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Elucidating performance failures in use of granular sludge for nutrient removal from domestic wastewater in a warm coastal climate region

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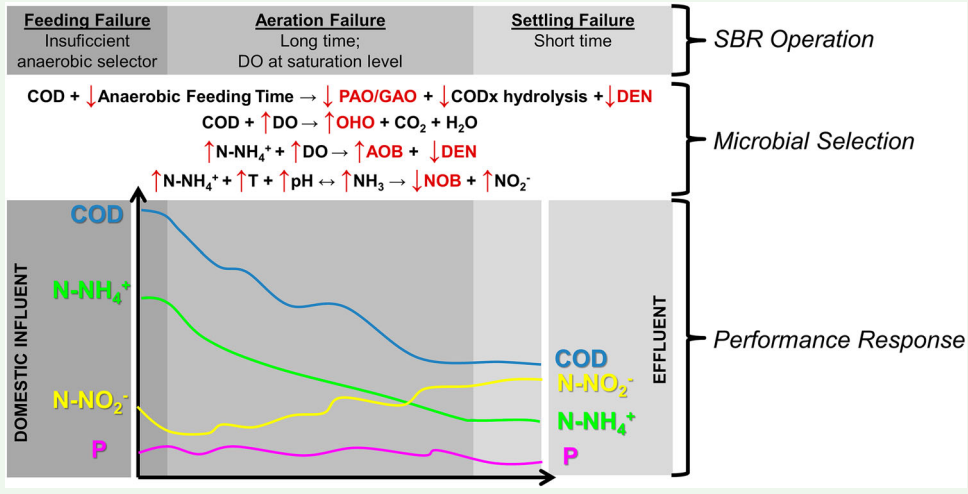
ABSTRACT

The effects of domestic wastewater and a coastal warm climate on granular sludge and biological nutrient removal were evaluated using a pilot-scale sequencing batch reactor (SBR). The reactor operation employed two different operational strategies (OS) based on up-flow feeding regimes, defined as fast (OS1, flow rate = 18.0 L min^{-1} and flow velocity = 22.0 m h^{-1}) and slow (OS2, flow rate = 3.5 L min^{-1} and flow velocity = 4.3 m h^{-1}). Under slow feeding, larger (OS1: 290 µm; OS2: 450 µm) and faster settling granules were obtained (OS1: 109; OS2: 74 mL g^{-1} TSS). The slow feeding regime was also advantageous for the hydrolysis of particulate organic matter (OS1: 1.3; OS2: 3.1 g COD_{tot} g^{-1} VSS d^{-1}) and for phosphorus removal (OS1: <33%; OS2: >97.5%). Neither strategy resulted in substantial biomass accumulation in the reactor (OS1: 0.7; OS2: 1.5 g VSS L^{-1}), and high concentrations of nitrite were observed in the effluent (9–27 mg NO_2^-–N L^{-1}). Ordinary heterotrophic organisms dominated the granular sludge developed under both feeding regimes (OS1: 30% of *Thauera*; OS2: 56% of *Comamonas*), while polyphosphate-accumulating organisms (PAOs) were only detected during OS2 (2.3–3.4% of total bacteria). A successful granular sludge process should be able to cope with high fluctuations in wastewater loads caused by rain events (82–182 mm $month^{-1}$ in Florianopolis, Brazil). In order to achieve higher water quality, strategies identified for an efficient granular sludge SBR operation included (i) management of an anaerobic phase for PAO selection, and (ii) aeration control for successful nitrification/denitrification.



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Feeding regime; granular sludge; low strength domestic wastewater; nutrient removal; SBR operation

Introduction

Full-scale plants using the granular sludge technology for biological nutrient removal (BNR) are increasingly installed worldwide for the treatment of municipal and industrial wastewaters [1–3]. The technology currently covers treatment capacities ranging from five thousands to more than 1 million population equivalent (PE) [4]. The method was first developed in Europe and has more

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recently been adopted in developing countries such as Brazil, so significant differences in sewage and climate conditions need to be considered in the design and operation of such processes. Although the granular sludge process has been successfully implemented for municipal wastewater [1], the complexity of real wastewaters and the effects of low loads are still a challenge in terms of process performance [5,6]. Domestic sewage contains a range of dissolved and particulate organic substances, whose impacts on granulation and BNR need to be understood. The effects of low strength and fluctuating carbon load have so far received little attention. Additional issues found with real sewage are long granulation times, unpredictable granule morphology, granule disintegration, and inefficient BNR [7].

The initial design criterion of many granular sludge investigations has been based on a reactor column geometry with a high height-to-diameter ratio ($H/D = 9$), assuming that granules would form and that flocs would be washed out from the system [8]. Operational conditions have included a high hydrodynamic shear force and a short settling time, which have been previously considered crucial for granulation [8]. The hydrodynamic shear force has been enhanced by superficial up-flow gas velocities above 1.0 cm s^{-1} [9]. Short settling times [10] have been achieved using hydraulic pressure to select for biomass particles with high settling velocities (>30 m h^{-1}).

Pilot designs have often been initially developed by analogy to traditional laboratory-scale systems used to cultivate 'aerobic granules' [7]. An SBR operation involving feeding, reaction, and settling offers easy and flexible operation, while generating substrate gradients that stimulate granulation. A high aeration flowrate in a shallow column design induces granulation by shear force, but can affect BNR efficiency by resulting in too high concentration of dissolved oxygen (DO) if not controlled. Feeding and aeration regimes can be adapted to tune phosphorus (P) removal and denitrification [11,12]. However, challenges faced by domestic wastewater systems are high load fluctuations and much lower COD and P concentrations (200–600 mg COD L^{-1}; 2–4 mg P_{Tot} L^{-1}) [13] than often experienced under synthetic laboratory conditions (400–800 mg COD L^{-1}; 10–200 mg P L^{-1}). Therefore, the growth of polyphosphate-accumulating organisms (PAOs) is limited, so P removal is consequently impaired under such conditions [7]. Volatile fatty acids (VFAs) can be used to enhance N and P removal [14], but this approach has significant cost implications for large-scale operations. Use of a static feeding regime, under anaerobic conditions, is a practical and economically viable option for promoting PAO selection, granule stability, and BNR [11]. When enhanced biological P removal (EBPR) is not targeted, selection for glycogen-accumulating organisms (GAOs) can foster granulation together with C and N removal [15].

In most subtropical climate regions, low strength municipal wastewaters present loads that can fluctuate widely, due to frequent and heavy rain events. This is the case in Florianopolis, Brazil, where precipitation showed a median of 110 mm $month^{-1}$ and 1st and 3rd quartiles of 87 and 182 mm $month^{-1}$, respectively [16]. The configuration of SBR operation with low strength municipal wastewater has been based on a 4-h cycle, with fast feeding followed by an extended aerobic reaction, settling, and withdrawal [5,17–19]. Variation of wastewater temperature in warm humid subtropical coastal climate regions, such as Florianopolis (median and 1st and 3rd quartiles of 25, 21, and 27°C, respectively) can be an important factor affecting process performance, especially in relation to PAO and GAO competition [20].

In Brazil, earlier pilot investigations with domestic wastewater in the Florianopolis catchment area [5,19,21] achieved granule formation together with removal of organic matter (expressed hereafter as chemical and biochemical oxygen demand equivalents: COD and BOD), as well as ammonium, resulting in compliance with national quality criteria [22] (Table 1). However, granule disintegration was observed and the effluent BOD and nitrogen (N) concentrations could still be considered high, since the Brazilian discharge limits (120 mg BOD L_{Eff}^{-1} or 60% removal; <20 mg NH_4-N L_{Eff}^{-1}) are higher than European requirements (25 mg BOD L_{Eff}^{-1} or 70–90% removal; 10 mg N_{Tot} L_{Eff}^{-1} or 70–80% removal) [23]. In addition, nitrite reached 15 mg $NO_2^- - N$ L_{Eff}^{-1}, similar to earlier reports for granular sludge, including with the use of synthetic wastewater [24]. Phosphorus removal has not been targeted in developing countries, such as Brazil, since no national discharge limit exists for this nutrient (Table 1).

The results of previous studies with low strength municipal wastewater under variable load conditions and high weather variations [5,19,21] showed that process improvements are still needed. Despite complying with the Brazilian national standards [22], the treated effluent concentrations for some parameters remain high, relative to international standards [23,25] (Table 1). Process failures such as granule disintegration, nitrite accumulation, and unsuccessful EBPR make the system vulnerable, requiring operational modifications. SBR cycle configurations vary among studies, with granulation having been reported under pulse [17], slow up-flow [1,11,26], and fill-and-draw [6] feeding regimes.

In the present study, two feeding regimes and their effects on granulation and BNR performance were

Table 1. Directives for emission quality standards of Brazil, Santa Catarina State (Brazil), European Union, and Switzerland, comparing from less to more stringent limits on wastewater discharge.

	Effluent quality standards directives (if not specified: mg L_{Eff}^{-1}) *% removal versus raw influent load*			
Parameter	Brazil [a]	Santa Catarina State [b]	European Union [c]	Switzerland [d]
Total suspended solids	1 mL L_{Eff}^{-1} in 1 h	Locally	60[c1] / 35[c2] *70%* [c1] */ 90%* [c2]	20[d2] / 15[d3] n/a
Total COD	n/s *n/s*	n/s *n/s*	125 *75%*	60[d2] / 45[d3] *80%* [d2] */ 85%* [d3]
Soluble COD	n/s *n/s*	n/s *n/s*	n/s *n/s*	10 mg DOC L^{-1} *85% DOC* [d1]
BOD_5	120 *60%*	60 *80%*	25 *70–90%*	20 *90%*
Total nitrogen[e]	n/s *n/s*	n/s *n/s*	15[c3] / 10[c4] *70–80%*	ALAP *AMAP*
Ammonium/ammonia-nitrogen	20 *n/a*	n/s *n/a*	n/s *n/s*	2 *90%*
Nitrite-nitrogen	n/s	n/s	n/s	0.3
Total phosphorus	n/s *n/s*	4 *75%*	2[c3] / 1[c4] *80%*	0.8 *80%*

[a]CONAMA (2011); Criterion for settling materials: 1 mL L_{Eff}^{-1} (1 h, Imhoff cone).
[b]CONSEMA-SC (2009): Criterion for suspended solids defined by the local environmental licensing authority.
[c]EUR-Lex (1991): Different criteria vs. plant size: [c1] 2k-10k p.e.; [c2] >10k p.e.; [c3] 10–100k p.e.; [c4] >100k p.e..
[d]WPO (1998/2016): Different criteria vs. plant size: [d1] >2k p.e.; [d2] <10k p.e.; [d3] >10k p.e.. For soluble organic matter, a discharge limit for dissolved organic carbon (DOC) is set at 10 mg DOC L_{Eff}^{-1}, with 85% removal of DOC. Nitrogen should be removed as maximum as possible (AMAP) to reach a residual concentration as low as possible (ALAP). Directives are also specific to plant connection to catchment area of the North Sea (sensitive area; nitrate to be removed) or the Mediterranean Sea (less sensitive area).
[e]Total nitrogen is determined as the sum of total Kjeldahl nitrogen (N_{Org} + NH_4-N), nitrite-nitrogen, and nitrate-nitrogen (NO_x-N).
n/s: not specified.

