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Corrigendum

Corrigendum to "Determining the mechanisms for aerobic granulation from mixed seed of floccular and crushed granules in activated sludge wastewater treatment" [Water Res. 46 (2012) 761–771]

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Determining the mechanisms for aerobic granulation from mixed seed of floccular and crushed granules in activated sludge wastewater treatment

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ABSTRACT

Aerobic granulation is a novel and promising technology for wastewater treatment. However, long start-up periods required for the development of granules from floccular sludge, and the loss of biomass in this period leading to poor nutrient removal performance are key challenges. In a recent study the addition of crushed granules to a floccular sludge significantly reduced the start-up period, and also maintained the nutrient removal performance during granulation. In this study, we examined the mechanisms responsible for the fast granulation from a mixture of floccular and granular sludges. Fluorescent microbead particles (4 μm diameter) were successfully applied to differentially label the surfaces of floccular and crushed granular aggregates. Labelled flocs and crushed granules were added to a laboratory scale wastewater treatment reactor, and the granule formation process was monitored using confocal laser scanning microscopy over an 80 day period. Flocs were observed to attach to the surface of the seeding granules, resulting in reduced biomass washout during granulation. This mechanism not only reduces the granulation period, but also maintains the nutrient removal performance of the reactor. The results indicate that the granules acted as nuclei for floccular particle attachment, which accelerated granule formation.

1. Introduction

Aerobic granulation is an emerging technology for secondary wastewater treatment. Compared to conventional floccular sludge, aerobic granules form larger and denser biofilm aggregates, which settle much faster and maintain higher biomass levels. Thus, the application of aerobic sludge granules has both operational and financial advantages (de Bruin et al., 2004; de Kreuk and de Bruin, 2004). Aerobic granules are normally generated directly from floccular sludge. Several operating parameters such as the aeration rate, substrate feeding mode, organic loading rate, and settling time have been manipulated to stimulate aerobic granulation (Beun et al., 1999; de Kreuk et al., 2007; Liu and Tay, 2002; McSwain et al., 2004; Wang et al., 2004). However, the mechanisms involved in aerobic granule formation in general are poorly understood.

Many studies have been successfully performed in laboratory scale reactors to develop granules from floccular sludge. However, large variations in start-up periods are evident (Table 1). Granules can be produced after only several days of operation when treating synthetic wastewater with very simple constituents (e.g. acetate) and with only carbon removal as the main performance objective (Tay et al., 2001b,

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Table 1 – Summary of length of start-up periods to achieve a granular system using laboratory SBRs.

Reactor type	Wastewater type	Process performance	[a]Period to achieve granular system (days)	References
SBR (lab-scale)	Synthetic	Carbon removal	7	(Tay et al., 2001b, 2001c)
SBR (lab-scale)	Abattoir	BNR	4	(Cassidy and Belia, 2005)
SBR (lab-scale)	Synthetic to Abattoir	BNR	170	(Yilmaz et al., 2008)
SBR (lab-scale)	Dairy effluent	COD and N removal	21	(Arrojo et al., 2004)
SBR (pilot-scale)	Domestic	COD and N removal	300 (85% granular system)	(Ni et al., 2009)
SBR (lab-scale)	Malting wastewater	Carbon removal	147	(Schwarzenbeck et al., 2004)
SBR (lab-scale)	Effluent from anaerobic digestor treating fish canning factory wastewater	N removal	75	(Figureroa et al., 2008)
SBR (pilot-scale)	Real wastewater (40% domestic sewage, 60% industrial)	COD and N removal	400	(Liu et al., 2010)
SBR (lab-scale)	Soybean processing wastewater	Carbon removal	14	(Su and Yu, 2005)
SBR (lab-scale)	Domestic	COD and BNR	80	(Verawaty et al., 2010)
SBR (lab-scale)	Abattoir	COD and BNR	18–133	(Pijuan et al., 2011)

a The definition of a granular system is defined here to be when the median diameter of the particle is >200 μm, however the definition may vary in the studies listed in this table.

2001c). Much longer times, up to 300 days, have been reported for the development of granular sludge when treating real wastewaters with biological nutrient removal capabilities (Table 1). Some of these studies report dramatic loss of biomass during granulation resulting in poor nutrient removal performance (Guo et al., 2007; Pijuan et al., 2011; Verawaty et al., 2010). Different reactor seeding strategies have been studied to reduce start-up periods for granulation. Microbial strains have been added to enhance cell aggregation in aerobic granular reactors (Ivanov et al., 2008; Jiang et al., 2006). Recently we developed a novel strategy by adding crushed granules to floccular sludge as the seed, to reduce the start-up time to as low as 18 days (Pijuan et al., 2011). The strategy minimizes biomass loss during the granule formation period, and hence maintains the nutrient removal performance during granulation. This strategy has been successfully applied to treat both nutrient-rich abattoir wastewater (Pijuan et al., 2011) and domestic wastewater (Coma et al., 2011). However, the fundamental understanding of this granulation technology is currently missing.In particular, it is currently not known how flocs and crushed granules interact during granule formation. An understanding of such interactions will provide support to further optimise the technology.

Currently there is a range of suggestions to explain the mechanisms of aerobic granule formation. This includes mechanisms of fungi or protozoa acting as a matrix for granule formation (Beun et al., 1999); a gradual transformation from floccular sludge to compact aggregates, then to mature granules (Tay et al., 2001a); and microcolony outgrowth to form granules dominated by particular species (Barr et al., 2010). Additionally, aggregation of microcolonies is suggested to cause particle size increase and development of aerobic granules (Ahn et al., 2009; Barr et al., 2010) and this mechanism is supported by heterogeneities in cell arrangement and extracellular polymeric substances (EPS) appearance in granule ultra structure (Lemaire et al., 2008). However, many of these suggestions are based on observations and lack direct supportive evidence of the mechanism for the granule formation.

