Bioresource Technology 239 (2017) 180–189

Contents lists available at ScienceDirect

Bioresource Technology

journal homepage: www.elsevier.com/locate/biortech

Start-up and operation of an aerobic granular sludge system under low working temperature inoculated with cold-adapted activated sludge from Finland

Alejandro Gonzalez-Martinez [a,*], Barbara Muñoz-Palazon [b], Alejandro Rodriguez-Sanchez [b], Paula Maza-Márquez [b], Anna Mikola [a], Jesus Gonzalez-Lopez [b], Riku Vahala [a]

[a] Department of Built Environment, University of Aalto, P.O. Box 15200, Aalto, FI-00076 Espoo, Finland
[b] Institute of Water Research, University of Granada, C/Ramon y Cajal, 4, 18071 Granada, Spain

HIGHLIGHTS

- The granulation process at 7 °C can be done using cold-adapted sludge.
- An aerobic granular sludge system was operated at 7 °C.
- A cold-adapted inoculum from an activated sludge system in Finland was used.
- Cold-adapted inoculum led to superior performance than warm-adapted inoculum at 7 °C.
- *Microbacteriaceae* genera *Leucobacter* and *Microbacterium* dominated the granules.

ARTICLE INFO

Article history:
Received 17 February 2017
Received in revised form 4 May 2017
Accepted 7 May 2017
Available online 10 May 2017

Keywords:
Aerobic granular sludge
Cold-adapted sludge
Bacterial community dynamics
Granulation
Low temperature
Wastewater treatment

ABSTRACT

An aerobic granular sludge system has been started-up and operated at 7 °C temperature using cold-adapted activated sludge as inoculum. The system could form granular biomass due to batch operation allowing for just 5–3 min of biomass sedimentation. Scanning electron microscopy showed that fungi helped in the granular biomass formation in the early stages of the granule formation. The removal performance of the system was of 92–95% in BOD5, 75–80% in COD, 70–76% in total nitrogen and 50–60% in total phosphorous. The bacterial community structure from cold-adapted activated sludge changed during the operational time, leading to a final configuration dominated by Microbacteriaceae members Microbacterium and Leucobacter, which were strongly correlated to biomass settling velocity and bioreactor performance, as suggested by multivariate redundancy analyses. This experiment showed that aerobic granular sludge systems could be successfully started-up and operated, with high performance, under low operational temperatures when using cold-adapted biomass as inoculum.

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1. Introduction

Temperature is a key parameter that affects microbial metabolism and community structure in all environments. The biotransformation processes can be modified by temperature and therefore it is necessary to research bacterial behavior generated by changes in this parameter to understand its role in natural and engineered ecosystem (Adams et al., 2010). Most of the biosphere is at low temperature, below 5 °C, so research on adaptation of microorganisms at low temperature is relevant (Lewin et al., 2013). Microbes growing in cold habitats around freezing point need to deal with diverse challenges such as persistently low temperatures and low abundance of nutrients, as well as salinity fluctuations, desiccation and very different light conditions between seasons (Varin et al., 2012). In this way, low environmental temperature can trigger inhibition of the metabolism and activity of the microorganisms. With respect to wastewater treatment systems, especially biological nitrogen removal processes have a strong negative correlation with temperature, with complete inhibition of mild temperature (around 20 °C) adapted activated sludge when subjected to temperatures of 10 °C or lower (Gnida et al., 2016). Low temperature leads to several problems in biological wastewater treatment processes, such as higher waste activated sludge production, higher presence of filamentous microorganisms, lower settleability of sludge and worse effluent quality (He

* Corresponding author.
E-mail address: alejandro.gonzalezmartinez@aalto.fi (A. Gonzalez-Martinez).

http://dx.doi.org/10.1016/j.biortech.2017.05.037
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et al., 2016). Despite the possible inhibition of their activity, the processes of elimination of organic matter and nutrients from wastewater in Nordic countries such as Finland, have high removal efficiencies.

In the last years, one promising wastewater treatment technology has been developed based on the spontaneous formation of granular sludge biomass under aerobic conditions (Wan et al., 2015). Aerobic granular sludge systems were designed to treat different wastewater effluents such as organic matter, nutrients, antibiotics and hypersaline water, among others. The highly superior properties of granular sludge in terms of compactness and sedimentation properties with respect to floc biomass enhances the biomass retention capacity of these technology (Aqeel et al., 2016). Therefore, an important advantage of the aerobic granular sludge systems is that this bioprocess takes place in a single bioreactor with a very high biomass retention efficiency, eliminating the costs of biomass recycling and therefore yielding lower costs in the treatment of wastewater. An aerobic granular sludge bioreactor occupies 20% less surface compared to a conventional activated sludge system, meaning less investments and operational costs (Devlin et al., 2017).

Nevertheless, the effect of low temperature in an aerobic granular sludge system can generate problems such as washout of biomass and destruction of granules, so it is difficult to start-up this bioprocess under cold environmental temperature conditions (De Kreuk et al., 2005). Temperature has shown to influence settling properties of granules, increasing up to two-fold the necessary settling time (Winkler et al., 2012). Additionally, operation at temperatures as low as 8 °C have shown a process instability due to excessive growth of filamentous microorganisms when no cold-adapted inoculum was used (Adav et al., 2008; Bao et al., 2009; Jiang et al., 2015). Despite this fact, biological processes in cold regions, such as the Nordic countries, attain very high pollutant removal efficiencies at low temperature. This must be caused by the adaptation to low temperatures of microbial communities thriving in these systems. Therefore, it is of pivotal importance to investigate the aerobic granules technologies under low temperature conditions.

For these reasons, in the present study, the start-up, granulation process and operation of a sequential aerobic granular sludge system were done at low temperature conditions (7 °C), inoculating with cold adapted sludge from Finland. Moreover, the characterization of the bacterial populations involved in these low temperature processes were studied during the experiment using massive parallel sequencing techniques.

2. Materials and methods

2.1. Reactor start-up and operation condition

The experiment was performed in a column-type sequencing batch reactor (SBR). The reactor had 90 cm height and 7 cm diameter (2.5 L volume). The bioreactor was inoculated in winter with 2 L mixed liquor from the low temperature full-scale activated sludge system of Porvoo WWTP in Finland (average winter temperature of −6.9 °C). The aeration was introduced by the means of a fine bubble diffuser at the bottom of the reactor. The air was measured by a rotameter (2.5 L min^{-1}) and the dissolved oxygen (DO) was not controlled in the reactor because it was close to saturation (the saturation rate at 7 °C was around 11,5 mg/L). During the experiment, the temperature of the system was controlled at 7 °C thanks to a temperature-controlled chamber. The reactor underwent cyclical operation with a 50% volumetric exchange per cycle. The cycle phases are showed in Fig. S1.

