Granular biomass structure and population dynamics in Sequencing Batch Biofilter Granular Reactor (SBBGR)

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ABSTRACT

The aim of this paper is to study the microbial and structural changes occurring during the transition from flocculent (used as inoculum) to biofilm and granular sludge in a Sequencing Batch Biofilter Granular Reactor (SBBGR). SBBGR is a new and promising technology characterised by low sludge production (5–6 times lower than in conventional treatment plants), high biomass concentration (up to 35 g TSS/L_{bed}), high COD conversion capacity, high effluent quality and operation flexibility. Molecular *in situ* detection methods and microscopy staining procedures were employed in combination with the traditional measurements (i.e., oxygen uptake rate, COD removal efficiency) to evaluate the microbial activity and composition of the granular biomass. The granules structure was investigated by electron scanning microscopy, phase contrast analysis of granule sections and specific extracellular polymeric substances (EPS) stainings. Evident changes in biomass composition was observed during the shift from activated to granular sludge while a stable presence of active bacterial populations (mainly Proteobacteria) was found within mature granules.

1. Introduction

Aerobic granulation, a recently introduced biotechnological process, is increasingly drawing the interest of researchers working in the field of wastewater treatment because it deals with some of the frequent drawbacks associated with traditional biological processes, such as large area requirement, high sludge production, poor sludge settling properties (Horan, 1990). One of the most promising granular biomass based systems is Sequencing Batch Biofilter Granular Reactor (SBBGR) technology. In this system, the granules are not suspended as in Sequencing Batch Granular Reactor (SBGR), but retained in the pores produced by packing the reactor with a filling material (a secondary settler is therefore no longer necessary). This allows greater biomass retention in the reactor to be obtained (up to one magnitude order higher than that recorded in conventional biological systems). As a result, a notable increase in sludge age is achieved with consequent reduction in sludge production (Di Iaconi et al., in press).

In the SBBGR system, granular biomass generation takes place during the reactor start-up period (around 3 months). A gradual shift from biofilm growing on the carrier surface to granular biomass is observed during this phase. In particular, four steps can be distinguished in this process (Di Iaconi et al., 2005a): (1) the for-mation of a thin biofilm that fully covers the carrier surface, (2) an increase in biofilm thickness, (3) the detachment of a portion of the biofilm that covers the carriers, thus releasing biofilm particles, (4) the rearrangement of biofilm particles in smooth granules.

Three factors play a decisive role in SBBGR granular biomass generation: the trend of the hydrodynamic shear forces, the start-up operative conditions (i.e., the pattern of organic loading rate increase), and bed material features (Di Iaconi et al., 2005b). In particular, the authors found that during the first two steps, the reactor is characterised by rather weak shear force values. Under these weak forces, the biofilm, which is regulated primarily by the organic substrate loading rate, continuously increases in thickness. This produces a corresponding increase in the shear forces with negative effects on biomass stability, causing the detachment of biofilm particles (step 3). This, in turn, triggers a further sharp increase in the shear forces, promoting the rearrangement of the detached biofilm particles into smooth granules by continuous removal of protuberances (step 4). When biofilm detachment takes place, however, the filling material characteristics are crucial in granule generation since they must guarantee the retention of the detached biofilm particles. In particular, if a filling material which generates beds characterised by large pore volumes is used, biomass granulation cannot be achieved because of the expulsion of biofilm particles from the bed (Di Iaconi et al., in press).

Taking into account SBBGR performances recorded during the treatment of municipal and industrial wastewater (Di Iaconi

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et al., 2003, 2005a), more recently, the European Commission has financed the technological transfer of this technology to demonstrative scale (Life Project PERBIOF).

Nevertheless, due to its technological novelty, little is known about the microbiological aspects of SBBGR biomass. Therefore, in order to study the microbial changes during the evolution from flocculent (used as inoculum) to biofilm and granular sludge, a laboratory scale SBBGR reactor with a volume of 10-l was operated for 7 months.

2. Methods

2.1. Lab-scale SBBGR and operation

The investigation was carried out using a lab-scale SBBGR, a diagram and photograph of which are shown in Fig. 1. It consisted of a cylindrical reactor (geometric volume: 10 L; diameter: 18 cm; height: 40 cm) partly filled (fixed bed volume: 3.5 L) with biomass support material (wheel shaped plastic elements; features: 7 mm high, 8 mm diameter, specific area 690 m^2/m^3, 0.95 density, and bed porosity 0.74) packed between two sieves and aerated by air injection (at a flow rate of 200 L/h) through porous stones placed close to the upper sieve. An external loop allowed wastewater re-circulation (at a flow rate of 90 L/h) through the filling material by means of pump P$_R$ in order to assure a homogeneous distribution of substrate and oxygen.