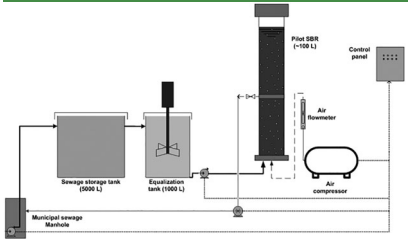
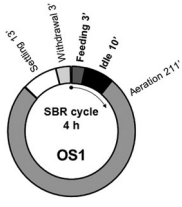
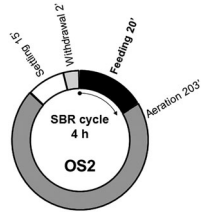
evaluated using a pilot SBR treating municipal sewage under warm coastal conditions. The effects of the two operational strategies were assessed in terms of granule properties, biomass accumulation, BNR, and bacterial selection. The results are thoroughly explored, considering the challenges resulting from process failures, together with engineering aspects for improving the technology.

Materials and methods

Operational strategies

Two operational strategies (OS1 and OS2) were carried out using a pilot SBR. Cycles of 4 h (Table 2) were performed with either (i) pulse feeding followed by an idle phase (OS1) or (ii) slow up-flow feeding (OS2), considering the effects on granulation and BNR applied to

Table 2. SBR operational strategies and domestic wastewater characteristics and loading.

Operational set-up and cycle configuration	OS1	OS2
		
Organic loading rate (kg COD_{Tot} m^{-3} d^{-1})	1.1 ± 0.4	2.1 ± 0.6
Organic loading rate (kg COD_{Sol} m^{-3} d^{-1})	0.6 ± 0.3	1.0 ± 0.3
Ammonium loading rate (kg NH_4^+ — N m^{-3} d^{-1})	0.16 ± 0.05	0.28 ± 0.07
Phosphorus loading rate (kg P_{Tot} m^{-3} d^{-1})	0.018 ± 0.006	0.027 ± 0.006
Hydraulic retention time (days)	0.30	0.28
Domestic wastewater characteristics		
Total COD (mg COD_{Tot} L^{-1})	335.0 ± 123.8	595.6 ± 164.7
Soluble COD (mg COD_{Sol} L^{-1})	202.4 ± 97.4	292.7 ± 86.1
Ammonium nitrogen (mg NH_4^+ — N L^{-1})	51.8 ± 10.9	78.8 ± 18.5
Total phosphorus (mg P_{Tot} L^{-1})	5.5 ± 1.8	7.6 ± 1.9
COD:N:P ratio (g COD_s: g NH_4^+ — N: g TP)	100:27:2.8	100:27:2.5
pH (-)	7.2 ± 0.2	6.9 ± 0.2
Temperature (°C)	26 ± 3	26 ± 2

domestic wastewater from the Florianopolis catchment area. The idle and slow feeding phases were performed without mixing and were intended to provide putative anaerobic conditions to select for PAOs and EBPR. During aeration, an up-flow superficial air velocity of 1.1 cm s^{-1} (39.6 m h^{-1}) was applied under both strategies, which ensured complete mixing (without mechanical mixers) and DO close to saturation (8–10 mg O_2 L^{-1}) inside the SBR column. The settling time was decreased stepwise from 30 to 13 min (OS1; working height of 2.18 m) and from 30 to 15 min (OS2; 2.42 m) during start-up, in order to wash out flocs and select for a rapidly settling biomass.

Granular sludge characteristics and BNR performance

The characteristics of the biomass were determined by measurements of the sludge volume index after 5, 10, and 30 min ($SVI_{5,10,30}$) [27], observation of the shapes of the aggregates by optical microscopy (BX40 microscope, Olympus, Japan), and analysis of particle size distributions by laser diffraction (MasterSizer Series 2000, Malvern Instruments, UK). The volume percentage of sludge with particle size below 200 µm was used to determine the time when the biomass switched to a predominance of granular sludge (SVP-SB200 < 50%) [26].

Temperature, pH, and DO were not controlled but were recorded in the bulk medium of the reactor using an on-line multi-parameter probe (Model 6600 V2, YSI, USA). Periodic analyses of suspended solids, COD, ammonium-nitrogen (NH_4^+- N), and total phosphorus (TP) were performed according to standard methods [27]. The organic substrate (determined as COD) in the influent and effluent was divided into two main fractions in terms of form and size: particulate (COD_x) and soluble (COD_{sol}) [28]. In this study, COD_{Tot} was measured in the raw influent and in the effluent containing the suspended organic solids particulate fraction, while COD_{sol} was measured after filtration through a cellulose acetate membrane (0.45 µm pore size). The COD_x fraction was obtained by subtracting COD_{sol} from COD_{Tot} and was used to calculate the hydrolysis time (see below). Nitrate (NO_3^-- N), nitrite (NO_2^-- N) and phosphate ($PO_4^{3-}-$ P) were measured by ion chromatography (Dionex Corporation, USA). Free ammonia (FA) was determined as described by Anthonisen [29].

Calculations

Process data were used to compute the following main kinetic parameters of interest [30]: volumetric rates (r_{COD}, r_{NH_4}, r_{Ptot}) and biomass specific rates (q_{COD}, q_{NH_4}, q_{Ptot}) of nutrient removal; observed biomass specific growth rate (μ_{obs}); observed saturation constant (Ks_{obs}); and decay rate (k_d).

Volumetric rates of nutrient removal (r_{COD}, r_{NH_4}, r_{Ptot})

$$r_{COD,\ NH_4,Ptot} = \frac{(C_i - C_e)V_e/t_c}{V_r} \quad (1)$$

Where:
r_{COD}, NH_4, Ptot are the volumetric rates of nutrient removal (COD, NH_4^+- N, or P_{Tot}, in mg d^{-1} L_R^{-1});
C_i is the concentration in the influent (COD, N, or P, in mg L^{-1});
C_e is the concentration in the effluent (COD, N, or P, in mg L^{-1});
V_e is the effluent volume in the SBR operating cycle (L);
t_c is the cycle time of the SBR operation (d);
V_r is the working volume of the SBR reactor (L_R).
Biomass specific rates of nutrient removal (q_{COD}, q_{NH_4}, q_{Ptot})

$$q_{COD,\ NH_4,Ptot} = \frac{(C_i - C_e)V_e/t_c}{V_r X_{VSS}} \quad (2)$$

Where:
q_{COD}, NH_4, Ptot are the observed biomass specific rates of nutrient removal (COD, NH_4^+- N, or P_{Tot}, in mg d^{-1} g VSS^{-1});
X_{VSS} is the concentration of volatile suspended solids in the reactor (g VSS L^{-1}).

Observed specific biomass growth rate (μ_{obs})

$$\frac{1}{\mu_{obs}} = \theta \quad (3)$$

Where:
μ_{obs} is the observed biomass specific growth rate (d^{-1});
θ is the solids retention time (d).
Observed half-saturation constant (Ks_{obs})

$$\frac{1}{\mu_{obs}} = \frac{Ks_{obs}}{\mu_{max}} \times \frac{1}{C_i} + \frac{1}{\mu_{max}} \quad (4)$$

Where:
Ks_{obs} is the substrate concentration at which the growth rate corresponds to half the μ_{max} (mg COD L^{-1});
μ_{obs} is the observed biomass specific growth rate (d^{-1});
μ_{max} is the maximum biomass specific growth rate of the microorganisms (d^{-1});
C_i is the influent substrate concentration (mg COD L^{-1}).

According to the Lineweaver–Burk plot (or double reciprocal plot), Ks_{obs} can be obtained by plotting $1/\mu_{obs}$ versus $1/C_i$, with a slope of Ks_{obs}/μ_{max} and y-axis intercept of $1/\mu_{max}$.

Biomass specific decay rate (k_d)

$$\frac{1}{\theta} = Y \times q_{obs} - k_d \quad (5)$$

Assuming that Y and k_d are constant for a steady state system, their values can be obtained by plotting $1/\theta$ versus q_{obs}, with a slope of Y and y-axis intercept of k_d.

Additional calculations were performed to determine the settling velocity and the theoretical contact time between the influent and the biomass required to promote the hydrolysis of organic matter [31].

Settling velocity ($v_{settling}$)

The sludge settling velocity was measured by adding 1 L of mixed liquor to a measuring cylinder.

$$\text{Where: } v_{settling} = \frac{(V_{Biomass,i} - V_{Biomass,f})/t_{settling}}{A_c} \quad (6)$$

$v_{settling}$ is the sludge settling velocity (cm min^{-1});
$V_{biomass,i}$ is the initial volume filled by the mixed liquor (mL);
$V_{biomass,f}$ is the volume filled by the mixed liquor after settling (mL);
$t_{settling}$ is the time taken by the biomass to settle (min);
A_C is the end area of the cylinder used for the measurement (cm^2).

Theoretical contact time for hydrolysis (t_H)

$$t_H = \frac{Xs/V_r}{q_H} \quad (7)$$

Where:
t_H is the contact time between the biomass and the influent particulate matter required to promote hydrolysis (d);
X_s is the mass of particulate matter in the influent (g X_s);
V_r is the working volume of the SBR reactor (L_R);
q_H is the specific rate of particulate matter hydrolysis (g X_s L^{-1} d^{-1}).