The reactor was fed with a synthetic wastewater medium simulating urban wastewater. In this way, the synthetic wastewater composition used through the experimental period was: 1 g L^{-1} NaAc, 0.06 g L^{-1} $MgSO_4 \cdot 7H_2O$, 0.20 g L^{-1} NH_4Cl, 0.064 g L^{-1} $CaCl_2 \cdot 2H_2O$, 0.035 g L^{-1} KH_2PO_4, 0.3 g L^{-1} $NaHCO_3$ and 0.3 mL L^{-1} of trace element solution. This trace solution was composed of 10 mg L^{-1} EDTA; 0.18 mg L^{-1} KI; 0.12 mg L^{-1} $ZnSO_4 \cdot 7H_2O$; 0.15 mg L^{-1} H_3BO_3; 0.12 mg L^{-1} $MnCl_2$; 1.5 mg L^{-1} $FeCl_3 \cdot 6H_2O$; 0.04 mg L^{-1} $(NH4)Mo_7O_{24} \cdot 4H_2O$; 0.03 mg L^{-1} $CuSO_4 \cdot 5H_2O$; and 0.15 mg L^{-1} $CoCl_2 \cdot 6H_2O$.

2.2. Physic-chemical determinations

During the whole study, the samples of the influent, effluent and excess activated sludge were taken on a regular basis to investigate the performance of the bioprocess. The Chemical Oxygen Demand (COD), Biological Oxygen Demand (BOD), Suspended Solids (SS), Total Nitrogen (TN), Nitrogen oxides (NO_2^-, NO_3^-) and Total Phosphorus (TP) were measured according to the standard methods (APHA, 2005). Determinations were done in duplicates. The temperature was controlled by operation inside a temperature-controlled chamber set up at 7 °C. The settling time of the biomass was determined by measuring the sedimentation of biomass in a 2 m column. The size of the granular biomass was measured according to Laguna et al., 1999. The pH was measured using an inoLab pH 720 pH-meter equipped with a Sentix 81 Plus probe.

2.3. Collection of biological samples, DNA extraction and iTag sequencing procedure

For the purpose of bacterial identification of the granular biomass in the aerobic granular sludge bioreactor during the start-up and operation time, 200 mL of inoculum and 200 mL of mixed liquor containing granular biomass were collected at operational days 0, 7, 15, 30, 60, 120 and 240. The samples collected were immediately taken to the laboratory and then centrifuged at 3500 rpm during 10 min at room temperature. After the process the liquid supernatant was discarded and the pelleted biomass was stored at −20 °C for further DNA extraction procedure.

The extraction of DNA for each of the biological samples collected was replicated five times. For each replication, 350 mg biomass were subjected to DNA extraction using the FastDNA SPIN Kit for Soil (MP Biomedicals, Solon, OH, USA) according to the manufacturer's protocol. The extracted DNA from replicates derived from the same biological sample were then merged into a DNA pool for future *iTag* sequencing procedure.

The DNA pools were kept at −20 °C and sent to Research & Testing Laboratory (Lubbock, TX, USA) to proceed with the *iTag* sequencing process. This was done using the Illumina MiSeq equipment and the Illumina Miseq Reagent Kit v3. The primer pair 28F-519R (50-GAGTTTGATCNTGGCTCAG-30 and 50-GTNTTACNGCGGCKGCTG-30, respectively) (Gonzalez-Martinez et al., 2016a) was used for the amplification of the hypervariable regions V1-V2-V3 of the 16S rDNA gene of Bacteria. The PCR conditions for the *iTag* sequencing process were: 3 min at 94 °C, then 32 cycles of: 30 s at 94 °C, 40 s at 60 °C, and 60 s at 72 °C; final elongation step of 5 min at 72 °C.

2.4. iTag sequencing post-process

The raw data derived from the *iTag* sequencing process was treated using the software mothur v1.3.4.4 (Schloss et al., 2009). Raw paired-end reads from *iTag* sequencing process were made into contigs. These contigs were then subjected to a process consisting on quality trimming eliminating sequences with any

ambiguous bases or with more than 8 homopolymers. Then, remaining sequences were aligned against SiLVA database. Those that failed to align with the forward primer position or those that ended past the 95% of all samples were considered as alignment failures and thus eliminated. The remaining sequences then underwent a preclustering in a two bases difference threshold. Then the remaining sequences were checked for detection and removal of chimeras using UCHIME v.4.2.40 implemented in mothur v1.34.4. To carry out the ecological analysis of all *iTag* sequencing samples, all of them were rarified and cut to 14405 reads. Each of the subsamples then was subjected to the ecological analysis. First, a Phylip distance matrix was calculated for all sequences within a subsample, which then were clustered in a 97% similarity threshold to conform OTUs. Then, representative sequences from each OTU were taken and taxonomically affiliated based on SiLVA database. Finally, all taxonomically-identified OTUs were taken to conform the consensus taxonomy of the subsample using the similarity cutoff of 80%.

2.5. Study of sequencing coverage

The ecological coverage provided by the *iTag* sequencing subsamples was checked using two different methods. These were the conformation of complexity curves and the calculation of the redundancy abundance-weighted coverage of the subsample. The complexity curves were calculated using the software aRarefactWin and taking the consensus taxonomy of OTUs in each of the *iTag* sequencing subsamples (Gonzalez-Martinez et al., 2016a). The redundancy abundance-weighted coverage analysis was performed taking all sequences from the *iTag* sequencing subsamples and using Nonpareil software setting a 50% overlap of sequences, a 95% similarity threshold between sequences and a query set size of 1000 sequences, as they were the default parameters for calculation offered by the software.

2.6. α-Diversity and β-diversity of biological samples

The study of α-diversity of the *iTag* sequencing subsamples was done using the software PAST v.3.06 and considering the indices of Good's coverage, Chao-1, Shannon-Wiener, Simpson, Pielou's evenness and Berger-Parker evenness, which were calculated with a 95% confidence range by 1000 bootstrap replications.

The Morisita-Horn and symmetric indices, which had been reported as the most robust indices to capture β-diversity of dominant phylotypes and rare phylotypes, respectively, were used to estimate the β-diversity among pairs of *iTag* sequencing subsamples. These indices were calculated using the packages vegan v2.0 and vegetarian implemented in R-project software.

2.7. Multivariate redundancy analysis

A multivariate redundancy analysis (RDA) was done to link the bacterial species and the operational and performance parameters. Another RDA was done to link the bacterial species with the parameters of the biomass in the system. The calculation of the RDAs was made by 499 unconstrained Monte-Carlo simulation and run using the software CANOCO 4.5 for Windows (Gonzalez-Martinez at al., 2014). The relative abundance of the consensus taxonomy OTUs with at least >1.00% relative abundance of *iTag* sequencing subsamples was taken for the multivariate redundancy analysis.