A pressure meter set at the bottom of the reactor measured on-line biofilter headlosses due to biomass growth. When a fixed value of headloss was reached (i.e., 100 cm), a washing step was carried out by means of compressed air until the headloss decreased to a predefined value (i.e., 60 cm). The washing water was collected and measured (as TSS, total suspended solids) in order to calculate the specific sludge production.

The SBBGR operation was based on a succession of three treatment cycles per day, each consisting of three consecutive phases: filling, reaction and drawing. During the filling phase (length: 10 min), 2.5 L synthetic wastewater were pumped (by means of pump P$_F$) into the SBBGR. In the reaction phase (length: 450 min), the filled wastewater was continuously aerated and recycled through the biomass support material. Finally, the treated wastewater was withdrawn (length: 10 min) by gravity from the reactor (by opening the valve V$_M$) and the plant was ready to start a new treatment cycle. The operation of the system was completely automated using a programmable logic controller (PLC).

Activated sludge from the municipal wastewater treatment plant of Bari was used as inoculum.

2.2. SBBGR operative schedule

The experimental activities were split in two periods (A, B). Period A referred to the granular biomass generation, whereas period B to steady state conditions. Table 1 reports the operative conditions of the plant in both periods.

Concentrated synthetic wastewater was used to prepare the reactor feed. The composition of the concentrated media was as follows: glucose, 120 g/L; NH$_4$Cl, 38 g/L; Na$_2$HPO$_4$, 5.5 g/L. The SBBGR feed during period B, obtained diluting synthetic wastewater with tap water, resulted in an influent COD (chemical oxygen demand), ammonia and phosphorus concentration of 960 mg/L, 80 mgNH$_4$-N/L and 9.6 mg PO$_4$-P/L, respectively.

In the start-up period (i.e., period A), during which the organic load value was progressively increased from 0.25 to 2 g COD/L$_{bed}$ d (through six steps, 0.25, 0.5, 1.0, 1.25, 1.5, 2.0 g COD/L$_{bed}$ d, respectively), a higher dilution ratio of concentrated synthetic wastewater was used.

2.3. Analytical methods and procedures

COD, ammonia, nitrite, nitrate, phosphorus, TSS and VSS (volatile suspended solids) were determined according to standard methods (APHA, 1998). DO and pH were on-line measured using selective probes.

The specific excess sludge production (as g TSS/g COD$_{removed}$) was calculated by dividing TSS leaving the system (i.e., TSS discharged with the effluent + TSS removed during the washing operation + TSS sampled for microbial and physic-chemical analysis) by the amount of COD removed in the period between two washing operations.

Biomass concentration was evaluated in terms of TSS and VSS, on representative samples of biomass detached from a bed volume previously determined by counting the carrier elements and relating them to carrier element number in 1 L of bed (i.e., 1023). Biomass density, expressed as grams of TSS per litre of biomass, was determined with dextran blue according to the method recently developed by Beun et al., 2002.
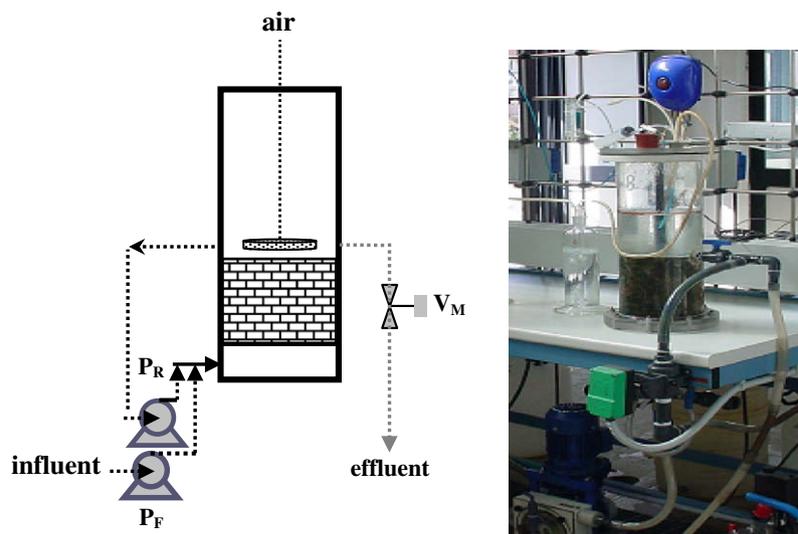

Fig. 1. Diagram and photograph of SBBGR.