Biomass specific hydrolysis rate of particulate organic matter (q_H)

$$q_H = k_H \times \frac{X_S/X_H}{K_X + X_S/X_H} \times X_H \quad (8)$$

Where:
k_H is the hydrolysis rate constant (g X_s g^{-1} X_H d^{-1});
X_H is the concentration of heterotrophic biomass responsible for X_s hydrolysis (g X_H L^{-1});
K_x is the saturation coefficient for particulate substrates, considered as 1 g X_s g^{-1} X_h for the temperature range of the present study (10–30°C).

Hydrolysis rate constant (k_H)

$$k_H(T) = k(20\ ^\circ C) \times e^{(\theta_T \times (T - 20\ ^\circ C))} \quad (9)$$

Where:
$k(20°C) = 3$ g X_s g^{-1} X_H d^{-1};
$\theta_T = 0.04$, considering that $k(10°C) = 2$ g X_s g^{-1} X_H d^{-1} [31].

Molecular analyses of bacterial population dynamics

Mixed liquor samples were collected during aeration. DNA was extracted using PowerSoil® DNA isolation kits (MoBio Laboratories Inc., USA), according to the manufacturer's instructions. Bacterial community compositions and population dynamics were analyzed by V1-V3 16S rRNA gene-based amplicon sequencing, using the MiDAS field guide [32]. The two universal bacterial primers 27F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 534R (5′-ATTACCGCGGCTGCTGG-3′) were used for PCR amplification. The pools of amplicons were sequenced at a depth of 23,600 ± 5,400 reads per sample (min: 12,100; max: 34,400), using a MiSeq desktop sequencer (Illumina, USA). Mapping was conducted against the curated MiDAS reference database to assign reads forming operational taxonomic units (OTUs) to closest bacterial relatives. Each sample dataset was rarefied to 10,000 reads and was processed in R using ampvis [33].

Analyses of 16S rRNA-targeted fluorescence *in situ* hybridization (FISH) [34,35], coupled with epifluorescence microscopy (EFM), were conducted to visually confirm shifts in predominant bacterial guilds and populations of interest, using the oligonucleotide probes EUBmix (eubacteria), NSO190 (β-proteobacterial ammonium-oxidizing bacteria), NEU (*Nitrosomonas* spp.), NIT3 (*Nitrobacter* spp.), Ntspa662 (genus *Nitrospira*), AMX820 (anaerobic ammonium-oxidizing bacteria), and PAOmix ('*Candidatus* Accumulibacter'), as defined in ProbeBase [36].

Results and discussion

Granulation under pulse and slow up-flow feeding regimes

Under both operational strategies with pulse feeding (OS1) and with slow up-flow feeding (OS2), granulation was achieved in 56 days (SVP-SB200 < 50%) in the pilot SBR fed with the low strength and warm domestic sewage from Florianopolis (Table 3). The biomass of OS2 displayed a higher level of granulation, larger aggregates, and better settling properties (Table 3).

However, neither strategy resulted in substantial biomass accumulation in the system (OS1: 0.7 ± 0.3; OS2: 1.5 ± 0.3 g VSS L^{-1}; Figure 1A). In both cases, the sludge retention time (SRT) was quite short, with values of 6.5 (OS1) and 15.5 days (OS2). A much higher biomass concentration of up to 8–10 g VSS L^{-1} is required for process scale-up and intensification, while SRT exceeding 20 days is typical for a well-performing granular sludge system [1].

The strategy OS2 resulted in a slightly higher biomass concentration, together with better physical properties

Table 3. Physical properties of the granular sludge at steady state for the two operational strategies (OS), compared to the activated sludge inoculum.

Sludge physical properties	Inoculum of OS1	Granular sludge OS1	Inoculum of OS2	Granular sludge OS2
SVP-SB200 (%)*	94	45 ± 4	94	22 ± 11
Mean size (μm)	93	289 ± 25	88	449 ± 90
SVI_{30} (mL g^{-1} TSS)	220	109 ± 27	296	74 ± 16
SVI_{10} (mL g^{-1} TSS)	n.a.	141 ± 46	n.a.	85 ± 22
SVI_5 (mL g^{-1} TSS)	n.a.	216 ± 110	n.a.	109 ± 31
SVI_{30}/SVI_{10}	-	0.8 ± 0.1	-	0.9 ± 0.1
SVI_{30}/SVI_5	-	0.6 ± 0.1	-	0.7 ± 0.1

*The volume percentage of sludge with particle size below 200 μm was used to identify a predominance of granular sludge in the reactor (SVP-SB200 < 50%) [26].
n.a. – not analyzed.

of the granular sludge. This could be ascribed to the higher COD concentration in the wastewater for OS2 (596 ± 165 mg COD_{Tot} L^{-1}; 2.1 ± 0.6 kg COD_{Tot} m^{-3} d^{-1}), compared to OS1 (335 ± 124 mg COD_{Tot} L^{-1}, 1.1 ± 0.4 kg COD_{Tot} m^{-3} d^{-1}) (Figure 1A, Table 2). Wagner and Costa [37] have compared the effects of organic loading rates (1.4, 1.0, and 2.0 kg COD m^{-3} d^{-1}) on granulation in an SBR treating domestic sewage, obtaining higher VSS concentrations and more compact granules

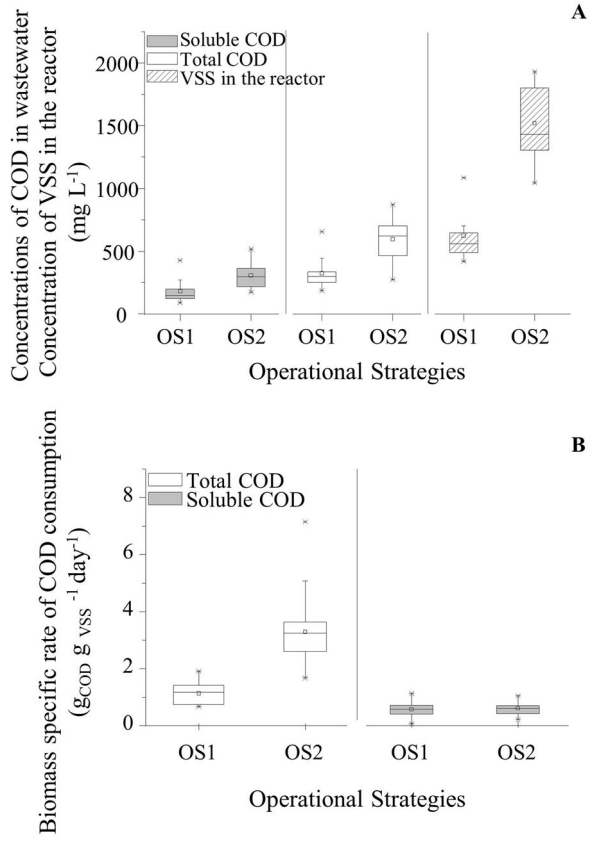


Figure 1. Impact of influent organic matter and operational strategies on granular biomass accumulation and COD removal: Loading fluctuations in the real wastewater (A); Biomass specific rates of COD removal (B).

at the highest rate. The biomass specific rate of total COD removal (q_{CODtot}) was concomitantly higher for OS2 (3.1 ± 1.4 g COD_{Tot} day^{-1} g^{-1} VSS) than for OS1 (1.3 ± 0.7 g COD_{Tot} day^{-1} g^{-1} VSS) (Figure 1B). Such a relationship between higher concentration and higher removal rate of COD was not observed for the soluble COD fraction (Figure 1A and B). This indicated a primary involvement of consumption of the particulate organic fraction (COD_x) by the higher amount of biomass that accumulated under the operational conditions and higher influent concentrations of total COD prevailing under OS2.

During up-flow feeding, the contact time between the influent and the biomass was extremely short under OS1 (24 ± 12 s) and was only moderately longer under OS2 (2.1 ± 0.4 min), due to the low biomass concentrations and relatively high influent up-flow rates and velocity (OS1: flow rate = 18.0 L min^{-1} and flow velocity = 22.0 m h^{-1}; OS2: flow rate = 3.5 L min^{-1} and flow velocity = 4.3 m h^{-1}). The slightly longer contact time provided by OS2 may have increased the retention of particulate organic matter, hence enhancing its hydrolysis [38]. A higher quantity of dissolved and readily biodegradable COD (COD_{rb}) was likely available for uptake and biomass growth under OS2. In laboratory-scale systems fed with mixtures of dissolved and particulate organics, a higher biomass concentration and compact granules with better settling abilities have been obtained when the slow (and putatively anaerobic) up-flow feeding period was extended in order to enhance hydrolysis [38]. Therefore, the design of the anaerobic selector should be based on optimization of the contact time, rather than the feeding phase duration *per se* [39].

Kinetics of biomass development

Kinetic calculations indicated that a theoretical contact time between the wastewater and the biomass of 11–56 min was required for full hydrolysis of the particulate organic matter during feeding, considering fluctuations in the particulate organic matter and the wastewater temperature (14–30°C). Therefore, contact times of 24 ± 12 s (OS1) and 2.1 ± 0.4 min (OS2) were not substantially different, and too low to stimulate hydrolysis. Strategy OS2 achieved higher biomass, which could potentially lead to increases of adsorption of particulate organic matter [40], of hydrolysis, and of biomass specific rates of total COD removal. Methods for the quantification of adsorption and hydrolysis should be developed to validate these hypotheses.

The observed granulation kinetics was influenced by the type of operation (Table 4). According to the Monod theory, at low substrate availabilities, as in the

Table 4. Averages and standard deviations for removal efficiencies, effluent concentrations, and kinetic parameters, for both strategies, during the entire operational periods.