2.8. Preparation of samples for scanning electron microscopy (SEM) analysis

The handling, preparation and visualization of biomass samples was done in the Center of Scientific Instrumentation of the University of Granada by using a scanning electron microscope (SEM, Carl Zeiss LEO 906E).

To prepare the samples, granular biomass was fixated in a mix solution of 2.5% glutaraldehyde in pH 7.4 cacodylate buffer 0.1 M at 4 °C during 2 h. Then, they were washed thrice with pH 7.4 cacodylate buffer during 20 min. After this, the samples were postfixated with 1% osmium tetroxide for 2 h, postfixation with 1% osmium tetroxide was for 1 h in darkness and at room temperature, followed by an additional wash with distilled water (three washes of 5 min each). Afterward, samples were dehydrated by successive baths in ethanol of 15 min: one at 50%, 70% and 90%, and two at 100%. For scanning electron microscopy, the samples, after ethanol dehydration, were desiccated through the critical point method using carbon dioxide in a Polaron CPD 7501 desiccator. Finally, the samples were covered with EMITECH K975X carbon cells for its following SEM microscopy observation.

3. Results and discussion

3.1. Operation at 7 °C

The SBR was inoculated with low temperature activated sludge inoculum from a full-scale WWTP located in Porvoo (Finland), and was started-up at 7 °C for the granulation of biomass. The impact of temperature over the aerobic granular sludge system initiated using 1 L activated sludge and 20 mL crushed granular biomass has been reported as negative in earlier studies. The formation of granules at 7 °C was reported not to be possible due to the growth of filamentous microorganisms which did not allow a correct washout of the biomass, and due to the operational instability of the reactor that caused the biomass not to grow (Adav et al., 2008). However, in this experiment the granulation of biomass and successful operation during a period of 8 months of a pilot-scale aerobic granular sludge system under 7 °C was achieved (Fig. S2). Although there was presence of filamentous microorganisms in the early stages of the start-up process, these were washed out by the means of controlling the settling time in the range of 5–3 min. Other experiments for granulation at low temperature (10 °C) using activated sludge from Harbin (China) found the growth of filamentous bacteria with the ongoing operation (De Kreuk et al., 2005; Bao et al., 2009), while in the present experiment this was not found to be a problem when cold-acclimated sludge was used. In addition, cold-adapted activated sludge as inoculum showed a final granule diameter larger than those systems inoculated with warm-adapted inoculum (De Kreuk et al., 2005; Bao et al., 2009). The evolution of the nature of the biomass during the whole operational time is shown in Table 1. Granular biomass was observed within an one week period of operation, similar than in other experiments performed for aerobic granulation at 20 °C temperature; nevertheless, the stability of granular biomass diameter was achieved at two months of operation, 30 days later than those reported at 20 °C; however, the particle diameter at operational days 60 and 120 were similar, even larger, than those obtained in similar experiments operating at 20 °C temperature (Beun et al., 2002; De Kreuk and van Loosdrecht, 2004). Cultivation of aerobic granules at low temperature has been done by addition of Mg^{+2} and Al^{+3} over an inoculum taken from a regular activated sludge system in China (Wang et al., 2012). In this case, granular biomass formation took 30 days to be achieved. The use of low temperature activated sludge showed granular biomass for-

Table 1
Parameters of the biomass during the whole operational time at 7 °C temperature.

Operational day	Day 0	Day 7	Day15	Day 30	Day 60	Day 120	Day 240
MLSS (g L^{-1})	3.49 ± 0.88	1.16 ± 0.27	2.19 ± 0.36	3.66 ± 0.62	8.78 ± 1.09	8.88 ± 0.69	8.38 ± 0.22
MLVSS (g L^{-1})	2.511 ± 0.43	1.027 ± 0.13	2.02 ± 0.07	2.68 ± 0.14	6.557 ± 0.24	6.69 ± 0.10	6.512 ± 0.06
MLVSS/MLSS	0.72	0.88	0.92	0.732	0.746	0.753	0.777
Diameter (mm)	–	<0.3 ± 0.2	1.1 ± 0.8	5.2 ± 0.6	4.2 ± 0.5	4.0 ± 0.6	3.9 ± 0.4
Settling velocity (m h^{-1})	–	>10	22.7	43	48.2	51.4	51.8

mation within 20 days, which showed the important impact that the acclimation of the inoculum has for the formation of granular biomass at low temperature. The settling velocity of the granular biomass at 7 °C using cold-adapted inoculum was around 25% faster than those obtained for operation at low temperature using warm-adapted inoculum (Winkler et al., 2012).

3.2. Physic-chemical parameters

Figs. 1–3 showed the performance of the aerobic granular sludge SBR reactor during the whole experimentation time. In terms of organic matter removal, expressed as COD and BOD_5, the system steeply acquired better removal performances from day 0 to day 20. From this point and until the end of the experiment both COD and BOD_5 performances increased slowly, reaching final stable values in the range of 75–80% for COD and 92–95% for BOD_5. Thus, the aerobic granular sludge operating at low temperature and initiated with cold-adapted inoculum could efficiently remove organic matter within three weeks of operation. The same results were observed for similar systems started-up at low temperature inoculated with warm-adapted inoculum (Jiang et al., 2015). On the other hand, nitrogen removal took longer to achieve good removal performances. During the first 25–30 days of operation the system showed small removal values for total nitrogen caused by the low rates of ammonium oxidation within the system. Once the ammonium started a successful oxidation in the bioprocess, a complete oxidation to nitrate was observed around day 40 of operation. Denitrification improved gradually from day 100 until the end of the experiment. Nitrite concentrations were of small importance during the whole experiment time except during the operational days 30 to 40. The trends observed in the nitrogen removal during the start-up of the system showed a three-step process, with achievement of ammonium oxidation at day 30, accumulation of nitrite until day 40, oxidation of nitrite from day 40 and accumulation until day 100 from which the system removed nitrogen successfully until the end of the experimentation period in the range of 70–76%. With respect to phosphorous, the aerobic granular sludge at low temperature underwent a consistent improvement in total phosphate removal from day 0 to day 80, from which the removal efficiency remained stable until the end of the experiment in the 50–60%. Stable values of nitrogen removal were lower for other aerobic granular sludge systems initiated at low temperature using warm-adapted inoculum, but in those cases the removal of phosphate was higher (De Kreuk et al., 2005; Bao et al., 2009). This must be caused by more efficient nitrifying-denitrifying communities grown from Finland activated sludge than from warm-adapted activated sludge, as well as the opposite case for phosphate-consuming microorganisms. As future prospective, longer anaerobic periods are recommended to achieve better phosphate removal, since oxygen periods of feast and famine have been defended as good approach for the development of phosphate accumulating organisms and therefore lead to higher phosphate removal performances (Henriet et al., 2016). Overall, the system could successfully eliminate organic matter, nitrogen and phosphorous under 7 °C temperature. The organic matter was efficiently removed within the first three weeks of operation, while the nutrients needed for three months of operation to be appropriately removed. Compared to other aerobic granular systems at 30 °C temperatures, the removal of COD was similar, but the removal of total nitrogen and phosphorous were around 20% lower (Ab Halim et al., 2015).