Table 1
Plant operative conditions during the start up (period A) and steady state conditions (period B).

Parameter	Period	
	A	B
Duration (d)	120	90
Organic load (g COD/L_{bed} d)	0.25–2	2
Hydraulic load (L/d)	7.5	7.5
Daily treatment cycle number	3	3
Re-circulation flow (L/h)	90	90
Air flow rate (L/h)	200	200
Frequency washing operation (d)	0	[a]

[a] Whenever headloss value reached 100 cm.

2.3.1. Respirometric tests

The maximum specific oxygen uptake rate ($SOUR_{max}$) of the biomass was measured using a 1 L closed completely mixed reactor with a high liquid/gas volume ratio ($V_L/V_G = 100$). OUR tests were carried out by adding an appropriate quantity of biomass (previously aerated for 12 h) to the reactor, in order to get a VSS concentration around 1000 mg/L. The biomass was aerated until the saturation value was reached; soon after, a large fixed volume of synthetic wastewater was added and the DO profile was recorded by means of an oxygen electrode (WTW OXI cell 325) interfaced with a PC for automatic recording and processing of data.

2.3.2. INT test

A specific activity test using 2(p-iodophenyl)-3-(p-nitrophenyl)-5-phenyl tetrazolium chloride (INT) reduction to INT-formazan crystals was applied on the sample taken at the end of the experimentation period according to the method described in Bitton and Koopman, 1981. In this test, INT is utilised by cells as the final electron acceptor instead of oxygen, so insoluble intracellular INT-formazan crystals are only formed in active cells.

2.3.3. FISH (fluorescence in situ hybridization) analysis

Biomass samples were periodically taken in order to investigate the main physical and microbiological properties. FISH analysis was performed on granular sludge samples fixed in paraformaldehyde according to the procedure described in Amann et al. (1995). Oligonucleotide probes specific for *Alpha-*, *Beta-* and *Gamma-proteobacteria* (ALF968, Bet-42a and Gam-42a probes, respectively), most *Flavobacteria* (CF319a probe), *Actinobacteria* (HGC69A probe), *Firmicutes* (LGC354mix), *Chloroflexi* (CFX1223), *Thauera/Azoarcus* group (THAU646 and AZO644) and for *Archaea* (ARC915) were used. Ammonia (Nso1225 probe) and nitrite oxidisers (Ntspa662, NSR1156 and NIT3 probes) were also monitored by FISH. All the hybridizations with group specific probes were carried out simultaneously with probes EUB338, EUB338-II and EUB338-III combined in a mixture (EUB338mix) for the detection of most bacteria and with DAPI staining for quantifying the total number of cells. To estimate the amount of bacteria from the total number of cells, DAPI was directly added to the hybridization buffer at a final concentration of 1 µg/ml. Details on oligonucleotide probes are available at probeBase (Loy et al., 2007). All the probes were synthesised with 5′-FITC and -Cy3 labels and purchased from MWG AG Biotech (Germany). Slides were examined under an epifluorescence microscope (Olympus BX51) and CLSM (Leica SP5).

All microscopic analyses were performed on both disaggregated and sectioned granules. After fixation, granules were rinsed with 1× phosphate buffered saline (PBS) and transferred into 2 mL tubes and embedded in Tissue-Tek OCT compound (Sakura Finetek Inc., USA). The embedding was performed at room temperature and the embedded samples were left at 4 °C overnight to ensure good penetration of the OCT throughout each granule. Afterwards, samples were frozen at −20 °C and kept at this temperature until cryosectioned (14–18 µm sections).