In this study we aimed to determine the mechanism of granule formation using a labelled biofilm approach. Here we used the advanced granule formation strategy that employs a mixture of floccular sludge and crushed granules. Both flocs and crushed granules were labelled with fluorescent microbeads prior to being mixed in a laboratory scale sequencing batch reactor (SBR) treating abattoir wastewater. Throughout the experiment the labelled particles were monitored to reveal the interactions between flocs and crushed granules. This is for the first time providing detail of the granule formation mechanism, especially in relation to the innovative accelerated strategy which is leading towards the full-scale application of this technology.

2. Materials and methods

2.1. *Microbead labelling of flocs and crushed granules*

Floccular sludge was obtained from a full-scale wastewater treatment plant (WWTP) in Queensland, Australia performing chemical oxygen demand (COD), nitrogen (N) and phosphorus (P) removal from domestic wastewater. Granular sludge was sourced from a laboratory scale reactor treating abattoir wastewater (Pijuan et al., 2011). Crushed granules were obtained by pressing intact granules through a sieve with an aperture of 500 μm. Flocs and crushed granules were then labelled with fluorescence microbeads. Flocs were incubated with red florescence microbeads (Invitrogen, CA, USA) catalogue number: F8858, with diameter of 4 μm and excitation/emission maximums of 580/605 nm. Crushed granules were incubated with green florescence microbeads (Invitrogen, CA, USA) with catalogue number: F8859, a diameter of 4 μm and

excitation/emission maximums of 505/515 nm. Microbeads were added to cover between 20 and 40% of the crushed granule and floc surface areas, which were determined as previously described (Beun et al., 1999). The suspensions were vortexed briefly and incubated in 25 ml flasks while shaken horizontally (100 rpm) for up to 8 days. Samples of the biofilms were taken daily and viewed by fluorescence microscopy. 100 μl samples were taken, washed 3 times with phosphate buffer saline (PBS) to remove unbound beads, and then applied to gelatine coated glass slides and air dried. The dried samples were counterstained with the EPS stain Concanavalin A (ConA), with excitation/emission maximums of 543/560 nm (Invitrogen Co., Carlsbad, CA, USA) as previously used (Chen et al., 2007) and viewed by fluorescence microscopy at 10 times magnification (Olympus SZH10). The successfully labelled flocs and crushed granules were collected and washed with PBS to remove the unbound beads before used in the batch tests or the laboratory scale sequencing batch reactor described below.

2.2. Batch tests to assess the stability of the microbead labelling

Batch tests were conducted to assess the efficiency and stability of the microbead adherence to the flocs and crushed granules. Separate suspensions of flocs and crushed granules, both at 1 $gVSS.L^{-1}$ (VSS – volatile suspended solids), were labelled with microbeads as described above. The labelled granules and flocs were mixed at a volume ratio of 3:7 respectively, and then 13 ml of the mixture was introduced to 12 ml of wastewater and incubated with horizontal shaking (100 rpm) for 7 days. Biofilm samples from the batch tests were taken daily and viewed by fluorescence microscopy as described above. Additionally, samples were viewed by confocal laser scanning microscopy (CLSM) (LSM 510, Zeiss, Germany) and images were analysed to determine the spatial arrangement of the labelled biofilms as described below (see section on Microscopy of biofilms).

2.3. Reactor operation for granule formation

A laboratory scale sequencing batch reactor (SBR) with a 2 l working volume was operated for nutrient removal while treating abattoir wastewater as previously described (Pijuan et al., 2011). The SBR was inoculated with microbead labelled aggregates, prepared as described above, consisting of 35% crushed granules (1.05 $gVSS.L^{-1}$) and 65% of floccular sludge (1.95 $gVSS.L^{-1}$). The wastewater feed comprising: soluble COD of 862–1137 mg L^{-1}; VFA of 650–800 mg L^{-1}; N–NH_4 of 200–254 mg. L^{-1}; and P–PO_4 at 31–40 mg L^{-1}; was collected weekly and kept at 4 °C. The wastewater temperature was adjusted to the SBR operating temperature of 20–23 °C when fed to the reactor. The SBR was operated with a cycle time of 8 h, each cycle comprising feed, anaerobic, aerobic, anoxic, settling and decant phases as described (Pijuan et al., 2011). During the initial SBR operation, 250 ml of wastewater was fed in each cycle, resulting in a volume exchange ratio (VER) of 12.5% and a hydraulic retention time (HRT) of 64 h. Providing a nutrient removal efficiency of 80% Nitrogen and 95% COD during the SBR operation, the VER was increased each week until reaching 50%. Additionally, the settling performance, the time required for the biomass to settle to the final decant level of the reactor, of the sludge was monitored and the settling time was progressively reduced such that a small fraction of the slower settling biomass was removed in the effluent. Consequently, the settling time was sequentially lowered from the initial 20 min down to 5 min, such that at the latter stage a completely granular system had developed.