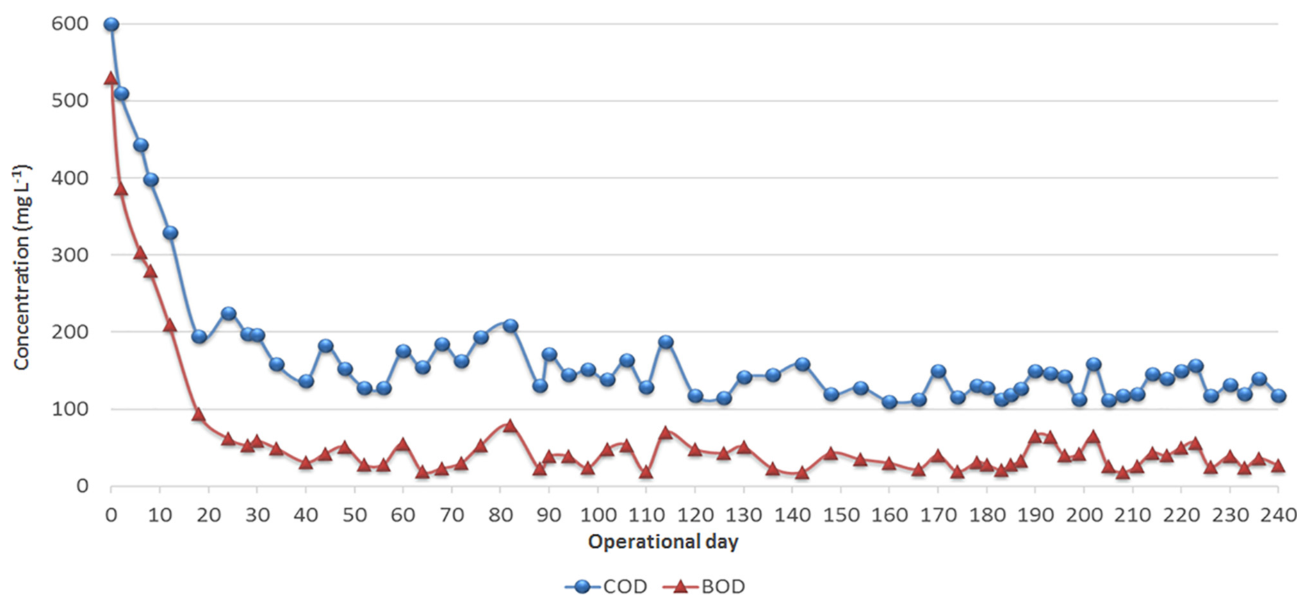

Fig. 1. Effluent concentrations of COD and BOD during the start-up and operation of the aerobic granular sludge bioreactor.

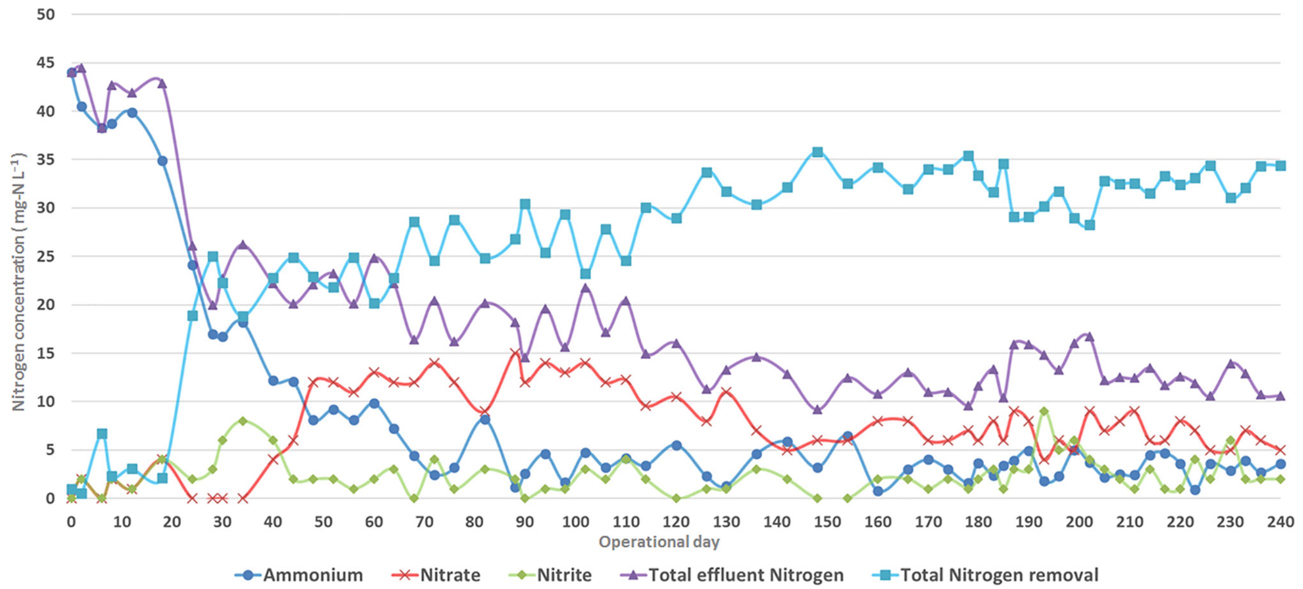

Fig. 2. Effluent ammonium, nitrite, nitrate and total nitrogen removal during the start-up and operation of the aerobic granular sludge bioreactor.