Removal efficiencies	OS1	OS2
COD_{Tot} (%)	64 ± 28	67 ± 19
COD_{Sol} (%)	67 ± 19	78 ± 9
NH_4^+ — N (%)	73 ± 8	78 ± 15
P_{Tot} (%)	18 ± 14	48 ± 29
Effluent concentrations		
COD_{Tot} (mg L^{-1})	105 ± 54	188 ± 120
COD_{Sol} (mg L^{-1})	55 ± 19	62 ± 27
NH_4^+ — N (mg L^{-1})	13 ± 4	17 ± 12
NO_2^- — N (mg L^{-1})	17 ± 8	20 ± 7
NO_3^- — N (mg L^{-1})	8 ± 7	0.7 ± 0.3
P_{Tot} (mg L^{-1})	4.5 ± 1.5	4.1 ± 2.9
Kinetic parameters		
Ks_{obs} (mg COD L^{-1})[a]	110	410
μ_{obs} (d^{-1})	0.18 ± 0.07	0.08 ± 0.03
K_d (d^{-1})[a]	0.154	0.038
r_{CODTot} (g COD_{Tot} day^{-1} L_R^{-1})	0.80 ± 0.41	1.36 ± 0.57
q_{CODTot} (g COD_{Tot} day^{-1} g^{-1} VSS)	1.05 ± 0.47	0.85 ± 0.43
r_{CODSol} (g COD_{Sol} day^{-1} L_R^{-1})	0.49 ± 0.33	0.83 ± 0.30
q_{CODSol} (g COD_{Sol} day^{-1} g^{-1} VSS)	0.58 ± 0.25	0.51 ± 0.25
r_{NH_4} (g NH_4^+ — N day^{-1} L_R^{-1})	0.12 ± 0.03	0.21 ± 0.06
q_{NH_4} (g NH_4^+ — N day^{-1} g^{-1} VSS)	0.18 ± 0.08	0.10 ± 0.06
$r_{NO_2^-}$ (g NO_2^- — N day^{-1} L_R^{-1})[b]	0.05 ± 0.04	0.08 ± 0.05
$r_{NO_3^-}$ (g NO_3^- — N day^{-1} L_R^{-1})[b]	0.03 ± 0.03	0.003 ± 0.002
$q_{NO_2^-}$ (g NO_2^- — N day^{-1} g^{-1} VSS)[b]	0.10 ± 0.08	0.05 ± 0.03
$q_{NO_3^-}$ (g NO_3^- — N day^{-1} g^{-1} VSS)[b]	0.07 ± 0.06	0.002 ± 0.002
r_{TP} (g P_{Tot} day^{-1} L_R^{-1})	0.004 ± 0.003	0.013 ± 0.008
q_{TP} (g P_{Tot} day^{-1} g^{-1} VSS)	0.005 ± 0.004	0.008 ± 0.006

[a]No standard deviation because *Ks* and K_d were obtained by plotting a linear curve and the values were obtained from the slope and y-axis intercept.
[b]Volumetric and biomass specific rates of NO_x^- production.

present study, the growth kinetics becomes substrate limited. The half-saturation constant (*Ks*) is an important parameter reflecting the growth rate. According to Lineweaver-Burke linearization, the observed value (Ks_{obs}) here was lower for OS1 (110 mg COD_{Sol} L^{-1}) than for OS2 (410 mg COD_{Sol} L^{-1}). The Ks_{obs} results encountered in the present study are much higher than *Ks* value of 20 mg COD L^{-1} for municipal wastewater given in early ASM1-3 models [42]. Organisms that present lower K_S have higher affinity for the substrate and exhibit higher growth rates at low substrate conditions, consequently outcompeting the other organisms present in the reactor [41]. However, the difference in *Ks* here may relate more to the different diffusion limitation effects depending on the size of the bioaggregates present under OS1 (290 µm) and OS2 (450 µm) than to difference in microorganism physiologies [41].

The observed biomass specific growth rate (μ_{obs}) for OS1 (0.18 d^{-1}) was two-fold higher than for OS2 (0.08 d^{-1}). However, the biomass specific decay rate (k_d) indicated a loss of biomass that was four times higher for OS1 (0.154 d^{-1}) than for OS2 (0.038 d^{-1}). The k_d values for OS1 were above those generally found for activated sludge systems (0.06–0.1 d^{-1}) [43], corroborating the low accumulation of biomass in this system.

Different mechanisms can explain biomass decay and loss, including endogenous metabolism, lysis, and predation processes [30]. Endogenous respiration [31] may have resulted from long periods of starvation under full aeration at DO close to saturation (8–10 mg O_2 L^{-1}). The contact time between the wastewater and the biomass was minimal and did not allow for full COD_{sol} uptake for storage. For both strategies, most of the COD was consumed (oxidized) during the first 30–40 min of aeration (designated as COD feast time) (Figure 3A and B). The processes subsequently continued during 170 min of aeration under COD starvation. The starvation times were considerably longer than 80% of the duration of the aeration phase. This probably affected the growth of the ordinary heterotrophic organisms (OHOs) that populated the sludge under both OS (see below). This caused extra energy consumption by over-aeration, in addition to low reactor capacity. Most of laboratory studies have indicated stable granulation under a feast/famine regime at high DO concentrations [44]. Here, we praise that a reactor regime dedicated to biomass accumulation and stable granulation should involve alternation of an anaerobic C-feast phase followed by an aerobic C-famine phase [15]. Under such regime, slow-growing and C-N-P-removing organisms store the organic substrates (after hydrolysis and pre-fermentation as volatile fatty acids) as intracellular polymers (poly-β-hydroxyalkanoates), prior to using them for growth when the external substrate is depleted. In this way the organisms are capable of balancing their growth.

Nitrogen removal

Ammonium removal increased concomitantly with granulation under both OS1 (73 ± 8%, 13 ± 4 mg NH_4^+ — N L_{Eff}^{-1}) and OS2 (78 ± 15%, 17 ± 12 mg NH_4^+ — N L_{Eff}^{-1}) (Table 4). Maximum removal efficiencies reached 83% (6.6 mg NH_4^+ — N L_{Eff}^{-1}) on day 133, for OS1, and 95 ± 5% (5 ± 5 mg NH_4^+ — N L_{Eff}^{-1}) from day 175 to 200, for OS2. Higher efficiency of 97%, with 1.1 mg NH_4^+ — N L_{Eff}^{-1}, was previously achieved for a full-scale SBR operating with simultaneous feeding and effluent withdrawal (60 min under dry weather; 90 min under rainy weather), followed by aeration to maintain dissolved oxygen between 1.8 and 2.5 mg L^{-1} (300 min under dry weather; 60 min under rainy weather), and finally a settling/sludge withdrawal/idle period (30 min) [1].

The volumetric rate of ammonium conversion (r_{NH_4}) was almost two-fold lower for OS1 (0.12 ± 0.03 g NH_4^+ — N day^{-1} L_R^{-1}) than for OS2 (0.21 ± 0.06 g NH_4^+ — N day^{-1} L_R^{-1}) (Table 4), which was possibly linked to the different biomass concentrations. The r_{NH_4}

values were lower than obtained previously under laboratory-scale synthetic conditions (0.4 g NH_4^+- N day^{-1} L_R^{-1}) [11], but similar to the performance of a full-scale system (0.17 g NH_4^+- N day^{-1} L_R^{-1}) [1]. Aeration can be extended to improve ammonium removal, while controlling DO (1.8–2.5 mg L^{-1}) [9], in order to favour N removal, while limiting endogenous respiration.

The biomass specific rate of ammonium removal (q_{NH4}) (Figure 2A) increased under both OS, indicating progressive establishment of the AOB guild and activity. Nonetheless, stable operation had not yet been reached by the end of the experimental periods. Slightly higher q_{NH4} was obtained for OS1 (0.02–0.32 g NH_4^+- N day^{-1} g^{-1} VSS) than for OS2 (0.02–0.28 g NH_4^+- N day^{-1} g^{-1} VSS). A similar value of 0.024 g NH_4^+- N day^{-1} g^{-1} VSS has been obtained previously for a pilot SBR reactor fed with real sewage, with anaerobic feeding (1 h), aeration (at saturation level, 180 min), settling (8–45 min), and discharge [26]. Higher q_{NH4} of 0.59 g NH_4^+- N day^{-1} g^{-1} VSS was achieved using low strength synthetic wastewater, but with a much higher ammonia concentration in the influent (292 ± 12 mg L^{-1}, COD/N = 1) and a longer SBR cycle (12 h), especially considering the aeration period (705 min) [45].

Nitrification remained incomplete, with unfavourable accumulation of nitrite for both OS. In the case of OS1, nitrite and nitrate fluctuated in the effluent with 17 ± 8 mg NO_2^-- N L^{-1} and 8 ± 7 mg NO_3^-- N L^{-1}, respectively (Table 4). The application of OS2 led to partial nitrification (20 ± 7 mg NO_2^-- N L^{-1}; 0.7 ± 0.3 mg NO_3^-- N L^{-1}). Nitrite accumulation has been found previously in laboratory-scale systems [24], as well as in earlier pilot investigations conducted in Florianopolis [5,21]. In such cases, AOB outcompeted nitrite-oxidizing bacteria (NOB) [19]. Granulation start-ups have shown nitrite accumulation prior to nitrate formation, correlating with the sequential development of AOB and NOB [46]. Studies of granular sludge have found NOB repression and nitrite accumulation resulting from the establishment of AOB colonies in the outer shell, which consumed the available oxygen [47]. However, this was probably not the case in the present study, since DO was maintained at the saturation level and the granule size was relatively small, which would not favour this limitation. Specific physical-chemical factors, such as elevated temperature and high concentration of free ammonia, could explain the repeated failure of nitrification in the present pilot system operated in a warm coastal environment.

AOB outcompeted NOB under mesophilic conditions above 25 °C, for both OS1 and OS2. Differences in the growth rates of AOB and NOB at high temperature (typically 35 °C) have been used to design single reactor systems for high ammonium removal over nitrite (SHARON®) [48]. In the present case, the warm weather conditions (18–30°C) contributed to nitrite accumulation by preferentially selecting for AOB, rather than NOB. The NO_x^- production values (Table 4) showed that for both strategies, the volumetric and biomass specific production rates were higher for nitrite (OS1: 0.05 ± 0.04 g NO_2^-- N day^{-1} L_R^{-1} and 0.10 ± 0.08 g NO_2^-- N day^{-1} g^{-1} VSS; OS2: 0.08 ± 0.05 g NO_2^-- N day^{-1} L_R^{-1} and 0.05 ± 0.03 g NO_2^-- N day^{-1} g^{-1} VSS) than for nitrate (OS1: 0.03 ± 0.03 g NO_3^-- N day^{-1} L_R^{-1} and 0.07 ± 0.06 g NO_3^-- N day^{-1} g^{-1} VSS; OS2: 0.003 ± 0.002 g NO_3^-- N day^{-1} L_R^{-1} and 0.002 ± 0.002 g NO_3^-- N day^{-1} g^{-1} VSS).