2.4. Chemical analysis of SBR samples

SBR samples were analysed three times per week for mixed liquor suspended solids (MLSS) and mixed liquor volatile suspended solids (MLVSS) using standard methods (APHA, 2006). Granule size was measured two times per week from 30 ml samples of SBR mixed liquor taken at the end of the aeration period on a Malvern Mastersizer (2000 series, version 5.60, Malvern Instruments Ltd, Malvern, UK). Biochemical transformations were monitored weekly through a SBR cycle. During a SBR cycle, mixed liquor samples were taken and filtered using millipore filters of 0.22 μm pore size (Millipore Ireland Ltd). The filtrate was analysed for ammonium (NH_4^+), nitrate (NO_3^-), nitrite (NO_2^-) and orthophosphate (PO_4^{3-}) on a Lachat QuickChem8000 Flow Injection Analyzer (Lachat Instrument, Milwaukee, USA) (APHA, 2006). The filtrate was also analysed for volatile fatty acids (VFAs) using a Perkin–Elmer gas chromatograph with a DB-FFAP column of dimensions 15 m × 0.53 mm × 1.0 μm (length × ID × film) operated at 140 °C, and injector and FID detector temperatures at 220 °C and 250 °C respectively (APHA, 2006).

2.5. Microscopy of SBR and batch test biofilms

Microscopic analyses were conducted for morphological characterization and for analysis of attached microbeads to cell aggregates over the time of SBR operation. Mixed liquor from reactor was sampled every two days and fixed for 8 min using Paraformaldehyde (PFA) (Amann et al., 1990) prior to EPS staining using ConA for 2 h (Chen et al., 2007). EPS staining was required for three-dimensional image reconstruction and image quantification analyses. Unfixed sludge samples were used for routine monitoring by epifluorescence (Olympus BX61) and stereo microscopy (Olympus SZH10). Fixed samples of granules were applied to glass slides after staining with ConA, air dried and the fluorescent signal was viewed by confocal laser scanning microscopy (CLSM) (LSM 510, Zeiss, Germany) equipped with argon, helium and neon lasers, and a plan-apochromatic objective (10×). Three-dimensional surface reconstructions of granules were obtained from image stacks obtained by CLSM and the Carl Zeiss LSM Imaging Software, using z-steps of 6.32 μm thickness.

Quantification of attached microbeads was performed on images obtained by CLSM on both fixed intact and cryosectioned granules. For each fixed intact granule sample, 100 μl was applied to at least 6 gelatine coated glass slides and air dried. For cryosectioning, granules were fixed (8 min) with paraformaldehyde (PFA), stained with ConA and then embedded into the optimal cutting temperature media (OCT) (TissueTek, Sakura Finetek, Torrance, CA). Those granules were frozen and sectioned into 10 μm thick slices using

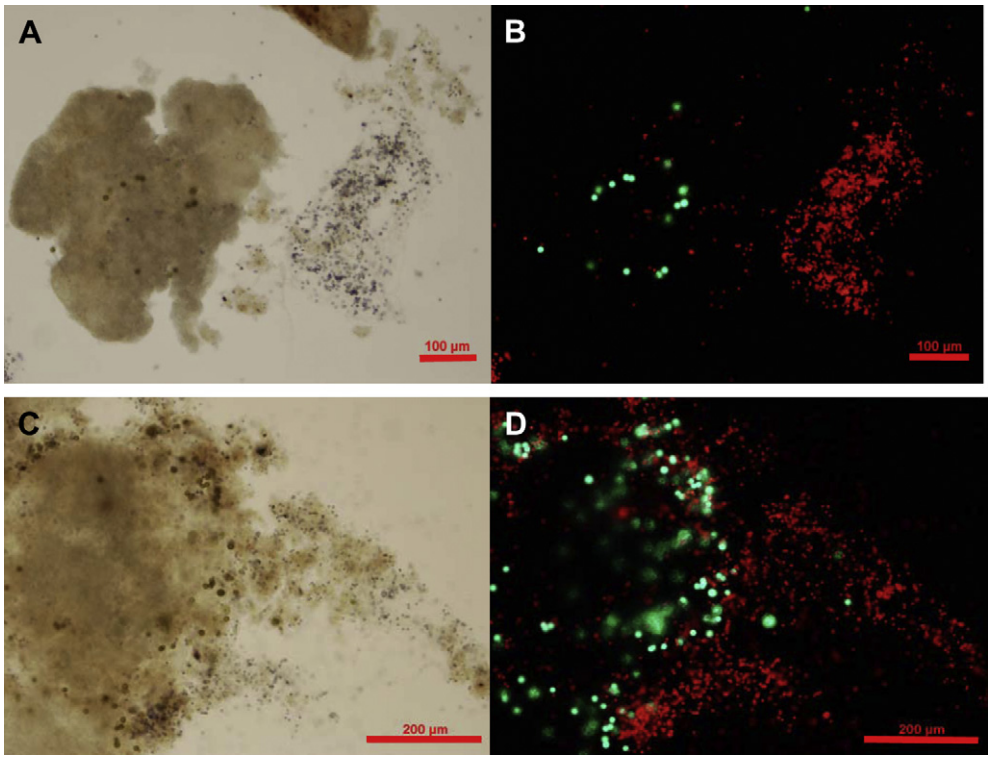

Fig. 1 – Images of light and epifluorescent microscopy of aggregates from the batch tests of red labelled flocs with green labelled crushed granules taken at day 0 A and B, and day 7, C and D. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

a cryotome operated at −20 °C (Kryo 1720; Leitz, Wetzler, Germany). The slices were collected onto SuperFrost Plus microscope slides (Menzel-Glaser, Braunschweig, Germany), then washed with MiliQ water and air dried (Chen et al., 2007). For each sample 20-30 digital images from randomly chosen fields were collected and quantification of microbeads was performed using the Daime software (Daims et al., 2006). Quantification of attached red and green microbeads was calculated as the biovolume fraction of the total biofilm area stained by ConA.