2.3.4. Granule porosity estimation by image analysis

Whole granule sections, obtained as described above, were analysed by phase contrast microscopy with an BX51 Olympus microscope at 100× magnification. Multiple images were captured
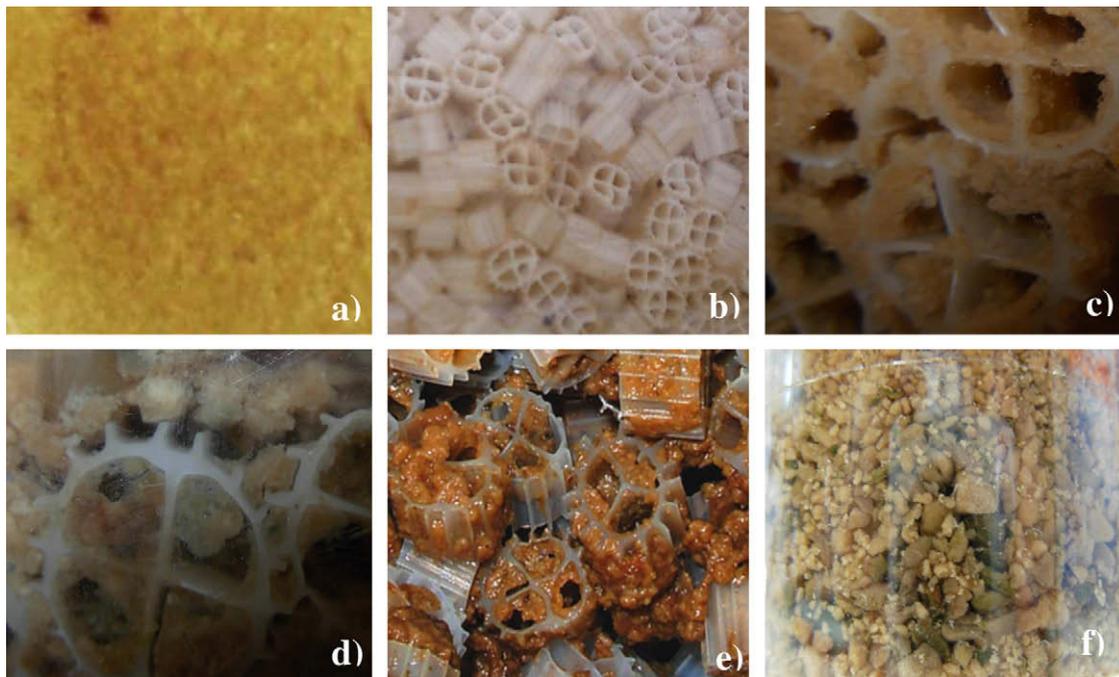
Fig. 2. Granular biomass generation during SBBGR start-up period. (a) activated sludge used as inoculum; (b) formation of a thin biofilm covering filling material surface; (c) thickening of the biofilm; (d) detachment of biofilm particles from filling material; (e) bed volume sample; (f) granules after suspending in a beaker containing water the sample shown in (e).

(about 28 per granule section) with an Olympus F-View II digital camera via AnalySIS image analysis software (Soft Imaging System GmbH, Münster, Germany). Digital images were manually analysed using ImageJ software (version 1.30 for Windows); area measurement tools available with ImageJ software were utilised for estimating the ratio of empty/total surface area of each captured image. For each granule, at least three sections were imaged and measured, and the mean and standard deviation values from all data were calculated.

2.3.5. Electron scanning microscope (SEM) analysis

The external structure of granules was observed by SEM analysis. The samples were treated as described in Van Langerak et al. (1998) and analysed by means of a scanning electron microscope (SEM, mod. LEO1450VP).

2.3.6. Biological staining for PHA

The presence of intracellular polymeric storage compounds (PHA) was evaluated by means of a specific fluorescent Nile Blue dye according to the method developed by Ostle and Holt (1982). Nile Blue was applied on all the biomass samples, biofilm and crushed granules. Slides were examined under an epifluorescence microscope (Olympus BX51) at 1000× magnification.

2.3.7. EPS analysis

The presence of EPS was investigated by both chemical analysis and microscopic techniques. Chemical analysis was performed by means of cation exchange resin extraction (Dowex) according to the method developed by Frolund et al. (1996). The proteins and carbohydrates present in the extract were determined according to reference methods (Lowry et al., 1951; Gaudy, 1962).

The use of microscopic techniques allowed the *in situ* detection of EPS in granular samples by means of epifluorescence microscopy. Three different EPS staining methods for the main EPS component were tested on both granule sections and crushed granules, and on biofilm: carbohydrate-containing EPS staining (mainly α-glucans) according to the method described in Strathmann et al. (2002); carbohydrate-containing EPS staining (mainly β-glucans) according the method described in De Beer et al. (1996); amino-sugars and proteins containing EPS using a modified form of the method described in Schmid et al. (2003).

3. Results and discussion

3.1. Granular biomass generation and plant performance

As reported in the Section 2, the reactor was started up by seeding activated sludge from the Bari municipal wastewater treatment plant.