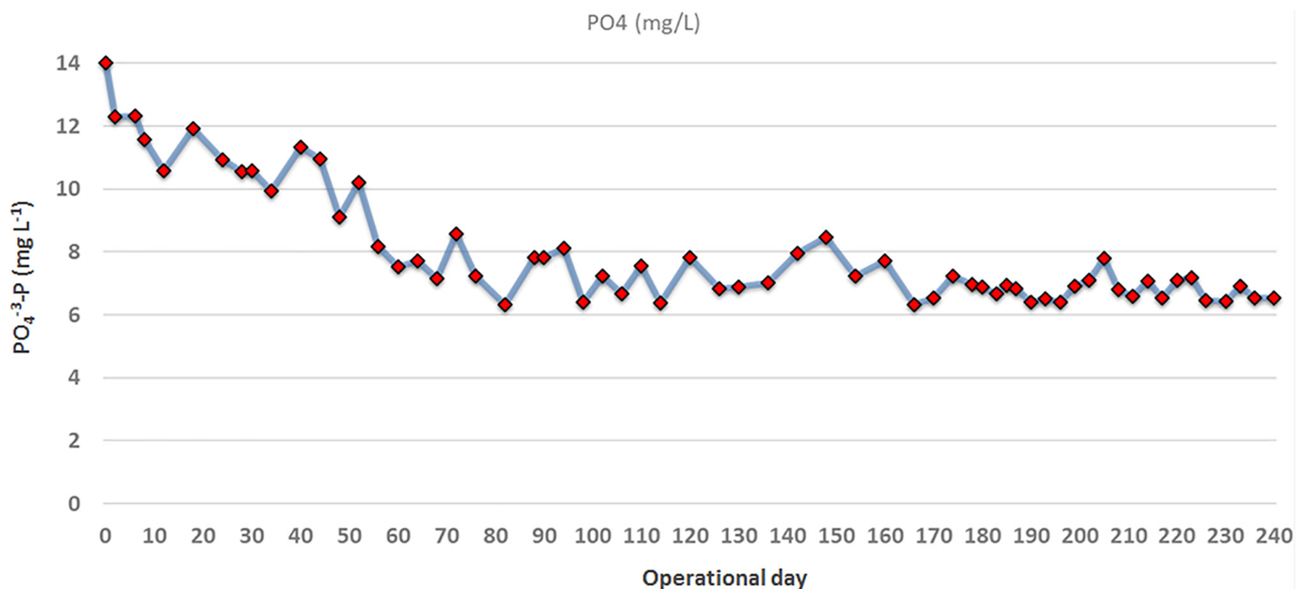

Fig. 3. Effluent phosphate concentration during the start-up and operation of the aerobic granular sludge bioreactor.

3.3. Study of sequencing coverage of iTag sequencing subsamples

The complexity curves of all bacterial *iTag* sequencing subsamples tended to a plateau for a maximum number of 14,405 reads (Fig. S3). The complexity curves showed higher values of species richness for the subsamples at day 0, day 7 and day 15 than for all other subsamples. This could be caused by the richness of bacterial diversity in the inoculum taken from the full-scale activated sludge system in Finland. In addition to the coverage estimation by complexity curves, the results of the redundancy abundance-weighted coverage analysis showed a high coverage for the *iTag* sequencing subsamples, higher than 87% for all *iTag* sequencing samples (Table 2). The higher diversity species in the low temperature full-scale activated sludge system could cause lower redundancy abundance-weighted coverage analysis in the inoculum biological subsample with respect to the others.

3.4. Study of α-diversity and β-diversity of the biological samples

In the *iTag* sequencing subsamples, the species richness showed higher values at operational days 0, 7, and 15, acquiring similar values between samples. In comparison, there was a decrease of species richness values with the ongoing experimentation in samples of days 30, 60, 120 and 240. The system displayed lower values of species richness until the bioreactor stabilized during operational days 120 and 240, which showed similar values of species richness.

The results of the α-diversity indices showed a decrease in diversity and evenness from operational day 0 to operational day 30, from which these increased until stabilization in the range of operational days 120–240 (Table 2). At day 0 the results of α-diversity indices were very high, was caused by the high diversity and evenness of the cold-adapted activated sludge inoculum. From

Table 2
Indices of coverage and α-diversity of the *iTag* sequencing subsamples.

Sample	Individuals	Species Richness	Good's Coverage	Redundancy Abundance-Weighted Coverage	Simpson	Shannon-Wiener	Pielou's Evenness	Berger-Parker	Chao-1
Day 0	14405	616	0.95723707	87.63%	0.982	4.825	1	0.0622	837.3
Day 7	14405	519	0.963970843	96.19%	0.9821	4.732	0.7569	0.05151	668.4
Day 15	14405	547	0.962027074	93.30%	0.8103	3.46	0.5488	0.4266	712.2
Day 30	14405	279	0.980631725	95.00%	0.7122	2.302	0.4088	0.5114	399.7
Day 60	14405	331	0.977021867	93.69%	0.9135	3.561	0.6137	0.26	432
Day 120	14405	281	0.980492884	95.97%	0.9188	3.344	0.593	0.1837	428
Day 240	14405	301	0.979104478	94.82%	0.9164	3.462	0.6067	0.2159	422.9

this day, the *iTag* sequencing subsamples showed a decrease in the Simpson, Shannon-Wiener, Pielou's evenness and Chao-1 indices, as well as an increase in the Berger-Parker index, until operational day 30. During this time the bacterial communities in the system experienced a decrease in both diversity and evenness caused by the adaptation of the inoculum to the bioreactor and the development of the granular biomass. However, since operational day 30 the α-diversity indices showed that the diversity and evenness of the granular biomass increased over operational time until stabilization between days 120 and 240. This increase in diversity and evenness was related to the adaptation of the microbial communities to the conditions of the aerobic granular sludge system, which concluded with the conformation of a stable bacterial community representing mature granular biomass at low temperature. In this sense, the α-diversity indices of the *iTag* sequencing subsamples showed a bacterial dynamics of adaptation of bacterial communities from the inoculum to mature granular biomass.

The values of the Morisita-Horn and symmetric indices for the pair of samples analyzed for β-diversity estimation are shown in Fig. S4. The differences in dominant phylotypes showed a pattern similar to that of the α-diversity indices. Changes were important from the inoculation to operational day 7, and deep changes occurred from day 7 to day 60 in terms of dominant phylotypes. Changes were less pronounced from day 60 to day 120, and almost negligible from day 120 to day 240. However, the results of the symmetric index showed deep changes in rare phylotypes from day 0 to day 15, less pronounced changes between days 30 and 120, and another drastic change from day 120 to day 240. The changes in rare phylotypes were caused by the adaptation of the biomass during the first days of experimentation and by the imposition of dominant phylotypes by the end of the experimentation, while intermediate operational days allowed for rare phylotypes to continue thriving in the system. Overall, the β-diversity indices used showed an adaptation of the biomass from the inoculum to the stabilization, accordingly to the results obtained through α-diversity analysis. In this sense, α- and β-diversity analyses showed that dominant bacterial communities in the bioreactor reached a stable composition around 120 days after inoculation.

3.5. Community structure dynamics

The community structure in the system during the experimentation period had a total number of 69 consensus taxonomy OTUs at genus level of major represented *Bacteria* (>1.00% total relative abundance in at least one of the subsamples), which is shown in Fig. 4. The phylum with higher representation was Proteobacteria with 34 genera. The second major represented phylum was Actinobacteria with a total number of 10 genera. The phylum *Firmicutes* and *Bacteroidetes* were affiliated with 16 and 5 genera, respectively. Finally, the phylum *Candidate_Division_T7*, *Chloroflexi*, *Acidobacteria* and *WCHB1-60* had 1 representative genus.