The spatial arrangement analysis was performed to determine if the arrangement of the fluorescent beads were random relative to each other (e.g. the position of red beads relative to the green beads). This was performed to further examine the stability of the bead labelling. If the adherence and detachment of the microbeads to the cell aggregates was dynamic, this would tend towards a random arrangement of microbeads over time. Samples were prepared as described for quantification of microbeads above and examined by CLSM. For each spatial arrangement analysis, 30 images containing green and red fluorescent signal were recorded at random positions and random focal planes. One population spatial arrangement (single microbead label experiment) and two population spatial arrangement (dual microbead label experiment) analyses were performed as described using Daime software (Daims et al., 2006). Briefly, images were imported into Daime, for dual label experiments the colour channels were separated, and the resulting image series were 2-D segmented. The pair cross-correlation function $g(r)$ of the fluorescent signal was then determined over distances between 0.6 and 600 um (Daims et al., 2006). This function determines whether the spatial distribution of the fluorescent beads in the image is random, attractive or repulsive over a range of distances (r). Such that $g(r) > 1$ indicates attractive, $g(r) < 1$ indicates repulsive, and $g(r) = 1$ indicates random spatial distributions.

For scanning electron microscopy (SEM) sludge samples were first stabilized using 2.5% glutaraldehyde and 75 mM lysine in 0.1 M cacodylate buffer for 10 min (Jacques and Graham, 1989). Then samples were fixed twice for 10 min in 3% glutaraldehyde in 0.1 M cacodylate buffer, and immediately washed with 0.1 M cacodylate. All subsequent preparations were performed in a Pelco Biowave microwave oven. Samples were treated in 1% osmium tetroxide, dehydrated in a graded ethanol series (20–100%) and infiltrated with the drying agent hexamethyldisilazane (Lemaire et al., 2008). After drying overnight the samples were sputter coated with platinum. Visualization of samples was conducted using a JEOL Neoscope Scanning Electron Microscope (Hoskin Scientific Tokyo, Japan).

3. Results

3.1. *Assessing the microbead labelling of cell aggregates*

Initial tests were conducted to observe the microbead labelling of the aggregates, in which the floc and granule

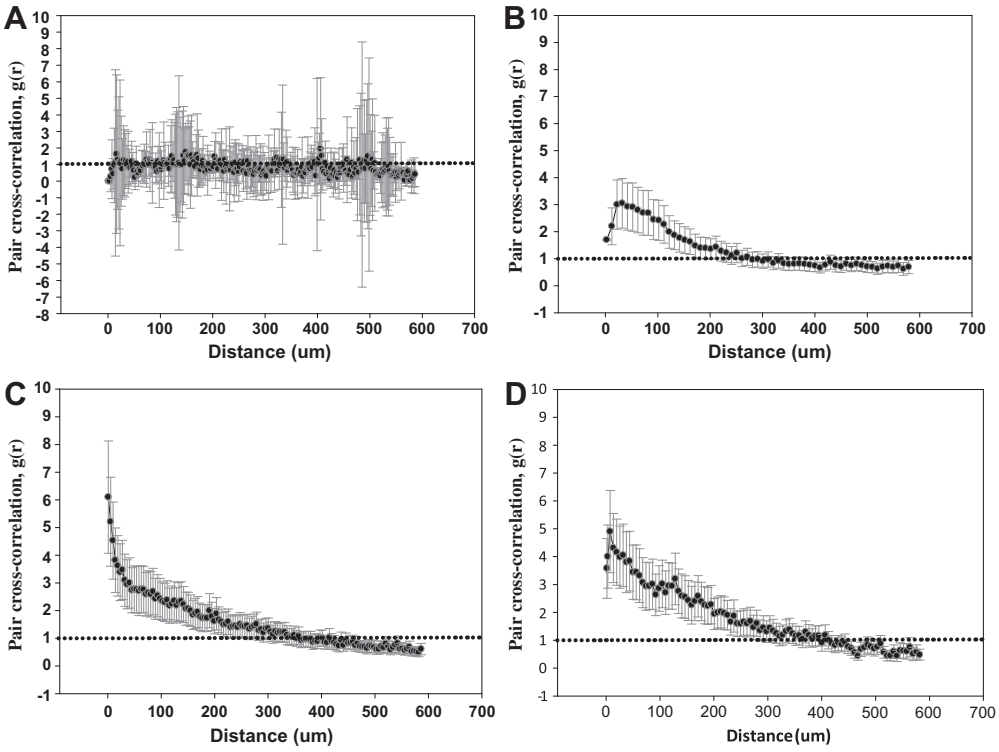

Fig. 2 – Determining the spatial arrangement of microbeads during experiments on the labelled aggregates. The analyses were performed to determine the pair cross-correlation function *g(r)* averaged over the set of images (represented by the black filled cirles) and determined over a range of distances between fluorescent beads. The standard errors are the 95% confidence intervals of *g(r)*. Spatial arrangement of one population was determined in a sample of microbeads only as a control (A) and in floccular sludge immediately labelled with microbeads (B) (see Materials and Methods). Spatial arrangement of two populations was determined in dual floccular sludge and crushed granule labelled experiments, from samples of the 7 day batch experiment (C), and on samples from the SBR on day 30 of operation (D). Pair cross-correlation values near 1 indicate the bead arrangement is random. Values above or below 1 indicate the beads are clustered or they are repulsed respectively at that particular distance.