During the first few days of the start-up period, a thin biofilm completely covering the surface of the carrier material was produced (see Fig. 2b) from the activated sludge used as inoculum (see Fig. 2a). Later, an increase in biofilm thickness was recorded which therefore led to an increase in biomass concentration (see Fig. 2c). Subsequently, a detachment of biofilm particles and their deposition inside the pores of the filling material was observed (see Fig. 2d). The particles entrapped inside the carrier continued to grow, reaching a size and shape similar to granules. Therefore, at the end of the start-up period (i.e., after four months), the biomass present in the reactor bed consisted of two different fractions: the biofilm attached to the carrier and the granules confined in the interstitial pores of the filling material. The separation of these two fractions was easily obtained by sampling a small volume of the bed (see Fig. 2e) and suspending it in a beaker containing water. As a result, the support material with the at-

tached biofilm floated, while the granules settled to the bottom of the beaker (see Fig. 2f).

During the start-up period the biomass concentration in the reactor bed gradually increased to 34 g TSS/L_{bed} (with a VSS/TSS ratio of about 90%). Fig. 3a shows the biomass concentration profile in the reactor bed throughout the experimentation period. Nevertheless, the high biomass concentration in the reactor did not affect the amount of solids, always lower than 30 mg/L, released into the effluent (see Fig. 3a).

At the end of the fourth month, the headloss along the bed increased to 100 cm, and a first washing step was carried out for a few minutes until the headloss decreased from 100 down to 60 cm. The successive working period, during which the headloss increased from 60 cm to 100 cm, lasted approximately 15 days;
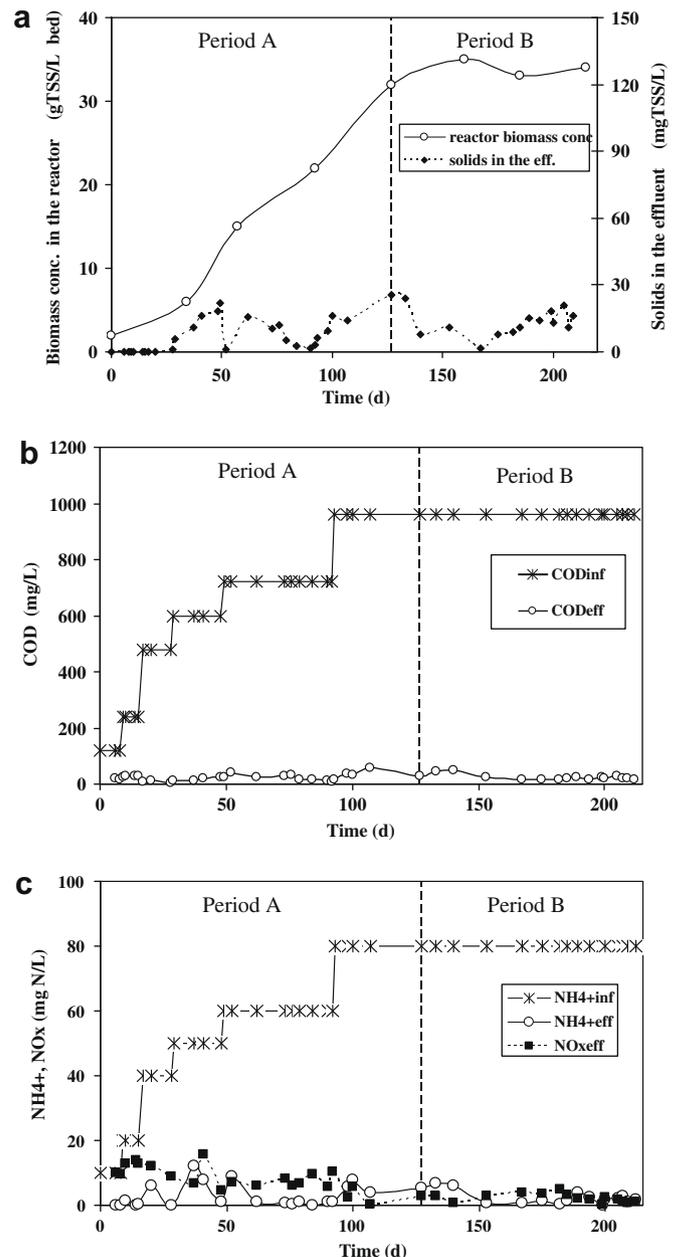

Fig. 3. SBBGR performances throughout the experimentation. (a) Biomass concentration in reactor bed and suspended solids content in the effluent; (b) influent and effluent COD concentration; (c) influent and effluent ammonia and effluent oxidised nitrogen.

then a washing step was again carried out. The sequence "working operation/washing step" was then repeated until the end of experimentation period.