The formation of free ammonia (FA) results in partial nitrification. Concentrations between 0.1 and 1.0 mg FA L^{-1} inhibit NOB activity. AOB are only inhibited at much higher concentrations of 10–150 mg FA L^{-1} [29]. The formation of FA depends on pH, temperature, and NH_4^+ concentration. Here, FA was correlated to nitrite accumulation. The evolution of FA during typical SBR cycle profiles was recorded for both strategies (Figure 3). For OS1 (day 170, Figure 3C), ammonium oxidation to nitrite occurred during 1.6 h from the start of aeration. The FA concentration then decreased from 1.8 to

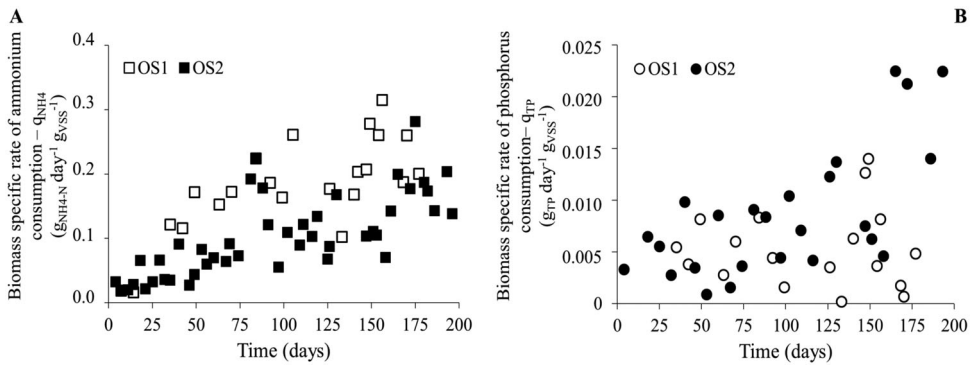


Figure 2. Ammonium specific removal rates (q_{NH_4}) (A); Total phosphorus specific removal rates (q_{TP}) (B).

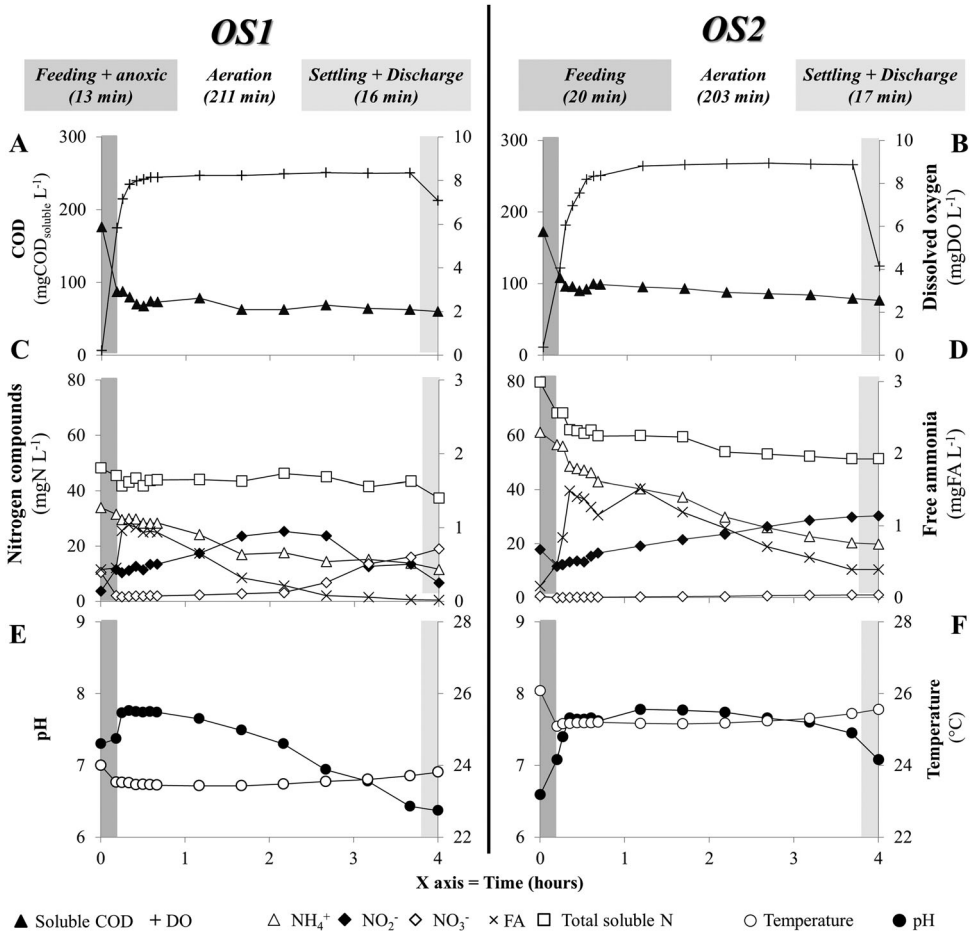


Figure 3. Typical operational cycle profiles of COD and DO (A, B), nitrogen compounds and free ammonia (C, D), and pH and temperature (E, F), under OS1 (left side) and OS2 (right side), after the granulation start-up period.

0.21 mg FA L^{-1} in 1.6 h. In the next 2.1 h, FA reached values below 0.1 mg FA L^{-1}, while r_{NO3} increased from 0.67 to 8.78 mg $NO_3^- - N\ L^{-1}\ h^{-1}$. In the case of OS2 (day 130, Figure 3D), a stable partial nitrification to nitrite was observed, leading to 0.4–1.5 mg FA L^{-1} over the full cycle. The combination of higher ammonium concentrations in the wastewater during OS2 (29–114 mg $NH_4^+ - N\ L^{-1}$), compared to OS1 (29–69 mg $NH_4^+ - N\ L^{-1}$), together with a slightly basic pH (7–7.3), resulted in higher FA concentrations and inhibition of NOB.

Granule fraction and size affect mass transfer phenomena, with diffusional restrictions leading to gradients of dissolved substrates and oxygen across the biofilm depth. Microbial guilds are established along substrate and redox gradients [11], while simultaneous nitrification and denitrification (SND) can occur during aeration, depending on granule metrics, depth of penetration of DO and nitrogenous electron acceptors, and microbial activity. Typical DO penetration depths of 300 µm have been measured using microsensors in nitrifying granules [49] and in COD- and N-removing granules [50]. This value represents the entire granule diameter recorded here. Under OS1, low granulation levels, small aggregates, and DO saturation probably resulted in solely aerated biovolumes. The DO saturation level during aeration (Figure 3A and B) promoted nitrification, but prevented simultaneous denitrification. NO_x decreased only slightly during idle under OS1 (from 48 to 41 mg $N_{soluble}\ L^{-1}$) and during feeding under OS2 (from 79 to 68 mg $N_{soluble}\ L^{-1}$). These two phases were intended to be anaerobic, but were anoxic due to NO_x accumulation. In OS1, both nitrate and nitrite could be used as terminal electron acceptors, while only nitrite could be used for this purpose in OS2 (Figure 3C and D).

Phosphorus removal

For both OS1 and OS2, only low biomass specific rates of total phosphorus removal (q_{TP}) (Figure 2B) were observed on more than 100 days. Nonetheless, a doubling trend was detected by the end of OS2 (0.022 g $P_{Tot}\ day^{-1}\ g^{-1}$ VSS), indicating the progressive establishment of PAOs. The period of highest q_{TP} matched that

with high EBPR efficiency (>97.5% and <0.2 mg P_{Tot} L_{Eff}^{-1} for days 165–193). Under OS1, no increase in q_{TP} was observed and the maximum EBPR efficiency was only 33%, with 3.9 mg P_{Tot} L_{Eff}^{-1} discharged.

The PAO biomass specific rates of polyphosphate storage ($q_{PP,PAO}$) used in EBPR modelling are 1.5 (20 °C) and 3.6 (28 °C) g P day^{-1} g^{-1} COD_{PAO} [31]. The maximum q_{TP} achieved here corresponded to 0.016 g P day^{-1} g^{-1} COD_x, using a conversion factor of 1.366 g COD_x g^{-1} VSS (as $C_1H_{1.8}O_{0.5}N_{0.2}$). Amplicon sequencing revealed good EBPR under OS2 with '*Candidatus* Accumulibacter' at 2.3% (total read count). This model PAO was not detected under OS1. Its relative abundance under OS2 corresponded to approximately 0.042 g VSS_{PAO} L^{-1} and 5 g VSS_{PAO} in the reactor, and to an effective q_{TP} of 0.5 g P day^{-1} g^{-1} COD_{PAO}. This activity is still low for an intensified and stable EBPR. Selecting for 3-fold higher amount and activity of PAOs in the VSS is required, employing an improved anaerobic selector [51].

The P loading rate and C:P ratio also affect EBPR. The values of these parameters were similar for OS1 (18 mg P_{Tot} L^{-1} day^{-1}; 71 g COD_{Tot} g^{-1} P_{Tot}) and OS2 (27 mg P_{Tot} L^{-1} day^{-1}; 84 g COD_{Tot} g^{-1} P_{Tot}). A typical C:P ratio below 20 g COD g^{-1} PO_4^{3-} − P has been used together with anaerobic-aerobic alternation to achieve enrichment of PAOs at bench scale, with the excess phosphorus being discharged through the outlet. High P limitation, corresponding to a C:P ratio of 200 g COD g^{-1} PO_4^{3-} − P, selects for GAOs [20]. A C:P ratio of around 50–100 g COD g^{-1} PO_4^{3-} − P sustains PAOs, given suitable environmental conditions (temperature and pH). The C:P ratio obtained here constituted a good basis for PAO selection.

The domestic wastewater presented low P concentrations (6.8 ± 2.0 mg P_{Tot} L^{-1}) and limited amounts of VFAs (>20 mg COD L^{-1}), which have presented challenges in other studies [52]. The influent COD was largely in particulate form (45; 38–55% of COD_{Tot}). The SBR cycle should be configured with a substantial anaerobic contact time inside the biomass to permit the hydrolysis of particulate matter, followed by VFA production and uptake by PAOs. This can be tested in different ways, with a simple mathematical model being useful for this purpose [39]. During the feeding phase, the influent flow rate should be adjusted according to the biomass bed height. Influent recirculation loops across the bed can be included in order to increase the frequency of contact between the wastewater and the biomass. An anaerobic mixed batch phase can be inserted after feeding, in order to extend the anaerobic contact time. During start-up, an amendment of phosphorus or VFAs (for example, from pre-fermented excess sludge) could be used to balance the C_{VFA}:P ratio, depending on the local sewage condition.