aggregates were labelled separately. The highest surface coverage of the flocs and granules was obtained after 3 and 8 days incubations respectively (results not shown). Thus, we concluded the microbead labelling of the aggregates was successful. Batch tests were then performed to assess the dynamics of the microbead attachment prior to conducting a long-term experiment using labelled flocs and granules into a SBR. The successfully labelled flocs and granules were mixed and incubated together. On day 0 of the batch test, the labelled flocs (red) and labelled granules (green) still show their original arrangement (Figs. 1A and 2B). After seven days of incubation, some labelled flocs had attached to the surface of the labelled granules. The red beads were observed as mostly on the surface of the aggregate, suggesting the attachment of flocs forming a layer of coverage on the labelled crushed granule surface (Fig. 1C and D). The spatial analysis of the freshly labelled floccular sludge indicated that the beads were clustered up to a distance of around 200 μm, and consequently the arrangement was not random; see comparison of Fig. 2A and B, which was also observed by microscopy (Fig. 1B). The distance limitation in the analysis (200 μm) results from the non-homogenous nature of activated sludge and the floc diameter (Daims et al., 2006). Then following the batch test incubation of the labelled flocs and granules the spatial analysis indicated a clustered arrangement remained between the microbeads (Fig. 2C). This was important as a random arrangement would suggest the bead attachment and detachment was dynamic. Consequently, the analysis showed that at least for this seven day period the microbead labelling was permanent, such that the beads remained attached to the type of biofilm to which they initially adhered. Importantly, this supported our approach of using the labelled aggregates to study the floc and crushed granule interactions during the formation of aerobic granules in an SBR operation.

3.2. *SBR operation for granule formation*

Granule formation within the SBR was monitored by observation of the labelled flocs and crushed granules over an 80 day operating period. The SBR was treating abattoir wastewater performing biological nutrient removal. To encourage granule selection the biomass settling performance was monitored and the settling period was progressively decreased from 20 min initially, to 7 min at day 21 and then finally to 5 min from day 28 to the end of operation. The VER was also altered from 12.5% to 50% VER after 25 days that

resulted in a decreased hydraulic retention time (HRT) from 64 to 16 h (Fig. 3A). The initial SBR biomass was 3 g L^{-1} MLVSS and this increased progressively to 7.33 g L^{-1} MLVSS after the 80 day period (Fig. 3A). The sludge retention time (SRT) also progressively decreased from its initial value of 100 to be between 20 and 18 days at the latter stage of operation. Granule formation was evident from particle size changes, these increased noticeably from around 85 μm (median particle size) on day 0, to around 980 μm (median) on day 80 (Fig. 3B). The SBR aggregates were mostly granular by day 50, as the median particle size had reached 625 μm. The system was completely granular from day 65 as the 10th percentile had risen above 200 μm (i.e. 90% of the particles were larger than 200 μm) (Fig. 3B).

The SBR was operated for biological nitrogen (N) and phosphorus (P) removal. Stable N removal was achieved throughout the operation with a removal efficiency of 82–93%, but stable P removal was not achieved. Biological phosphorus removal from abattoir wastewater is challenging due to the high levels of ammonia in the influent. This results in high levels of nitrite and nitrate during nitrification, which are detrimental for establishing stable biological phosphorus removal (Lemaire et al., 2009; Pijuan and Yuan, 2010).

3.3. *Microscopic evaluation of granule formation during the SBR operation*

Epifluorescent microscopy, SEM, and CLSM were used to extensively monitor and analyse the granule formation throughout the reactor operating period (Fig. 4). Aggregate morphology and microbead patterns were detected. Some self

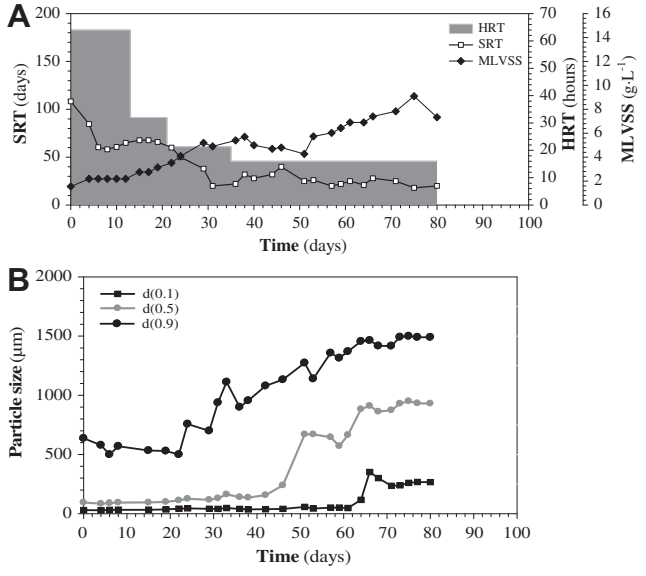

Fig. 3 – The SBR operation hydraulic retention times (HRT), sludge retention times (SRT) and mixed liquor volatile suspended solids (MLVSS) (A). Sludge particle size distribution during the SBR operation for granule formation (B). The filled black circles are the 90th percentile size (ie. 90% of the sludge is smaller than that value), the filled grey circles and the filled black squares are 50th and 10th percentiles of the particle sizes respectively.

aggregation of floccular material was observed to develop into larger aggregates. However, the major pattern observed was aggregation of floccular and granular biomass. Images taken on day 26 of operation represent the typical aggregation found in the reactor (Fig. 4D and E). Interestingly, in this experiment, the self aggregation of smaller crushed granules into larger granules was not observed. During the operation floccular biomass was detected in the effluent from the SBR and these washed out particles were labelled red, confirming that they were flocs (results not shown).

Floc and crushed granule aggregation was also evident from SEM images where layers of flocs (with an apparent loose structure) protruded from granule surfaces (Fig. 4A). This arrangement of the labelled floc material attaching to the labelled granule surface was also observed by CLSM (Fig. 4B and C, D and E). According to the spatial analysis the flocs and crushed granules were clustered and were not randomly arranged (Fig. 2D). This was detected up to a microbead distance of around 400 μm, representing the aggregate diameter (Daims et al., 2006). This supports the finding of the batch experiments, that the microbead labelling to the original aggregate was permanent. This is also evidence of a structural arrangement between the floc and crushed granule aggregates. Often the floc material was observed to attach to rough or indented regions of the granule surface, suggesting that shear forces influenced aggregation sites. Additionally, ciliates were frequently observed in the surface crevices of the aggregates (results not shown).