The bacterial community structure of the inoculum collected from the Porvoo WWTP showed a very high richness and diversity, as confirmed by the species richness and the α-diversity analysis of this subsample. The most represented genera were *Rhodobacter* (6.22%), *Blautia* (5.28%), *Subdoligranulum* (3.52%), *Pseudobutyrivibrio* (3.27%) and *Vasilyevaea* (3.26%). Interestingly, dominant phylotype *Rhodobacter* has a phototrophic metabolism in addition to aerobic chemoheterotrophic and nitrogen fixation metabolisms, and thus it could be possible that activated sludge systems at low temperature favor metabolically flexible phylotypes. *Blautia*, *Subdoligranulum* and *Pseudobutyrivibrio* are strictly anaerobic bacteria with fermentative metabolism which have been isolated from human gut and rumen (Kopecny et al., 2003; Hølmstrom et al., 2004; Liu et al., 2008). On the other hand, *Vasilyevaea* has been described as aerobic, heterotrophic bacterium (Yee et al., 2010). Among the bacterial genera with >1.00% relative abundance *Candidatus Nitrotoga* was found in a 1.51% relative abundance. It has been proposed that *Candidatus Nitrotoga* plays a crucial role in the nitrite oxidation in WWTPs at low temperature (Lücker et al., 2015). The dominant phylotypes found in the inoculum from the Finnish activated sludge system showed a high representation of bacterial genera associated with human and animal gut. In this sense, it has been reported that influent wastewater to WWTPs in Spain and The Netherlands showed bacterial genera associated with human gut, but that the bacterial community structure of the activated sludge systems receiving these influents are completely different with no human gut bacteria found at major phylotypes (Gonzalez-Martinez et al., 2016b). The presence of human gut bacteria in cold-adapted activated sludge systems may signify that low temperatures configure the ecology of an activated sludge system in such a way that strictly anaerobic bacteria might be of fundamental importance for the wastewater treatment process. Future research efforts should be spent in the examination of the microbiology of activated sludge systems in Finland and other extremely cold areas.

Bacterial community in the subsample at day 7 represents the community structure of activated sludge at low temperature in its early stage of acclimation to the new operational conditions. As corroborated by species richness and diversity indices values, both the diversity and evenness in the system were very high. The community structure was very diverse but accounted for some genera above 4% relative abundance, including the most represented phylotype *Iamia* (5.15%), which followed by *Simplicispira* (4.45%). The genus of *Rhodobacter* was represented with 4.43% relative abundance, and *Defluviimonas* and *Hyphomicrobium* with a 4%. Strains of the *Iamia* genus had been isolated from marine sediments and arctic environments, and therefore this genus contains some cold-adapted species.

The bacterial community structure in the subsample of granular biomass at day 15 was consistent with the results of the values of the α-diversity analysis because although its diversity was high, as it showed a total of 279 consensus taxonomy genera, however, evenness was more unbalanced than in the subsample of day 7. There was a sharp change in the community structure of day 7 to day 15, as the sample of the day 7 showed more similar relative

Domain	Phylum	Class	Order	Family	Genus	Code	Day 0	Day 7	Day 15	Day 30	Day 60	Day 120	Day 240
	Acidobacteria	Acidobacteria	Subgroup_4	Unknown_Family	Blastocatella	1							
				Acidimicrobiaceae	CL500-29_marine_group	2							
				Iamiaceae	Iamia	3							
		Acidimicrobiia	Acidimicrobiales	Sva0996_marine_group	unclassified	4							
				Intrasporangiaceae	Tetrasphaera	5							
					Diaminobutyricimonas	6							
	Actinobacteria				Leucobacter	7							
					Microbacterium	8							
				Microbacteriaceae	Okibacterium	9							
			Micrococcales	Micrococcaceae	Arthrobacter	10							
		Actinobacteria	Propionibacteriales	Nocardioidaceae	Aeromicrobium	11							
		Cytophagia	Cytophagales	Cytophagaceae	Chryseolinea	12							
					Chryseobacterium	13							
	Bacteroidetes	Flavobacteriia	Flavobacteriales	Flavobacteriaceae	Flavobacterium	14							
				Saprospiraceae	Saprospira	15							
		Sphingobacteriia	Sphingobacteriales	Sphingobacteriaceae	Pedobacter	16							
	Candidate_division_TM7	unclassified	unclassified	unclassified	unclassified	17							
	Chloroflexi	Caldilineae	Caldilineales	Caldilineaceae	Caldilinea	18							
			Bacillales	Bacillaceae	Bacillus	19							
				Carnobacteriaceae	Trichococcus	20							
				Enterococcaceae	Vagococcus	21							
		Bacilli	Lactobacillales	Streptococcaceae	Lactococcus	22							
					Anaerostipes	23							
					Blautia	24							
					Coprococcus	25							
	Firmicutes				Dorea	26							
					Incertae_Sedis	27							
					Pseudobutyrivibrio	28							
					Robinsoniella	29							
				Lachnospiraceae	Syntrophococcus	30							
				Peptostreptococcaceae	Incertae_Sedis	31							
					Faecalibacterium	32							
		Clostridia	Clostridiales	Ruminococcaceae	Subdoligranulum	33							
				A0839	unclassified	34							
Bacteria					Bosea	35							
					Nitrobacter	36							
				Bradyrhizobiaceae	Oligotropha	37							
					Filomicrobium	38							
					Hyphomicrobium	39							
				Hyphomicrobiaceae	Vasilyevaea	40							
				Methylobacteriaceae	Meganema	41							
				Methylocystaceae	Methylocystis	42							
			Rhizobiales	Rhizobiaceae	Shinella	43							
					Defluviimonas	44							
					Paracoccus	45							
					Pseudorhodobacter	46							
					Rhodobacter	47							
			Rhodobacterales	Rhodobacteraceae	Roseicitreum	48							
			Rhodospirillales	Acetobacteraceae	Oleomonas	49							
		Alphaproteobacteria	Sphingomonadales	Ellin6055	unclassified	50							
	Proteobacteria				Acidovorax	51							
					Alicycliphilus	52							
					Brachymonas	53							
					Comamonas	54							
					Diaphorobacter	55							
					Mitsuaria	56							
					Rhizobacter	57							
					Simplicispira	58							
			Burkholderiales	Comamonadaceae	Xenophilus	59							
					Methylobacillus	60							
			Methylophilales	Methylophilaceae	Methylotenera	61							
			Nitrosomonadales	Gallionellaceae	Candidatus_Nitrotoga	62							
		Betaproteobacteria	Rhodocyclales	Rhodocyclaceae	Ferribacterium	63							
			Enterobacteriales	Enterobacteriaceae	Citrobacter	64							
			Pseudomonadales	Moraxellaceae	Acinetobacter	65							
			Thiotrichales	Thiotrichaceae	Thiothrix	66							
				Xanthomonadaceae	Pseudoxanthomonas	67							
		Gammaproteobacteria	Xanthomonadales	Xanthomonadales_Incertae_Sedis	Steroidobacter	68							
	WCHB1-60	unclassified	unclassified	unclassified	unclassified	69							
	Others						39.89	40.29	26.10	11.81	20.55	14.88	17.91