Bacterial ecology signatures related to BNR

The combination of COD_{sol} leakage and aeration at DO saturation resulted in selection for faster-growing OHOs that were easily able to outcompete the slower-growing PAOs and GAOs. This can lead to the proliferation of filaments and finger-type bulking structures. Irregular granules with high SVI destabilize the process and hamper EBPR [11].

OS1 led to substantial selection for AOB, with 4–10% *Nitrosomonas* during days 140–177, associated with the highest q_{NH4} values. In OS2, this guild was almost not detected by amplicon sequencing (<0.03%) (Figure 4). Betaproteobacterial AOB were nonetheless detected for both OS1 (day 156) and OS2 (day 165), using FISH-EFM with the NSO190 oligonucleotide probe. For OS2, colonies of *Nitrosomonas* spp. were detected with the NEU probe (day 91) (Figure 5).

Ammonium oxidation can be performed by heterotrophic populations such as *Pseudomonas* [53]. This genus was detected for both OS1 (9–1%, on days 77–107) and OS2 (24–8%, on days 56–91). *Comamonas*, a known heterotrophic nitrifier [54], was more abundant for OS2 (4–56%, on days 123–186), compared to OS1 (3–23%, on days 49–98) (Figure 4). Ecophysiology analyses could be used to confirm the nitrification functional property of these potentially nitrifying-denitrifying organisms in the granules. The primers and FISH probes used were unable to detect ammonium-oxidizing archaea (AOA). Further investigations could screen for a broader set of ammonium-oxidizing organisms (AOOs).

Bradyrhizobiaceae populations were detected in OS1 during two periods (days 0–107: 0.9–3%; days 126–177: 0.1–0.5%) and in OS2 continuously (0.2–2%). Their OTU sequences were mapped against the NCBI nucleotide database using BLASTN [55] and were affiliated at 99% identity to the genus *Nitrobacter*. However, multiple other species also related to this genus at high identity. The 500 bp amplicons were too short to discriminate between phylotypes, with longer sequences being needed for this purpose. FISH highlighted the presence of both *Nitrobacter* (NIT3) and *Nitrospira* (Ntspa662) in OS1, where they formed small clusters, while these organisms were not detected in OS2. *Nitrospira* can also be involved in complete ammonium oxidation [56]. Granular sludge studies have highlighted occurrences of imbalance between AOB and NOB, with the latter being predominant [57,58] and showing either *Nitrobacter* or *Nitrospira* as the predominant NOB. Anaerobic ammonium oxidizing organisms (AMOs) were not detected by FISH and were not covered by amplicon sequencing, due to the primer pair used.

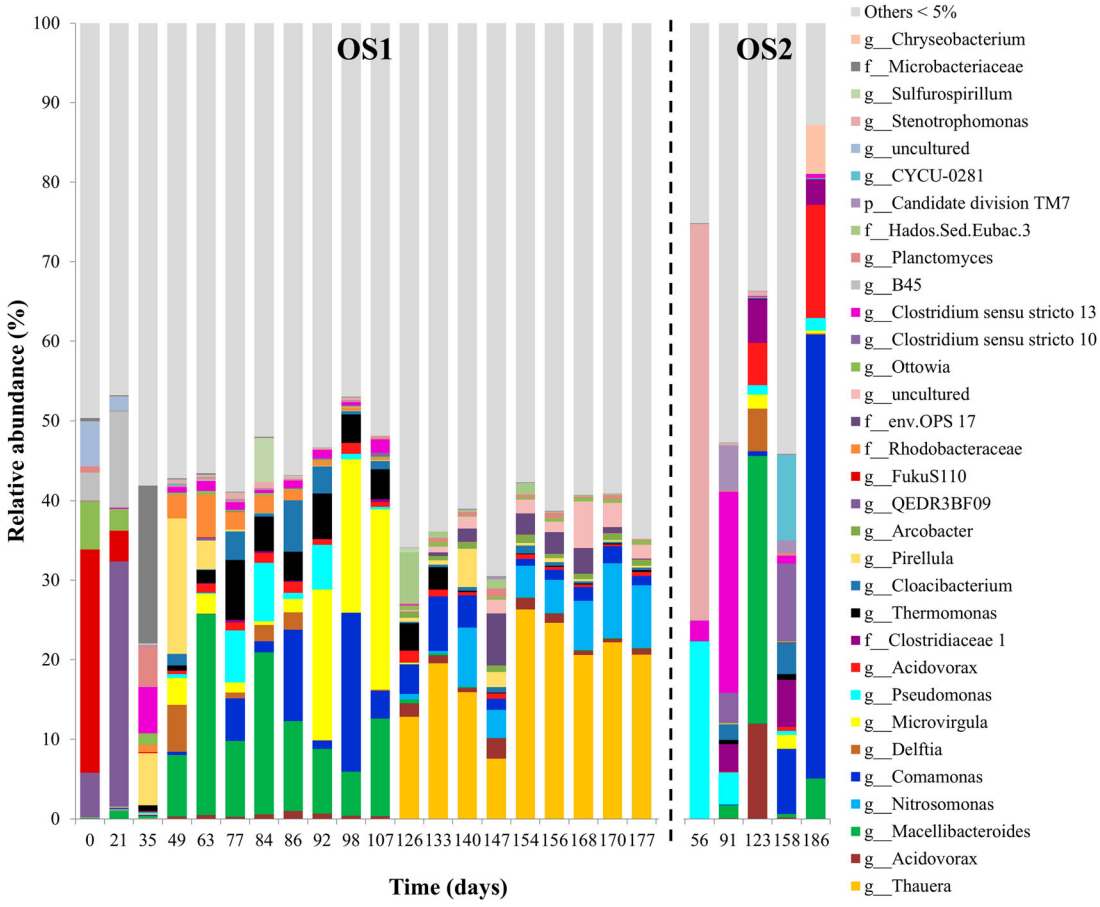


Figure 4. Bacterial community dynamics during the OS1 and OS2 operational periods. OTUs with relative abundance below 5% are included in 'Others'.

The two OS led to different compositions of the guild of putative denitrifying heterotrophic organisms (DHOs). The conditions of OS1 selected mainly for members of the *Rhodocyclaceae* family, with *Thauera* (7–29%) as the predominant genus (Figure 4). Under OS2, *Comamonas* (4–56%) and *Acidovorax* (0.7–17%) affiliates of the *Comamonadaceae* family were more abundant (Figure 4). Granules cultivated by Adav [59] have been able to consume high concentrations of nitrite, under predominance of *Comamonadaceae* relatives (52%) located at 100–200 µm of granule depth. Conversely, the granules described by Yan [60] decreased nitrite and nitrate, with *Thauera* (20%) as the dominant DHO. The DHO composition depends on the main carbon sources and electron donors contained in the wastewater. Here, the same sewage was used for both OS. The difference in the DHOs could have been related to the electron acceptor available and the operational conditions applied. This highlights the impact of operational conditions on bacterial ecology.

Operation with slower up-flow feeding under OS2 led to progressive selection of the genus *Macellibacteroides*, from 0.04% (day 56) to 34% (day 123) (Figure 4), with a concomitant increase in '*Ca.* Accumulibacter' (from 2.3 to 3.4%). In OS2, PAOs were detected by FISH, qualitatively confirming the sequencing result (Figure 5). *Macellibacteroides* showed a substantial presence in OS1 (8–25% for days 49–63). This fermentative organism can act as a useful indicator of hydrolytic and fermentative processes in the sludge [61]. Ecophysiological and genomic analyses could be used to elucidate the metabolism of this organism in this ecosystem.

Suggestions to improve granular biomass accumulation and BNR

The bacterial ecology of granular sludge was affected by the conditions imposed under the two different OS. Starting from process failures identified in the two strategies applied here, microbial ecology concepts are translated hereafter into possible ways to optimize granular sludge processes that handle low strength domestic wastewater under warm coastal climatic conditions. The recommendations may be applicable to

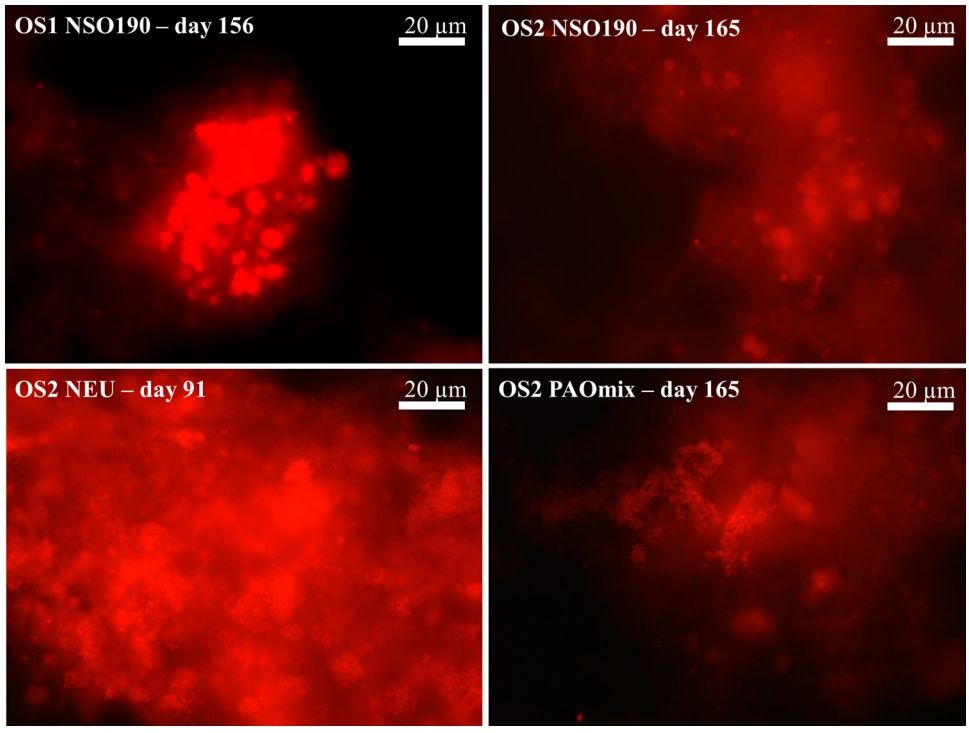


Figure 5. Fluorescence *in situ* hybridization of β-proteobacterial AOB (NSO190), *Nitrosomonas* spp. (NEU) and '*Ca.* Accumulibacter'-like PAOs (PAO mix) on bioaggregates formed under operational strategies OS1 and OS2.

most granular sludge systems handling municipal wastewater.