Using a quantitative approach to monitor the granule formation we measured the microbead fluorescence green to red (G/R) ratio from images of granules (>500 μm particle size) over the 80 days operation (Fig. 5). The G/R ratio decreased from 17 at the start of operation to 2.5 after 17 days of operation, and it remained low afterwards. As the average number of green beads on a granule is assumed stable the decreasing of G/R ratio suggests an increase of red bead labelled flocs attaching to the surface of the crushed granules. This indicates during the first 17 days there was extensive attachment of flocs to granules. The ratio remained fairly stable from day 22 onwards; indicating floc attachment to the crushed granules was permanent (Fig. 6).

4. Discussion

There is much interest to decrease the time required for granule formation, and to maintain biomass levels and reactor performance during reactor start-up periods (Ivanov et al., 2008; Jiang et al., 2006; Pijuan et al., 2011). Recently we have used a novel strategy to reduce the start-up time of an aerobic granular system by combining crushed granules with floccular sludge as the reactor seed (Coma et al., 2011; Pijuan et al., 2011). The main thrust of this study was to use this advanced method for granule formation and then to observe granule formation patterns between the floc and crushed granule starting material.

We considered some hypotheses to describe possible interactions between the flocs and crushed granules in the accelerated granule formation process (Fig. 6). These hypotheses explaining the early stages of increasing aggregate size

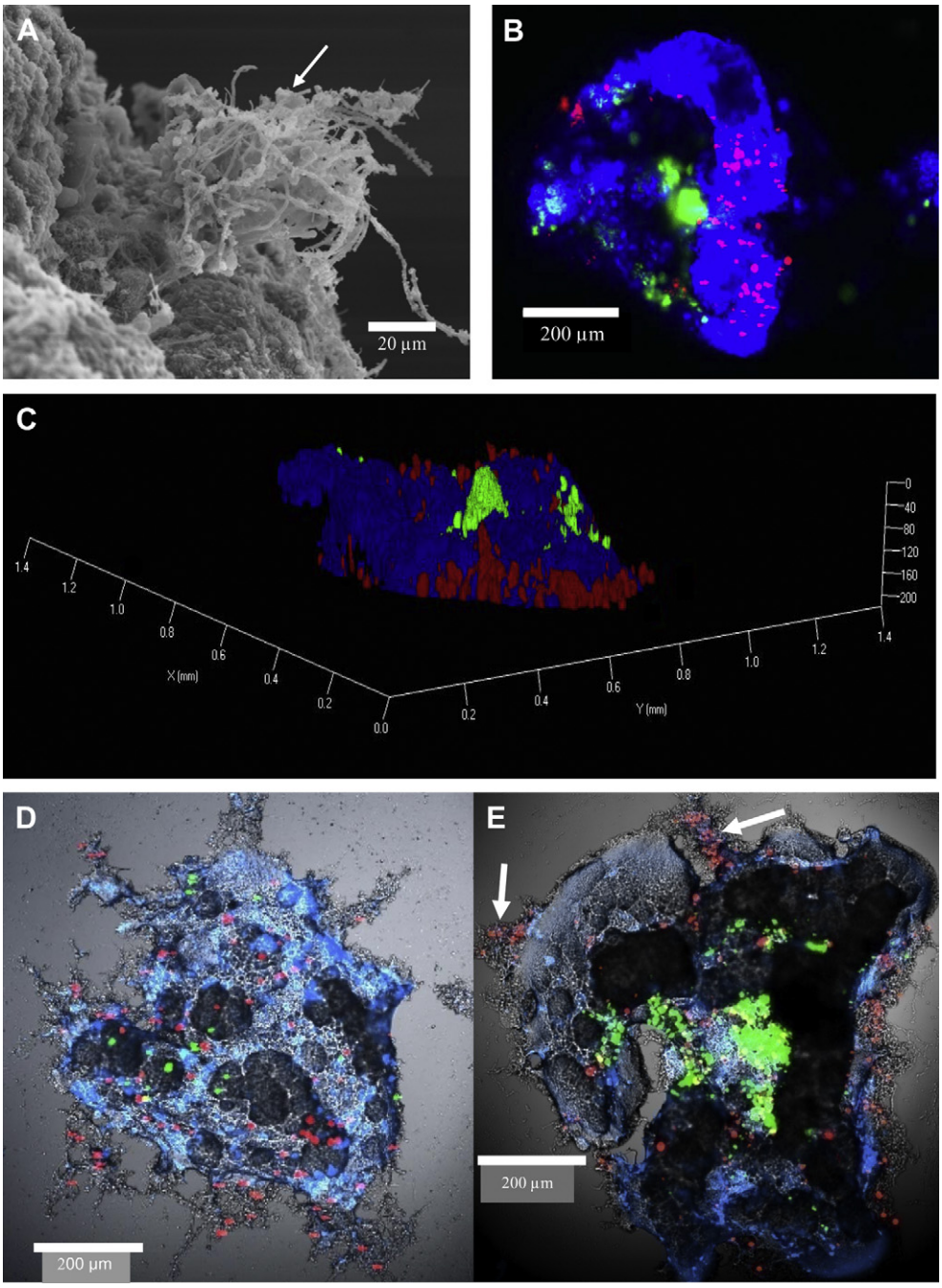

Fig. 4 – Evidence of attachment of flocs onto granule surfaces on day 26 of reactor operation. An SEM image of the granule surface with what appears to be protruding floc material, arrowed (A). The CLSM images of a section of a granule (B) and of a three-dimensional representation of a granule with attached flocs (C). The biofilm EPS is stained blue, flocs are labelled red and crushed granules are labelled green. Brightfield and fluorescence CLSM images showing the attachment of labelled flocs (red) to the surface of the labelled granules (green) (D and E). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