After the start-up period (period A), the experimental activities continued in order to assess plant performance and granule stability (period B). Fig. 3b and c report COD and nitrogen concentrations in the influent and effluent of the plant, respectively, throughout the experimentation period. Looking at these profiles, it is possible to observe that at steady state conditions (i.e., during period B), COD and ammonia removal efficiencies higher than 95% were obtained. Moreover, oxidised nitrogen concentration in the effluent was always lower than 5 mg/L. Considering the absence of a planned anoxic phase in the treatment cycle of the plant, this result indicates the occurrence, to a great extent, of simultaneous nitrification–denitrification in the plant. This can be ascribed to both high biomass concentration (i.e., around 35 g TSS/L_{bed}) and transient conditions (typical of sequential reactors). In fact, in these conditions, the ammonium oxidizers mainly situated in the granule/biofilm outer layers carry out the oxidation of ammonium to nitrite/nitrate, while denitrifying bacteria located in deeper layers, where oxygen cannot penetrate, reduce the nitrite/nitrate to nitrogen gas by using carbon sources coming from storage products.

Finally, the process was characterised by an excess sludge production as low as 0.1 g TSS/g $COD_{removed}$ (i.e., about 4–5 times lower than that recorded in conventional treatment systems).

Such a low sludge production value can be explained considering the very high age of the biomass in the reactor ($\theta_c > 100$ d). In these circumstances, the microorganisms spend more time than

in conventional systems in the endogenous metabolism phase, where the biomass decay rate is high, and thus the net biomass production rate is low.

3.2. Molecular characterization by FISH analysis

Microbial population dynamics. The samplings for the molecular characterization were performed monthly on granules and biofilm samples. The molecular analysis was carried out by FISH with a wide probe set (20 probes including those available for important functional bacterial groups, i.e., nitrifiers). Round shaped, compact and dense granules were obtained after the start-up period; mature granules, obtained under steady state conditions (after about 5 months of operation), were characterised by a size of 4–5 mm and by a density of 150 g TSS/$L_{biomass}$ (Fig. 2f).

More than 90% of the bacteria were identified by group specific probes during reactors operation (Fig. 4a) with the sole exception of sample 57 d, for which a value of only 80% was obtained. As shown in Fig. 4a, some evident changes in biomass composition were observed mainly during the first four months of experimentation. Instead, when the reactor reached steady state conditions after mature granules were formed (sampling points 176 d and 210 d), only slight differences were observed. In particular, *Betaproteobacteria* and members of the *Cytophaga–Flavobacteria–Bacteroidetes* group were enriched during operation, while a constant negligible presence of *Archaea* was observed. Most of the *Betaproteobacteria* in granules were identified as ammonia-oxidizers and members of the *Thauera/Azoarcus* group,
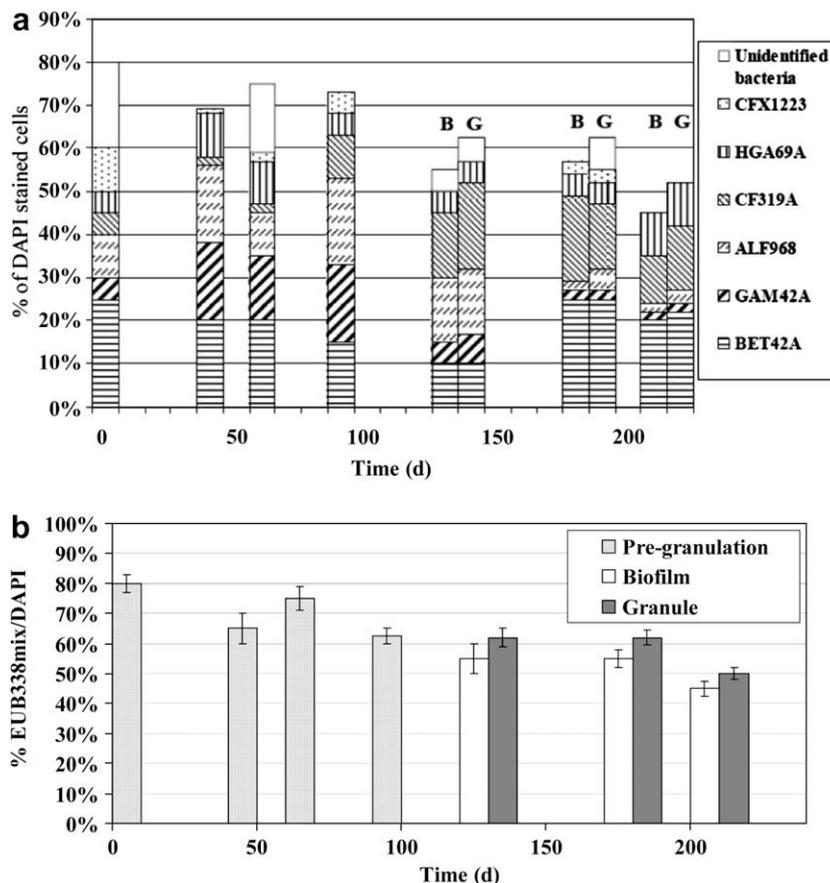
Fig. 4. (a) Microbial population dynamics estimated by FISH on monthly samples taken during SBBGR operation. At time "0 d" the composition of activated sludge used as inoculum is reported. Samples 34-57-91 d refer to biomass before granulation, while samples 132-176-210 d were taken after granulation occurred; (b) bacteria/total cells ratio estimated by FISH (%EUB338mix/DAPI stained cells) on monthly samples taken during seven months of SBBGR operation (samples 1-34-57-91 before granulation, samples 132-176-210 d after granulation occurred).

