Fig 2; p=0.001) and the weighted UniFrac distance metrics (Fig 2d; p=0.001). The clear gradient across sizes was contrary to our assumption that the diversity would increase with granule size, especially as larger biofilms contain more cells. Microbial populations within the most abundant taxa (Fig. 3) were dominated by methanogenic archaea, including acetoclastic methanogens from the genus Methanosaeta, hydrogenotrophic methanogens from the genera Methanolinea and Methanobacterium, as well as metabolically-diverse methanogens from the Methanosarcinaceae. In the largest granules 50% the community structure was made up by four methanogenic archaea: Methanosaeta, Methanolinea, and two taxonomic classifications of Methanobacterium beijingense.

Figure 3. Community structure based on relative abundance of the top-25 most abundant OTUs from across each size fraction, where ‘others’ refers to all OTUs not included in the ‘top-25’

Three hypotheses were developed to explain the seemingly shifting diversity across the size fractions: (1) the neutral explanation: communities are a balance between immigration and extinction; (2) the functional group explanation: a particular functional group will dominate due to a niche effect; and (3) the competition effect: within any, or all, functional groups present, better competitors emerge, which then dominate and reduce overall diversity. We found that as the granule size increased, the proportion of Euryarchaeota increased, but this group was not diverse (consisting of only 69 OTUs in total). The impact was reduced richness across the size fractions. Indeed, we tested this by calculating the rarefied Euryarchaeota richness, which remained fixed with granule size (data not shown), indicating the observed diversity may be associated with changing proportions of functional groups rather than reduced diversity within functional groups.

Growth hypothesis
The observations lead to the development of hypotheses on the life-cycle of biofilm growth and granule development in anaerobic bioreactors (Fig. 4). It is proposed that granules start as small, intact structures (1.a). Granule growth by replication and accumulation leads to medium-sized, highly active granules (1.b). As granules grow, however, they weaken (1.c) and eventually break (1.d). Broken pieces, now smaller and less settleable – although still active – rise through the sludge bed (1.e) and provide the foundation for small, ‘healed’ granules. A life-cycle model of such biofilms will provide an intensely interesting, new view on biofilm formation and development in anaerobic bioreactors.
Distributional shifts
To test this hypothesis, 12 identical, lab-scale (2L) EGSB reactors were then operated in four sets of triplicates: the first set (R_{S1}–R_{S3}) containing only small (S) granules (0.6–1 mm); the second set (R_{M1}–R_{M3}) containing only medium (M)-sized granules (1–1.4 mm); the third set (R_{L1}–R_{L3}) containing only large (L) granules (1.4–1.8 mm); and the fourth set (R_{N1}–R_{N3}) inoculated with the naturally distributed sludge (N). The EGSBs were operated identically, and after 51 days the sludge was removed for re-fractioning into XS, S, M, L and XL groups. The size distributions in each of the R_{S}, R_{M}, and R_{L} reactors had diversified during the trial (Fig. 5) indicating that granules do indeed grow, and that a life-cycle is probable.

Figure 5. Changes in size distribution in R_{S}, R_{M}, R_{L} and R_{N} bioreactors at takedown after a 51-day trial where (a) shows the initial and final distribution for the R_{S} – R_{S3}; (b) R_{M} – R_{M3}; (c) R_{L} – R_{L3}; (d) R_{N} – R_{N3} bioreactor sets and colours indicate the size of the emerging granules.

REFERENCES