include: (i) the crushed granule acts as a nuclei to which floccular material attaches; (ii) flocs attach together to form bigger particles; (iii) growth of microorganisms on the crushed granules and (iv) small crushed granules attach together to form larger granules. Our results detected various bead patterns by microscopy as the aggregates developed into granules in the reactor (Fig. 4). Some floccular material attached together to develop into larger aggregates; however the proportion of aggregates with this pattern was small. The major pattern observed was attributed to the aggregation of floccular and granular biomass. These results support hypotheses (i) that the crushed granules act as nuclei to which

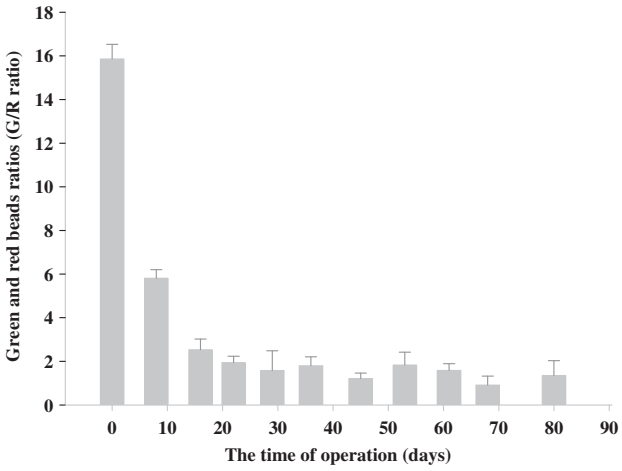

Fig. 5 – The average ratio of green to red beads measured from 25 to 30 individual growing granules taken from the SBR on sampling days during the 80 day operating period. Error bars are the standard error of the average. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

the floccular material attached and enhance the granule formation. This was the key mechanism detected to explain the accelerated granule formation process using a starting mixture of floc and crushed granule aggregates.

According to the analysis of the microbead labelled aggregates, this floc attachment mostly occurred during the first 20 days of reactor operation. During this time there was a slight increase in the granule size, although most of the size increase occurred after day 45. It is apparent that aggregation of the floc material did not increase the granule size substantially. However the reactor biomass did increase in that period, from approximately 2–4 g L^{-1}, indicating new growth was occurring possibly around these aggregates. Consequently, it seems the beneficial effect of the crushed granules is to aid retention of the floc material during the early stages of reactor start-up. At this time selecting forces, such as decreasing settling times, were applied for granule formation. This would aid to increase the granule size, and aid to maintain biomass and reactor performance (Pijuan et al., 2011).

Stable attachment of the flocs to the granules was detected. Crevices in the granules were frequently observed as favourable sites for floc attachment. Although, sequential attachment of further flocs past day 20 was not observed, and the ensuing increase in granule size was due to biomass growth. Possibly the easy attachment sites were used up. The nature of the attachment was not studied here, but may occur as through suggested biofilm development stages of reversible and irreversible attachment followed by EPS production (Kolenbrander et al., 1985; Zhang et al., 2007). Specific factors that influence biomass attachment may include the local chemistry and microbial community composition (Wang et al., 2005; Yu et al., 2009), the presence of indentations on the granule surface and fluid shear conditions (Tay et al., 2001a). Other studies have suggested that solids in the influent may serve as nuclei for granulation (Cassidy and Belia, 2005), or that fungi may serve this purpose while treating synthetic wastewaters without influent solids (Beun et al., 1999; Morgenroth et al., 1997). Furthermore, ciliates are reported to play a role in granule formation. We observed ciliates in granule crevices that were also favourable attachment sites for floc aggregates. Other studies implicate their importance in granule structure, suggesting they provide a backbone for the granule (Weber et al., 2007), and by improving fluid flow and localised nutrient availability (Zima-Kulisiewicz et al., 2009). We suggest the irregular surface of the crushed granules was important for floc attachment in the initial stage to form larger granules by providing a larger and rougher surface for the floccular biomass attachment under the existing shear forces. Similar positive effects of rough surfaces on biofilm development, bacterial attachment and secondary colonization, has been detected previously (Tijhuis et al., 1996). A number of these factors are likely to have influenced the floc and crushed granule aggregation and the subsequent granule size increase observed here.

Our results imply that the crushed granules act as nuclei for floccular particle attachment, which accelerates the granule formation. A challenge in the full-scale application of aerobic granules is maintaining treatment performance during the transition from a floc to a granule biomass (Weber et al., 2007), and there are few experiences reported regarding nutrient removal. However, recently nutrient removal was maintained during start-up of an aerobic granule reactor treating domestic wastewater (Coma et al., 2011). In that study the reactor was started from a mixture of crushed granules and floc biomass and the improved nutrient removal performance is suggested to result from the better retention of floc material in the early stages of granule formation. This study provides important supporting evidence for granule formation in this innovative technology, which is relevant for its application to full-scale wastewater treatment.