which have been reported in previous studies to be the main denitrifying bacteria in activated sludge systems (Morgan-Sagastume et al., 2008). Furthermore, no significant differences in terms of bacterial composition were found between the biofilm growing on the carrier surface and the granules, thus confirming the evidence that the granules derived from detached particles of biofilm as previously reported.

SBBGR biomass activity. Different approaches were utilised to determine to what extent such large bacterial aggregates were active. Initially, the ratio Bacteria/total number of cells (%EU-B338mix/DAPI stained cells) was estimated by FISH. The detectability of bacteria by FISH is indeed often utilised as a simple "gross parameter" to assess the overall physiological state of bacterial communities and found to be in agreement with reactor performances and with conventional respirometric tests applied for microbial activities measurements in previous experiences (Li et al., 2006; Kurisu et al., 2002; Witzig et al., 2002). In granules this parameter showed values (50–60%, Fig. 4b) similar to those (50–80%) typically recorded on activated sludge samples (i.e., Eschenhagena et al., 2003) characterised by a significative lower biomass concentration (3–4 g TSS/L against 30–40 g TSS/L of granular reactor) and sludge age.

This finding was also confirmed by traditional respirometric and INT (tetrazolium salt reduction test) tests applied on SBBGR biomass. Several respirometric tests were performed on the biomass taken at the end of the experimentation period (i.e., at 210 d) and the specific oxygen uptake rate (SOUR) obtained ranged between 25–30 mgO$_2$/gVSS × h. The INT test was performed on

both granular biomass and biofilm. When crushed samples were analysed by means of contrast phase microscopy, about 50–60% of the total cells were shown to be active by the presence of intracellular INT-formazan crystals. No relevant differences were found between granular biomass and biofilm.