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Fig. 4. Heat map of >1.00% relative abundance genera in the *iTag* sequencing subsamples collected from the aerobic granular sludge bioreactor.

abundances between genus compared to the sample of day 15, which was clearly dominated by genus *Methylotenera* with a relative abundance of 44.66%. This is a very common genus in wastewater and contaminated water (Kalyuzhnaya et al., 2006). The second major represented genus was an unclassified genus within the WCHBI-60 phylum, with 6.5% of relative abundance. The rest of phylotypes presented in the system at that time had a lower relative abundance of 4%.

The community structure of granular biomass subsample day 30 was in line with the values obtained from α-diversity, reflecting the lowest values of all registered in both evenness and diversity, as also observed in the species richness showed in the complexity curves analysis. The domination belonged to the phylotype *Diaphorobacter* with 51.13% relative abundance and the second most represented genus was *Alicycliphilus* with 12.06% relative abundance. Both these genera belong to the family *Comamonadaceae*. *Acidovorax* genus was represented with 9.06% of abundance relative in the community, followed by genus *Shinella* with 4.31%. The remaining phylotypes present in the system had less than 3% relative abundance.

A strong change was observed in the granular biomass at operational day 60, as shown by the increase in diversity and evenness expressed by the α-diversity indices in comparison with day 30. The major represented genus was *Thiothrix* with a relative abundance of 26%. This genus has stringy characteristics, adaptation to saline environments and is usually present in activated sludge when there are sources of sulfide. In addition, *Thiothrix* species have been reported as filamentous bacteria present in aerobic granular sludge, and therefore their ecological role in the formation of granular biomass has been identified as crucial (Su et al., 2012; Figueroa et al., 2015; Muszynski and Milobedzka, 2015). Besides the removal of sulfur, *Thiothrix* genus has been reported for the elimination of phosphate through biomineralization in aerobic granular sludge (Li et al., 2014), as well as its capacity for denitrification has been found (Desai et al., 2013; Trubitsyn et al., 2013, 2014). In this sense, the presence of *Thiothrix* could be related to the accumulation of sulphate in the aerobic granular sludge, their imposition as the main denitrifier in the system, or both. The genus *Paracoccus* was represented by 6.27% of the total abundance of the system. *Leucobacter* presented a 5.56% relative abundance of the system and *Comamonas* a total 4.11%.

The bacterial community structure after 120 days from start-up of the reactor was drastically different from that of the inoculum used. The results of α-diversity indices and species richness showed high values for diversity and evenness at that time. Since day 120 of operation the bioreactor showed a community structure much more stable in time within the granular biomass, whose dominant phylotypes belonged to the family of *Microbacteriaceae*

and which were of *Microbacterium* and *Leucobacter* genera. *Microbacterium* and *Leucobacter* were present in 18.36% and 14.71% of relative abundance in the system at that point. Also *Leucobacter* was described as a highly hydrophobic microorganism with strong negative superficial charges and thus it has been associated with high flocculation indices (Xie et al., 2010). High flocculation indices are linked to flocculation-granulation processes and to shorter settling time of biomass. In addition, having a negative superficial charge would lead to an increase in the interaction of electrostatic attraction between surfaces at intermediate or high concentrations of electrolytes such as Ca^{+2} (Zita and Hermansson, 1994) which would favor bioflocculation. *Flavobacteriaceae*-belonging *Chryseobacterium* genus, which has been described as a cold-tolerant genus in water systems, was present in 11.6%. This genus has been reported as capable of removing phosphorus in wastewater treatment systems at low temperature (8–12 °C) (Hantsis-Zacharov et al., 2008). Among the phylotypes with >2.00% relative abundance in the system *Arthrobacter*, *Shinella*, *Rhodobacter*, *Lactococcus* and *Bosea* were found.

The dominant bacterial community structure at day 240 remained stable with respect to that at day 90, as showed by the β-diversity analysis. This meant that by day 120 of operation the granular biomass achieved a stable dominant bacterial community structure operating at low temperature. At day 240 *Microbacteriaceae* was the dominant family with genera *Microbacterium* and *Leucobacter* having 21.59% and 15.62% relative abundance, respectively. *Chryseobacterium* continued as third major represented phylotype in the system with a 5.20%, which showed a decline in representation with respect to day 120. Other important genera present at this time were *Steroidobacter* with 4.75% relative abundance, *Acidovorax* with 3.52% and *Brachymonas* with 3.16%.

3.6. Multivariate analyses of operational variables and bacterial community structure in the granular biomass

The bacterial community structure of granules was correlated with two groups of variables. One was related to biomass characteristics such as MLSS, MLSSV, diameter of granules and settling velocity. The other RDA was done considering operational and performance data such as effluent ammonium, nitrite and nitrate concentrations; and removal of BOD5, COD, total nitrogen and phosphate.

The RDA linking bacterial genera with biomass characteristics showed that a group of phylotypes had a strong negative correlation with the MLSS, MLSSV, diameter of granule and settling velocity (Fig. 5A). This cluster of bacterial genera was related with the community structure of samples at days 0, 7 and 15, given that biomass was flocculated and not granular at that time. Representative genera in subsamples were *Iamia*, *Simpliscira*, *Defluviimonas*, *Hyphomicrobium*, *Filomicrobium*, *Aeromicrobium*, *Blastocatella*, *Chryseolinea*, *Subdoligranulum*, *Tetrasphaera*, *Nitrobacter*, and *Trichococcus*, some of which have been found in activated sludge systems. For the day 30 subsample there was a change in the community structure as suggested by α-diversity, β-diversity and dominant phylotypes structure analyses. This change started the granulation process, as the RDA suggested. The genera shown in this subsample were *Diaphorobacter*, *Alicycliphilus* and *Acidovorax*, all of them belonging to the *Comamonadaceae* family, and could favor the initial phases of biomass granulation. Finally, another bacterial genera cluster showed a strong positive correlation with MLSS, MLSSV, diameter of granules and settling velocity, which corresponded to subsamples of days 60, 120 and 240. In this sense, the RDA suggested that at day 60 the granulation process had concluded. However, day 60 subsample community structure was represented mainly by *Thiothrix* and *Paracoccus*, belonging to the family *Thiotrichaceae* and *Rhodobacteraceae*, respectively, while the subsamples of days 120 and 240 had a stable community structure dominated by *Mycobacterium*, *Leucobacter* and *Chryseobacterium*, among others.