Feeding issues

The cycle configuration should improve the supply of COD_{rb} by hydrolysis of raw organic matter, in order to increase biomass accumulation and P removal. Given the insufficient anaerobic contact time between the influent and the biomass, observed for both strategies (OS1: 24 ± 12 s; OS2: 2.1 ± 0.4 min), an extended anaerobic feeding would enable pre-fermentation of COD_{rb} into VFAs that are stored by PAOs and GAOs, as a prerequisite for good granulation [15,58]. This should benefit from the flexible SBR technology, using (i) a slow up-flow feeding regime through a substantial bed of biomass, (ii) a pulse feeding followed by a prolonged anaerobic mixed batch phase, or (iii) a combination of both [39]. In contrast to widely held beliefs, EBPR or full BNR in granular sludge does not occur directly during transport through granule layers *per se*: well-controlled alternation of substrate and redox selection pressures is required along SBR operation, as for activated sludge systems [39]. PAOs and GAOs become established by means of alternating anaerobic C-feast and aerobic C-starvation. While enhancing granulation, PAOs provide EBPR, effectively for free, and assist in enabling compliance with more stringent regional water quality standards.

Aeration issues

Aeration phase duration and DO control are crucial for controlling biomass accumulation, AOB, and BNR. Due to the insufficient AOB activity and DO at saturation under both strategies, optimization by extending this phase and reducing the level of DO could improve nitrification by consuming ammonium, lowering FA, and preventing nitrite accumulation. Control at moderate DO prevents endogenous respiration by over-oxygenation and promotes anoxic biovolumes for SND. Leakage of terminal electron acceptors (DO, nitrite, and nitrate) from the aeration to the anaerobic feeding or anaerobic batch phases should be suppressed by DO control for SND, or by alternated nitrification-denitrification (AND) and/or inclusion of a post-anoxic phase to reduce residual concentrations of NO_x^-. The use of AND should be considered for enhancing N removal [12]. Here, high residual concentrations of ammonium and nitrite, in an almost equimolar ratio, could be used to stimulate the growth of AMOs.

Settling considerations

The hydraulic selection pressure for granulation is determined by the settling time and the volume exchange ratio. Short settling times select for dense aggregates [8,10]. Bench-scale studies have used fast settling times shorter than 5 min, with reactor working heights of

about 1 m, hence selecting for aggregates with settling velocities above 12 m h^{-1}. The pilot start-ups involved stepwise decreases in settling time, nominally selecting for particles settling faster than 4.5–10 m h^{-1}. For both OS, the settling velocities increased from the inoculum stage (1.8 m h^{-1}) to the granules stage (3.3 m h^{-1}), but nonetheless remained low. The feeding flow rates (OS1:18 L min^{-1}; OS2: 3.5 L min^{-1}) and flow velocities (OS1: 22.0 m h^{-1}; OS2: 4.3 m h^{-1}) were too high for both, and should be lowered in order to maintain biomass in the system. An excessively strong hydraulic selection pressure can be problematic [1], notably due to a high fraction of slowly biodegradable particulate organic matter (Xs). Continuous wash-out of slowly settling biomass limits the growth of granules [6,38]. High Xs, as in real wastewater, can hinder biomass accumulation and substrate removal, so settling and feeding patterns should account for the type of substrate present. In full-scale operation, lower hydraulic selection pressure, with 30 min settling followed by a simultaneous fill/draw regime, enabled the biomass to settle for a longer time, with discharge at the reactor head lasting 60–90 min [1].

Effects of fluctuations in operational conditions

SBR configurations need to be adapted according to local wastewater and climate conditions, taking fluctuations into account. A suitable reactor loading is crucial for achieving satisfactory granulation and BNR rates. The low strength of wastewater with high variation, as observed in the present study (high standard deviations, Table 2), should be counteracted by adjusting SBR phase lengths as a function of the fluctuations of influent concentrations. The daily organic loading rate can be increased by shortening the cycle during rainy periods [1,2], with 50% extension of the feeding phase duration and substantial decrease of the aeration phase having been found to be effective for treatment of a 5-fold higher hydraulic load of wastewater. Consideration of yearly statistical time series of influent concentrations, rainfall, and temperature is essential for setting operational conditions. Temperature variations affect the contact time requirements in the SBR phases, by analogy to increase or decrease of redox volumes in flow-through activated sludge systems. Managing the effects of temperature fluctuations, rainfall, and infiltration of saltwater is crucial for the success of microbial selection, granulation and nutrient removal processes under coastal conditions.

Conclusions

The adoption of the granular sludge technology requires strong consideration of specific local conditions, in order to engineer the SBR operation for efficient microbial selection, granulation, and BNR. Failures in biomass accumulation and BNR performance were observed under the operational strategies applied here involving rapid and slow feeding regimes. Potential operational solutions were proposed to achieve a robust granular sludge process for the treatment of low-strength and highly-fluctuating domestic wastewater.

- Although biomass did not substantially accumulate in the reactor under either strategy, OS2 displayed a higher granulation level, larger aggregates, and better sludge settling properties. The slower feeding pattern permitted a slightly longer anaerobic contact time between the wastewater and the biomass, hence providing better conditions for particulate organic fraction hydrolysis, compared to OS1, although total anaerobic consumption of COD was still not achieved. Therefore, the design of the anaerobic selector must permit optimization of the contact time during the feeding phase, in order to improve total COD removal, specifically aiming at biomass accumulation and robust granulation.
- As a consequence of the inefficient anaerobic selector, COD was oxidized aerobically. Faster growth of heterotrophic microorganisms was observed for OS1 ($\mu_{obs} = 0.18$ d^{-1}), compared to OS2 ($\mu_{obs} = 0.08$ d^{-1}), but was associated with a faster decay rate (OS1: 0.154 d^{-1}; OS2: 0.038 d^{-1}) resulting from endogenous respiration due to long periods of starvation under aeration with DO at saturation concentration. Biomass accumulation should get fosterd under reactor regime involving anaerobic C-feast followed by aerobic C-famine, which would favour slow-growing and C-N-P-removing organisms. This strategy would enable the organisms to balance their growth.
- The removal of ammonium increased under both OS, indicating progressive establishment of the AOB guild and activity. However, effluent ammonium could reach even lower concentrations, while nitrification remained incomplete, with unfavourable accumulation of nitrite under both OS. High temperature and FA concentration were related to NOB inhibition and consequently to this failure in nitrification in the present pilot system operated in a warm climate region. For both strategies, the presence of DO at saturation levels also hampered denitrification. The removal of N could be improved by increasing the duration of the aeration phase, while maintaining low concentrations of oxygen, or by using additional post-phases.
- Strategy OS2 showed a progressive establishment of PAOs, achieving high EBPR efficiency (>97.5% and

<0.2 mg P_{Tot} L_{Eff}^{-1}) at the end of the operational period. Removal of P was impaired by the low P concentrations (6.8 ± 2.0 mg P_{Tot} L^{-1}) and limited amount of VFAs in the domestic wastewater, combined with incomplete anaerobic consumption. Therefore, an extended anaerobic feeding could enable pre-fermentation of COD_{rb} into VFAs stored by PAOs and GAOs, as a prerequisite for good granulation and P removal.
- A reduction of hydraulic selection pressure driven by the settling time would diminish biomass wash-out, while SBR cycle adaptations according to weather fluctuations would greatly assist in improving operational processes involving the treatment of municipal wastewaters in subtropical coastal regions.

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References

[1] Pronk M, de Kreuk MK, de Bruin B, et al. Full scale performance of the aerobic granular sludge process for sewage treatment. Water Res. 2015;84:207–217.
[2] Pronk M, Giesen A, Thompson A, et al. Aerobic granular biomass technology: Advancements in design, applications and further developments. Water Pract. Technol. 2017;12:987–996.
[3] Schwarzenbeck N, Erley R, McSwain BS, et al. Treatment of malting wastewater in a granular sludge sequencing batch reactor (SBR). Acta Hydrochim. Hydrobiol. 2004;32:16–24.
[4] Giesen A, Van Loosdrecht M, Pronk M, et al. Aerobic granular biomass technology : recent performance data, lessons learnt and retrofitting conventional treatment infrastructure. Proc. Water Environ. Fed. WEFTEC 2016 Sess. 300 Through Sess. 309. 2016;11:1913–1923.
[5] Wagner J, Guimarães LB, Akaboci TRV, et al. Aerobic granular sludge technology and nitrogen removal for domestic wastewater treatment. Water Sci. Technol. 2015;71:1040–1046.
[6] Derlon N, Wagner J, da Costa RHR, et al. Formation of aerobic granules for the treatment of real and low-strength municipal wastewater using a sequencing batch reactor operated at constant volume. Water Res. 2016;105:341–350.
[7] Sarma SJ, Tay JH, Chu A. Finding knowledge gaps in aerobic granulation technology. Trends Biotechnol. 2017;35:66–78.
[8] Figueroa M, del Rıo AV, Morales N, et al. Nitrogen removal in aerobic granular systems. Environ. Technol. to Treat nitrogen Pollut. London: IWA Publishing; 2009. p. 373–401.
[9] McSwain Sturm BS, Irvine RL. Dissolved oxygen as a key parameter to aerobic granule formation. Water Sci. Technol. 2008;58:781–787.
[10] Morgenroth E, Sherden T, van Loosdrecht MCM, et al. Aerobic granular sludge in a sequencing batch reactor. Water Res. 1997;31:3191–3194.
[11] de Kreuk MK, Heijnen JJ, van Loosdrecht MCM. Simultaneous COD, nitrogen, and phosphate removal by aerobic granular sludge. Biotechnol. Bioeng. 2005;90:761–769.
[12] Lochmatter S, Gonzalez-Gil G, Holliger C. Optimized aeration strategies for nitrogen and phosphorus removal with aerobic granular sludge. Water Res. 2013;47:6187–6197.
[13] Li J, Bin DL, Cai A, et al. Aerobic sludge granulation in a full-scale sequencing batch reactor. Biomed Res. Int. 2014;2014:1–12.
[14] Yang G, Wang D, Yang Q, et al. Effect of acetate to glycerol ratio on enhanced biological phosphorus removal. Chemosphere. 2018;196:78–86.
[15] de Kreuk MK, van Loosdrecht MCM. Selection of slow growing organisms as a means for improving aerobic granular sludge stability. Water Sci. Technol. 2004;49:9–17.
[16] INMET/BRASIL. Instituto Nacional de Metereologia [Internet]. [cited 2017 Jul 12]. Available from: http://www.inmet.gov.br/portal/index.php?r=home2/index.
[17] Ni BJ, Xie WM, Liu SG, et al. Granulation of activated sludge in a pilot-scale sequencing batch reactor for the treatment of low-strength municipal wastewater. Water Res. 2009;43:751–761.
[18] Jungles MK, Figueroa M, Morales N, et al. Start up of a pilot scale aerobic granular reactor for organic matter and nitrogen removal. J. Chem. Technol. Biotechnol. 2011;86:763–768.
[19] Guimarães LB, Mezzari MP, Daudt GC, et al. Microbial pathways of nitrogen removal in aerobic granular sludge treating domestic wastewater. J. Chem. Technol. Biotechnol. 2017;92:1756–1765.
[20] Weissbrodt DG, Schneiter GS, Fürbringer J-M, et al. Identification of trigger factors selecting for polyphosphate- and glycogen-accumulating organisms in aerobic granular sludge sequencing batch reactors. Water Res. 2013;47:7006–7018.