3.3. Granule structure

Microbial distribution within granules. Mature granules were sectioned and analysed by FISH to discover their internal structure. The analysis showed a multi-channel structure and identified the whole microbial composition present in the core and in the outer layers (edge) of mature granules. Slight differences were found in microbial composition within granules by applying group specific probes (Fig. 5a). Instead, an asymmetrical distribution of ammonia- and nitrite-oxidizers was found: they were more abundant in the outer layers (up to 15% of total number of cells), but were also present in the inner part of the granule (Fig. 5b). According to the data obtained from the analysis of crushed granules, no Archaea were detected, even in the granule cores. This finding, together with the presence of nitrifiers also in the inner part of the granules, suggests the absence of the strict anaerobic zone in SBBGR granules that is usually observed in granules obtained in Sequencing Batch Granular Reactors (SBGRs) (i.e., Tay et al., 2002; Jang et al., 2003). Some authors also reported the absence of active biomass in SBGR granule core (Toh et al., 2003). On the contrary, in the SBBGR system the presence of active cells was
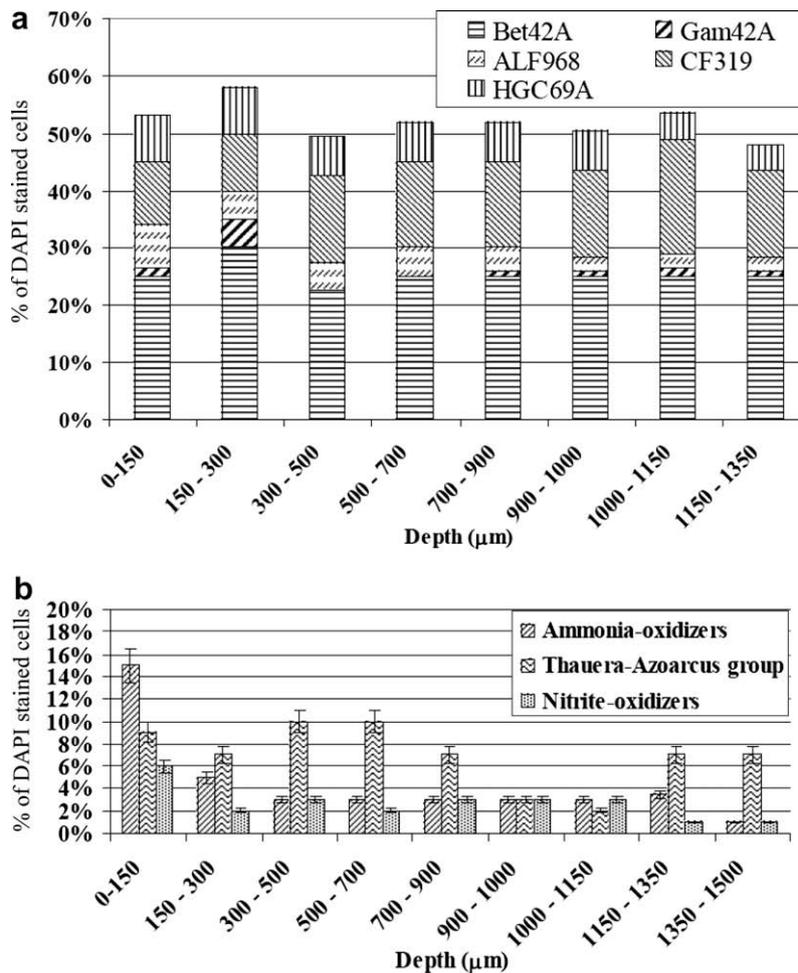

Fig. 5. Distribution of bacteria detected by FISH across granules (sample 210 d). (a) Bacterial group; (b) ammonia- nitrite-oxidizers and members of *Thauera-Azoarcus*.

Table 2
Granule porosity, defined as a percentage of the empty (no biomass) over total area of granule sections, estimated on granules taken during the three months of the granulation period.

% Empty space/total area	Edge	Core
132 d granules	18.9 ± 5.4	30.3 ± 6.3
176 d granules	17.4 ± 5.6	47.4 ± 6.9
210 d granules	19.1 ± 5.6	46.8 ± 8.5

found (EUB338mix/DAPI ratio of 40%) even in the central core of granules (at 2 mm depth).

Granule porosity. When mature granules were analysed by SEM, a dense and compact structure with the presence of multiple channels was highlighted. The granule porosity was estimated by measuring the area occupied by empty space (no biomass) and the total area of sections of granules taken after the fourth month of operation. An increased porosity was observed with time as summarised in Table 2. In particular, the granule structure was characterised by a denser external layer (about 3–400 μm thickness) and a multichannel core structure with higher porosity. The outer layer of granules maintained the same porosity (17–18%), while granule core porosity increased from 30.3% ± 6.3% (sample 132 d) to 46.8% ± 8.5% (samples 176–210 d). It is very likely that the high porosity values retrieved in the granule core guarantee oxygen and substrate diffusion within the inner part, as already shown by the molecular analysis (i.e., presence of nitrifiers, absence of Archaea and good EUB338/DAPI ratio).

EPS content and composition. As it is commonly reported that EPS can play an important role in conferring compactness to granules, these compounds were analysed by means of three specific staining techniques: Concanavalin A (for on α-glucans), Calcofluor White (for β-glucans) and FITC (for amino-acids and amino-sugars). Despite the well known limits associated to these available techniques, an increase in biomass EPS content during the granulation process was observed. Amino-sugars were found to be the main EPS component, which was also confirmed by chemical analysis by means of Dowex extraction. In fact, mainly proteins (41.8 mg/gVSS) and a lower presence of carbohydrates (1.2 mg/g VSS) were found in the EPS composition.

4. Conclusions

SBBGR is a novel and promising technology characterised by high treatment capability and low sludge production. The present paper was addressed to the characterization of granular biomass by means of molecular *in situ* detection methods, traditional tools and granule structure analysis.

The results demonstrate that:

– the granulation process involved a transition from biofilm growing on the carrier surface to granules;
– mature granules were characterised by a stable composition and activity;
– high cell activity was observed despite the high sludge concentration and age;
– simultaneous nitrification–denitrification occurred in mature granules and led to a high nitrogen removal.

Acknowledgements

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