The RDA linking the major bacterial genera (>1.00% relative abundance) and the bioreactor's performance is shown in Fig. 5B. The subsample of days 0 and 7 seem to have no strong correlation with any of the operational parameters. Days 0 and 7 subsamples had a weak negative correlation with effluent ammonium and nitrite, COD and BOD5 removal. This may be the caused by the adaptation process of the inoculum from day 0 to day 7. Effluent ammonium concentration had a positive correlation with genera *Flavobacterium*, *Trichococcus* and *Caldilinea*. Furthermore, the highest concentration of ammonium in the influent was related to the subsample at day 15. The subsample of day 30 was correlated with

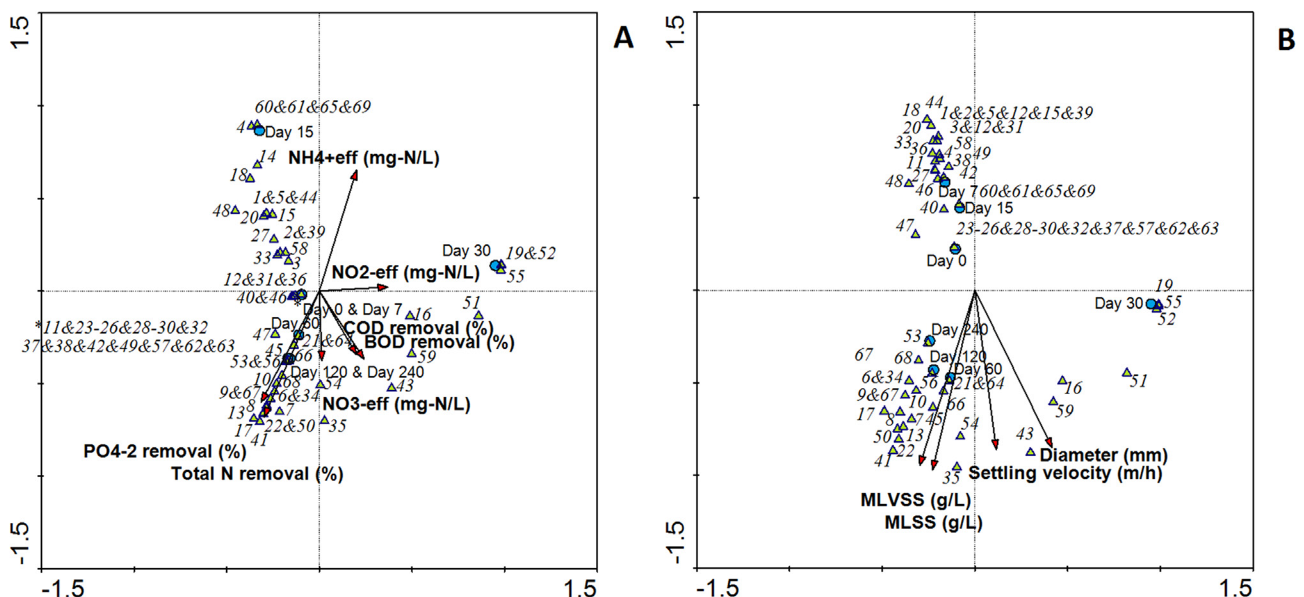

Fig. 5. Multivariate redundancy analyses linking the bacterial genera in the *iTag* sequencing subsamples with the operational parameters of the aerobic granular sludge bioreactor (A) and the characteristics of the biomass in the system (B).

high effluent nitrite concentrations and with genera Bacillus, *Diaphorobacter*, *Alicycliphilus* and *Acidovorax*, which are members of the *Comamonadaceae* family. Finally, subsample at days 60, 120 and 240 were positively correlated with effluent nitrate concentrations. In this way, the RDA suggested that the achievement of complete ammonium oxidation in the aerobic granular systems did not occur until 60 days after inoculation. The positive correlation with the percentage of total nitrogen removal and the elimination of orthophosphate occurred progressively from the sample of day 60, maintaining a stability on days 120 and 240. However, the strongest correlation with the parameters TN (% removal) and PO_4^{-3} (% removal) was shown by the subsample day 240, when the granular biomass was more mature and stable in terms of bacterial community structure. Therefore, it could be established that both the structure of the community that make up the granules as well as the performance were progressive since day 30 and stable since day 120. The cluster of most dominant and strongly correlated genera with removal efficiencies were *Chryseobacterium*, *Microbacterium*, *Leucobacter*, *Okibacterium*, *Pseudoxanthomonas* and *Comamonas*.

3.7. Scanning electron microscopy

The observation of the granules over SEM showed that the initial steps of granular biomass formation was aided by filamentous fungi, over which bacteria formed colonies (Fig. S5A). There was a great diversity bacterial morphology at this stage, although mainly the bacilli morphotypes stood out (Fig. S5B). Bacteria were colonizing the fungi hyphae by the means of adhesion through bacterial EPS. The SEM showed the fungi had broad hyphae growing up to 2–4 μm wide and more than 100 μm long. The presence of fungi showed that these microorganisms could be fundamental for the trigger of granular biomass formation at low temperature (Weber et al., 2007). Indeed, filamentous fungi have been reported as the main responsible for granular biomass formation (Wan et al., 2014).

With ongoing operation, the granular biomass lost the presence of fungi and developed extracellular polymeric substances (EPS) (Fig. S5C–F), which are very important for the maintenance and stability of the granules, since they allow to improve the stability of the shear force and are utilized as substrate for the microbial community that forms the granule. Bacterial morphotypes at the end of the experiment showed diverse morphotypes with coccoids and bacilli being the most dominant.

4. Conclusions

An aerobic granular sludge system was started-up and operated at 7 °C using cold-adapted inoculum. The bioprocess operated with high efficiencies, proving that these systems are a reliable technology operating at 7 °C. The cold-adapted inoculum showed superior capacity for granular biomass formation compared to warm-adapted inoculum. Granules bacterial community was dominated by *Microbacterium* and *Leucobacter*, which were also the most related genera to COD, BOD_5, nitrogen and phosphate removal, and biomass settling velocity, as suggested by multivariate redundancy analyses. Scanning electron microscopy showed that fungi helped in the granulation early stages as bacteria used them as surfaces for growth.

Acknowledgements

The authors would like to acknowledge the support given by Matti Lounio in the Built environment department of the Aalto University, as well as the Institute of Water Research of the University of Granada.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.biortech.2017.05.037.

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