In this study we used fluorescent microbeads to label cell aggregates, we then monitored the aggregation of the differentially labelled flocs and granules in a granulation reactor during an extended period (80 days). This is to our knowledge the first occasion to use this labelling approach to monitor biomass development over an extended period. It was seen in the batch tests, that for the 7 day period, the labelling was permanent and thus likely appropriate for an extended experiment within a reactor, which was consequently performed. Previously, fluorescent microbeads have been used as tracers for particle movement to examine the transport of particulate species within biofilms (Drury et al., 1993; Tijhuis et al., 1994), for investigating the dynamics of spatial distributions of particulate components in mixed population biofilms (Okabe et al., 1997) and also for microsphere adhesion to cells to measure cell hydrophobicity (Olofsson et al., 1998). This study for the first time provides detail of the granule formation mechanism, especially in relation to the innovative accelerated strategy which is leading towards the full-scale application of this technology. This novel use of fluorescent microbeads provided foundation methodology for studies of biofilm dynamics.

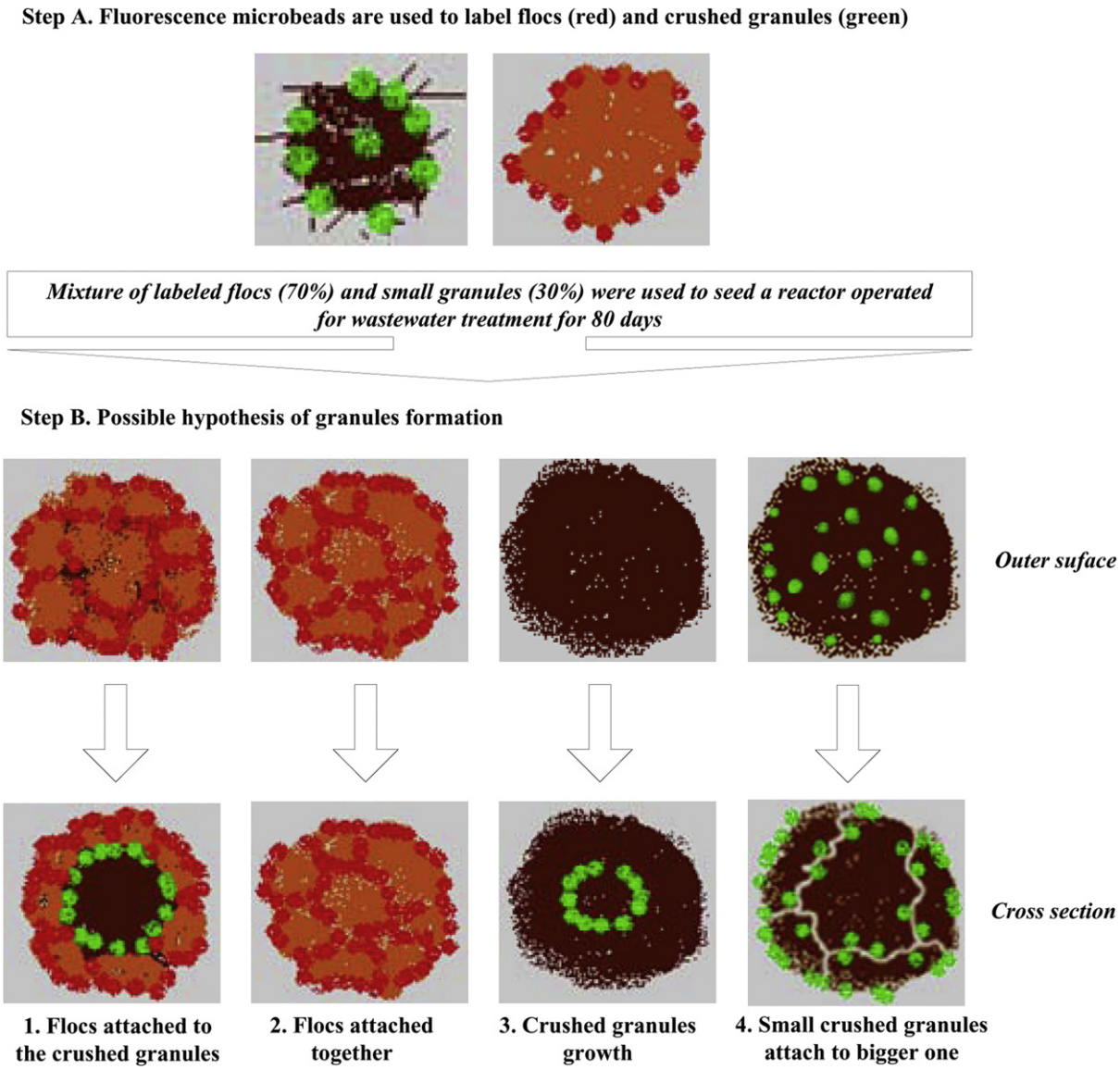

Fig. 6 – Outline of study using crushed granules and floccular biofilm that are fluorescently labelled and some hypothetical outcomes of granule formation mechanisms. Step A. The surface of flocs and granules are labelled with fluorescent microbeads (flocs red, granules green) and are mixed and used to seed a reactor. Step B. Larger granules form during the start-up period and the patterns of beads on the outside and within the granule are assessed to understand the mechanism of formation. The proposed aggregation patterns include: 1. The crushed granules act as nuclei where the flocs attach, 2. The flocs attach together to form bigger particles, 3. The crushed granules grow bigger due to biomass growth, and 4. The crushed granules attach to form larger granules. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

5. Conclusions

A novel method for crushed granules and flocs labelling was developed and applied to understand aerobic granules formation cultivated from the mixture of floccular and crushed granular seed sludge in SBR treating abattoir wastewater. It was demonstrated that crushed granules acted as nuclei for the attachment of floccular sludge during the initial stage of granule development. This physical attachment aided to avoid aggressive biomass loss as the system is pushed for granule development. Consequently, this assists for maintaining higher biomass and good BNR performance, and is the likely mechanism contributing to the enhanced granulation process.

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