[21] Jungles MK, Campos JL, Costa RHR. Sequencing batch reactor operation for treating wastewater with aerobic granular sludge. Brazilian J. Chem. Eng. 2014;31:27–33.
[22] Conselho Nacional do Meio Ambiente (CONAMA). Resolução CONAMA 430/2011 [Internet]. Ministério do Meio Ambient. 2011. p. 8. Available from: http://www2.mma.gov.br/port/conama/legiabre.cfm?codlegi=646.
[23] Council EEC. 91/271/EEC of 21 May 1991 concerning urban waste-water treatment. EEC Counc. Dir. 1991;135:40–52.
[24] Wang F, Qing XS, Liu Y, et al. Community analysis of ammonia and nitrite oxidizers in start-up of aerobic granular sludge reactor. J. Environ. Sci. 2007;19:996–1002.
[25] Swiss Federal Council. WPO 814.201 Waters Protection Ordinance of 28 October 1998 [Internet]. 814.201 Swiss; p. (Status as of 2 February 2016). Available from: https://www.admin.ch/opc/en/classified-compilation/19983281/index.html.
[26] Liu YQ, Moy B, Kong YH, et al. Formation, physical characteristics and microbial community structure of aerobic granules in a pilot-scale sequencing batch reactor for real wastewater treatment. Enzyme Microb. Technol. 2010;46:520–525.
[27] AWWA, APHA, WEF. Standard methods for the examination of water and wastewater. 22nd ed. Rice EW, Baird RB, Eaton, Andrew D, Clesceri LS, editors. Washington: AWWA, American Water Works Association APHA, American Public Works Association WEF, Water Environment Federation; 2012.
[28] von Sperling M, de lemos CA. Biological wastewater treatment in warm climate regions. London: IWA Publishing; 2005.
[29] Anthonisen a C, Srinath EG, Loehr RC, et al. Inhibition of nitrification and nitrous acid compounds. J. Water Pollut. Control Fed. 1976;48:835–852.
[30] Liu YQ, Tay JH. Influence of cycle time on kinetic behaviors of steady-state aerobic granules in sequencing batch reactors. Enzyme Microb. Technol. 2007;41:516–522.
[31] Gujer W, Henze M, Mino T, et al. Activated sludge model No. 3. Water Sci. Technol. 1999;39:183–193.
[32] McIlroy SJ, Saunders AM, Albertsen M, et al. MiDAS: The field guide to the microbes of activated sludge. Database. 2015;2015:1–8.
[33] Albertsen M, Karst SM, Ziegler AS, et al. Back to basics - The influence of DNA extraction and primer choice on phylogenetic analysis of activated sludge communities. PLoS One. 2015;10:1–15.
[34] Nielsen JL, Nielsen PH, Daims H, et al. FISH Handbook for biological wastewater treatment. London: IWA Publishing; 2009.
[35] Nielsen JL, Seviour RJ, Nielsen PH. Microscopy. Exp. methods wastewater Treat. London: IWA Publishing; 2016. p. 263–282.
[36] Greuter D, Loy A, Horn M, et al. ProbeBase-an online resource for rRNA-targeted oligonucleotide probes and primers: New features 2016. Nucleic Acids Res. 2016;44:D586–D589.
[37] Wagner J, Costa RHR. Aerobic granulation in a sequencing batch reactor using real domestic wastewater. J. Environ. Eng. 2013;139:1391–1396.
[38] Wagner J, Weissbrodt DG, Manguin V, et al. Effect of particulate organic substrate on aerobic granulation and operating conditions of sequencing batch reactors. Water Res. 2015;85:158–166.
[39] Weissbrodt DG, Holliger C, Morgenroth E. Modelling hydraulic transport and anaerobic uptake by PAOs and GAOs during wastewater feeding in EBPR granular sludge reactors. Biotechnol. Bioeng. 2017;114:1688–1702.
[40] de Kreuk MK, Kishida N, Tsuneda S, et al. Behavior of polymeric substrates in an aerobic granular sludge system. Water Res. 2010;44:5929–5938.
[41] Arnaldos M, Amerlinck Y, Rehman U, et al. From the affinity constant to the half-saturation index: Understanding conventional modeling concepts in novel wastewater treatment processes. Water Res. 2015;70:458–470.
[42] Gujer W, Henze M, Mino T, et al. The activated sludge model No. 2: biological phosphorus removal. Water Sci. Technol. 1995;31:1–11.
[43] Metcalf, Eddy. Wastewater engineering: treatment and reuse. 4th ed. Metcalf, Eddy I, editor. New York: McGraw-Hill; 2003.
[44] Beun JJ, Hendriks A, van Loosdrecht MCM, et al. Aerobic granulation in a sequencing batch reactor. Water Res. 1999;33:2283–2290.
[45] Cydzik-Kwiatkowska A, Wojnowska-Baryła I. Nitrifying granules cultivation in a sequencing batch reactor at a low organics-to-total nitrogen ratio in wastewater. Folia Microbiol. (Praha). 2011;56:201–208.
[46] Weissbrodt DG, Neu TR, Kuhlicke U, et al. Assessment of bacterial and structural dynamics in aerobic granular biofilms. Front. Microbiol. 2013;4:1–18.
[47] Poot V, Hoekstra M, Geleijnse M, et al. Effects of the residual ammonium concentration on NOB repression during partial nitritation with granular sludge. Water Res. 2016;106:518–530.
[48] Hellinga C, Schellen AAJC, Mulder JW, et al. The SHARON process: An innovative method for nitrogen removal from ammonium-rich waste water. Water Sci. Technol. 1998;37:135–142.
[49] Matsumoto S, Katoku M, Saeki G, et al. Microbial community structure in autotrophic nitrifying granules characterized by experimental and simulation analyses. Environ. Microbiol. 2010;12:192–206.
[50] Li J, Cai A, Wang D, et al. Structure analysis of aerobic granule from a sequencing batch reactor for organic matter and ammonia nitrogen removal. Int J Environ Res Public Health. 2014;11:2427–2436.
[51] Winkler M-KH, Meunier C, Henriet O, et al. An integrative review of granular sludge for the biological removal of nutrients and recalcitrant organic matter from wastewater. Chem. Eng. J. 2018;336:489–502.
[52] Wang D, Wang D, Fu Q, et al. Free nitrous acid-based nitrifying sludge treatment in a two-sludge system enhances nutrient removal from low-carbon wastewater bioresource technology free nitrous acid-based nitrifying sludge treatment in a two-sludge system enhances nutrient removal fro. Bioresour. Technol. 2017;244:920–928.
[53] Li C, Yang J, Wang X, et al. Removal of nitrogen by heterotrophic nitrification-aerobic denitrification of a phosphate accumulating bacterium Pseudomonas stutzeri YG-24. Bioresour. Technol. 2015;182:18–25.
[54] Patureau D, Davison J, Bernet N, et al. Dentrification under various aeration conditions in Comomonas sp., strain SGLY2. FEMS Microbiol. Lett. 1994;14:71–78.
[55] NCBI. Bethesda (MD): National Library of Medicine (US), National Center for Biotechnology Information.

[Internet]. 1988 [cited 2017 Jun 1]. Available from: https://www.ncbi.nlm.nih.gov/.

[56] Daims H, Lebedeva EV, Pjevac P, et al. Complete nitrification by Nitrospira bacteria. Nature. 2015;528:504–509.

[57] Winkler MKH, Kleerebezem R, Khunjar WO, et al. Evaluating the solid retention time of bacteria in flocculent and granular sludge. Water Res. 2012;46:4973–4980.

[58] Weissbrodt DG, Shani N, Holliger C. Linking bacterial population dynamics and nutrient removal in the granular sludge biofilm ecosystem engineered for wastewater treatment. FEMS Microbiol. Ecol. 2014;88:579–595.

[59] Adav SS, Lee DJ, Lai JY. Microbial community of acetate utilizing denitrifiers in aerobic granules. Appl. Microbiol. Biotechnol. 2010;85:753–762.

[60] Yan L, Zhang S, Hao G, et al. Simultaneous nitrification and denitrification by EPSs in aerobic granular sludge enhanced nitrogen removal of ammonium-nitrogen-rich wastewater. Bioresour. Technol. 2016;202:101–106.

[61] Jabari L, Gannoun H, Cayol JL, et al. Macellibacteroides fermentans gen. nov., sp. nov., a member of the family Porphyromonadaceae isolated from an upflow anaerobic filter treating abattoir wastewaters. Int. J. Syst. Evol. Microbiol. 2012;62:2522–2527.